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REPORT

DJ-1 linked parkinsonism (PARK7) is associated with Lewy body pathology

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Mutations in DJ-1 (encoded by *PARK7*) are a very rare cause of early-onset recessive Parkinson's disease. We describe a patient with early-onset parkinsonism, starting at the age of 22, with poor response to levodopa and additional features in progression (dystonia, pyramidal signs and dementia), who died when he was 49 years old. The neuropathological study showed severe substantia nigra and locus coeruleus neuronal loss, with diffuse Lewy body pathology (Lewy bodies, aberrant neurites, grain-like structures, spheroids and scattered glial pathology). Genetic analysis revealed a novel c.515T > A; p.L172Q mutation in the *PARK7* gene. To evaluate the pathogenicity of this new mutation we explored DJ-1 expression levels *in vitro* showing a massive reduction in DJ-1 protein levels due to a highly unstable and rapidly degraded L172Q mutant. DJ-1 immunohistochemistry of brain tissue revealed no staining in our case. This is the first neuropathological report of a brain from DJ-1-linked parkinsonism that, although based on a single case study, suggests that DJ-1 mutations are causative of α-synucleinopathy. These results can help in the understanding of Parkinson's disease pathophysiology, promote research studies to increase the knowledge on the pathways involved in the neurodegeneration process, and pave the way for new therapeutic interventions.

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Introduction

Parkinson's disease is the most common movement disorder and the second most common neurodegenerative disease after Alzheimer's disease, with a prevalence of 1–2% in the population over 65 years (de Rijk *et al.*, 2000). Early-onset Parkinson's disease, defined by age of onset between 20 and 40 years of age (Quinn *et al.*, 1987), accounts for 4–10% of all patients with Parkinson's disease. The pathological hallmarks are the selective and severe loss of dopaminergic neurons together with the accumulation of Lewy bodies (Forno, 1996). In the past decades important developments on the pathophysiology of Parkinson's disease were made, largely driven by genetics. The discovery of mutations in α-synuclein (PARK1, encoded by *SNCA*), responsible for autosomal dominant early-onset Parkinson's disease (Polymeropoulos *et al.*, 1997), and the establishment of α-synuclein as the major component of Lewy

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bodies (Spillantini *et al.*, 1997) was a cornerstone achievement. The identification of familial forms contributes to the understanding of the underlying pathways resulting in the Parkinson's disease phenotype. Mutations in DJ-1 (*PARK7*) are a very rare cause of early onset recessive Parkinson's disease (Bonifati *et al.*, 2003). Only a few patients with homozygous mutations in *PARK7* have been described. Until now no neuropathological data have been reported.

Materials and methods

Extended material and methods are available in Supplementary material.

Clinical characteristics

The patient was followed longitudinally at Centro Hospitalar do Porto (CHP). Written consent was obtained and the study was approved by the Ethics Committee of CHP.

Neuropathological analysis

Histological studies with haematoxylin and eosin, Klüver-Barrera, and immunohistochemistry [α-synuclein, ubiquitin, SQSTM1/p62, tau (AT8), TDP-43, GFAP, amyloid-β and DJ-1] were performed in 6 µm paraffin-embedded formalin-fixed sections from selected areas of the brain.

Molecular studies

A gene panel comprising *ATP13A2*, *DNAJC6*, *FBXO7*, *PARK2*, *PARK7*, *PINK1*, *PLA2G6* and *SYNJ1* was used to establish the molecular diagnosis through next generation sequencing (NGS). All variants were confirmed by Sanger sequencing. For mutation characterization, HEK293T cells were grown under standard conditions and transfected with either pcDNA3.1/GS, wild-type, L166P or L172Q DJ-1. Proteasome activity was inhibited with MG132. Transfected DJ-1 levels were analysed by western blot and real-time polymerase chain reaction (PCR) was used to assess mRNA levels.

Statistical analysis

All quantitative data are expressed as mean ± standard deviation (SD) of three independent experiments. Comparison of expression levels between wild-type and mutant protein or mRNA was carried out using ANOVA) with Tukey *post hoc* test.

Results

Case report

This patient was a 49-year-old male, born from consanguineous parents and history of mental retardation in the maternal family and early child deaths in siblings, who developed rest and postural left hand irregular tremor at the age of 22. Walking difficulties appeared early in the disease, with progressive postural instability and recurrent falls. Two years later levodopa treatment was started with poor response. At the age of 27 he developed a slowly progressive hypophonia and dysphagia and stopped working as a teacher 2 years later due to speech problems. Levodopa-related involuntary movements were described from the age of 30, starting in the left lower limb evolving to a generalized dystonia with facial predominance. He reported sexual impotence from the age of 30 and episodes of excessive sleepiness, hypothermia and sweating lasting for several days.

He was first seen in our centre at the age of 35. Neurological examination showed a hypo-mimic face, hypophonic voice, symmetrical parkinsonism with important postural instability and walking difficulties due to abnormal lower limb postures. There was rest and posture irregular hand tremor and craniocervical dystonia. The myotatic reflexes were globally reduced, with exception of a brisk patellar reflex. Neuropsychological assessment revealed mild difficulties in multiple cognitive domains, despite scoring 30/30 on the Mini-Mental State Examination. Extensive diagnostic work-up, including MRI, laboratory analysis and genetic testing (*PARK2*, *PANK2*, *FRAXA* and mitochondrial disorders) was performed and was normal/negative.

There was a gradual deterioration of his condition; at the age of 39 a new neuropsychological testing showed significant overall cognitive decline. Muscle wasting of upper and lower limbs became evident, bilateral Babinski's sign appeared, there were tonic-clonic seizures, and he became wheelchair bound. A second MRI performed at 43 years of age showed mild frontal atrophy (Fig. 1). Due to severe dysphagia he needed feeding gastrostomy by the age of 48 and died 1 year later without definitive diagnosis. (Supplementary Video 1)

Neuropathological study

The whole brain weighed 1491 g. Sequential coronal sections of the cerebral hemispheres revealed slight enlargement of the frontal horns of the lateral ventricles. Horizontal sections of the brainstem showed severe pigment loss of substantia nigra pars compacta (SNpc) (Fig. 2A) and locus coeruleus.

Histological examination revealed severe neuronal loss of SNpc accompanied by gliosis and extracellular pigment. Locus coeruleus was also severely depleted. Classical Lewy bodies were readily visible (Fig. 2B and C). The globus pallidus showed moderate neuronal loss and the putamen was less affected. Cortical Lewy bodies were also identified (Fig. 2D). Immunohistochemistry study for α-synuclein showed diffuse Lewy body, Lewy neurites and grains, some spheroids and glial inclusions (Fig. 2E–M) (Supplementary Table 1). There were moderate numbers of Lewy body and Lewy neurites in limbic and temporal cortices, particularly in deep cortical layers. Frontal and parietal cortices were mildly affected. In the hippocampal

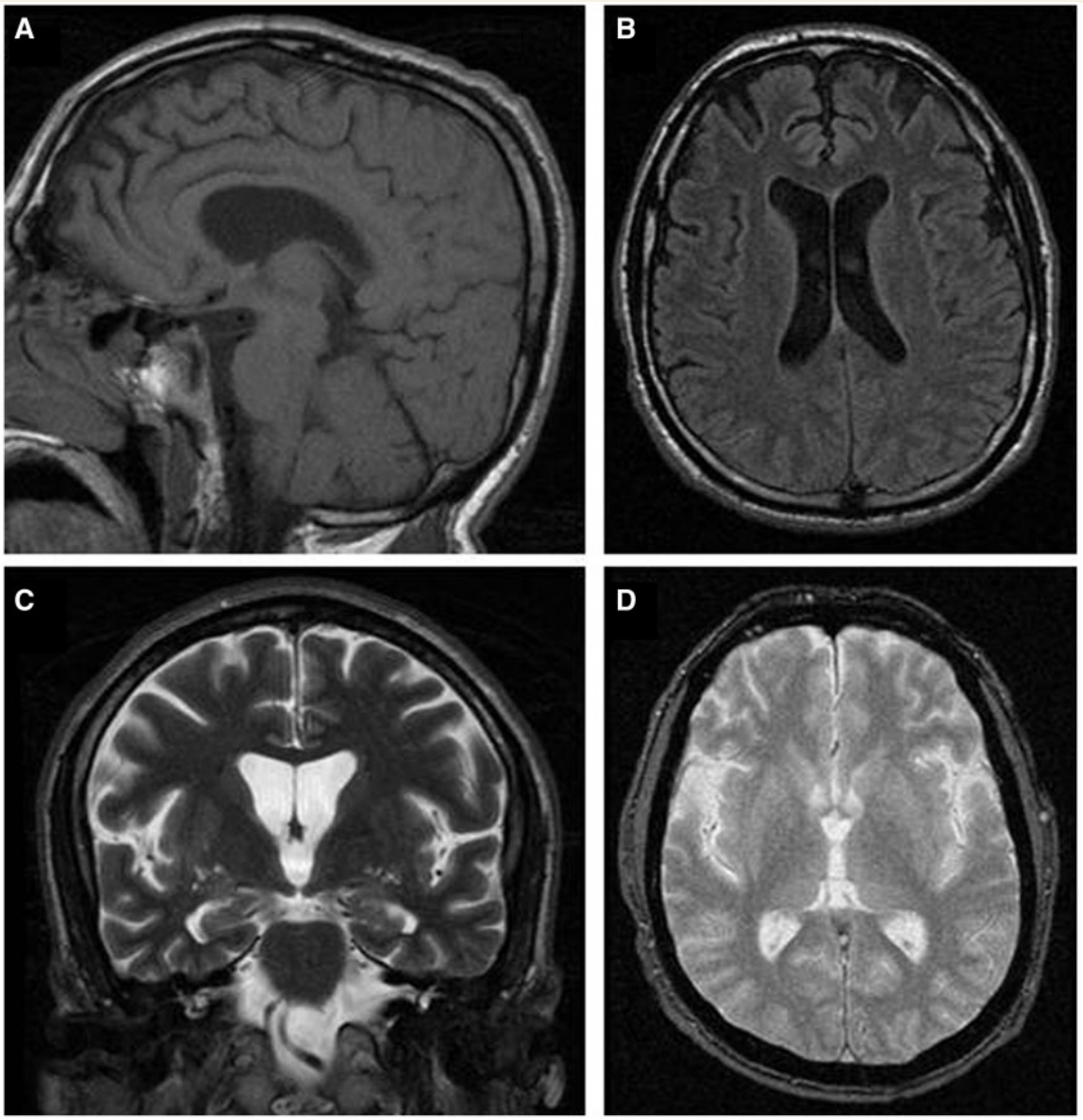

Figure 1 MRI of the patient. Sagittal T_1 (**A**) and axial fluid-attenuated inversion recovery (FLAIR) and (**B**) MRI showing mild frontal atrophy. Coronal T_2- (**C**) and axial T_2^*- (**D**) weighted acquisitions without basal ganglia hypointensities suggestive of iron deposition.

region, α-synuclein pathology involved severely the CA4 and CA2/3 regions, with much lesser degree across CA1 and subiculum. The dentate fascia was unaffected. The basal ganglia revealed mild density of α-synuclein pathology, with scattered spheroids. The thalamus showed dense α-synuclein pathology in the intralaminar nucleus regions with the nearby nuclear masses relatively free of pathology. The hypothalamus was also affected. The amygdala showed the highest α-synuclein pathology burden. The nucleus basalis of Meynert was severely affected with α-synuclein spheroids present. In the midbrain, SNpc and reticular formation showed severe α-synuclein pathology. Locus coeruleus and raphe nuclei were severely affected. Pontine nuclei were α-synuclein free. In the medulla, tegmentum was heavily affected and dorsal vagus nucleus showed mild α-synuclein pathology. The cerebellum was unaffected. The majority of α-synuclein pathology was immunoreactive for ubiquitin and p62. The immunohistochemistry study for tau showed scattered neurofibrillary tangles and dystrophic neurites in transentorhinal cortex, locus coeruleus and pontine raphe nuclei, consistent with Braak neurofibrillary stage I/primary age-related tauopathy. No abnormalities were seen with anti-amyloid-β and anti-TDP-43 antibodies in any region. The neuropathological diagnosis at this point was of a predominant nigral neurodegenerative disease with Lewy body (α-synuclein) diffuse pathology.

Mutation analysis

Mutation screening, performed post-mortem, revealed the presence of a novel homozygous mutation, c.515T > A in

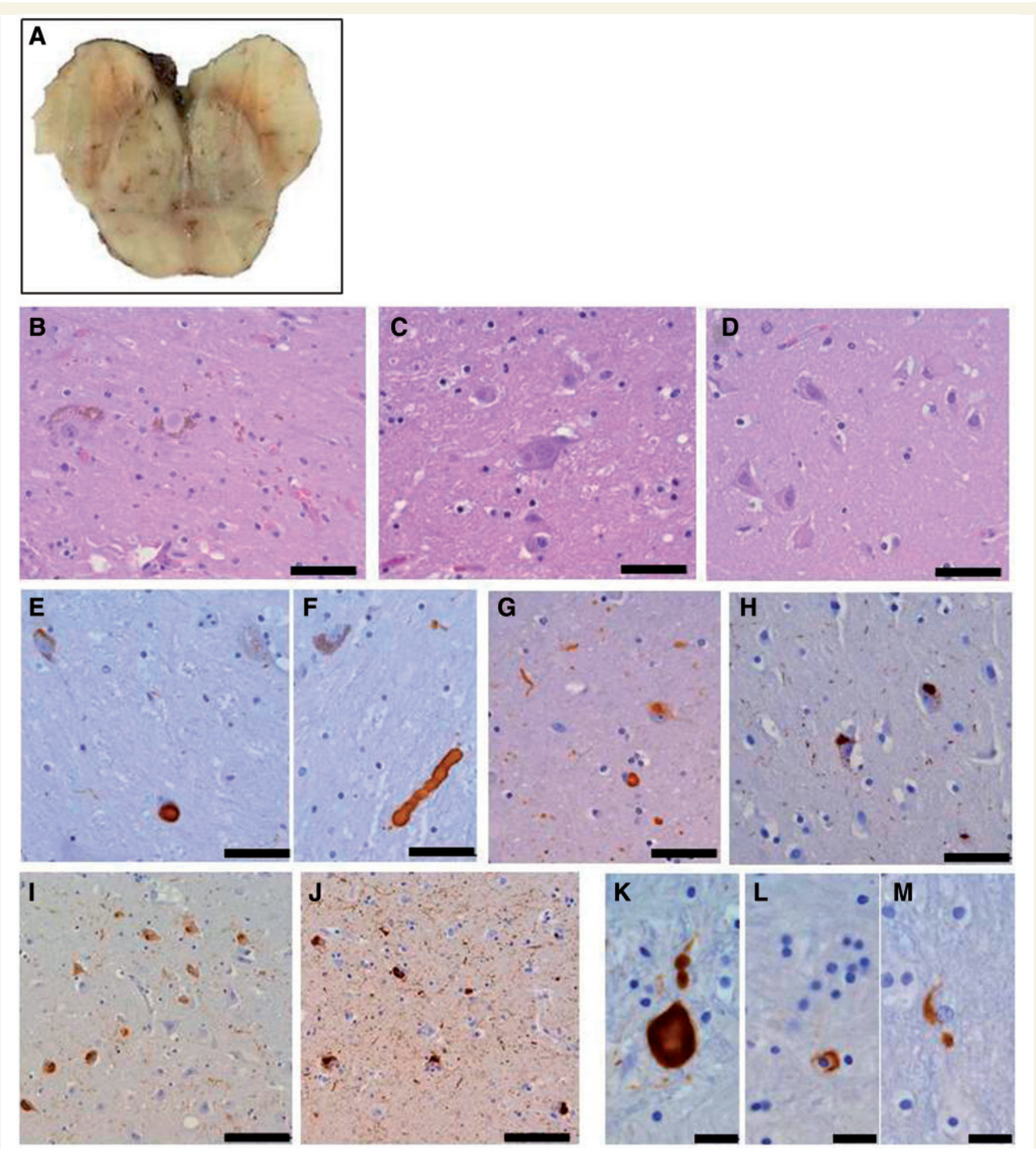

Figure 2 Neuropathological findings. (**A**) Macroscopic imaging of the midbrain showing marked depigmentation of the substantia nigra. (**B**–**D**) Classical Lewy bodies in substantia nigra (**B**) and locus coeruleus (**C**), and cortical Lewy bodies in cingulate cortex (**D**); (**E**–**M**) α-synuclein immunohistochemistry showing Lewy bodies and Lewy neurites in substantia nigra (**E**), nucleus basalis of Meynert (**G**), cingulate cortex (**H**), CA4 area of the hippocampus (**I**) and amygdala (**J**); spheroids α-synuclein immunoreactive in substantia nigra (**F**) and globus pallidus (**K**); α-synuclein glial pathology in putamen (**L**) and frontal white matter (**M**). Scale bars: **B**–**H** = 50 μm; **I** and **J** = 100 μm; **K**–**M** = 20 μm.

PARK7, which replaces a highly conserved positively charged leucine by a neutral glutamine (L172Q) (Fig. 3B) predicted to be probably damaging by different bioinformatic analysis software.

In vitro studies

The newly identified mutation is present in the last α-helical stretch of the DJ-1 protein, close to the previously reported

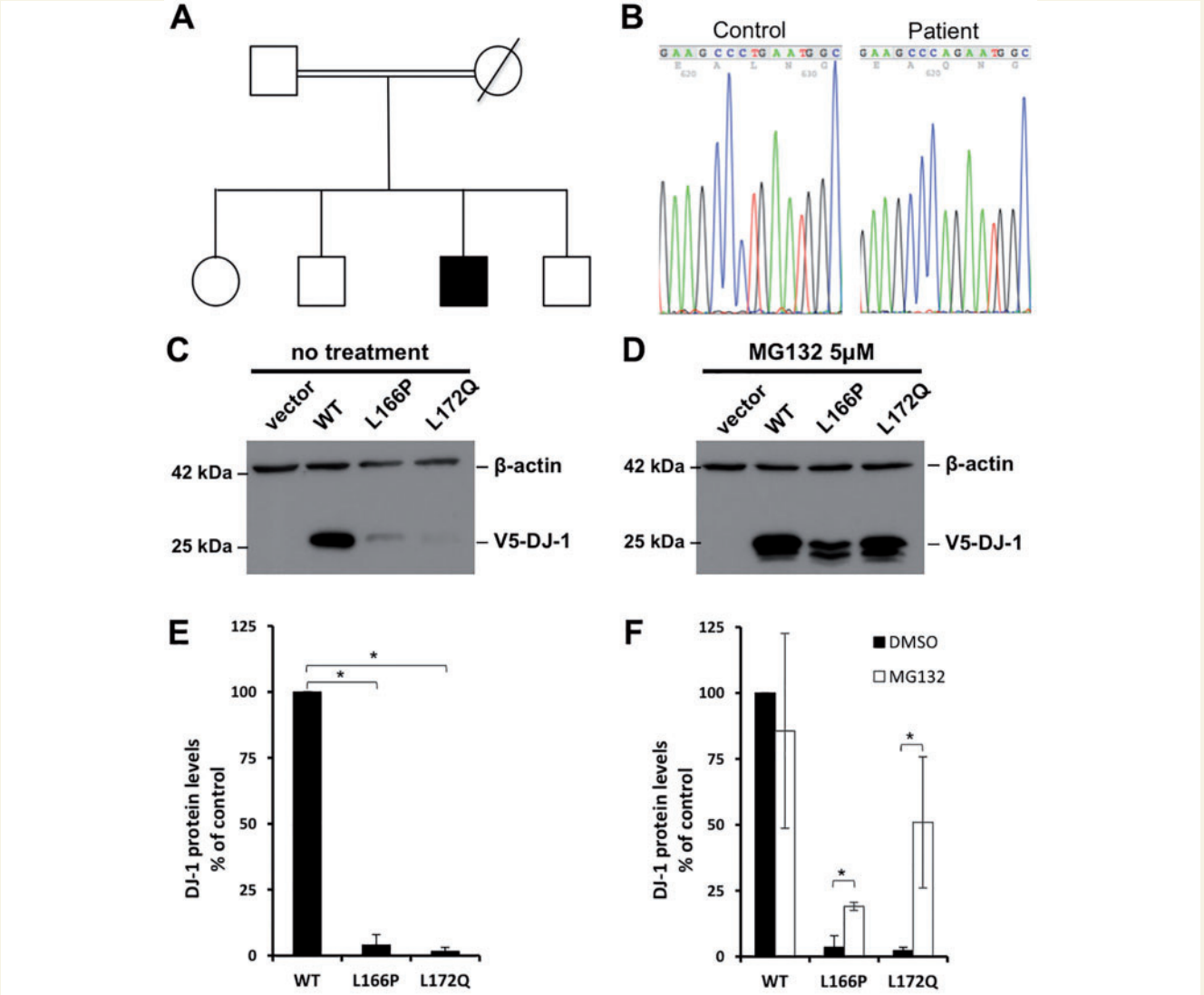

Figure 3 Pedigree and DJ-1 analysis. (**A**) Pedigree of the family described. Black square indicates the affected proband. (**B**) Sequencing of exon 7 in control and patient. A T-to-A substitution was detected at position 515, causing a leucine-to-glutamine change at codon 172. (**C** and **E**) DJ-1 protein expression in HEK293 cells. Reduction on mutant protein levels detected by western blot analysis and quantitative analysis of normal and mutant DJ-1 protein levels. (**D** and **F**) DJ-1 protein expression in HEK293 cells in the presence of MG132 known to inhibit proteasome function. MG132 restores mutant DJ-1 levels with a greater effect in the L172Q mutation; data are expressed as percentage of control. Means ± SD of three independent quantifications are shown. $^{*}P < 0.05$ from three independent experiments compared with normal protein levels.

L166P, known to be pathogenic. To clarify the pathogenicity of the mutation and confirm PARK7 diagnosis, we transfected HEK293T cells with plasmids containing C-terminal V5 tagged wild-type, L166P or L172Q mutant DJ-1, or control empty plasmid followed by western blotting using anti-V5 antibody. At 48 h post-transfection, and even earlier, similar to the L166P, the novel L172Q mutant shows a dramatic reduction in protein levels when compared to the V5 tagged wild-type DJ-1 ($P < 0.001$; variances homogeneity assumption not fulfilled) (Fig. 3C and E).

Quantitative real-time PCR showed no significant reduction of either L172Q or L166P mutant mRNA compared to wild-type DJ-1 when probing exclusively the transfected version of DJ-1 (V5 tag), showing that decreased protein levels are not due to reduction or instability of the mRNA (data not shown).

These data thus suggest that the L172Q mutation resulting from a single nucleotide substitution does not reduce the expression of *PARK7* mRNA, but somehow destabilizes the protein to a point where it is barely detectable by western blot.

To determine whether proteasome degradation is the major cause of the diminished L172Q mutant protein levels, as it has been previously suggested for the L166P DJ-1, we selectively inhibited proteasome activity. HEK293T cells were

transfected with V5-tagged DJ-1 (wild-type, L166P or L172Q) or control plasmid and 24 h post-transfection were treated for the following 24 h with either MG132 (5 μM) or control vehicle (dimethyl sulphoxide). Treatment of cells with MG132 greatly restores mutant DJ-1 levels with a greater effect in the L172Q mutation (Fig. 3D and F). After inhibiting the proteasome, mutant protein levels do not return to wild-type levels possibly indicating that other protein degradation mechanisms may also be involved in DJ-1 clearance.

L172Q DJ-1 in the human brain

After the DJ-1 *in vitro* studies pointing towards a highly unstable L172Q mutant that is rapidly degraded by the proteasome, we performed immunohistochemistry for the DJ-1 protein in the brain of the patient. DJ-1 probing revealed no staining in the case when compared to the prominent immunoreactivity in astrocytes and astrocytic processes in the control (Fig. 4), thus confirming our previous findings with the human cell line.

Discussion

Clinical features

PARK7 mutations are a rare cause of early-onset Parkinson's disease with an estimated frequency of ~1% of all cases (Abou-Sleiman *et al.*, 2003). Early onset parkinsonism, with slow disease progression, early leg dystonia and good response to levodopa were initially described (Bonifati *et al.*, 2003). The age of onset in PARK7 reported cases ranges from 24 (Hague *et al.*, 2003) to 40 years (van Duijn *et al.*, 2001; Bonifati *et al.*, 2002) (Supplementary Table 2). Our patient presented earlier, without early leg dystonia and with a poor levodopa response. Dementia, bulbar signs (with speech problems and dysphagia) and signs of involvement of upper and lower motor neuron have been reported as associated features in an Italian family with *PARK7* mutations (Annesi *et al.*, 2005). Interestingly, our patient had cognitive deterioration and bulbar symptoms with pyramidal signs appearing early in the disease course although EMG, repeated several times, never showed denervation. Family history and the atypical clinical features diverted our attention from familial parkinsonism.

Neuropathology of DJ-1-linked parkinsonism

Similar to sporadic Parkinson's disease, we found neuronal loss in SNpc and Lewy body pathology (Gelb *et al.*, 1999) closely resembling Braak stage 6 (Braak *et al.*, 2003). Interestingly, there were other characteristics commonly observed in sporadic Parkinson's disease, such as the dense burden of Lewy body-related pathology in the intralaminar regions of the thalamus sparing most of the other thalamic regions (Brooks and Halliday, 2009), the α-synuclein pathology distribution in the hippocampus (Spillantini *et al.*, 1997; Galvin *et al.*, 1999) and the predominance of Lewy bodies in deep cortical layers (Braak *et al.*, 2003). However, some particularities were found that differ from the typical pathology seen in sporadic Parkinson's disease. We found axonal spheroids immunoreactive for α-synuclein, classically described in infantile neuroaxonal dystrophy due to mutations in the phospholipase A2, group VI (*PLA2G6*) (Paisan-Ruiz *et al.*, 2012), and in some pathological descriptions of patients with mutations in the gene encoding α-synuclein (*SNCA*) (Duda *et al.*, 2002), but not in sporadic Parkinson's disease. Furthermore, the relatively mild involvement of dorsal motor nucleus of the vagus nerve, usually severely affected at the end stage of the disease (Braak *et al.*, 2003), the neuronal loss and the presence of Lewy body-related pathology in basal ganglia, usually spared (Dickson, 2012), was also atypical for sporadic Parkinson's disease. These findings (neuronal loss and Lewy body-related pathology in basal ganglia) could have contributed to the partial clinical response to levodopa.

DJ-1 is a ubiquitously expressed protein that participates predominantly in the protection of cells against oxidative stress by various mechanisms (Zondler *et al.*, 2014; van der Merwe *et al.*, 2015). Recent evidence links *PARK2* (parkin), *PINK1* and *PARK7* (DJ-1), the three genes responsible for the majority of cases of autosomal recessive Parkinson's disease, in a common biological pathway (van der Merwe *et al.*, 2015). Similar to our description, neuropathological studies from patients with *PARK2*-linked parkinsonism consistently showed neuronal loss in substantia nigra and locus coeruleus but only rarely associated to Lewy body pathology (Singleton *et al.*, 2013). Samaranch *et al.* (2010) reported the neuropathological study of a patient with *PINK1* mutations also showing neuronal loss, Lewy bodies and aberrant neurites in the SNpc, but contrary to our findings the locus coeruleus, amygdala, hippocampus and neocortex were unaffected. Interestingly, the authors hypothesized that the preservation of the locus coeruleus could be related to the slowly progressive parkinsonism observed. Our case had a rapid and complex progression of the disease with dementia. The involvement of this nucleus, as well as the nucleus basalis of Meynert and the neocortex could help explain the dementia symptoms. Thalamic, hypothalamic and raphe nuclei pathological involvement could also be responsible for the excessive somnolence periods. Contrary to the presumption of the reported DJ-1 case associated to amyotrophic lateral sclerosis (ALS), that suggested a possible link between DJ-1 and tau protein taking into account the clinical phenotype of FTDP17 cases (Annesi *et al.*, 2005), the neuropathology of our case does not support this hypothesis. Furthermore, there was no evidence of TDP43 pathology, the principal protein involved in ALS (Neumann *et al.*, 2006). We found spheroid α-synuclein pathology similar to some pathological descriptions of patients with *SNCA* mutations

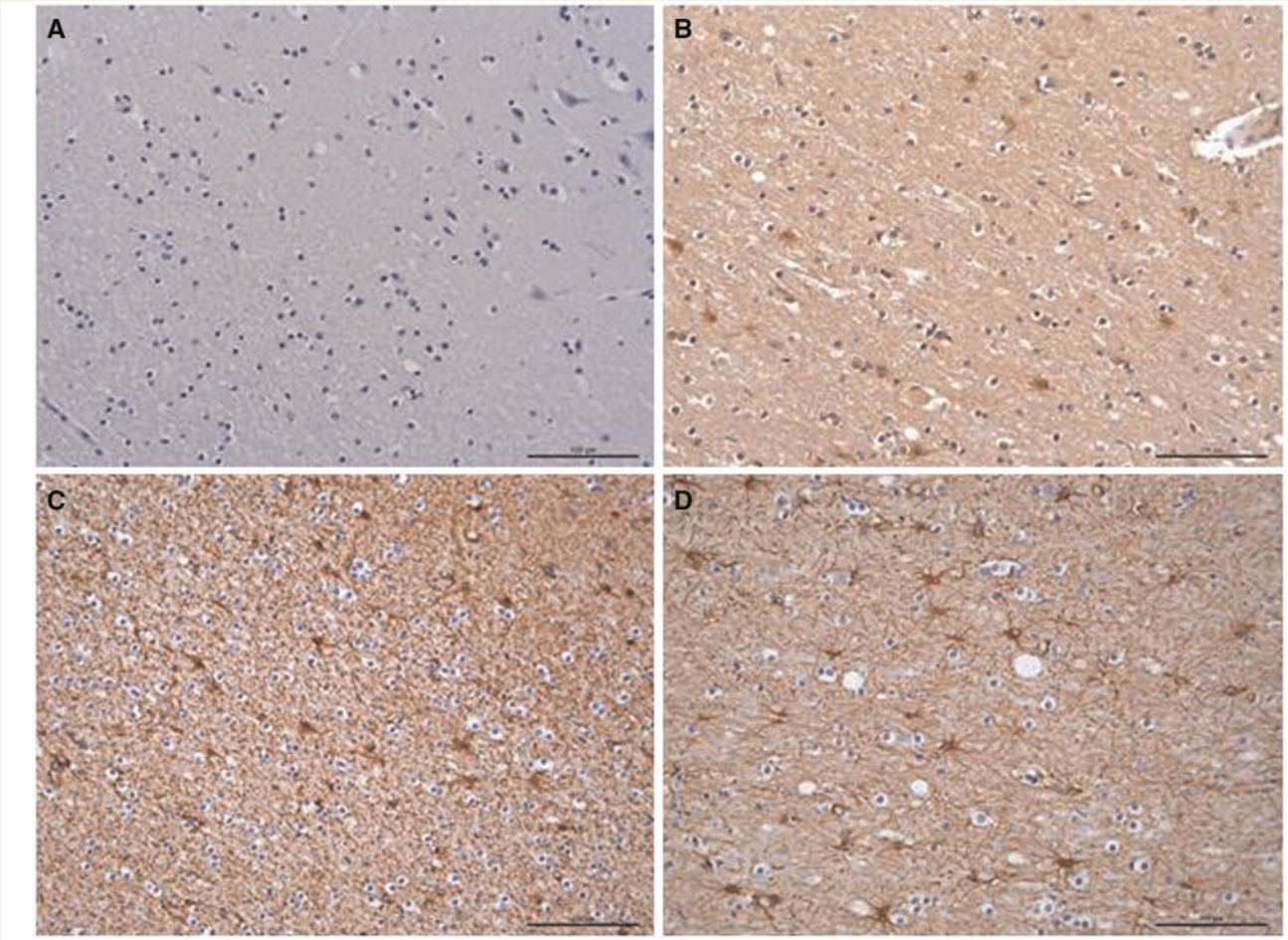

Figure 4 DJ-1 immunohistochemistry study. DJ-1 immunohistochemistry from frontal subcortical white matter showing no staining in our case (**A**) compared to the prominent immunoreactivity in astrocytes and astrocytic processes in the control (**B**). The same study for GFAP shows the astrocytes in both PARK7 case (**C**) and control (**D**). Scale bars = 100 μm.

(Duda *et al.*, 2002), but contrary to their findings we did not find evidence of significant concurrent tau pathology beyond age related descriptions (Braak *et al.*, 2006; Elobeid *et al.*, 2012). However, we cannot exclude a role of DJ-1 in the tau pathology found.

DJ-1 mutations associated with early-onset Parkinson's disease

Genetic analysis revealed a novel homozygous mutation in the *PARK7* gene. To evaluate the pathogenicity of this new mutation we explored DJ-1 expression levels *in vitro*, under several conditions showing a massive reduction in DJ-1 protein levels that was caused by a highly unstable and rapidly degraded L172Q mutant, similar to that of the previously reported L166P mutation (Moore *et al.*, 2003). Both L172Q and L166P mutations effectively mimic knock-out of DJ-1.

Most importantly, DJ-1 immunohistochemistry of brain tissue, revealed no staining in our PARK7 case when compared to the prominent immunoreactivity in astrocytes and astrocytic processes of the control. The latter, as it had been observed before, with control subjects and cases with sporadic Parkinson's disease (Bandopadhyay *et al.*, 2004). Also, the extensive α-synuclein aggregation found in this patient's brain fits well in the model proposed by previous *in vitro* studies showing that L166P mutation abolishes DJ-1 chaperone activity over α-synuclein, which facilitates its aggregation (Shendelman *et al.*, 2004; Zondler *et al.*, 2014). Recent work shows that DJ-1 directly binds α-synuclein mono- and oligomers, actively reducing dimerization and reversing α-synuclein dependent cellular toxicity. Several DJ-1 mutants are impaired in this chaperoning activity; however a stabilized version of L166P was demonstrated to retain this function (Zondler *et al.*, 2014). We have established that L172Q behaves very similarly to L166P and possibly also retains chaperoning activity, but this is overridden by protein instability, as *in vitro* and brain pathology shows.

Therefore, we are able for the first time to validate *in vitro* experiments, especially those regarding L166P and L172Q mutants' depletion from cells, confirming that the

most probable pathogenic mechanism in play is the absence of protein to perform its normal function.

In summary, our clinical, genetic and pathological findings, although based on a single case study, suggest that DJ-1 mutations are causative of α-synucleinopathy. These results can help in the understanding of Parkinson's disease pathophysiology, promote research studies to increase the knowledge on the pathways involved in the neurodegeneration process and pave the way for new therapeutic interventions.

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Supplementary material

Supplementary material is available at *Brain* online.

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