Human Molecular Genetics, 2005, Vol. 14, No. 14 **2063–2073**
doi:10.1093/hmg/ddi211
Advance Access published on June 8, 2005

Mitochondrial localization of the Parkinson's disease related protein DJ-1: implications for pathogenesis

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Received March 16, 2005; Revised May 22, 2005; Accepted June 5, 2005

Both homozygous (L166P, M26I, deletion) and heterozygous mutations (D149A, A104T) in the *DJ-1* gene have been identified in Parkinson's disease (PD) patients. The biochemical function and subcellular localization of DJ-1 protein have not been clarified. To date the localization of DJ-1 protein has largely been described in studies over-expressing tagged DJ-1 protein *in vitro*. It is not known whether the subcellular localization of over-expressed DJ-1 protein is identical to that of endogenously expressed DJ-1 protein both *in vitro* and *in vivo*. To clarify the subcellular localization and function of DJ-1, we generated three highly specific antibodies to DJ-1 protein and investigated the subcellular localization of endogenous DJ-1 protein in both mouse brain tissues and human neuroblastoma cells. We have found that DJ-1 is widely distributed and is highly expressed in the brain. By cell fractionation and immunogold electron microscopy, we have identified an endogenous pool of DJ-1 in mitochondrial matrix and inter-membrane space. To further investigate whether pathogenic mutations might prevent the distribution of DJ-1 to mitochondria, we generated human neuroblastoma cells stably transfected with wild-type (WT) or mutant (M26I, L166P, A104T, D149A) DJ-1 and performed mitochondrial fractionation and confocal co-localization imaging studies. When compared with WT and other mutants, L166P mutant exhibits largely reduced protein level. However, the pathogenic mutations do not alter the distribution of DJ-1 to mitochondria. Thus, DJ-1 is an integral mitochondrial protein that may have important functions in regulating mitochondrial physiology. Our findings of DJ-1's mitochondrial localization may have important implications for understanding the pathogenesis of PD.

INTRODUCTION

Parkinson's disease (PD) is a common progressive neurodegenerative disorder. A hallmark feature of the disease is the relatively selective loss of nigrostriatal dopaminergic neurons (1,2). The molecular mechanisms underlying the pathogenesis of PD are not understood, but epidemiologic studies, neuropathologic investigations, new experimental models of PD and genetic analyses are yielding important new insights into the causes of PD. The majority of PD is sporadic and studies in human post-mortem brain indicate that reactive oxygen species (ROS) and free radical stress play important roles in the pathogenesis of PD. In particular, there are consistent findings of decrements in mitochondrial complex-I (complex-I) (3–5). Defects in complex-I lead to increased free radical stress and increased neuronal vulnerability to glutamate excitotoxicity (3). At least three complex-I inhibitors cause selective dopaminergic cell death and lead to

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the formation of inclusions similar to Lewy bodies, the pathologic hallmark of sporadic PD (6–9). Thus, derangements in complex-I may be central to the pathogenesis of sporadic PD (10).

Genetic defects have been identified in rare familial cases of PD (11). At least 10 distinct loci are responsible for rare Mendelian forms of PD. To date, mutations in five genes are linked to familial PD. Missense mutations or triplications of α-*synuclein* cause autosomal dominantly inherited PD (12,13). Mutations in the ubiquitin E3-ligase, *parkin*, are the major cause of autosomal recessive PD and are considered to be one of the major causes of familial PD (14). Recently, mutations in *DJ-1* and *PINK1* are linked with autosomal recessive early onset PD (15,16), and mutations in *LRRK2* are linked with autosomal dominantly inherited PD (17,18).

DJ-1 belongs to the ThiJ/PfpI protein superfamily and is present in a diverse number of organisms from humans to bacteria (15). The biologic function of DJ-1 remains unknown, but recent studies suggest that it may function as a redox sensitive molecular chaperone (19,20). DJ-1 has been crystallized and structurally resembles the heat shock protein 31 of bacteria and yeast (21,22). Over-expression of DJ-1 protects against oxidative stress-induced injury and knockdown of DJ-1 by RNA interference renders cells more susceptible to oxidative injury (23). Both homozygous mutations (L166P, M26I, deletion) and heterozygous mutations (D149A, A104T) have been identified in PD patients. How mutations in DJ-1 cause dopaminergic cell death and lead to PD is not known. However, the L166P mutant has been suggested to cause PD due to its instability at protein level and its inability to accomplish required cellular functions (21,23–28). The biochemical function and subcellular localization of DJ-1 protein have not been clarified. To date, the localization of DJ-1 protein has largely been described in studies over-expressing tagged DJ-1 protein *in vitro* (24,29,30). It is not clear whether the subcellular localization of over-expressed DJ-1 protein is identical to that of endogenously expressed DJ-1 protein both *in vitro* and *in vivo*. To begin to dissect the molecular mechanisms by which derangements in DJ-1 lead to the pathogenesis of PD, we generated highly specific DJ-1 antibodies to determine the cellular and subcellular localization of DJ-1. We also generated human neuroblastoma cells stably transfected with wild-type (WT) or mutant DJ-1 to investigate whether pathogenic DJ-1 mutations might interfere with the distribution of DJ-1 to its target organelles. Here, we show that DJ-1 is expressed in a variety of tissues including brain and is localized to mitochondrial matrix and inter-membrane space (IMS) and the pathogenic mutations do not prevent the distribution of DJ-1 to mitochondria. DJ-1's mitochondrial localization may have important implications for understanding the pathogenesis of PD.

RESULTS

Generation of polyclonal antisera to DJ-1

A C-terminal peptide corresponding to amino acids 179–189 of human DJ-1 and a N-terminal peptide based on amino acids 1–13 of human DJ-1 were synthesized and conjugated to KLH and injected into rabbits to raise antiserum (28). A recombinant full-length mouse DJ-1 protein was generated and injected into rabbits to produce antiserum against full-length DJ-1 protein. All three antibodies to DJ-1 reveal a discrete band at 23 kDa in whole mouse brain lysate (Fig. 1A). The specificity of all three DJ-1 antibodies was evaluated by comparing immunoreactivity in WT mice versus DJ-1 knockout (KO) mice whole brain lysates. All three antibodies robustly recognize DJ-1 in WT mice, whereas there is no immunoreactivity in DJ-1 KO mice (Fig. 1B).

The tissue distribution of DJ-1 was examined and compared with other PD associated genes, parkin and α-synuclein (Fig. 2). DJ-1 is widely distributed and highly expressed in the brain and a variety of peripheral organs, including the testes, ovaries, skeletal muscle (SM), spleen, adrenal gland, kidney, liver, lung and heart, whereas there are very low levels of DJ-1 in adipose tissue (Fig. 2). In contrast, α-synuclein is enriched in the brain and lung and parkin is expressed in the brain, heart, liver, kidney, adrenal gland, SM and ovaries (Fig. 2).

Immunohistochemical localization of DJ-1 in brain

At low magnification, immunohistochemical visualization reveals that DJ-1 is expressed throughout the brain. It is particularly enriched in the pyramidal cells of the hippocampus and in the cerebellum, as well as in the olfactory bulb (Fig. 3A). In the cerebral cortex, DJ-1 staining is evident throughout all laminae with minimal staining in the corpus callosum (Fig. 3C). DJ-1 staining is also present in the striatum (Fig. 3E). In the hippocampus, pyramidal cells demonstrate DJ-1 immunoreactivity throughout their perikarya in all CA subfields (Fig. 3D). In the substantia nigra (SN), DJ-1 immunoreactivity is present both in the pars compacta and pars reticulata (Fig. 3F). The specificity of DJ-1 staining was confirmed by the absence of immunoreactivity in DJ-1 KO mouse brain (Fig. 3B). High power views of DJ-1 staining reveal that DJ-1 is enriched and concentrated primarily within the perikarya of cortical pyramidal cells (Fig. 4A). In the striatum, DJ-1 is enriched within the cell bodies of small-medium-sized spiny neurons (Fig. 4B). In the hippocampus, DJ-1 is localized to cell bodies and there is evidence of faint staining in dendrites of CA1 pyramidal cells (Fig. 4C). In the SN, DJ-1 is widely distributed throughout the cell bodies of the SN pars compacta and pars reticulata (Fig. 4D).

Localization of DJ-1 to mitochondria

As DJ-1 immunostaining was enriched within the perikarya of neurons, we performed subcellular fractionation studies to identify the subcellular compartments where DJ-1 is localized and compared the subcellular fractionation of DJ-1 with parkin and α-synuclein. These observations were further compared with the synaptic vesicle-enriched protein synaptophysin-1 (Fig. 5A). DJ-1, parkin, α-synuclein and synaptophysin-1 are all detectable within the total homogenate (H) and fractionate within the S1 fraction, which contains mitochondria, as well as other subcellular organelles. Further, fractionation reveals that DJ-1 is absent from the P1 fraction, which contains crude nuclei, cell debris and myelin fragments (Fig. 5A).

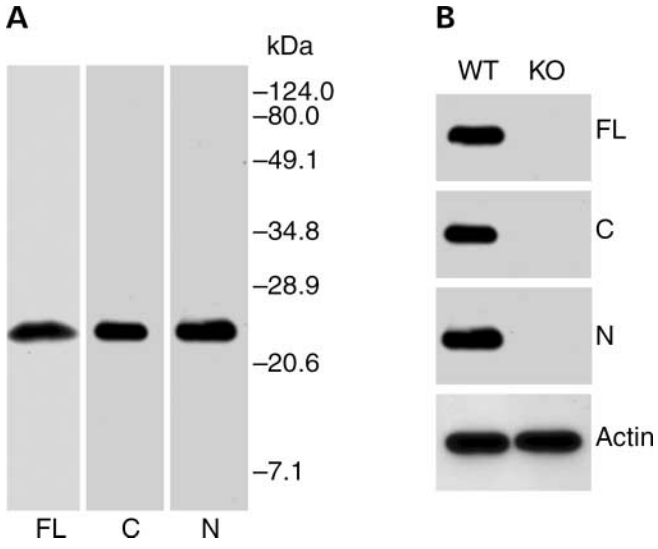

Figure 1. Characterization of anti-DJ-1 protein antibodies by western blot analysis. (**A**) Homogenates of DJ-1 WT mouse brain (20 μg per lane) were subjected to SDS–PAGE, blotted to PVDF membranes and probed with anti-DJ-1-FL (FL), anti-DJ-1-C (C) or anti-DJ-1-N (N) antibodies. All three antibodies detected a single band (Mr 23 kDa). (**B**) Homogenates of DJ-1 WT or DJ-1 KO mouse brain (20 μg per lane) were subjected to western blot analysis with three anti-DJ-1 antibodies and an anti-actin antibody. All three anti-DJ-1 antibodies detected a band in WT but not in KO mouse brain homogenates. These experiments were run three times with similar results.

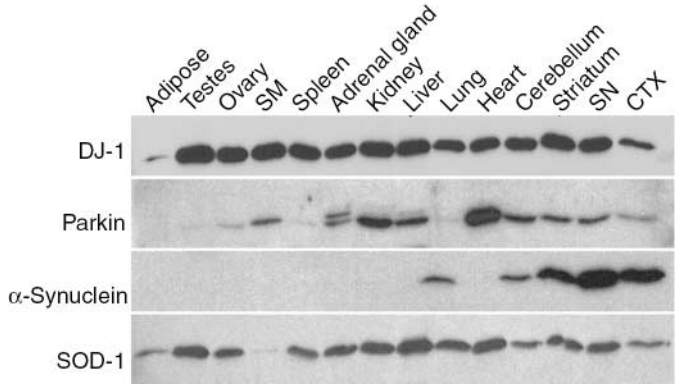

Figure 2. Western blot analysis of DJ-1 protein expression in mouse brain and peripheral tissues. Homogenates prepared from various mouse tissues were resolved by SDS–PAGE, blotted to PVDF membranes and probed with anti-DJ-1-FL, anti-parkin, anti- α-synuclein or anti-SOD-1 antibodies. DJ-1 was present in essentially all tissues, α-synuclein was mainly expressed in brain. These experiments were repeated two times with similar results.

DJ-1, parkin and α-synuclein are present in both the medium speed (P2) and high speed (P3) pellets. Both DJ-1 and α-synuclein are present in the high-speed supernatant (S3), whereas parkin is absent from the high speed S3 fraction. When the P2 fraction containing mitochondria and synaptosomes was subfractionated after hypotonic lysis according to the method of Huttner *et al.* (31), synaptophysin, an integral synaptic vesicle protein, was highly enriched in the LP2 fraction as expected. DJ-1 and α-synuclein are found in the soluble (LS2) fraction. DJ-1 is found in the synaptic vesicle-enriched LP2 fraction, whereas α-synuclein is barely detectable in this fraction.

Because DJ-1 is potentially localized to the mitochondrial compartment based on the subcellular fractionation studies, we further explored the possibility that DJ-1 may be localized to the mitochondria. Accordingly, we performed mitochondrial subfractionation studies and demonstrate that DJ-1 is localized to the mitochondria and that DJ-1 co-segregates with manganese superoxide dismutase (MnSOD), an integral mitochondrial protein (Fig. 5B). The integrity of our subfractionation studies was confirmed by the absence of immunoreactivity for the nuclear protein, histone, in our mitochondrial fraction (Fig. 5B). These results taken together suggest that DJ-1 is distributed in a variety of subcellular compartments of the cell, but that one pool of this protein is localized to the mitochondria.

Mitochondria have several compartments. To determine the mitochondrial localization of DJ-1, submitochondrial fractionation studies were conducted. DJ-1 protein is located in the mitochondrial IMS and matrix. Little, if any, DJ-1 protein is associated with outer or inner mitochondrial membranes (Fig. 5C). The identity of the different submitochondrial fractions was demonstrated by enrichment of marker proteins in expected fractions. We observe the outer membrane (OM) marker voltage-dependent anion channel (VDAC) in the inner membrane (IM) fraction, which suggests that the separation of the OM from IM was incomplete. This might be a result of the localization of VDAC to specialized contact points in mitochondria where IM and OMs are associated. Nevertheless, our results clearly reveal that DJ-1 protein is not associated with mitochondrial membrane fractions but is localized to the IMS and matrix.

To further confirm that DJ-1 is localized to mitochondria, we performed immunogold electron microscopy to examine the subcellular distribution of DJ-1 at the ultrastructural level. Consistent with our fractionation studies, immunogold particles signaling DJ-1 are found to be associated with mitochondria (Fig. 6A). The distance between the center of a gold particle and the respective epitope may be up to 20–25 nm [corresponding to the sizes of the interposed immunoglobulins (32)]. Thus, the immunogold analysis does not permit conclusions as to the precise submitochondrial localization of DJ-1. However, the pattern of immunogold labeling is consistent with the idea that the epitope is associated with the intermembrane space, as gold particles tended to cluster over the mitochondrial cristae (Fig. 6A). Gold particles are also superimposed on the matrix. In contrast, gold particles are rarely found in association with the outer mitochondrial membrane. The specificity of DJ-1 staining was confirmed by the absence of immunogold particles in DJ-1 KO mouse tissue (Fig. 6B). As both cell fractionation and immunogold electron microscopy studies showed an endogenous pool of DJ-1 in mitochondria, we further investigated whether pathogenic mutations might prevent the distribution of DJ-1 to mitochondria. We first evaluated the distribution of DJ-1 in human SH-SY5Y neuroblastoma cells by cell fractionation analysis and detected an endogenous pool of DJ-1 in mitochondria (Fig. 7A). Approximately 25% of total DJ-1

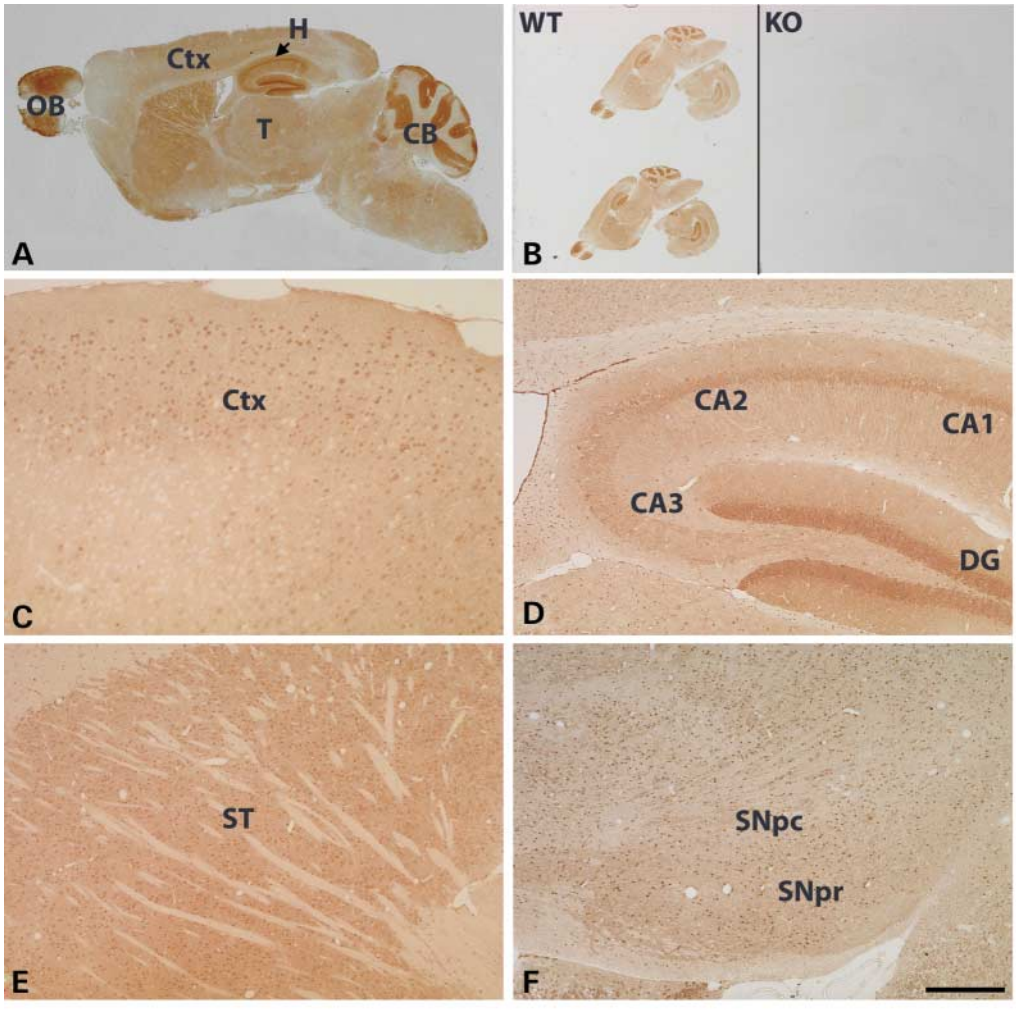

Figure 3. Immunohistochemical distribution of the DJ-1 protein in mouse brain. Sagittal sections (**A**) or coronal sections from DJ-1 WT mouse brain including cerebral cortex (**C**), hippocampus (**D**), striatum (**E**) and SN (**F**) were stained with anti-DJ-1-FL. DJ-1 immunoreactivity was present throughout the brain and was particularly enriched in the hippocampus. The immunoreactivity was absent in DJ-1 KO mouse brain (**B**). Bar, 200 μm. These experiments were run three times with similar results.

is localized to mitochondria (Fig. 7B). To ascertain whether oxidative stress causes an increase in the mitochondrial pool of DJ-1, we treated SH-SY5Y cells with paraquat. We failed to detect any significant increase of DJ-1 in the mitochondrial fraction (Fig. 7C). We then generated human SH-SY5Y neuroblastoma cells containing myc-tagged WT or mutant (L166P, M26I, A104T or D149A) human DJ-1 or control plasmid, and performed mitochondrial fractionation studies and confocal co-localization imaging analysis. The mitochondrial fractionation studies show that, compared with WT DJ-1 and other mutants, L166P mutant exhibits largely reduced protein levels. However, both WT and mutant DJ-1 proteins were detectable in the mitochondria fraction (Fig. 7D). Confocal imaging analysis also shows that both WT and mutant DJ-1 immunoreactivity significantly overlaps and co-localizes with MitoTracker fluorescence (Fig. 8). These data taken together indicate that the pathogenic mutations investigated in this study do not alter the distribution of DJ-1 to mitochondria.

DISCUSSION

The major finding of this study is that a pool of endogenous DJ-1 is localized to the mitochondrial matrix and IMS of neurons. DJ-1 is expressed throughout the body in a variety of organs except for minimal expression in adipose tissue. In the brain, DJ-1 is found in a broad range of brain regions. The distribution of DJ-1 is more extensive than that of parkin and α-synuclein, which have restricted tissue distributions.

The specificity of anti-DJ-1 antibodies is evident from the failure to observe any immunoreactivity in mice lacking the *DJ-1* gene. Using these highly specific antibodies, we show that endogenous DJ-1 is exclusively cytoplasmic with no detectable immunoreactivity in the nucleus. This contrasts with other reports, which suggest that DJ-1 may be localized to the nucleus. We suspect that this may be due to antibody specificity issues, but we cannot exclude the possibility of differences in methodologies. This study indicates that DJ-1

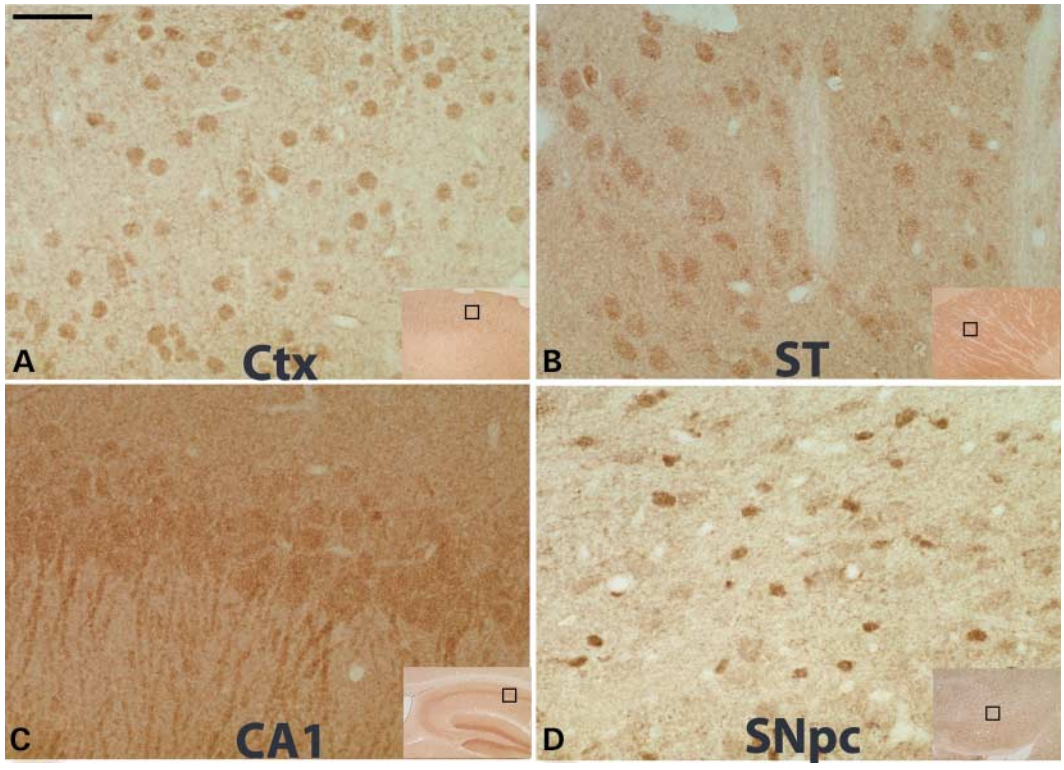

Figure 4. Higher magnification of anti-DJ-1 protein immunoperoxidase reaction product in mouse brain. Sections of mouse cerebral cortex (Ctx), striatum (ST), CA1 of hippocampus and pars compacta of substantia nigra (SNpc) were stained with anti-DJ-1-FL. In the cerebral cortex, DJ-1 immunoreactivity appeared primarily within the perikarya of cortical pyramidal cells (**A**). In the striatum, DJ-1 was enriched within the cell bodies of small-medium spiny neurons (**B**). In the hippocampus, DJ-1 was primarily localized to cell bodies and there was faint staining in dendrites of CA-1 pyramidal cells (**C**). In the substantia, DJ-1 was widely distributed throughout the cell bodies of the SN pars compacta (**D**). (A–D) are higher magnification views of the boxed area in the inset. Bar, 40 μm.

is expressed as a 23 kDa protein with widespread distribution in both brain and peripheral tissues. Within the brain, this protein is present in the SN, but it is not enriched where the pathology is greatest in PD. It is expressed in neurons at moderate to high levels and in glia at much lower levels, if at all. In neurons, it is expressed in cell bodies and dendrites including small caliber processes as evident by neuropil staining. We were unable to directly compare the immunostaining properties of DJ-1 and parkin as currently available specific and selective parkin antibodies are not adequate for immunohistochemical localization of parkin (33). We did not directly compare DJ-1 immunoreactivity with α-synuclein as prior studies indicate that α-synuclein is a synaptic terminal enriched protein (34).

The subcellular distribution of DJ-1 indicates that it is present in both the high-speed supernatant and the high-speed pellet and moderately enriched in the LP2 fraction. This subcellular distribution differs from that of parkin and α-synuclein. Parkin is primarily enriched in the high-speed pellet and LP1 fraction, whereas synuclein is primarily enriched in the high-speed pellet and the LP1 and LS2 fractions. The fact that parkin is enriched in the LP1 fraction is consistent with the recent observations suggesting that parkin plays an important role in mitochondrial function, including the observation that *Drosophila* lacking parkin have mitochondrial defects (35). Furthermore, parkin may be localized to the outer mitochondrial membrane (36) and parkin KO mice have mitochondrial defects (37). The localization of α-synuclein to the LP1 fraction is consistent with the observations that over-expression of α-synuclein impairs mitochondrial function (38), and its enrichment in the LS2 fraction is consistent with it being a synaptic terminal associated protein (34).

The localization of DJ-1 to the mitochondrial fraction and its co-localization with MitoTracker and its localization ultrastructurally to the mitochondria suggest that DJ-1 may play important roles in mitochondrial function. The precise role of DJ-1 in mitochondria remains to be elucidated. The fact that DJ-1 is not indispensable for cell development observed in DJ-1 KO mouse (39) (L. Zhang, M. Sasaki, T.M. Dawson and V.L. Dawson, unpublished data) and in PD patients with DJ-1 mutations suggests that DJ-1 may not play a critical role in energy metabolism, the most common function of mitochondria. Rather, DJ-1 might contribute to the maintenance of a stable microenvironment inside mitochondria allowing mitochondria to function properly in physiological conditions. Multiple *in vitro* studies implicate DJ-1 as an antioxidant protein or a free radical scavenger as it can protect against cell death induced by oxidative stress (23,30,40,41). It is possible that under physiologic conditions, DJ-1 may function as a free radical scavenger to balance the free radical level within mitochondria. Consistent with this notion is the observation

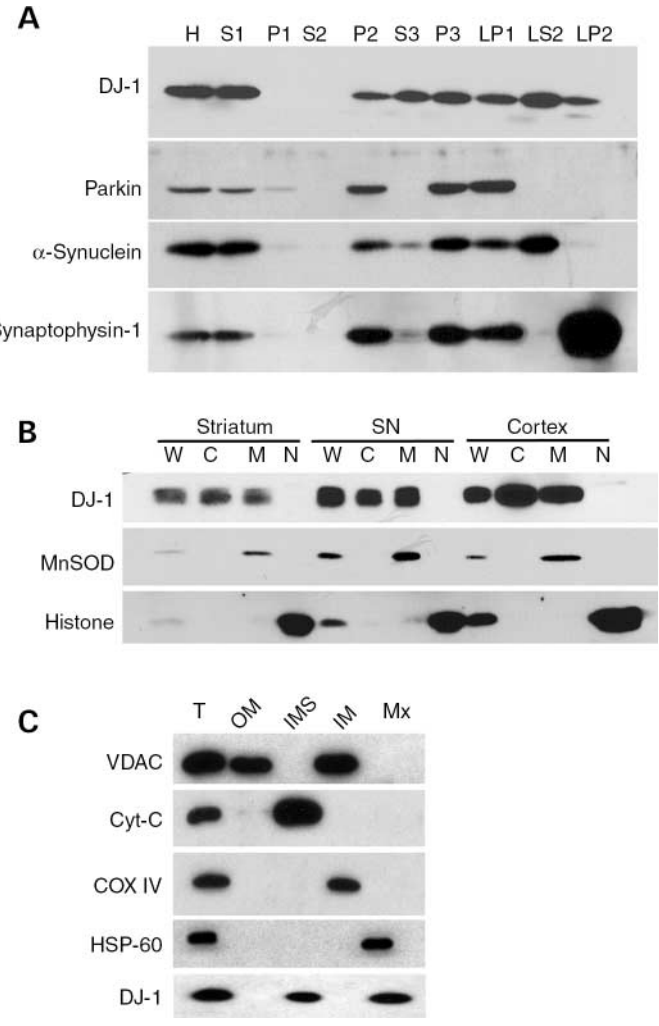

Figure 5. Subcellular fractionation of mouse brain. (**A**) Subcellular fractions were prepared from mouse brain tissue, resolved by SDS–PAGE, blotted to PVDF membranes and incubated with anti-DJ1-C, anti-parkin or anti-α-synuclein antibodies. The blot was also probed with an antibody to synaptophysin, yielding a band at the appropriate indicated molecular masses. (**B**) Whole brain lysate (W) and different subcellular fractions [cytosol (C), mitochondria (M) and nucleus (N)] prepared from various mouse brain tissues (striatum, SN and cerebral cortex) were subjected to western blot analysis with anti-DJ-1 antibodies. Of all the three tissues tested, DJ-1 immunoactivity was present in whole brain lysate, cytosol and mitochondria but not in nucleus. The blot was also probed with antibodies to the indicated proteins (MnSOD for mitochondria, Histone for nucleus), yielding bands at the appropriate indicated molecular masses. (**C**) Submitochondrial fractionation of mouse brain. Mitochondria isolated from the mouse brain were fractionated into OM, IMS, IM and matrix (Mx). About 10 μg proteins from each subfraction and the total mitochondrial homogenate (T) were subjected to western blot analysis using antibodies to DJ-1 and markers for different mitochondrial subfractions (see Materials and Methods). DJ-1 is mainly located in the intermembrane space and matrix of mitochondria.

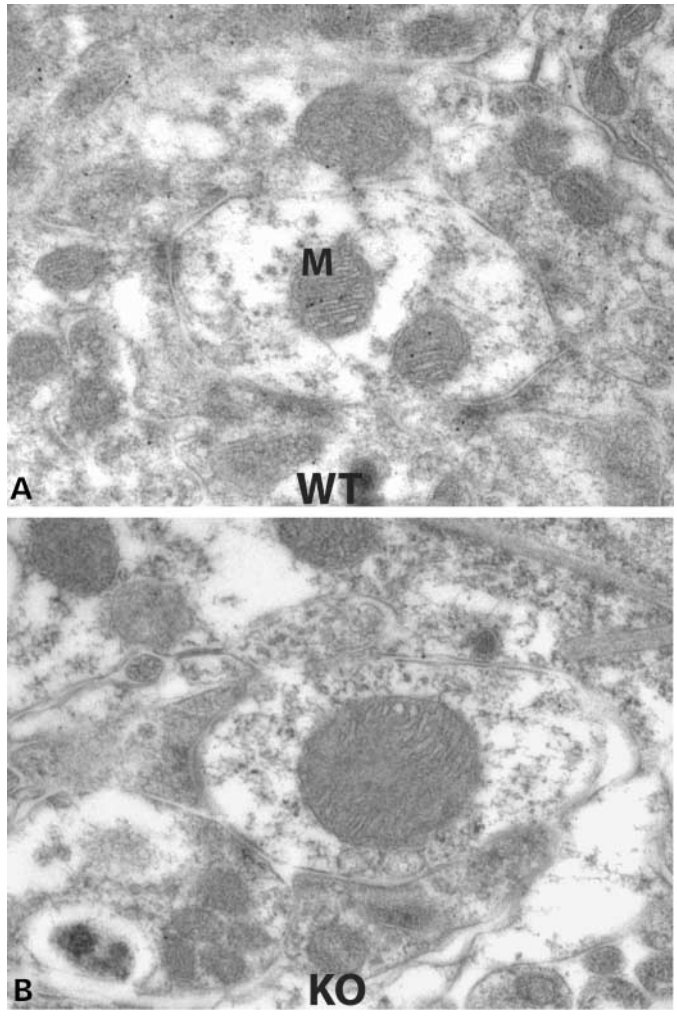

Figure 6. DJ-1 immunogold labeling in mouse SN (pars reticulata). Ultrathin Lowicryl sections from DJ-1 WT (**A**) or KO (**B**) mouse SN were subjected to immunogold cytochemistry with anti-DJ-1-FL. The majority of gold particles were detected in DJ-1 WT but not in KO mouse mitochondria (M). The labeled mitochondria shown here were localized in major dendrites but immunopositive mitochondria also occurred in cell bodies and in small caliber processes, consistent with the neuropil staining demonstrated at the light microscope level.

that the DJ-1 KO mice are more sensitive to the toxic effects of MPTP (42). Loss of this protective effect of DJ-1 could render mitochondria and cells more susceptible to free radical injuries. The accumulation of free radical injuries and oxidative stress damage might eventually result in an initiation of an irreversible cell death process. This is particularly important in light of the evidence that dopamine and its metabolite can generate ROS and enhance oxidative stress.

In *in vitro* studies over-expressing a tagged DJ-1, investigators report that oxidizing conditions favor both WT and pathogenic mutant DJ-1 relocalization from cytoplasm to the outer mitochondrial membrane (30,43) and oxidative stress induced relocalization of DJ-1 protein is required for its protective role against oxidative stress (30). However, as shown in our present studies, endogenous DJ-1 is localized to the mitochondrial matrix and IMS in addition to a cytosolic localization. Moreover, we fail to observe translocation of endogenous DJ-1 following oxidative stress. Thus, it is likely that translocation of DJ-1 is not required for its protective properties. Our failure to observe translocation may be due to methodological considerations, which requires further investigation to clarify the role, if any, for DJ-1 translocation

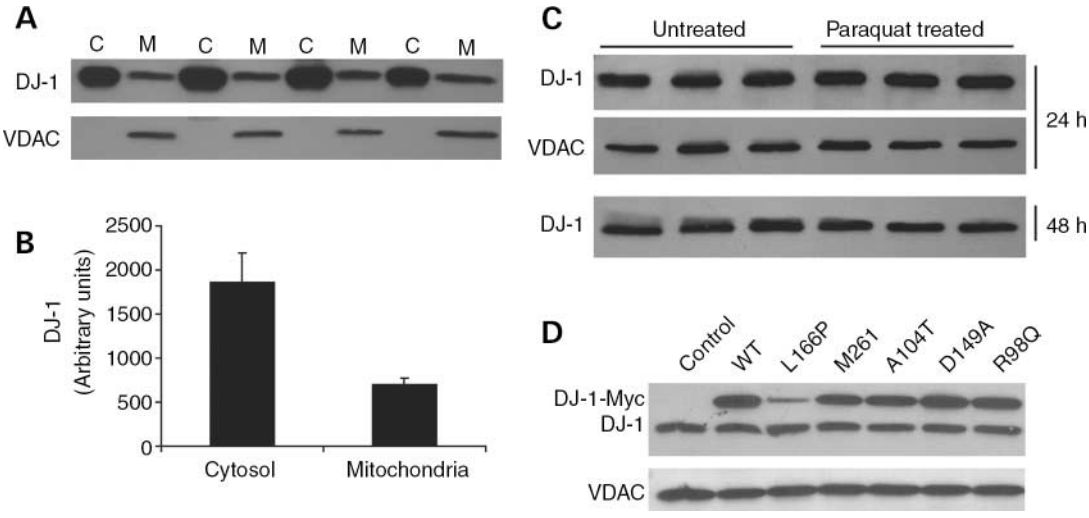

Figure 7. Distribution of WT and mutant DJ-1 in human neuroblastoma cells. (**A**) Cytosol and mitochondria prepared from human SH-SY5Y neurobalstoma cells were subjected to SDS–PAGE analysis with anti-DJ-1-N. The blot was also probed with an antibody to VDAC, a marker of mitochondria, yielding a band at the appropriate indicated molecular masses. (**B**) The levels of DJ-1 protein from (A) were quantified by densitometry analysis. The bar represents the mean ± SE of four independent experiments. (**C**) Mitochondria were prepared from untreated SH-SY5Y cells or cells treated with 100 μM of paraquat for 24 or 48 h. The proteins were resolved by SDS–PAGE, blotted to PVDF membranes and incubated with anti-DJ1-N. The blot was also probed with an antibody to VDAC for loading control. (**D**) Mitochondria were prepared from human SH-SY5Y neuroblastoma cells stably transfected with myc-tagged WT or mutant (L166P, M26I, A104T and D149A) DJ-1, a polymorphic variant of DJ-1 (R98Q) or control plasmid, subjected to SDS–PAGE analysis with anti-DJ1-N. The corresponding position of myc-tagged (DJ-1-Myc) or endogenous (DJ-1) DJ-1 protein is indicated. The blot was also probed with an antibody to VDAC.

in protection against oxidative stress. It is unclear which pool of DJ-1 protein contributes to protection against oxidative stress. On the basis of the central role of mitochondria in free radical generation and oxidative stress, it is possible that under physiological conditions, it is mainly the mitochondrial DJ-1 that helps to control the level of free radicals in mitochondria.

In summary, the localization of DJ-1 in mitochondria and the potential function of DJ-1 as an antioxidant imply that DJ-1 might play a role in pathologic processes involving oxidative stress. Increased oxidative stress is a common feature of PD and might be a link between sporadic PD and familial PD due to DJ-1 mutations (44). As evidence from this study shows that the pathogenic DJ-1 mutations (L166P, M26I, A104T or D149A) do not alter the distribution of DJ-1 to mitochondria, further studies are needed to directly test the antioxidant actions of DJ-1 and its mutants and to investigate the antioxidant capacity of mitochondria from DJ-1 null mice and in tissue from familial PD patients due to DJ-1 mutations. The fact that DJ-1 is widely distributed in neurons and cells in the periphery and that DJ-1 is also present in other subcellular fractions other than mitochondria suggests that it is also important to investigate other functions of DJ-1 throughout the body.

MATERIALS AND METHODS

Generation and characterization of DJ-1 antibodies

N-terminal (anti-DJ-1-N) and C-terminal (anti-DJ-1-C) DJ-1 antibodies were generated as described previously (28). These two antibodies recognize both human and mouse DJ-1 protein. To generate an antibody to full-length DJ-1 protein, the cDNA encoding the full-length open reading frame of mouse *DJ-1* gene was subcloned in frame with a glutathione *S*-transferase (GST) gene in a pGEX-6P vector (Amersham Biosciences, Piscataway, NJ, USA). The recombinant GST–DJ-1 fusion protein was then expressed in *Escherichia coli* BL21 cells and purified through a glutathione agarose column. After digestion by PreScission™ protease (Amersham) to remove the GST tag, the untagged DJ-1 protein was then injected into a rabbit to generate antibodies. Full-length DJ-1 antibody (anti-DJ-1-FL) was then affinity-purified from crude rabbit serum.

A DJ-1 KO mouse was generated by deletion of partial exon 2 and complete exon 3 of *DJ-1* gene (L. Zhang, M. Sasaki, T.M. Dawson and V.L. Dawson, unpublished data). To test the specificity of anti-DJ-1 antibodies, proteins from either WT or DJ-1 KO mouse brain tissue were resolved by 15% SDS–PAGE and blotted to a PVDF membrane. The membrane was probed with rabbit polyclonal anti-DJ-1-N, anti-DJ-1-C or anti-DJ-1-FL at 4°C for overnight. The membrane was then probed with a horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG antibody (Pierce Biotechnology, Rockford, IL, USA) at room temperature (RT) for 1 h. The signals were detected with enhanced chemiluminescence method (Pierce). The membrane was re-probed with a mouse monoclonal anti-actin antibody (Sigma, St Louis, MO, USA) for loading control.

Cell lines and transfections

Human SH-SY5Y neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin and incubated at 37°C in a 5% CO_2 atmosphere. Cells were

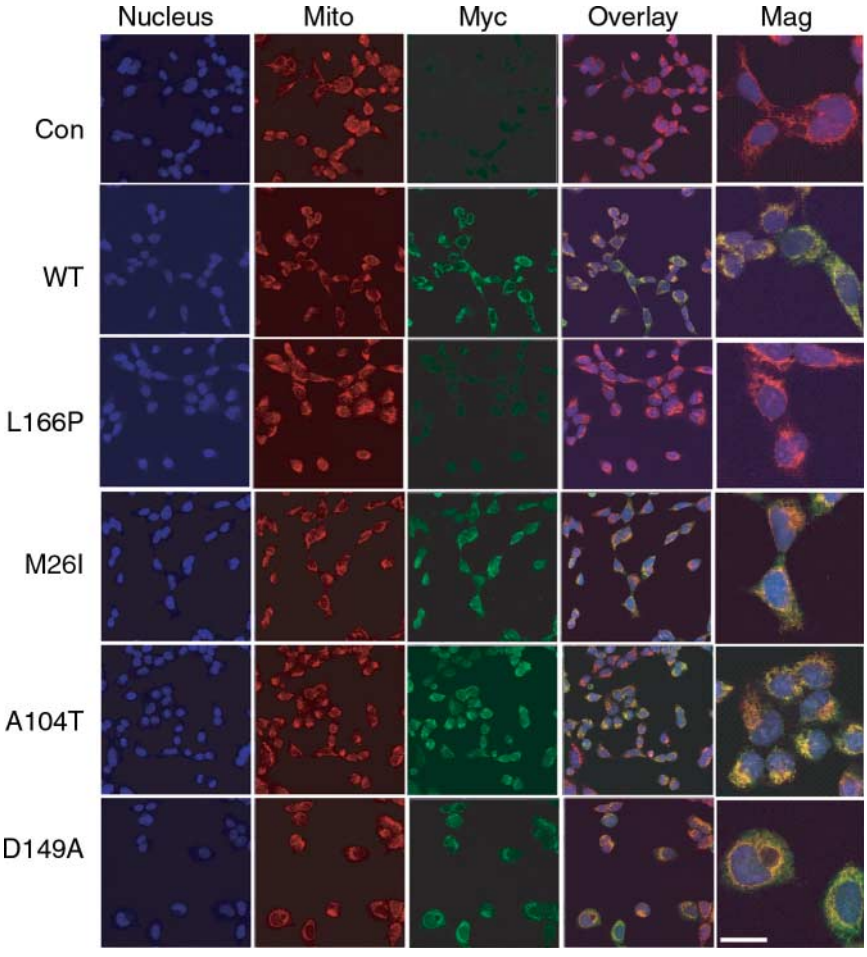

Figure 8. Co-localization of WT and mutant DJ-1 with MitoTracker in stably tranfected human neuroblastoma cells. Human SH-SY5Y neuroblastoma cells stably transfected with myc-tagged WT or mutant (L166P, M26I, A104T and D149A) DJ-1 or control plasmid (Con) were stained with anti-Myc antibody (Green), MitoTracker (Red) and TOTO-3 (Blue). Both WT and mutant DJ-1 immunoreactivity overlaps with MitoTracker fluorescence. Bar, 13 μm for higher magnification views (Mag) and 40 μm for the others.

stably transfected with C-terminal myc-tagged WT or mutant (L166P, M26I, A104T or D149A) human DJ-1 or control plasmid, by using Lipofectamine 2000 transfection reagent (Invitrogen). The stably transfected cells were then maintained in the medium containing G418 (Invitrogen).

Regional tissue distribution

To analyze the distribution of DJ-1 protein, various tissues, including cerebral cortex (CTX), SN, striatum, cerebellum, heart, lung, liver, kidney, adrenal grand, spleen, SM, ovary, testes and adipose tissue from adult male and female C57BL6 mice were collected for western blot analysis. Each tissue sample was homogenized in cell lysis buffer [10 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 × complete mini protease inhibitor cocktail (Roche, IN, USA), pH 7.4]. Total protein in the lysates was quantified by the BCA kit (Pierce) with bovine serum albumin (BSA) standards. About 25 μg of protein from each tissue sample was resolved by SDS–PAGE and subjected to western blot analysis with rabbit polyclonal anti-DJ-1-FL (1:2000), mouse monoclonal anti-parkin (1:250), mouse monoclonal anti-α-synuclein (1:2000, BD Biosciences, Palo Alto, CA, USA) or rabbit polyclonal anti-superoxide dismutase-1 (SOD-1) (1:2000, kindly provided by D. Borchelt, Johns Hopkins University, USA) at 4°C overnight. The membrane was then probed with a HRP conjugated goat anti-rabbit IgG (Pierce) or a goat anti-mouse IgG (Sigma) antibody at RT for 1 h. The signals were detected with enhanced chemiluminescence method (Pierce).

Mitochondrial and nuclear fractions

For the subcellular fractionation assay, cerebral cortex or striatum from eight adult C57BL6 mice were pooled and processed. Subcellular fractioning of mouse brain tissue was performed as described (31,45). Proteins from each fraction were resolved by SDS-gradient gel (4–20%) and subjected to western blot analysis with anti-DJ-1-C, anti-parkin, anti-α-synuclein or anti-synaptophysin-I (1:2000, Sigma) antibodies.

Isolation of intact mitochondria from brain tissues was performed as described (46). To isolate intact nuclei, brain tissues dissected from 10 adult C57BL6 mice were subjected to

differential centrifugations. After centrifugation at 2000*g* at 4°C for 3 min, the supernatant was discarded and the pellet was resuspended in 1 ml SEE media (250 mM sucrose; 1 mg/ml BSA; 0.5 mM EDTA; 0.5 mM EGTA; 10 mM HEPES, pH 7.4) and mixed with 4 ml sucrose buffer II [2 M sucrose; 5 mM magnesium acetate; 0.1 mM EDTA; 1 mM dithiothreitol (DTT); 10 mM Tris–HCl, pH 8.0]. The sample was then layered onto 4.4 ml sucrose buffer II, topped off with sucrose buffer I (0.32 M sucrose; 3 mM $CaCl_2$; 2 mM magnesium acetate; 0.1 mM EDTA; 1 mM DTT; 0.5% NP-40; 10 mM Tris–HCl, pH 8.0) and ultracentrifuged at 30 000*g* at 4°C for 45 min. After centrifugation, the supernatant was removed and the nuclei pellet was resuspended with glycerol storage buffer (5 mM $MgCl_2$; 0.1 mM EDTA; 50% glycerol; 0.1 mM PMSF; 50 mM Tris–HCl, pH 8.0). Proteins from mitochondria and nucleus were then subjected to western blot analysis with anti-DJ-1-FL, rabbit polyclonal anti-superoxide dismutase-1 or sheep polyclonal anti-human histone (1:100, USBiological, Swampscott, MA, USA) antibodies.

Further sub-fractionations of mouse brain mitochondria were prepared as described (47) with minor modifications. In brief, mouse whole brain was removed and homogenized in buffer A (300 mM sucrose, 0.1 mM EGTA, 10 mM HEPES, pH 7.4) with a glass Potter-Elvehjem homogenizer and a PTFE pestle. The homogenate was then centrifuged at 600*g* for 5 min. The supernatant was collected and filtered through four layers of cheesecloth and centrifuged at 3300*g* for 10 min. The pellet was suspended in buffer B (10 mM KH_2PO_4, pH 7.4) and the mixture was shaken at 4°C for 15 min. Equal volume of buffer C [32% (w/v) sucrose, 30% glycerol (v/v), 10 mM $MgCl_2$, 10 mM KH_2PO_4, pH 7.4] was added to the mixture and shaken for another 15 min. The mixture was then centrifuged at 12 000*g* for 10 min and supernatant (S1) and pellet (P1) were collected separately. P1 was resuspended in 3 ml of buffer B, shaken at 4°C for 30 min, and subjected to ultracentrifugation at 160 000*g* for 30 min. The supernatant (S2) and pellet (P2) were collected. S2 was the fraction of mitochondrial matrix (Mx), and P2 mitochondria IM. S1 was also subjected to ultracentrifugation at 160 000*g* for 30 min, and the resulting supernatant (S3) and pellet (P3) were fractions containing mitochondria IMS and mitochondria OM, respectively. Protein was then subjected to western blot analysis with antibodies to DJ-1 (anti-DJ-1-C) and markers for different mitochondrial subfractions, i.e. VDAC (EMD Biosciences, San Diego, CA, USA) for mitochondria OM, cytochrome *c* oxidase subunit IV (COX IV) (Molecular Probes, Eugene, OR, USA) for mitochondrial inner membrane, HSP-60 (Stressgen, San Diego, CA, USA) for mitochondrial matrix and cytochrome *C* (Cyt-C) (BD Pharmingen, San Diego, CA, USA) for mitochondrial IMS.

To investigate mitochondrial distribution of WT and mutant DJ-1 in cells, cytosol and mitochondria were prepared, as previously described (48), from human SH-SY5Y neuroblastoma cells treated with 100 μM of paraquat (Sigma) for 24 or 48 h, and from SH-SY5Y cells stably transfected with myc-tagged WT or mutant (L166P, M26I, A104T or D149A) DJ-1 or control plasmid. Proteins were then resolved by SDS–PAGE, blotted to PVDF membranes and incubated with anti-DJ-1-N and VDAC. Quantitation of protein expression was performed using densitometry analysis software (AlphaImager, Alpha Innotech Corp., San Leandro, CA, USA).

Immunocytochemistry

For immunostaining and light microscopy studies, mice were perfused with phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS. Brains were removed and post-fixed at 4°C overnight in the same fixative. Paraffin sections were prepared at 5 μm thickness following standard protocol. The sections were blocked with 4% goat serum in PBS and 0.2% Triton X-100, incubated with anti-DJ-1-FL (1:2000), followed by incubations with a biotin-conjugated polyclonal goat anti-rabbit antibody (1:1000, Jackson Immunoresearch Laboratories, West Grove, PA, USA), a HRP-conjugated avidin/biotin complex (Vector Laboratories, Burlingame, CA, USA) and a SigmaFast™ DAB Peroxidase Substrate (Sigma). Sections were dehydrated through graded ethanol, cleared in xylene and mounted on glass slides before coverslipping.

For post-embedding immunogold labeling, ultrathin Lowicryl HM20 sections of freeze-substituted specimens obtained from perfusion fixed mouse brain were mounted on formvar-coated grids and processed for immunogold cytochemistry as described (49). In brief, sections were immersed in a saturated solution of NaOH in absolute ethanol for 2–3 s and incubated at 27°C first for 10 min in 0.1% sodium borohydride and 50 mM glycine in 5 mM Tris buffer containing 0.3% NaCl and 0.1% Trition X-100 (TBNT), followed by incubation in 2% human serum albumin (HSA) in TBNT, and then for 12–16 h in mixtures of anti-DJ-1-FL in TBNT containing 2% HSA. Next, sections were placed in 2% HSA in TBNT for 10 min and, finally, in goat anti-rabbit Fab fragments coupled to 10 nM gold particles (GFAR10; British BioCell International, Cardiff, UK) diluted 1:20 in TBNT containing 2% HSA and polyethylene glycol (0.5 mg/ml) for 2 h. Sections were examined and photographed in a Tecnai electron microscope at 20 000 × primary magnification.

For double labeling and confocal microscopy studies, stably transfected human SH-SY5Y neuroblastoma cells were grown in DMEM containing 10% FBS and penicillin/streptomycin/G418 at 37°C in a 5% CO_2 atmosphere. Cells were incubated with 500 nM MitoTracker Orange CMTMRos (Molecular Probes) at 37°C for 45 min, washed briefly with ice-cold Tris-buffered saline (TBS), fixed with methanol at −20°C for 10 min, blocked with 4% goat serum in TBS at RT for 30 min and incubated with anti-DJ-1-FL (2% goat serum, 0.02% NaN_3 in TBS) at 4°C for overnight. The cells were then incubated with fluorescein (FITC)-conjugated goat anti-rabbit IgG (1:100, Jackson Immunoresearch Laboratories) at RT for 1 h and stained with dimeric cyanine nucleic acid stains (TOTO-3) (1:1000, Molecular Probes) for 10 min. The fluorescent signals were examined with a Zeiss confocal microscope with LSM 510 software system.

ACKNOWLEDGEMENTS

This work was supported by grants from the USPHS NS38377 and NS47565, Sylvia Nachlas Trust and the Lee Martin Trust.

L.Z. is a recipient of the Veola Kerr Postdoctoral Fellowship from the Parkinson's Disease Foundation. D.M. is the Herbert Freiberg Fellow in Parkinson's Disease. T.M.D. is the Leonard and Madlyn Abramson Professor in Neurodegenerative Diseases. R.T. and O.P.O. are supported by the Norwegian Research Council and FUGE.

Conflict of Interest statement. None declared.

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