The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson’s disease

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Summary

Two mutations in the DJ-1 gene on chromosome1p36 have been identified recently to cause early-onset, autosomal recessive Parkinson’s disease. As no information is available regarding the distribution of DJ-1 protein in the human brain, in this study we used a monoclonal antibody for DJ-1 to map its distribution in frontal cortex and substantia nigra, regions invariably involved in Parkinson’s disease. Western blotting of human frontal cortex showed DJ-1 to be an abundant protein in control, idiopathic Parkinson’s disease, cases with clinical and pathological phenotypes of Parkinson’s disease with R98Q polymorphism for DJ-1, and in progressive supranuclear palsy (PSP) brains. We also showed that DJ-1 immunoreactivity (IR) was particularly prominent in astrocytes and astrocytic processes in both control and Parkinson’s disease frontal cortex, whereas neurons showed light or no DJ-1 IR. Only occasional Lewy bodies (LBs), the pathological hallmarks of Parkinson’s disease, showed faint DJ-1 IR, localized to the outer halo. In preclinical studies we showed that DJ-1 is expressed in primary hippocampal and astrocyte cultures of mouse brain. By 2D gel analysis we also showed multiple pI isoforms for DJ-1 ranging between 5.5–6.6 in both control and Parkinson’s disease brains, whilst exposure of M17 cells to the oxidizing agent paraquat was manifested as a shift in pI of endogenous DJ-1 towards more acidic isoforms. We conclude that DJ-1 is not an essential component of LBs and Lewy neurites, is expressed mainly by astrocytes in human brain tissue and is sensitive to oxidative stress conditions. These results are consistent with the hypothesis that neuronal–glial interactions are important in the pathophysiology of Parkinson’s disease.

Keywords: DJ-1; Parkinson’s disease; immunohistochemistry; 2D gel electrophoresis; paraquat

Abbreviations: DA = dopaminergic; 2DGE = two-dimensional gel electrophoresis; GFAP = glial fibrillary acidic protein; IR = immunoreactivity, immunoreactive; LB = Lewy body; LN =Lewy neurite; PSP = progressive supranuclear palsy; SN = substantia nigra

Introduction
Parkinson’s disease is an incurable, inexorably progressive neurodegenerative disorder affecting around 2% of the population over the age of 65 (de Rijk et al., 1997). The cardinal presenting clinical features comprise bradykinesia, rigidity and resting tremor, with a therapeutic response to L-dopa (Yahr et al., 1969). Selective and severe loss of dopaminergic (DA) neurons projecting from the substantia nigra (SN) to the striatum is responsible for the major motor handicaps. The pathological hallmark is the accumulation of eosinophilic, proteinaceous, intracytoplasmic inclusions known as Lewy bodies (LBs) in the substantia nigra, locus coeruleus, dorsal nucleus of the vagus, parahippocampal gyrus and other brainstem and cortical regions (Forno, 1996).

Currently, three Parkinson’s disease genes, two candidate genes and a further six distinct loci responsible for rare Mendelian forms of Parkinson’s disease have been identified (Dawson and Dawson, 2003; Hardy et al., 2003). However, the molecular mechanisms leading to neurodegeneration in Parkinson’s disease remain poorly understood.

Two point mutations (A30P, A53T) in the α-synuclein (PARK1) gene cause autosomal dominant, early-onset Parkinson’s disease in some families (Polymeropoulos et al., 1997), and α-synuclein is a major component of LBs (Spillantini et al., 1997), thus implicating its involvement in the aetiopathology of sporadic Parkinson’s disease. The production of α-synuclein in transgenic mice and transgenic fruit flies may induce disturbances of motor behaviour (Maslia et al., 2000) and LB-like inclusions (Feany and Bender, 2000). A variety of mutations (homozygous deletions, multiplications of exons, point-mutations and insertions) in the parkin gene (PARK2) cause autosomal recessive juvenile Parkinson’s disease (Kitada et al., 1998). Parkin functions as an E3 ubiquitin protein ligase (Shimura et al., 2000) and its levels are low or absent in patients with autosomal recessive juvenile Parkinson’s disease (Shimura et al., 1999). Neuropathological examination shows that neuronal death occurs preferentially in the SN and locus coeruleus and LBs are absent in the brains of most parkin-mutated cases (Mori et al., 1998). Recently parkin has been reported to be a component of LBs and Lewy neurites (LN)s (Schlossmacher et al., 2002), suggesting its possible involvement in the pathological processes mediating idiopathic Parkinson’s disease. A point mutation in the ubiquitin carboxy terminal hydrolase L1 gene (UCH-L1) (Leroy et al., 1998) has been reported to cause autosomal dominant Parkinson’s disease in two affected siblings in a German family. Brain pathology is not yet available from these patients but UCH-L1 has been shown to be a component of LBs (Lowe et al., 1990).

Recently, two homozygous mutations in the DJ-1 gene (PARK7) have been shown to cause early-onset autosomal recessive Parkinson’s disease in families in the Netherlands and Italy (Bonifati et al., 2003). In the Dutch family isolate there was a deletion in exons 1–5 that included the promoter start site of the DJ-1 gene. In the Italian family, a Leu-Pro substitution at position 166 segregated with Parkinson’s disease. It is thought that the Dutch patients are unlikely to produce any DJ-1, whereas the point mutation in the Italian family could lead to an impairment of its normal function (Bonifati et al., 2003). The neuropathological changes occurring in individuals affected by these mutations are not known. Other DJ-1 mutations, with homozygous, compound heterozygous and heterozygous genotypes, may confer disease susceptibility in young-onset Parkinson’s disease, as shown by further recent genetic studies (Abou-Sleiman et al., 2003; Hague et al., 2003). Some of these mutations may be population-specific (Abou-Sleiman et al., 2003).

DJ-1 is an 189 amino acid protein with multiple functions. It was first identified and cloned as a c-myc protein interactor by yeast-two hybrid screening from a HeLa cDNA library (Nagakubo et al., 1997). In association with ras, it transformed NIH3T3 cells, suggesting it has oncogenic potential and may be involved in ras-mediated signalling pathways (Nagakubo et al., 1997). The rat DJ-1 homologue, CAP1/ sP22, was cloned from rat sperm and is important in fertilization (Wagenfeld et al., 1998). An RNA-binding regulatory subunit, RS, purified from rat hepatoma cells (Hod et al., 1999), is almost identical to DJ-1 and has the capacity to bind and inhibit RNA-binding activity. The structure of DJ-1 has some homology to the bacterial proteins Thj and Pfp1, which are involved in thiamine synthesis and protease activity respectively (Lee et al., 2003).

DJ-1 may be involved in the regulation of transcription (Takahashi et al., 2001). By binding with protein inhibitor of activated STAT (signal transducers and activators of transcription) (PIAS), a family of (SUMO-1) ligases, DJ-1 can positively regulate transcription of androgen-responsive genes (Takahashi et al., 2001). It can be post-translationally modified by SUMO, a small ubiquitin-like modifier at amino acid position 130 (Takahashi et al., 2001). One DJ-1 molecule interacts with another as seen in yeast-two hybrid system, and L166P mutation down-regulates this interaction (Miller et al., 2003). DJ-1 appears to be sensitive to oxidative stress conditions and undergoes an alteration in its pI (from 6.2 to 5.8) when cultured endothelial cells are treated with paraquat (Mitsumoto et al., 2001). The crystal structure of DJ-1 has been reported, and it suggests that DJ-1 proteins may only function as dimers and that the L166P mutation may disrupt the dimerization of the protein (Honbou et al., 2003; Tao and Tong, 2003).

DJ-1 is expressed in many tissues, including the brain (Nagakubo et al., 1997). DJ-1 mRNA expression is greater in subcortical regions (Bonifati et al., 2003), raising the possibility that it may be important in basal ganglia function. DJ-1 is also expressed by a number of cell lines, is associated with microtubules and localizes to both the nucleus and the cytoplasm (Hod et al., 1999).
The function and detailed distribution of DJ-1 in the brain is unknown. It is also not known whether it is associated with LBs and LNs. The aim of this study was to delineate the distribution of endogenous DJ-1 protein in human brain and cultured cells. We also examined the distribution of the protein in normal and pathological states and examined the pI shift of this protein in response to oxidative stress and in Parkinson’s disease.

**Material and methods**

**Cases**

Brain tissue was obtained from the Queen Square Brain Bank for Neurological Disorders. Human tissue was collected with the informed consent of next of kin and with the permission of the National Hospital for Neurology and Neurosurgery and the Institute of Neurology Joint Research Ethics Committee. All brains were neuropathologically evaluated to confirm the clinical diagnosis and to exclude undiagnosed pathology in the neurologically normal controls. Details of all cases are outlined in Table 1. Three of our cases harbour a heterozygous G to A substitution at position 98 in exon 5, and had the classical clinical and pathological phenotype of Parkinson’s disease. This polymorphism results in the substitution of an alanine for a glycine residue (Abou-Sleiman et al., 2003).

**Western bloting**

Human brain tissue from the frontal cortex of control, Parkinson’s disease, Parkinson’s disease with DJ-1 heterozygous mutations and progressive supranuclear palsy (PSP) were homogenized in isotonic sucrose (10% homogenates) buffered with HEPES (Sigma, Poole, UK) and spun at 12 000 g for 10 min to remove cellular debris. Protein from the supernatants was measured by the bicinchoninic acid method (Biorad, Hemel Hempstead, UK) using BSA as standard. Ten micrograms of protein from each supernatant was

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**Table 1 Clinical and post-mortem details of cases included in the study**

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age (years)</th>
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<th>Cause of death</th>
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<tr>
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<td>F</td>
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</tr>
<tr>
<td>C5</td>
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<td>92</td>
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<td>MI, IHD</td>
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<td>F</td>
<td>78</td>
<td>50</td>
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</table>

*From death to fixation of brain. Cause of death was as stated on death certificate or hospital post-mortem report. COAD = chronic obstructive airway disease; MI = myocardial infarction; IHD = ischaemic heart disease, PSP = progressive supranuclear palsy.
solubilized in NuPAGE (Invitrogen, Paisley, UK) sample buffer and loaded onto 10% Bis-Tris gels and run with the MES (morpholinopropane sulfonic acid) buffer system (Invitrogen). Protein bands were subsequently electroblotted onto Hybond P (Pharmacia Biotech, UK) membrane. Duplicate membranes were blocked with 5% milk (Marvel) in phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20 (PBS-T). Western blot analysis was carried out using DJ-1 antibody (clone 3E8; Stressgen, San Diego, CA, USA) at 1:5000 dilution or β-tubulin (clone SAP.4G5; Sigma) also diluted 1:5000, followed by incubation with horseshadish peroxidase-conjugated mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:10,000 dilution. Three 5 min washes with PBS-T were performed between each antibody treatment. The blots were developed by the enhanced chemiluminescence (ECL) method using standard procedures and captured onto Kodak Biomax (Amersham, UK) autoradiography film.

Production of recombinant DJ-1 and antibody preabsorption

DJ-1 cDNA clone was purchased from HGMP (Cambridge, UK; clone No-1309796, GB.Acc – AA770265) and the plasmid DNA was used as the template for the polymerase chain reaction (PCR). The full-length DJ-1 cDNA was constructed using the forward primer 5’-AAAAGAGCCTGTCATCCTG-3’ and the reverse primer 5’-GTCTTTAAGAACAAGTGGACG-3’. The PCR reaction was run on a 60–50°C touchdown programme followed by 20 cycles at 95°C for 30 s, 50°C for 30 s and 72°C for 30 s. The correct sequence was verified by sequencing. The PCR product was subcloned into pTrcHis2-topo-cloning/expression vector (Invitrogen) and transformed into one-shot E.coli competent cells (Invitrogen). DJ-1 protein was expressed by induction with IPTG (an inducer of β-galactosidase activity in bacteria, Promega, Southampton, UK) (1 mM). The DJ-1 protein was purified using His-select HC nickel affinity gel (Sigma) according to the manufacturer’s instructions. The purified fractions were run on SDS–PAGE for analysis, and dialysed against PBS containing 0.1 M PMSF. For preabsorption studies, DJ-1 antibody (1:3000, 1:3000) was incubated with 50 μg of recombinant DJ-1 fusion protein at 37°C for 1 h. The sample was further incubated sequentially at 4°C overnight. The solution was centrifuged for 10 min at 13,000 r.p.m. and the supernatant was used for blocking specific band on immunoblots and tissue sections.

Immunohistochemistry and immunofluorescence on brain sections

Formalin-fixed sections (6 μm) were first dewaxed in xylene. Endogenous peroxidase was blocked with 0.6% hydrogen peroxide in methanol for 10 min. Following rehydration, antigens were retrieved by autoclaving in citrate buffer (10 mM, pH 6.0) for 10 min. Sections were blocked with 10% normal goat serum (Sigma) before treating with monoclonal antibody to DJ-1 (Stressgen) and glial fibrillary acidic protein (GFAP; rabbit polyclonal; Dako, UK) at 1:3000 dilution overnight at 4°C. Biotinylated secondary antibody to horseradish peroxidase-activated DAB (diaminobenzidine) was used. Three 5 min washes in PBS were carried out between each step. Tissue sections were lightly counterstained with haematoxylin, dehydrated through graded alcohols, cleared with xylene and mounted in mounting medium (Merck, UK). For specificity of staining, some sections were stained in the absence of primary antibody or with antibody that had been preabsorbed with recombinant DJ-1.

For double immunolabelling of DJ-1 and GFAP in astrocytes, antigens were retrieved by microwaving for 20 min in citrate buffer (10 mM, pH 6.0). DJ-1 and GFAP antibodies were used as above. The DJ-1 signal was visualized using the tetramethyl rhodamine and GFAP with the fluorescein signal amplification kit (Perkin Elmer, UK). Sections were washed thoroughly in PBS and mounted in Aquamount (Merck) and scanned using a Leica TCS40 laser confocal microscope.

Immuno-electron microscopy

Small blocks of substantia nigra were obtained from fresh tissue and fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.15 M Sorenson’s phosphate buffer (pH 7.4) for 3 h. After fixation, the tissue was washed and stored in 0.15 M phosphate buffer (pH 7.4) overnight at 4°C, followed by dehydration in graded ethanol and embedded in London resin white (Agar Scientific, Stansted, UK). Ultrathin sections mounted on nickel grids were floated on a droplet of ammonium chloride (0.5 M) for 1 h, followed by incubating buffer containing 1.0% BSA, 0.1% normal goat serum, 0.1% sodium azide and 0.1% Tween 20 in PBS (pH 8.2) for 30 min. The sections were incubated in anti-DJ-1 (1 μg/ml, Stressgen) overnight at 4°C, washed and treated with goat anti-mouse gold conjugate (20 nm, BB International, Cardiff, UK) for 4 h at room temperature. After incubation with secondary antibody, the sections were washed in series of droplets of distilled water, stained with 0.5% uranyl acetate and examined in a Philips CM10 electron microscope operating at 80 kV. Control sections were prepared by omitting the primary antibody.

Primary cell culture and staining

Primary neurons and astrocytes from postnatal day 2 mouse pups were dissociated as described previously (Petrucelli et al., 2002). Gial cells were cultured in feeder layers alone for 2–4 weeks and stained for GFAP using a monoclonal antibody G-A-5 (Sigma). These glial-enriched cultures were >95% GFAP-positive. Neuron-enriched cultures were prepared by dissecting and dissociating hippocampi, also from P2 mouse pups, which were plated in serum-free medium for 3 days prior to staining. Neurons were identified by staining with monoclonal antibody to microtubule-associated protein 2 (MAP2) (clone AP-20; Sigma) and represented approximately 75% of the cells in these cultures, the rest being contaminating glial cells. For DJ-1 staining, cells were preincubated with 500 nM Mitotracker CMTMRos (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C prior to staining. Cells were fixed in 4% paraformaldehyde in Dulbecco’s PS (DPBS) for 30 min at room temperature, permeabilized with 0.1% Triton X-100 and quenched with 0.1 M glycine. After washing in DPBS, non-specific immunoreactivity was blocked with DPBS containing 10% foetal bovine serum and 0.1% Triton X-100 and incubated with sheep polyclonal anti-DJ-1 (gift of Gary Klinefelter; 1:1000) overnight at 4°C. This antibody was used in preference to the monoclonal antibody used for human tissue, which did not recognize DJ-1 in mouse brain extracts (data not shown). Cells were incubated with
AlexaFluor 488-conjugated donkey anti-sheep IgG conjugated prior to mounting under ProLong Antifade medium (Molecular Probes, Eugene, OR, USA). Slides were examined using a Zeiss LSM510 confocal microscope using independent excitation for both channels. Omission of primary antibody was used to evaluate non-specific fluorescence and in all cases gave no signal.

2D gel electrophoresis (2DGE)

M17 human dopaminergic neuroblastoma cells were grown in Optimem (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and either left untreated or exposed to 100 μM paraquat (Sigma) for 20 h. Cytosolic protein extracts were made as described previously (Allen et al., 2003). For 2DGE, 500 μg of protein was separated first on 13 cm immobilized pH gradient (IPG; Amersham Biosciences) strips using 3.0–10.0 linear gradients according to the manufacturer’s instructions on the IPGPhor system (Amersham Biosciences). The second dimension was resolved on 10–20% SDS–PAGE gels (Jule, Milford, CT, USA). Gels were blotted to Immobilon polyvinylidene difluoride (PVDF) (Amersham Biosciences) membranes and probed with monoclonal antibody to DJ-1 (Stressgen; 1:1000). Blots were developed using peroxidase-labelled secondary antibodies (Jackson Immunochemicals; 1:5000) and ECL-plus (Amersham Biosciences). pI was calibrated using creatine phosphokinase carbamylated standards (Amersham Biosciences) and molecular weight using Precision prestained markers (Biorad). A similar protocol was used for 2DGE for human extracts as above with some modifications. Briefly, for the first dimension, human brain homogenates (10 μg) from control and Parkinson’s disease subjects, including those with heterozygous changes in the DJ-1 gene, were applied to IPG strips and separated as above. Proteins were blotted onto nitrocellulose membranes (Hybond ECL; Amersham Biosciences) which were probed for DJ-1 as detailed in the western blotting section. Protein spots were visualized with an ECL kit (Amersham Biosciences) according to the manufacturer’s instructions.

Results

Western blotting

Immunoblotting of homogenized tissue from frontal cortex of control, idiopathic Parkinson’s disease and PSP cases with

![Fig. 1](A) Immunoblot analysis of DJ-1 in human brain. Lysates of brain tissue of control (C), Parkinson’s disease and PSP cases were analysed by immunoblot assay with DJ-1 antibody (upper panel). Each lane contained 10 μg of protein. Equivalent loadings were confirmed in replicate blots probed with β-tubulin (lower panel). Markers on the right of the blot indicate molecular weights in kilodaltons. (B) Preabsorption of DJ-1 antibody with recombinant DJ-1. The specific signal for DJ-1 (lane 1) shown is blocked by an excess of recombinant DJ1 (lane 2).

![Fig. 2](Immunohistochemistry for DJ-1 in control, and Parkinson’s disease tissue. Examples of DJ-1 IR in astrocytes (arrows) in the grey matter from control cortex (A) and the white matter in a Parkinson’s disease case (B). This staining was specific, as a preabsorbed DJ-1 antibody with recombinant DJ-1 shows no positive astrocytic labelling in the white matter from a case of idiopathic Parkinson’s disease (C). A high-power picture of astrocytes shows both cytoplasmic and nuclear staining for DJ-1 (D). Astrocyte morphology was demonstrated with GFAP staining in white matter of a case of idiopathic Parkinson’s disease (E). In some non-DA neurons from the nigra of a Parkinson’s disease case there was cytoplasmic and axonal staining for DJ-1 (F). Scale bars = 20 μm for A, B and C, and 10 μm for D, E and F.)
DJ-1 antibody demonstrated a single band at ~20 kDa (Fig. 1A), which was not detected when the antibody was preabsorbed with excess (50 μg) recombinant DJ-1 (Fig. 1B). Omission of primary antibody also did not give any band for DJ-1 (data not shown). There was no obvious difference in expression levels of DJ-1 in controls, Parkinson’s disease or PSP cases. Our Parkinson’s disease cases with DJ-1 R98Q polymorphism also gave a similar intensity band (data not shown).

**DJ-1 IR in control brain tissue**

**Frontal cortex**

Strong DJ-1 IR was observed in a proportion of glial cells with morphological attributes of astrocytes and DJ-1 IR glia were present in both grey (Fig. 2A) and white matter. In the grey matter, staining for DJ-1 was diffuse throughout the brain parenchyma with some individual glial profiles that were most common in the deep layers, along the white matter border. They were also seen at the cortical surface. DJ-1 IR in glia was present throughout the cell, in cytosol and glial processes. The morphology and distribution of DJ-1 IR cells was similar to that of GFAP IR cells, supporting the view that most DJ-1 IR cells were astrocytes (Fig. 2E), but the dense network of fibres revealed by GFAP staining was not seen with DJ-1 immunohistochemistry (Fig. 2A, B). Glial nuclear staining was seen in both grey and white matter (Fig. 2). There were more astrocytes positive for DJ-1 in the white matter than in the grey matter. Occasional cortical neurons of the deep layers were weakly DJ-1 immunoreactive. Specificity of glial staining by DJ-1 was demonstrated by incubation following pre-absorption of the antibody with recombinant DJ-1 (Fig. 2C) or omission of primary antibody when no staining was seen. Colocalization of DJ-1 and GFAP in astrocytes was also evident by double-labelling immunofluorescence.

**Midbrain**

Immunopositive cells with astrocyte morphology were observed in the neuropil surrounding DA nigral neurons. Very few were seen among the nigral cell groups. In some cases there was diffuse staining for DJ-1 throughout the nigral neuropil, with no distinct glial morphology; however, in these cases we observed a fair proportion of positively stained glial nuclei. Neuramelin-containing neurons of the substantia nigra were negative for DJ-1 IR.

**DJ-1 IR in Parkinson’s disease**

**Frontal cortex**

The pattern of staining for DJ-1 IR in Parkinson’s disease frontal cortex was similar to that observed in control brain tissue. DJ-1 IR was present in glial cells in both white matter and grey matter, with the same distribution pattern as described above (Fig. 2B, D). Glial cell processes were positive for DJ-1 and staining was also seen in their nuclei and cytoplasm. DJ-1 and GFAP were found to colocalize in a number of astrocytes, as seen by double- immunofluorescence labelling (Fig. 3A–C). The majority of neuronal perikarya remained negative for DJ-1 IR but occasional neurons in the deep layers were DJ-1-positive. We did not observe any DJ-1-positive cortical LBs or LNIs. A similar pattern of staining was seen in brains of Parkinson’s disease subjects with DJ-1 R98Q polymorphisms.
Midbrain

In Parkinson’s disease nigra, in addition to glial staining, DJ-1 IR was observed only in occasional LBs and a single LN. However the majority of LBs and pale bodies were negative. In the few LBs that were stained, DJ-1 was mainly localized to the periphery (Fig. 4B). The cytoplasm of DA neurons in Parkinson’s disease brains was lightly labelled and staining was also seen in neurons of the third nerve nucleus. Some non-melanized neurons and their processes were also lightly labelled (Fig. 2F). In immunoelectron microscope studies, some scattered immunogold labelling of DJ-1 was observed within a small number of nigral LBs. In some, immunogold...
particles were found to be concentrated around circular structures (Fig. 4A) In addition, some conjugated gold particles were observed on structures resembling mitochondria around the periphery of the LB (Fig. 4A). No immunogold labelling of the fibrillar material per se was detected. No labelling was seen when the primary antibody was omitted (data not shown).

**DJ-1 expression in mouse primary astrocytes and hippocampal neurons**

Given that DJ-1 expression was found in glial cells in vivo, we also cultured cortical astrocytes. DJ-1 was also expressed endogenously in both mouse hippocampal neurons and astrocytes (Fig. 5). Previous results (Bonifati et al., 2003) have shown a possible colocalization of DJ-1, especially the L166P mutant, with mitochondria. However, in both these cultured cell types, endogenous DJ-1 signal (green) did not appear to colocalize with the Mitotracker probe (red) (Fig. 5 B, D).

**Multiple isoforms of DJ-1 in oxidative stress models and human brain**

Previous results in non-neuronal cell lines have indicated that exposure to oxidative stress produces a shift in pI of DJ-1, but no results in neural tissue have been reported to date. We first replicated previous results showing a paraquat-induced pI shift for DJ-1 in the human dopaminergic neuroblastoma cell line M17. These cells express readily detectable endogenous amounts of DJ-1 with a range of pI isoforms from approximately 6.0 to 6.2 (Fig. 6A). Exposure to 100 μM paraquat resulted in the accumulation of acidic isoforms with a pI of 5.8 (Fig. 6B). We next performed 2DGE of human frontal cortex extracts and found at least six different pI isoforms of DJ-1. In control brain, they ranged from 5.5 to 6.6, the 6.4 pI isoform being the most prominent. Compared with controls (Fig. 6C), in Parkinson’s disease samples the 6.6 pI isoform appears to be missing or diminished (Fig. 6D). Additionally, a marked difference in the distribution of the more acidic DJ-1 isoforms was seen in the Parkinson’s disease subjects with DJ-1 R98Q polymorphism compared with both control subjects and other Parkinson’s disease cases (Fig. 6E).

**Discussion**

This is the first report on the distribution of DJ-1 protein in the human brain. We have used an antibody for DJ-1 that recognizes a single band corresponding to DJ-1 from human frontal cortex homogenates by western blotting, but not rodent DJ-1 (data not shown). DJ-1 is expressed in high amounts in the frontal cortex, a region of predilection for LB deposition, in control, PSP and Parkinson’s disease cases (Fig. 1A). Using this highly specific antibody, we have shown that the major cell type expressing DJ-1 IR in human brain is glial rather than neuronal (Fig. 2A, B, D) We have corroborated the findings on DJ-1 immunohistochemistry by staining with GFAP (Fig. 2E), which produces a similar staining pattern with respect to the morphology and distribution of glial cells. Further confirmation of the localization of DJ-1 in astrocytes has been shown by double-labelling immunofluorescence and confocal microscopy (Fig. 3A–C). The localization of DJ-1 IR in both the cytoplasm and nucleus of glial cells is in keeping with the known distribution of this protein in various cell lines (Nagakubo et al., 1997). In addition, we show that some neurons are weakly DJ-1 immunopositive (Fig. 2F). However, the relative concentrations of DJ-1 protein in neurons may be much lower compared with astrocytes. Staining was specific, as recombinant DJ-1 protein was able to abolish immunoreactivity in both blotting and staining techniques.

The presence of significant amounts of DJ-1 in glial cells in the brain is of interest as the two other Parkinson’s disease genes, α-synuclein and parkin, are predominantly neuronal. However, it has been recently shown that parkin expression in glial cells is up-regulated during unfolded protein stress (Ledesma et al., 2002). We have shown that DJ-1 is expressed by mouse primary astrocytes and neurons (Fig. 5). A difference between in vivo and in vitro results is that cultured mouse hippocampal neurons did express detectable DJ-1, suggesting either that there are species differences or that the tissue culture environment promotes the expression of this stress-responsive protein. As DJ-1 is responsive to oxidative stress (see below), it is possible that DJ-1 is up-regulated in the culture environment due to exposure to free radicals.

The brain regions chosen for this study are known to be vulnerable to pathological damage in Parkinson’s disease, including LB and LN accumulation. In both the typical Parkinson’s disease cases and in the cases of Parkinson’s disease with DJ-1 R98Q polymorphism examined here, DJ-1 IR was localized to only a few nigral LBs, indicating that DJ-1 protein is not an essential component of LBs and is unlikely to be important in their formation in Parkinson’s disease. This result is in contrast to the localization of α-synuclein and parkin, which are present in most LBs and LNs (Spillantini et al., 1998; Schlossmacher et al., 2002).

Our 2D gel analysis of human brain extracts and cells exposed to oxidative stress, shows the existence of a range of pI isoforms for DJ-1 protein. This may be specific to brain tissue as only two pI isoforms for DJ-1 have been reported in human endothelial cell cultures and in mouse lung tissue (Mitsumoto and Nakagawa, 2001). In addition, we show that DJ-1 protein responds to the oxidative stressor paraquat by exhibiting more acidic pI isoforms in a neuroblastoma cell line (Fig. 6A), in agreement with a previous study showing DJ-1 sensitivity to paraquat (Mitsumoto et al., 2001). Oxidative stress factors are believed to be implicated in the pathogenesis of Parkinson’s disease (Jenner, 2003) and it has been suggested that the shift in pI of DJ-1 is a useful indicator of oxidative stress status both in vivo and in vitro (Mitsumoto and Nakagawa, 2001). Thus the presence of multiple isoforms...
of DJ-1 in control and Parkinson’s disease brains and the tendency of the most alkaline pI isofrom to be absent from Parkinson’s disease cases (Fig. 6D,E) could point to the involvement of oxidative stress in (Abbas et al., 1999) Parkinson’s disease. Whether these factors are the cause of the differences in the DJ-1 pI isofrom distribution in the brain between cases of Parkinson’s disease and cases with DJ-1 R98Q polymorphism remains to be determined, but it is clear that there is greater complexity in DJ-1 expression in the human brain compared with other systems. Our Parkinson’s disease cases heterozygous for DJ-1 mutation showed a similar expression pattern for DJ-1 protein compared with idiopathic Parkinson’s disease cases and phenotypically they were similar to late-onset Parkinson’s disease cases. It is possible that in these individuals, the DJ-1 R98Q polymorphism is non-pathogenic or that the DJ-1 variant together with other yet unknown variants are responsible for the disease phenotype (Abou-Sleiman et al., 2003). In this respect, the DJ-1 R98Q polymorphism may be similar to parkin mutation, for which a single heterozygous mutation may confer disease susceptibility (Abbas et al., 1999) indistinguishable from idiopathic Parkinson’s disease. The effect of the G to A heterozygous DJ-1 mutation on the biological function of the protein, however, remains to be studied.

There is increasing interest in the possibility that glial cells may be major contributors to oxidative stress in Parkinson’s disease (Czlonkowska et al., 2002; Teismann et al., 2003). The post-mortem Parkinson’s disease brain exhibits some increase in astrogia, as seen by GFAP staining (Forno et al., 1992; Mirza et al., 2000). Furthermore, there is an inverse correlation between numbers of GFAP-positive astrocytes and DA cell loss, and areas with a sparse astrocytic response show greater cell loss (Damier et al., 1993). In addition, histological examination of familial Parkinson’s disease cases with parkin mutations has also shown gliosis in the nigra (Ishikawa and Takahashi, 1998; Hayashi et al., 2000; Hishikawa et al., 2001). Astrocytes, however, are known for their protective effects on neurons through their capacity to secrete glial cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), and may be protective (Czlonkowska et al., 2002). Addition of astrocytes to neuronal cultures prevents cell death caused by a number of neurotoxic compounds (Hou et al., 1997; Tieu et al., 2001; Mena et al., 2002). Furthermore, astrocytes are rich in glutathione peroxidase, which catalyses the removal of hydrogen peroxide by formation of oxidized glutathione and prevents the formation of hydroxyl radicals by a reaction between hydrogen peroxide and heavy metals. The levels of this important enzyme have been demonstrated to be reduced in homogenates of substantia nigra in Parkinson’s disease (Sotic et al., 1992; Sian et al., 1994; Pearce et al., 1997). It is possible that some of this reduction in glutathione peroxidase takes place in nigral glia. Any change in the normal physiological role of astrocytes may therefore contribute to DA cell death by reduced secretion of neurotrophic factors and a reduced capacity of glia to cope with free radical production. The abundance of DJ-1 in astroglia suggests a prominent role for this protein in glial biology and in some neurodegenerative processes, and may also indicate that astrocytes are subjected to oxidative stress in Parkinson’s disease.

Present concepts of disease pathogenesis suggest a unifying pathway involving α-synuclein, parkin and UCH-L1, the three genes associated with familial forms of Parkinson’s disease, within the ubiquitin–proteasome system (UPS) (Cookson, 2003; Hardy et al., 2003). In this context, reduced proteasome function in Parkinson’s disease cases, indicating a defect in the UPS, has been reported (McNaught and Jenner, 2001). How this relates to oxidative stress is unclear, but there is some evidence that the proteasome has a major role in the degradation of oxidatively damaged proteins (Grune et al., 2003; Shringarpure et al., 2003). Furthermore, oxidative stress can inhibit proteasome function (Ding and Keller, 2001) and formation of intracellular protein aggregates is dependent on oxidative events (Demasi and Davies, 2003). Therefore, the pathways involving proteasome function and oxidative stress may intersect, as has been highlighted by others (Chung et al., 2001; Jenner, 2003). It is not yet clear whether DJ-1 will be part of this pathway or whether it will involve new pathways contributing to a common end-point (Cookson, 2003). Interestingly, mutant DJ-1, but not the wild-type protein, is degraded by the UPS in cell culture (Miller et al., 2003). Further work on the cell biology of DJ-1 is needed to establish its precise role in the loss of DA neurons. However, our study provides further data to suggest that glia may be important in the pathogenesis of Parkinson’s disease and that interactions between neuronal and glial function should be investigated in greater depth.

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