PINK1 protein in normal human brain and Parkinson's disease

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Parkinson's disease is a common incurable neurodegenerative disease whose molecular aetiology remains
unclear. The identification of Mendelian genes causing rare familial forms of Parkinson's disease has revealed
novel proteins and pathways that are likely to be relevant in the pathogenesis of sporadic Parkinson's disease.
Recently, mutations in a novel gene, PINK1, encoding a 581 amino acid protein with both mitochondrial
targeting and serine/threonine kinase domains, were identified as a cause of autosomal recessive parkinsonism.
This provided important evidence for the role of the mitochondrial dysfunction and kinase pathways in neuro-
degeneration. In this study, we report the first characterization of the PINK1 protein in normal human and
sporadic Parkinson's brains, in addition to Parkinson's cases with heterozygous PINK1 mutations. The possible
role of the PINK1 protein was also assessed in a number of neurodegenerative diseases characterized by
proteinaceous inclusions. For these studies, rabbit polyclonal antibodies were raised against two peptide
sequences within the N-terminal hydrophilic loops of PINK1 protein. Using immunohistochemistry and
western blotting we were able to demonstrate that PINK1 is a ubiquitous protein expressed throughout
the human brain and it is found in all cell types showing a punctate cytoplasmic staining pattern consistent
with mitochondrial localization. Fractionation studies of human and rat brain confirm that PINK1 is localized to
the mitochondrial membranes. In addition, we show that PINK1 is detected in a proportion of Lewy bodies in
cases of sporadic Parkinson's disease and Parkinson's disease associated with heterozygous mutations in the
PINK1 gene, which are clinically and pathologically indistinguishable from the sporadic cases. PINK1 was absent
in cortical Lewy bodies, in neurofibrillary tangles in Alzheimer's disease, progressive supranuclear palsy and
corticobasal degeneration, and in the glial and neuronal α-synuclein positive inclusions in multiple system
atrophy. These studies provide for the first time in vivo morphological and biochemical evidence to support
a mitochondrial localization of PINK1 and underpin the significance of mitochondrial dysfunction in the patho-
genesis of nigral cell degeneration in Parkinson's disease.

Keywords: Parkinson's disease; PINK1; immunohistochemistry; genetics; brain

Abbreviations: CBD = corticobasal degeneration; IM = inner mitochondrial membrane; LB = Lewy body;
MSA = multiple system atrophy; OM = outer mitochondrial membrane; PINK1-IR = PINK1 immunoreactivity;
PSP = progressive supranuclear palsy

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Introduction

Parkinson's disease is a common and incurable progressive neurodegenerative disorder. It is characterized pathologically
by the pronounced and selective loss of dopaminergic neurons projecting from the substantia nigra pars compacta
to the striatum, and by the accumulation of proteinaceous intracytoplasmic inclusions known as Lewy bodies (LBs)
(Braak et al., 2003). The molecular pathways that result in this pathological picture and concomitant motor deficits remain unknown. However, the identification of several genes that cause rare inherited forms of Parkinson’s disease has provided important insights into the molecular pathogenesis of the sporadic illness.

PINK1, the gene responsible for PARK6-associated autosomal recessive parkinsonism, was identified in 2004 after a homozygous missense mutation (G309D) and a homozygous truncating mutation (W437X) were discovered in Spanish and Italian kindreds with Parkinson’s disease, respectively (Valente et al., 2004b). Subsequently, a number of further point mutations, frameshift and truncating mutations have been reported in the PINK1 gene (Hatano et al., 2004; Li et al., 2005; Rogueva et al., 2004; Rohe et al., 2004; Valente et al., 2004a; Bonifati et al., 2005). The PINK1 gene encodes a 581 amino acid protein with an N-terminal mitochondrial targeting motif and a highly conserved kinase domain homologous to the serine/threonine kinases of the Ca2+/calmodulin family. To date, several groups have demonstrated that PINK1 can be localized to mitochondria in vitro (Valente et al., 2004b; Bellina et al., 2005; Silvestri et al., 2005). Functional studies have shown that PINK1 protein may have a neuroprotective role as wild-type PINK1 protects cells against proteasomal inhibition (Valente et al., 2004b), as well as staurosporine-induced apoptosis (Petit et al., 2005). This protective effect is abrogated by mutations in the PINK1 gene.

The discovery that mutations in the PINK1 gene could result in parkinsonism highlights two points: first, it provides a potential molecular link between the mitochondria and neurodegeneration in Parkinson’s disease, and secondly, it was the first evidence that a kinase signalling pathway may be important in the pathogenesis of dopaminergic nigral cell death. However, it has not yet been established whether the in vitro localization of overexpressed PINK1 is identical to that of the endogenously expressed PINK1 either in vitro or in vivo. Ultimately, it is important to understand the site and function of the endogenous PINK1 protein in human brain. Using novel polyclonal antibodies, we have therefore characterized PINK1 protein expression in normal human brain, and, furthermore, studied its expression in cases of sporadic Parkinson’s disease. As post-mortem human brain has limited tissue preservation, we have demonstrated the subcellular location of the PINK1 protein using fresh rat brain. Finally, we have identified four post-mortem brains that harbour heterozygous mutations in the PINK1 gene and performed a detailed clinicopathological analysis in these cases to investigate the potential pathology associated with PINK1 mutations.

Material and methods

Cases

Brain tissue was obtained from the Queen Square Brain Bank (where it had been donated with the informed consent of next of kin) with approval from the National Hospital for Neurology and Neurosurgery/Institute of Neurology Joint Ethics Committee. Four post-mortem brains with Parkinson’s disease associated with heterozygous mutations in the PINK1 gene were identified following an extensive mutation screen (P.M. Sleiman et al., personal communication). Of these cases, two had flash frozen tissue and two slow frozen tissue. All four cases were re-examined by two neuropathologists, and detailed medical records were obtained from the Queen Square Brain Bank. The extent of Lewy body and Alzheimer’s disease pathology was established using standard criteria (Braak et al., 1991; Mirra et al., 1991; McKeith et al., 1996; National Institute on Aging, 1997).

Immunohistochemistry for PINK1 was performed on two normal control brains, two cases of sporadic Parkinson’s disease and two cases with heterozygous PINK1 mutations (the two cases with available frozen material). Six further cases of sporadic Parkinson’s disease were used to investigate PINK1 expression specifically in the substantia nigra. Disease-specific regions of two cases each of multiple system atrophy (MSA), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Alzheimer’s disease and neocortical Lewy body disease (Parkinson’s disease with dementia) were also studied. Two of the control human brains and one of the Parkinson’s disease brains from the immunohistochemical study were used for tissue fractionation and immunoblotting.

Antibody preparation

Rabbit polyclonal antibodies (PINK1-49 and PINK1-48) were raised by immunizing two rabbits with synthetic peptides corresponding to unique hydrophilic amino acid residues at positions 135–149 and 194–209, respectively. The peptides were coupled to keyhole limpet haemocyanin before immunization. Anti-serum was affinity purified on columns against each of the synthetic peptides (Eurogentec, Liege, Belgium). Other antibodies used in this study included anti-α-synuclein antibody (Santa Cruz Biotechnology, Santa Cruz, USA), anti-Tim23 (BD Biosciences, Pharmingen, USA), anti-GAPDH (Chemicon, CA, USA), anti-complex I antibody (Molecular Probes, Leiden, The Netherlands), anti-mitochondrial antibody raised to the non-glycosylated 60-kDa mitochondrial antibody (Santa Cruz Biotechnology, Santa Cruz, USA), anti-α-synuclein antibody (Santa Cruz Biotechnology, Santa Cruz, USA), anti-Tim23 (BD Biosciences, Pharmingen, USA), anti-GAPDH (Chemicon, CA, USA), anti-complex I antibody (Molecular Probes, Leiden, The Netherlands), anti-mitochondrial antibody raised to the non-glycosylated 60-kDa portion of the mitochondria (Abcam 3298, clone MTC02, Cambridge, UK), anti-hsp60 (Stressgen SPA-806, clone LK-1, San Diego, USA) and anti-cytochrome c antibody (BD Biosciences, Pharmingen, USA).

Immunohistochemistry

The immunohistochemical appearance of the PINK1-48 and PINK1-49 was successfully optimized on frozen tissue sections. Ten-micrometre-thick frozen sections were cut using a cryostat from the following brain regions: frontal cortex, hippocampus, putamen, caudate, thalamus, subthalamic nucleus, cerebellum,pons, medulla and midbrain. Sections were fixed in 1% paraformaldehyde for 5 min and then treated with 0.3% H2O2 in methanol for 10 min to inhibit the endogenous peroxidase activity. Sections were blocked with horse serum (Vector Laboratories, UK) for 30 min and incubated overnight at 4°C with either PINK1-49 antibody (1:50 dilution), or PINK1-48 antibody (1:20) or at room temperature for 1 h with anti-mitochondrial antibody (1:200), anti-hsp60 antibody (1:100) or anti-α-synuclein antibody (1:100). Biotinylated secondary antibody (VectorKit) was applied for 1 h at room temperature, followed by avidin–biotin peroxidase complex...
(VectorKit) for 30 min. Immunoreactivity was visualized using the chromagen 3,3’-diaminobenzidine-nickel. The sections were counterstained either with 0.2% methyl green or with Mayer’s haematoxylin blue.

Negative controls performed in parallel included omission of the primary antibody and application of PINK1-49 antibody preabsorbed with the PINK1 peptide.

**Immunoblotting**

Tissue blocks weighing ~50 mg were cut from different brain regions (frontal cortex, temporal cortex, caudate, putamen, substantia nigra, cerebellum) of four flash frozen normal human brains. The samples were homogenized in 0.1% Tween-20 buffer with protease inhibitors [50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 0.1% Tween-20; 10% (v/v) glycerol] using a hand-held polytron homogenizer, incubated on ice for 30 min and centrifuged at 13 000 g for 30 min at 4°C. Protein concentration of the supernatant (detent-soluble fraction) was determined using the Bradford assay (Pierce). Forty micrograms of protein from the detergent-soluble fractions and the detergent-insoluble fractions was heated to 100°C for 5 min in Laemmmi buffer. The lysates were separated by electrophoresis on a 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gel, transferred to a nitrocellulose membrane and probed with the primary antibody (PINK1-49 antibody, 1 : 500; PINK1-48, 1 : 100) overnight at 4°C followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgGs (Dako, 1 : 2000) for 1 h at room temperature. The membranes were washed three times for 5 min with 0.1% PBS-Tween. The membranes were developed using electrochemiluminescence reagent (Amersham Biosciences, UK) and exposed to radiographic film.

Protein loading of detergent-soluble fractions was confirmed by re-probing membranes with anti-GAPDH antibody; protein loading of detergent-insoluble fractions was confirmed by re-probing with anti-β-actin antibody.

**Fractionation of human brain**

Frozen blocks of human frontal cortex were weighed and placed in a ground-glass hand-held homogenizer on ice, and 9 μl/mg of isolation medium was added (320 mM sucrose, 1 mM EDTA, 10 mM Trizma-base, pH 7.4). The material was homogenized thoroughly on ice, transferred to microcentrifuge tubes and centrifuged at 3000 g for 5 min, and the pellet (unhomogenized material) was discarded (step 1). The supernatant was centrifuged at 12 000 g for 10 min (step 2). The resulting pellet (crude mitochondrial fraction) was re-suspended in 1 ml isolation media, and repeat centrifugation at 3000 g was performed to remove nuclear contamination (step 3). The resulting pellet was discarded and the supernatant was centrifuged at 12 000 g for a further 10 min (step 4). Steps 3–4 were repeated twice to purify the mitochondrial fraction further. The resulting pellet from the 12 000 g spin constituted the mitochondria-enriched fraction. The supernatant obtained from step 2 was centrifuged at 70 000 g for 60 min (step 5). The resulting supernatant constituted the soluble cytosolic fraction, and the pellet constituted the microsomal (including the endoplasmic reticulum) enriched fraction. The pellet fractions were re-suspended in 1 ml isolation media, and all fractions were stored at −70°C.

The purity of the fractions was tested in two ways: first, by measuring the enzyme activity of the mitochondrial enzymes citrate synthase and complex II/complex III, and second, by immunoblotting with antibodies to mitochondrial proteins such as complex I (1 : 1000) and Tim23 (1 : 2500). Protein loading was confirmed in the cytosolic fraction by immunoblotting with the anti-GAPDH antibody (1 : 3000).

Protein concentrations of the mitochondria-enriched fraction (M), the cytosolic fraction (C) and the microsomal fraction (P) were determined using the Bradford assay, and the fractions were subjected to immunoblotting with the PINK1-49 antibody.

**Rat brain suborganelle fractionation**

Animals used for isolating mitochondria were adult male rats of the Wistar strain weighing ~250 g (B & K Universal Ltd., Hull, Yorkshire, UK). Animals were kept under 12 h light/dark cycles, fed a stock laboratory diet and provided with water ad libitum.

Isolation of non-synaptic rat brain mitochondria was performed in homogenization buffer comprising 320 mM sucrose, 1 mM K+ EDTA and 10 mM Tris–HCl, pH 7.4, as described by Lai and Clark (1979). Following isolation, mitochondria were re-suspended in a minimal volume of ice-cold isolation medium before subfractionation. Mitochondria were subfractionated according to the method of Kuzela and Golgberg (1994) with slight modifications.

Isolated mitochondria were incubated with varying concentrations of ice-cold digitonin (0.1–0.4 mg per milligram mitochondrial protein) for 15 min at 4°C to disrupt the mitochondrial outer membrane. The mitochondrial suspension was then centrifuged for 10 min at 9800 g at 4°C to generate a pellet of mitoplasts (inner membrane plus matrix) and a supernatant containing the outer membrane and intermembrane space. The supernatant was removed and the mitoplasts disrupted by sonication on ice for 1 min (5 s on/off intervals) using a MSE Soniprep 150 set at an amplitude of 14 μA. Sonicated mitoplasts and supernatants containing outer membrane plus intermembrane space were then centrifuged for 1 h at 100 000 g using a Beckman TLX optima ultracentrifuge at 4°C. This generated supernatants containing the mitochondrial matrix and intermembrane space, respectively, and pellets of inner membrane and outer membrane, respectively. Inner and outer membrane preparations were further purified by washing in isolation media followed by centrifugation for 10 min at 9800 g at 4°C, and this procedure was repeated a further two times to remove contaminating proteins.

Submitochondrial fractionation was confirmed by immunoblotting the fractions with antibodies to (i) an outer mitochondrial membrane marker (VDAC1); (ii) an inner mitochondrial membrane marker (Tim23); (iii) a matrix protein (hsp60); and (iv) an intermembrane space protein (cytochrome c). Submitochondrial fractions were then probed with the PINK1-48 antibody (1 : 100).

**Treatment of mitochondria with sodium carbonate**

To determine membrane integration of PINK1, soluble and peripheral membrane proteins were extracted from integral membrane proteins through the use of carbonate extraction (Fujiki et al., 1982). Briefly, mitochondrial pellets were re-suspended in 0.1 M sodium carbonate, pH 11.5. Following incubation on ice for 30 min, mitochondrial membranes were re-isolated by ultracentrifugation at 100 000 g for 30 min at 4°C. The presence of PINK1 in the fractions was determined by immunoblotting.
Results

Antibodies

PINK1-49 recognized a ~68–70 kDa band in the detergent-soluble and detergent-insoluble fractions of human brain. In addition, a smaller band at ~51–52 kDa was detected in the detergent-soluble fraction of frontal cortex (Fig. 1). Both of these bands were abolished on preabsorption of the antibody with the synthetic peptide, and thus both species are thought to represent the PINK1 protein. Characterization of the PINK1-48 antibody revealed that it recognized the same bands in human frontal cortex on immunoblotting and produced an identical appearance on immunohistochemistry to the PINK1-49 antibody (data not shown). Therefore, the remainder of the study of human brain was continued using PINK1-49 antibody alone. In rat brain, the PINK1-48 antibody recognizes a band at ~68 kDa representing the full-length PINK1 protein, and thus this antibody was used for the submitochondrial fractionation studies in rat tissue.

PINK1 protein in normal brain

PINK1 immunoreactivity (PINK1-IR) was observed in both the grey and white matter of all brain regions studied, although it was considerably more pronounced in the grey matter. Staining was positive in cells with both neuronal and glial morphology. Endothelial cells and smooth muscle cells of blood vessels were also positively stained for PINK1.

Within the neuronal cells, the PINK1-IR displayed a punctate appearance in the cytoplasm of the cell body and within the axonal processes. In the glial cells, PINK1-IR was concentrated in a rim around the nucleus. Astrocytes stained strongly with PINK1, in both a diffuse pattern within the cell body and a granular pattern along the astrocytic processes (Fig. 2). The same pattern of staining was demonstrated in all brain regions studied. The strongly punctate PINK1 immunoreactivity was similar to the immunohistochemical appearance of the anti-mitochondrial antibody (data not shown). Staining was absent in both negative controls, that is, application of the preabsorbed PINK1-49 antibody and omission of the PINK1-49 antibody.

Regional distribution

Immunoblotting of normal human brain revealed a band at ~51–52 kDa in the detergent-soluble fraction, corresponding to a cleaved form of PINK1. The ratio of PINK1 band optical density/GAPDH band optical density demonstrated equal levels of expression of the detergent-soluble cleaved PINK1 protein in frontal cortex, temporal cortex, caudate, putamen and cerebellum (Fig. 3B). Immunoblotting of the detergent-insoluble fraction revealed a product of ~68–70 kDa corresponding to the full-length PINK1 protein, which was detected in all regions of human brain (data not shown).

PINK1 protein in Parkinson’s disease brain

The distribution of the PINK1-IR and the pattern of neuronal and glial staining in sporadic Parkinson’s disease brain were similar to normal brain. In the substantia nigra, the neuromelanin containing nigral neurons demonstrated the typical granular staining in the neuronal cytoplasm. PINK1-IR was observed in a small proportion of intraneuronal inclusions with morphological features of LBs (Fig. 4). In such inclusions, the staining was for the most part concentrated in the halo, but was occasionally seen in the core of the inclusion. The proportion of PINK1 positive LBs was determined by counting α-synuclein positive inclusions in serial sections. This revealed that 5–10% of all brainstem α-synuclein containing LBs demonstrate PINK1-IR. Cortical LBs and Lewy neurites were negative for PINK1-IR.

For comparison, we used two other mitochondrial markers in the same cases of Parkinson’s disease substantia nigra (anti-mitochondrial antibody and an antibody to hsp60). In contrast to the PINK1-IR within LBs, we did not detect diffuse positivity with either of these mitochondrial markers within the halo or core of any of the inclusions. However, occasional scattered punctate immunostaining was visible in the LB, possibly representing intact mitochondria overlying the inclusion or within it. Further studies will be required to determine whether PINK1 localization to the LB is due solely to aggregation or whether it is in part mediated by recruitment of intact mitochondria.
Human brain fractionation

Prolonged hypoxia prior to freezing, combined with the freeze–thaw cycle causes loss of integrity of cellular organelles (in particular, the mitochondria) and therefore limits the purity of the subcellular fractions that can be generated from post-mortem frozen brain. Nonetheless, using both protein markers and enzymatic analysis of the fractions we are able to confirm the generation of mitochondria-enriched and mitochondria-depleted fractions (Fig. 5). The mitochondrial markers Tim23 (and complex 1) were detected in the mitochondrial fraction and were absent in
the cytosolic fraction. Similarly, significant levels of citrate synthase and complex II/III activity were detected in the mitochondria fraction and were markedly reduced or absent in the cytosolic fraction. Thus, these crude fractions may be referred to as mitochondria-enriched and mitochondria-depleted fractions. The residual pellet after centrifugation at 70,000 g is the microsomal fraction, classically consisting of fragments of plasma membrane, endoplasmic reticulum, and large ribosomes. We detected both mitochondrial markers and mitochondrial enzyme activity in this fraction, suggesting that the fragmented mitochondria from frozen brain may also appear after high-speed centrifugation. Thus, this fraction represents a mitochondria-contaminated microsomal fraction.

Immunoblotting with PINK1-49 demonstrated full-length PINK1 protein in the mitochondria-enriched fraction and confirmed the absence of the full-length PINK1 protein in the cytosolic fraction (Fig. 5). PINK1 was also detected in the mitochondria-contaminated microsomal fraction, which may either be related to its location in the mitochondria or to a potential location within microsomes.

**Rat brain fractionation**

To determine the exact location of PINK1 in mitochondria, we prepared submitochondrial fractions. The identity of the submitochondrial fractions was demonstrated by the enrichment of expected marker proteins in the fractions. The outer mitochondrial membrane (OM) fraction was free of any inner mitochondrial membrane (IM) and thus represents a pure OM fraction. However, we detect the OM proteinVDAC1 in the IM fraction, reflecting incomplete separation of the OM and IM by digitonin. VDAC1 persisted in the IM fraction even when the highest dose of digitonin (0.4 mg/mg) was used. The well-recognized presence of OM proteins such as VDAC1 in the IM fraction is usually explained by the presence of OM fragments that remain attached to the IM at specialized contact points where the OM and IM are associated. VDAC1 is known to be a protein at these contact sites. There was minimal contamination of the IM fraction by the matrix marker hsp60. Cytochrome c was enriched in the intermembrane space and was faintly detectable in the inner membrane.

Probing for the PINK1 protein using the PINK1-48 antibody revealed that PINK1 was clearly present in the whole mitochondrial preparations, the OM fraction and the IM fraction. Moreover, amongst the IM fractions tested, PINK1 was most strongly detected in the IM fraction generated after using the highest dose of digitonin, which releases...
the greatest amount of the OM (data not shown). No PINK1 protein was observed in the matrix or intermembrane space fraction (Fig. 6A).

**Treatment with sodium carbonate**
Following treatment of the rat brain mitochondrial fractions with sodium carbonate, PINK1 was detected in the residual membrane fraction, suggesting that PINK1 is an integral membrane protein (Fig. 6B).

**Heterozygous mutations in the PINK1 gene**
Following the identification of the PINK1 gene, a mutation screen was performed on all cases of Parkinson’s disease within the Queen Square Brain Bank (P.M. Sleiman et al., personal communication). Four cases of apparently idiopathic Parkinson’s disease were identified with heterozygous mutations in the PINK1 gene. The four mutations are A339T, Y431H, N451S, C575R. The first three mutations map to conserved amino acids in the kinase domain of the PINK1 protein, while C575R maps to the C-terminal extension beyond the kinase domain. The clinical features of these cases are detailed in Table 1. Briefly, the age of onset, asymmetrical motor features and response to L-dopa therapy were all indistinguishable from that seen in idiopathic forms of Parkinson’s disease. Of note, psychiatric features to varying degrees were present in all four cases; significant cognitive impairment was present in two cases, one of which was correlated with a coexisting pathological diagnosis of Alzheimer’s disease. The pathological features of the cases are summarized in Table 2. All cases demonstrated the typical hallmarks of nigral cell loss, and brainstem and cortical LBs. Immunohistochemistry was performed on Cases 2 and 4, in which the PINK1-IR in the neurons and glia was identical to the staining pattern seen in brain with idiopathic Parkinson’s disease and controls. Furthermore, the brainstem LBs, but not the cortical LBs, showed positive staining with the PINK1 antibody in a similar proportion to that already seen in idiopathic Parkinson’s disease.

**PINK1 protein in inclusion body diseases**
To examine the expression of PINK1 in other synucleinopathies, immunohistochemistry was performed on brain sections from cases of MSA and neocortical Lewy body disease. In both diseases, a similar granular appearance was demonstrated in the cytoplasm of neuronal cells and that surrounding the nucleus in gliial cells, as was found in normal controls and Parkinson’s disease. Reactive astrocytes showed strong PINK1 immunoreactivity. In both cases of MSA, the glial cytoplasmic inclusions were negative for PINK1. In the case of diffuse Lewy body disease, a number of LBs in the substantia nigra were immunoreactive for PINK1 in the halo of the body. Cortical LBs in the same case were negative for PINK1-IR.

To examine the expression of PINK1 in tauopathies, immunohistochemistry was performed on brain sections from cases with Alzheimer’s disease, CBD and PSP. In the cases of Alzheimer’s disease, the tangles were negative for PINK1. The PINK1-49 antibody positively labelled Aβ-positive plaques, and, therefore, PINK1 may represent an amyloid-associated protein. Neurofibrillary tangles and glial inclusions were unstained with the PINK1 antibody in the cases of CBD and PSP. However, reactive astrocytes stained strongly positive in these cases.

**Discussion**
**PINK1 in normal brain**
To characterize the expression of PINK1 in human brain, we generated two novel rabbit polyclonal antibodies to unique
peptide sequences within the PINK1 protein: PINK1-49 recognizes residues 135–149 at the N-terminus of the protein. PINK1-48 recognizes residues 194–209 within the kinase domain of the protein. PINK1-49 and PINK1-48 both recognize a 68–70 kDa band in human frontal cortex. The antibodies used in this study, as well as several commercially available anti-PINK1 antibodies, recognize the full-length PINK1 protein at a higher molecular weight than that predicted by the amino acid sequence of the protein (predicted molecular weight 63 kDa). This may be due to post-translational covalent modifications of the PINK1 protein, which are able to alter the electrophoretic mobility to a greater extent than the actual mass of the modification itself, as a result of alterations of the charge of the protein. Bioinformatic analysis (Predictprotein: Rost, 1996) predicts several potential sites for post-translational modifications in the form of glycosylation, phosphorylation and myristoylation sites within the PINK1 protein.

In addition, the PINK1-49 and PINK1-48 antibodies recognize a smaller band at ~51–52 kDa in the detergent-soluble fraction of frontal cortex. Detergents such as Tween solubilize membranes and therefore expose the antibody to antigens that may be otherwise sequestered within membranes or within organelles surrounded by membranes. It is recognized that PINK1 has a predicted N-terminal mitochondrial targeting motif and such mitochondrial signalling peptides may be cleaved by mitochondrial peptidases after import of the protein into the mitochondria. Bioinformatic analysis using the protein database SwissProt predicts a molecular weight of 54 kDa for the kinase chain alone following putative cleavage of the N-terminal mitochondrial targeting motif. To date, there is evidence from cellular studies that PINK1 may be cleaved: Beilina et al. (2005) reported that cells transfected with N-terminal or C-terminal tagged PINK1 protein consistently showed a full-length PINK1 protein and an additional fragment

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Onset (years)</th>
<th>Family history</th>
<th>Motor features at presentation</th>
<th>Other</th>
<th>Response to l-dopa</th>
<th>Psychiatric</th>
<th>Cognitive</th>
<th>Progression</th>
<th>Length of illness (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>58</td>
<td>Nil</td>
<td>Hypomimia, bradykinesia, rigidity, minimal rest tremor</td>
<td>Postural instability</td>
<td>Modest with dyskinesia</td>
<td>Depression</td>
<td>Nil</td>
<td>Moderate; H&amp;Y = 3</td>
<td>10 at presentation and H&amp;Y = 4 after 6 years</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>59</td>
<td>Nil</td>
<td>Tremor—predominant, bradykinesia, rigidity</td>
<td>Tremor—predominant, bradykinesia, rigidity</td>
<td>Excellent with dyskinesia and on/off fluctuations</td>
<td>Depression</td>
<td>Nil</td>
<td>Slow; H&amp;Y = 4</td>
<td>17 years</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>64</td>
<td>Nil</td>
<td>Hypomimia, mild rigidity, tremor</td>
<td>Postural instability with frequent falls</td>
<td>Excellent</td>
<td>Previous history of flat affect</td>
<td>Cognitive decline after 7 years</td>
<td>Rapid progression of motor features within 4 years</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>64</td>
<td>Nil</td>
<td>Shuffling gait, tremor, rigidity</td>
<td>Hypophonia</td>
<td>Excellent</td>
<td>Previous paranoia and anxiety; progressive confusion, visual hallucinations, paranoia</td>
<td>Cognitive decline after 5 years</td>
<td>Slow progression of motor features: H&amp;Y = 3.5 after 7 years</td>
<td>7</td>
</tr>
</tbody>
</table>

H&Y = Hoehn and Yahr staging.

### Table 2 The neuropathological findings of Parkinson’s disease cases associated with heterozygous PINK1 mutations

<table>
<thead>
<tr>
<th>Case</th>
<th>Mutation</th>
<th>Brainstem LB</th>
<th>Cortical LB</th>
<th>Neuritic plaques</th>
<th>Age-related plaque score</th>
<th>CERAD diagnosis</th>
<th>Braak and Braak stage</th>
<th>NIA/Reagan category</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N451S</td>
<td>Yes</td>
<td>Neocortical</td>
<td>None (diffuse Aβ deposits)</td>
<td>0</td>
<td>Normal</td>
<td>I</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>Y431H</td>
<td>Yes</td>
<td>Neocortical</td>
<td>Sparse Aβ deposits</td>
<td>A</td>
<td>Normal</td>
<td>IV</td>
<td>Low/intermediate likelihood</td>
</tr>
<tr>
<td>3</td>
<td>A339T</td>
<td>Yes</td>
<td>Neocortical</td>
<td>None (diffuse Aβ deposits)</td>
<td>0</td>
<td>Normal</td>
<td>I</td>
<td>Normal</td>
</tr>
<tr>
<td>4</td>
<td>C575R</td>
<td>Yes</td>
<td>Neocortical</td>
<td>Moderate Aβ deposits</td>
<td>C</td>
<td>Definite AD</td>
<td>V</td>
<td>High likelihood</td>
</tr>
</tbody>
</table>

LB = Lewy body; CERAD = Consortium to Establish a Registry for Alzheimer’s Disease; NIA = National Institute of Aging; Aβ = amyloid-beta peptide.
that was ~10 kDa smaller than the full-length protein. The smaller fragment was only detected by antibodies to the C-terminal tag of the fusion protein, and thus the authors concluded that cleavage at the N-terminus was responsible for this smaller fragment. Our laboratory has also found similar results (Muqit et al., 2006).

It is established that PINK1 mRNA is present in all adult tissues with most abundant levels of mRNA expression in the heart, skeletal muscle and testis (Unoki et al., 2001). In this study, we show that the PINK1 protein is detected in many brain regions including frontal cortex, temporal cortex, hippocampus, caudate, putamen, thalamus, hypothalamus, substantia nigra and cerebellum, and that the level of expression of the cleaved PINK1 protein is equal across these regions. This suggests that PINK1 has a function in all brain regions and is not specifically associated with areas affected in Parkinson’s disease. Immunohistochemistry of normal human brain further confirmed the ubiquitous nature of the PINK1 protein within both neurons and glia in all major neuronal groups throughout the brain. The characteristic granular appearance of PINK1 immunoreactivity would be consistent with the presence of PINK1 either in the mitochondria or within microsomes in neuronal and non-neuronal cells.

Fractionation studies on human brain enabled us to define the localization of PINK1 in human brain. Full-length PINK1 was present in the mitochondrial-enriched fraction but was absent from the cytosolic fraction. PINK1 was also detected in the final microsomal fraction (although this may be attributed in part to contamination by mitochondrial fragments in this fraction). Having identified PINK1 in mitochondrial-enriched fractions in humans we sought to further localize the PINK1 protein within the different mitochondrial compartments isolated from rat brain. The PINK1 protein was detected as an integral membrane protein in the mitochondrial membrane fraction. This is consistent with the bioinformatic prediction of several transmembrane domains in the PINK1 protein at residues 91–111, 274–293 and 436–452 (Tmpred: Hoffman and Stoffel, 1993). Further separation into sub-mitochondrial fractions revealed its presence in the OM and IM fractions. However, the presence of PINK1 in the IM fraction of rat brain must be interpreted cautiously as it may be explained in part or wholly by the presence of OM fragments contaminating the inner mitochondrial fraction. Alternatively, it may reflect that PINK1 is present at sites where the OM and IMs are associated. Ultimately, confirmation of its exact location within the mitochondrial membranes will require immunogold electron microscopy of brain tissue. Such immunogold electron microscopy of overexpressed PINK1 in cells in vitro has shown the presence of PINK1 on the mitochondrial cristae (Silvestri et al., 2005) or both in the IMs and OMs (Muqit et al., 2006).

To date the localization of PINK1 has been studied only in vitro. Active import into the mitochondria was confirmed using in vitro translated PINK1 precursors incubated in the presence of isolated human mitochondria (Silvestri et al., 2005). Furthermore, N-terminal tagged PINK1 co-localized with a mitochondrial marker and was found in the mitochondrial fraction in some cell models. In contrast, two studies (Belina et al., 2005; Petit et al., 2005) have demonstrated that C-terminal tagged PINK1 localized to punctate cytoplasmic structures that did not necessarily overlap with the mitochondrial marker, and that PINK1 appeared also in the cytoplasmic and microsomal fractions of these cells. Our data strongly support a mitochondrial location for the PINK1 protein. Some of our evidence putatively suggests an extramitochondrial pool of PINK1 protein: we have detected PINK1 in the microsomal fraction on tissue fractionation studies, and we note that in contrast to neurons, reactive astrocytes demonstrate a diffuse staining pattern of PINK1. However, confirmation of an additional intracellular location for PINK1 requires further characterization.

A combination of post-mortem brain studies, genetic and toxin-based models already points toward an aetiological role for mitochondria in the pathogenesis of Parkinson’s disease. Reduced levels of complex I activity and increased levels of oxidative stress products have been found in the brains of cases of sporadic Parkinson’s disease (Schapira et al., 1990; Anderson, 2004). Administration of complex I inhibitors, such as MPTP and rotenone, in rodent and primate models produces selective nigrostriatal neuronal loss and α-synuclein positive inclusions typical of Parkinson’s disease (Betarbet et al., 2000; Fornai et al., 2005). Parkin is a gene known to cause the autosomal recessive disease (Kitada et al., 1998) and parkin knockout models in Drosophila display predominantly mitochondrial pathology (Greene et al., 2003). Mutations in the DJ-1 gene also cause autosomal recessive Parkinson’s disease (Bonifati et al., 2003): DJ-1 has been shown to have a mitochondrial location in vitro, where it protects neurons from oxidative stress and mitochondrial damage (Canet-Aviles et al., 2004). Thus, localizing the PINK1 protein to the mitochondria is an important observation as it provides further molecular evidence that primary mitochondrial dysfunction may cause dopaminergic neuronal death.

**PINK1 protein in sporadic Parkinson’s disease**

The immunohistochemical appearance and regional and cellular localization of PINK1 in brains with sporadic Parkinson’s disease is indistinguishable from normal human brain. Interestingly, PINK1 was detected in a subset (5–10%) of brainstem LBs where the immunoreactivity was concentrated in the halo of the body independent of the appearance of intact mitochondria around or within the LB. LBs are recognized as one of the major pathological hallmarks of idiopathic Parkinson’s disease, although their role in promoting neuroprotection or neurotoxicity remains unclear. Ultrastructurally nigral LBs are spherical...
cytoplasmic inclusions with a dense granular core and an outer region of radiating filaments. Of the proteins encoded by genes known to cause Parkinson’s disease, α-synuclein, parkin and UCHL1 have all been demonstrated in LBs (Spillantini et al., 1997; Leroy et al., 1998; Schlossmacher et al., 2002). DJ-1 conversely is not present in LBs (Bandopadhyay et al., 2004). The main molecular composition, other than α-synuclein, appears to be proteins related to the ubiquitin–proteasome system [E3 ubiquitin ligase, ubiquitin C-terminal hydroxylase L1, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme UbcH7 (E2), proteasome activators PA700 and PA28, free ubiquitin and ubiquitinated protein conjugates, protein folding chaperones (heat shock proteins HSP70 and HSP90), and interactors of α-synuclein (synphilin-1) and parkin].

Early electron microscopy studies have demonstrated remnants of mitochondria in LBs (Morimura et al., 1985). The presence of PINK1 in the LB demonstrates that mitochondrial proteins, including kinases, may also be detected in the LB.

The small percentage of PINK1 positive LBs suggests that PINK1 is not a prerequisite to their formation. Furthermore, the presence of PINK1 in inclusions appears to be region-specific, that is, it occurs in brainstem LBs but not in cortical LBs. It is well established that there is inter-regional and intra-regional heterogeneity of LBs both in their morphology and their molecular composition (Sakamoto et al., 2002). This heterogeneity may reflect different stages of maturation of the inclusion body such that proteins accumulating late in the dying neuron would be present only in a small subset. In support of this, PINK1 was not seen in pale bodies, which are believed to reflect an early stage in LB formation. In addition, the detection of a protein within any inclusion body (and thus the molecular heterogeneity of inclusion bodies) is influenced by several factors such as the degree of unmasking of the antigen, the accessibility of the antibody to the antigen and the potential altered conformation of the epitope causing reduced recognition by the antibody.

PINK1 was not detected in other α-synuclein positive inclusions such as the glial cytoplasmic inclusions associated with MSA. It was not detected in tau-positive filamentous inclusions in either CBD or PSP. PINK1 immunoreactivity was detected within plaques in Alzheimer’s disease. However, plaques are well recognized to show immunoreactivity for a variety of proteins (Alexandrescu et al., 2005; Lashley et al., 2006), some of which may have a role in protein fibrillogenesis. Thus, the significance of PINK1 as an amyloid-associated protein in Alzheimer’s disease remains to be investigated.

Mutations in the PINK1 gene: clinicopathological analysis

A variety of nonsense truncating and missense mutations have now been reported in the PINK1 gene. However, to date there has been no neuropathological data regarding any individual affected with a homozygous mutation in the PINK1 gene. Our laboratory (P.M. Sleiman et al., personal communication) identified four heterozygous mutations in the PINK1 gene in cases of sporadic Parkinson’s disease with available pathological material (A339T, Y431H, N451S, C575R). Clinically, the four cases presented with an age of onset of 58–64 years, and with the cardinal symptoms of asymmetric tremor, bradykinesia and rigidity. There was no family history of Parkinson’s disease in any of the cases. There was a moderate-to-excellent response to L-dopa therapy, and dyskinesias developed late as a complication of drug therapy. Significant cognitive decline was reported in two cases, one of which was correlated with a concomitant pathological diagnosis of Alzheimer’s disease. All four cases displayed psychiatric symptomatology predominantly consisting of anxiety and depression.

All four brains with heterozygous mutations in the PINK1 gene displayed the typical pathological hallmarks of idiopathic Parkinson’s disease with significant nigral neuronal cell loss, and the presence of LBs in the brainstem and neocortex. Immunohistochemistry of two of these cases revealed an identical distribution and cellular localization of the PINK1 protein as compared with idiopathic Parkinson’s disease brain or normal brain. In addition, the PINK1 protein was detected in a similar proportion of brainstem LBs as in idiopathic Parkinson’s disease cases. Therefore, we conclude that (i) there are no pathological or clinical differences between Parkinson’s disease associated with PINK1 heterozygous mutations or sporadic Parkinson’s disease without any mutations in the PINK1 gene; and (ii) the presence of the described heterozygous mutations in the PINK1 gene does not alter the expression or localization of the protein in vivo.

Heterozygous mutations in genes causing autosomal recessive forms of parkinsonism, that is DJ-1, parkin and PINK1, have been identified in cases of sporadic Parkinson’s disease where their contribution to disease causation remains unclear. In our laboratory, we have identified a significantly higher frequency of heterozygous mutations in the PINK1 gene in sporadic Parkinson’s disease cases compared with controls (P.M. Sleiman et al., personal communication). One important hypothesis is that heterozygous mutations may have a functional effect on the encoded protein as a result of haploinsufficiency. Positron emission tomography studies (Khan et al., 2002) of clinically unaffected relatives of PINK1 cases who carry heterozygous mutations in the PINK1 gene have revealed a reduction of 18F-dopa uptake in their nigrostriatal neurons, indicating a degree of dopaminergic dysfunction. Thus, it appears that the presence of a heterozygous mutation in the PINK1 gene is able to exert a functional effect on the PINK1 protein and subsequently on dopaminergic neuronal function.

In summary, we report the first characterization of the PINK1 protein in normal human brain and in Parkinson’s disease brain. We demonstrate that the PINK1 protein is
expressed in all regions of the brain and in all cell types. In human and rat brain we confirm the location of PINK1 in the mitochondrial fraction, and further localize PINK1 to the outer, and putatively also to the inner, membranes of the mitochondria. In Parkinson’s disease brain we show that PINK1 is detected in a minority of LBs. Finally, we demonstrate that Parkinson’s disease associated with heterozygous mutations in the PINK1 gene presents with a late-onset typical parkinsonian phenotype associated with psychiatric symptomatology. Pathologically these cases are indistinguishable from idiopathic Parkinson’s disease and display no discernible difference in the expression of the PINK1 protein.

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