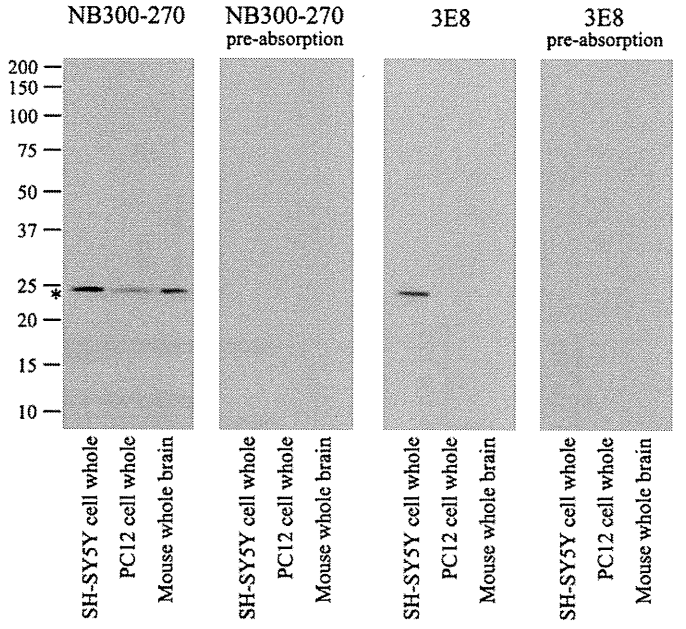


Fig. 1. Characterization of anti-DJ-1 antibodies. (A) Immunoblot of lysates from SH-SY5Y cells, PC12 cells, and mouse whole brain. Commercially available DJ-1 rabbit polyclonal antibody and mouse monoclonal antibody were used, as mentioned in Materials and methods. Specificities of these antibodies were confirmed by pre-absorption tests.

reacted membrane by centrifugation 5 times. PK-treated membranes were subjected to electrophoresis through Tris–HCl polyacrylamide gels (BIO-CRAFT) followed by staining with the GelCode SilverSNAP Stain Kit (Pierce).

In vitro binding assay by PC12 membranes

Recombinant DJ-1 WT, fused at its N terminus to the GST protein, or GST protein for negative control, were reacted with PC12 membranes, or PK-treated membranes (120 μl of PK beads concentration), at 30 °C for 1 h. The reacted samples were centrifuged at 100,000*g* for 1 h at 4 °C, and divided into supernatant and pellet. Both supernatant and pellet were subjected to SDS–PAGE followed by immunoblotting.

In vitro binding assay by LS1 fraction from DJ-1 KO mice

GST–DJ-1 WT recombinant protein (500 nM) or GST–DJ-1 mutant recombinant protein (M26I, A104T, D149A, and L166P) were combined with 200 μl of the LS1 fraction from DJ-1 KO mice ($n=3$), and rotated at 30 °C for 20 min. After treatment, the samples were centrifuged at 260,000*g* for 2 h at 4 °C. The supernatants were extracted and equal volumes of each fraction were subjected to immunoblot with anti-GST antibodies. The pellets were resuspended with the buffer (0.32 M sucrose-HEPES (pH 7.4) buffer) of equal volume, and equal volumes of each fraction were also subjected to immunoblot.

Immunoblotting

Cell lysates were mixed with LDS sample buffer (Invitrogen), heated for 5 min at 95 °C, and incubated on ice. The samples were resolved on 10–20% Tris–HCl gel (BIO CRAFT) in 1% SDS buffer and transferred onto polyvinylidene fluoride membranes (Bio-Rad Bioscience; Hercules, CA). The membranes were blocked for 1 h in TBS containing 0.1% Tween-20 (TBS-T) and 5% non-fat milk (BD Disco), and then incubated overnight at 4 °C with the primary antibody. The membranes were washed with TBS-T 3 times, followed by incubation for 1 h at RT with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG. Immunoreactivity (IR) was assessed by a chemiluminescence reaction using Western Lightning (Perkin Elmer-Cetus, Foster City, CA) or ECL Plus reagent (GE Health Care Bio-Sciences).

Results

Characterization of anti-DJ-1 antibodies

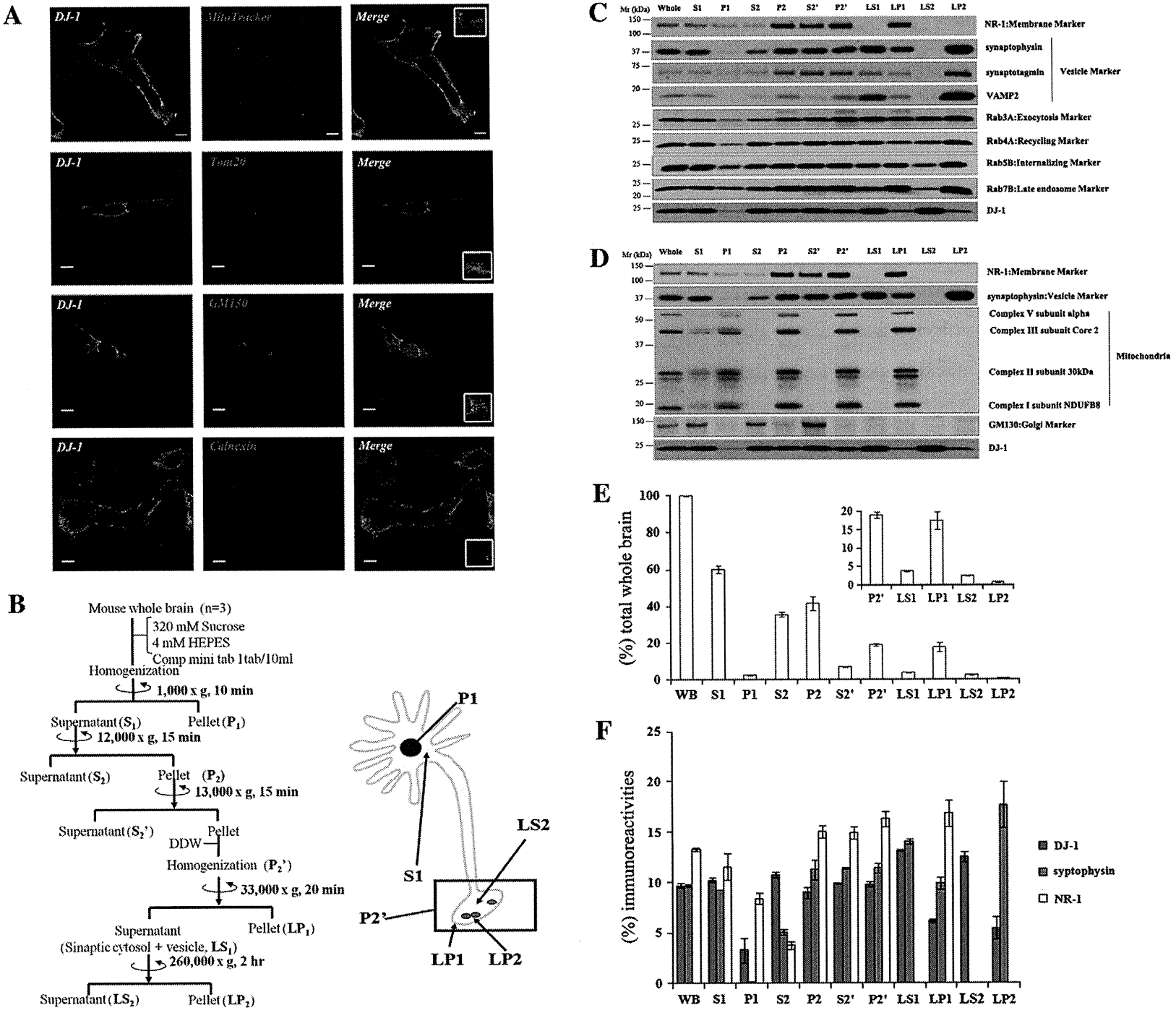
To determine the endogenous localization of DJ-1, we employed the use of the commercially available mouse monoclonal and rabbit polyclonal DJ-1 antibodies. Immunoblot analyses using NB300-270 antibody (a rabbit polyclonal antibody) revealed a single band around 22 kDa corresponding to endogenous DJ-1 in the extracts from SH-SY5Y cells, PC12 cells, and mouse brain (Fig. 1). The 3E8 antibody, a mouse monoclonal antibody, also recognized a single band corresponding to the human DJ-1 protein in SH-SY5Y cells. However, this antibody did not detect the rodent DJ-1 protein in PC12 cells and mouse brain (Fig. 1). The band corresponding to DJ-1 disappeared when the antibody was pre-incubated with an excess amount of the antigen, confirming the specificity of the antibody (Fig. 1). Based on these results, the 3E8 antibody, which specifically recognizes endogenous human DJ-1, was used for immunocytochemistry, and the polyclonal NB300-270 antibody, which recognizes the mouse and rodent DJ-1 protein, was used for immunoblotting and immunocytochemistry of primary cortical neuronal cells obtained from mouse brain.

DJ-1 diffusely distributes with main membranous organelles

To examine the subcellular localization of DJ-1, SH-SY5Y cells were double-stained with the DJ-1 antibody and organelle-specific antibodies. Microscopic observation revealed diffuse DJ-1 immunostaining and the protein partly colocalized with GM130, a marker for Golgi apparatus. A small portion of DJ-1 colocalized with Mito Tracker and Tom20, both mitochondrial markers, and calnexin, an ER marker (Fig. 2A).

Based on the immunocytochemical data showing diffuse distribution of DJ-1 in cultured cells, we investigated the precise localization of DJ-1 using biochemical methods. To elucidate DJ-1 distribution in neuronal cells, mouse brain samples were fractionated by differential centrifugation and the fractions were analyzed for the presence of DJ-1 by immunoblotting (Fig. 2B). DJ-1 was present at considerable levels in the synaptosomes (P2′), which consisted of synaptic terminals including synaptic plasma membranes (LP1) and synaptic vesicles (LP2), and co-fractionated with synaptophysin, synaptotagmin, and

Fig. 2. DJ-1 was widely distributed with the main membranous organelles and synaptosomes. (A) SH-SY5Y cells were double-stained with antibodies to DJ-1 (green) and Mito Tracker, Tom20 (mitochondria), GM130 (Golgi apparatus), or calnexin (ER). Scale bars = 10 μm. (B) The experimental design of the synaptosome preparation is shown. (C) Subcellular fractionation of the mouse brain is described in Materials and methods. Aliquots of the subcellular fractions, containing 5 μg of protein, were analyzed by immunoblotting. NR-1 (membrane marker) was recognized in the LP1 fraction, and synaptophysin, synaptotagmin, and VAMP2 (vesicle marker) were detected in the LP2 fraction. Rab3A, Rab4A, Rab5B and Rab7B were widely concentrated in various subcellular fractions. DJ-1 was found in various fractions in conjunction with the Rab proteins. (D) Complex I–V (mitochondria), and GM130 (Golgi apparatus) organelle markers were investigated. Mitochondria were present in the P2′ and the LP1 fractions, but mitochondria were barely evident in the synaptic fraction. The Golgi fraction did appear in the cytosolic fraction (S2). (E) The amount of each fraction was quantified and graphed as a percentage for the estimated amount of whole brain protein. Data were the average ± SD of three independent experiments. (F) Using the results from panel C, immunoreactivity (IR) of each fraction was quantified and graphed as a percentage of each IR to the total immunoreactivities in DJ-1. Synaptophysin and NR-1 were compared with DJ-1 as well.



VAMP2 (Fig. 2C). To further characterize the distribution of DJ-1 within synaptosomes, we investigated members of the family of monomeric GTPases called Rab proteins, such as Rab3A (exocytosis marker), Rab4A (recycling marker), Rab5B (endosome marker), and Rab7B (late endosome marker). As shown in Fig. 2B, these Rab proteins co-fractionated with DJ-1. However, mitochondrial respiratory complex proteins (Complex I subunit NDUFB8, Complex II subunit 30 kDa, Complex III subunit Core 2, and ATP synthase (Complex V) subunit α), which are mitochondrial markers, and the Golgi apparatus protein GM130, were not concentrated in the synaptic vesicle fraction (LP2) (Fig. 2D). The amount of each fraction was quantified and expressed as a percentage for the estimated amount of whole brain protein. The percentage of the P1 fraction was $2.69 \pm 0.20\%$, and DJ-1 was present in the nucleus, even though it was small. The percentages of P2′, LS1, LP1, LS2, and LP2, were $19.07 \pm 0.80\%$, $3.71 \pm 0.08\%$, $17.58 \pm 2.36\%$, $2.46 \pm 0.11\%$, and $0.75 \pm 0.19\%$, respectively (Fig. 2E). The amount of protein in the LP2 fraction was much less than that of the whole brain. DJ-1 IR of each fraction was quantified and shown as a percentage of each IR to total immunoreactivities. The percentage of DJ-1 IR of each fraction was $9.82 \pm 0.22\%$ (P2′), $13.19 \pm 0.07\%$ (LS1), $6.18 \pm 0.20\%$ (LP1), $12.54 \pm 0.50\%$ (LS2), and $5.43 \pm 1.08\%$ (LP2) (Fig. 2F).

DJ-1 localized on synaptic vesicles associated with synaptophysin and Rab3A

DJ-1 distributed with synaptic vesicles in the mouse brain (Fig. 2C). To elucidate the vesicle localization of DJ-1, the LS1 fraction containing synaptic vesicles and cytosol from mouse brain was further fractionated by sucrose density gradients centrifugation. Synaptophysin, VAMP2, synaptotagmin, and several Rab proteins were seen in fractions 9–18, and therefore, synaptic vesicles were collected in these fractions (Fig. 3A, B). Otherwise, the immunoreactivities of the synaptic vesicle markers were absent in fractions 1–8, suggesting that they were cytosolic fractions. The distribution of DJ-1 displayed biphasic peaks of both cytosolic fractions (fractions 1–8) and vesicle fractions (fractions 12–14). Coincidentally, the peak of DJ-1 IR agreed with the latter peak of synaptophysin and Rab3A (Fig. 3A, B). To further investigate the colocalization between DJ-1 and synaptic vesicles in neurons, primary cortical neuronal cells obtained from mouse brain were double-stained for DJ-1, and for synaptophysin or Rab3A. DJ-1 immunostaining appeared as punctate structures in the cytosol, axon, and synaptic terminals. DJ-1 was found to partly colocalize with synaptophysin and Rab3A, which play important roles in exocytosis (Edelmann et al., 1995; Handley et al., 2007) (Fig. 3C).

To gain further insight into the vesicle localization of DJ-1, immunoisolation was performed, as previously described (Burre et al., 2007; Morciano et al., 2005), with the LS1 fraction containing synaptic cytosol and vesicles from the mouse brain (Fig. 4A). To remove the nonspecifically interacting material, the LS1 fraction was treated with antibody-linked magnetic beads (Dyna-beads), which are cross-linked with normal rabbit or mouse IgG, and then the beads were removed. It was confirmed that DJ-1 and synaptophysin were not lost under this condition (Fig. 4B). Pre-cleaned LS1 was incubated with the Dyna-beads cross-linked with the DJ-1 antibodies, and then the vesicle isolates containing DJ-1 were subjected to immunoblotting with the DJ-1 antibody. Interestingly, synaptophysin and VAMP2 also localized with the DJ-1-associated vesicles (Fig. 4C). HSP70, which is known as a nuclear and cytosolic protein (Daugaard et al., 2007), was not isolated by this procedure (Fig. 4C). This indicates that the synaptic vesicle fraction was not contaminated with the cytosolic fraction. Therefore, this suggests that DJ-1, synaptophysin, and VAMP2 might localize on the surface of the same vesicle. In addition, it was further investigated whether DJ-1 directly interacts with synaptophysin and/or VAMP2. The LS1 fraction treated with RIPA buffer was immunoprecipitated with pull-down beads cross-linked

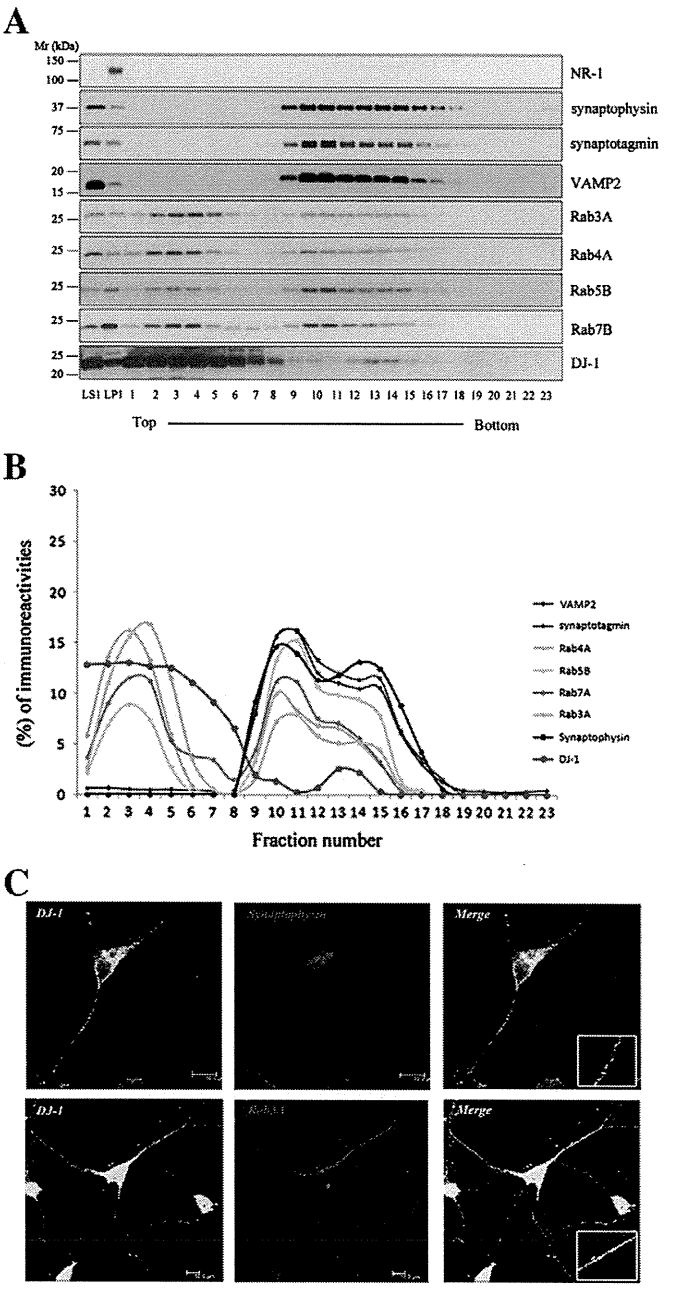


Fig. 3. DJ-1 associates with synaptic vesicles and colocalizes with synaptophysin and Rab3A. (A) The LS1 fraction was layered on top of a linear sucrose density gradient ranging from 0.2–2.0 M sucrose dissolved in HEPES buffer. Fractions were collected and 15 μl of each fraction were subjected to SDS–PAGE followed by immunoblotting using various markers. (B) Using the results from panel A, IR of each fraction was quantified and graphed as a percentage of each IR to the total immunoreactivities in each marker. DJ-1 had a biphasic profile of the immunoreactivities in fractions 1–8 and fractions 12–14, which indicated that there was some cytosolic fraction and some vesicle fractions. The peak of DJ-1 IR was in agreement with the latter peak of synaptophysin and Rab3A. (C) Primary cortical neurons from the mouse brain were fixed, permeabilized, and immunostained with DJ-1 antibody, and double-stained for synaptophysin and Rab3A. DJ-1 overlapped with synaptophysin and Rab3A. Scale bars = 10 μm.

with the synaptophysin antibody. It was found that VAMP2 interacts with synaptophysin as previous studies had reported (Baumert et al., 1989; Edelmann et al., 1995; Trimble et al., 1988). Immunoblotting with DJ-1 antibodies did not reveal endogenous DJ-1 in the resultant immunoprecipitates (Fig. 4C), whereas, endogenous synaptophysin and VAMP2 were not immunoprecipitated with the DJ-1 antibody.

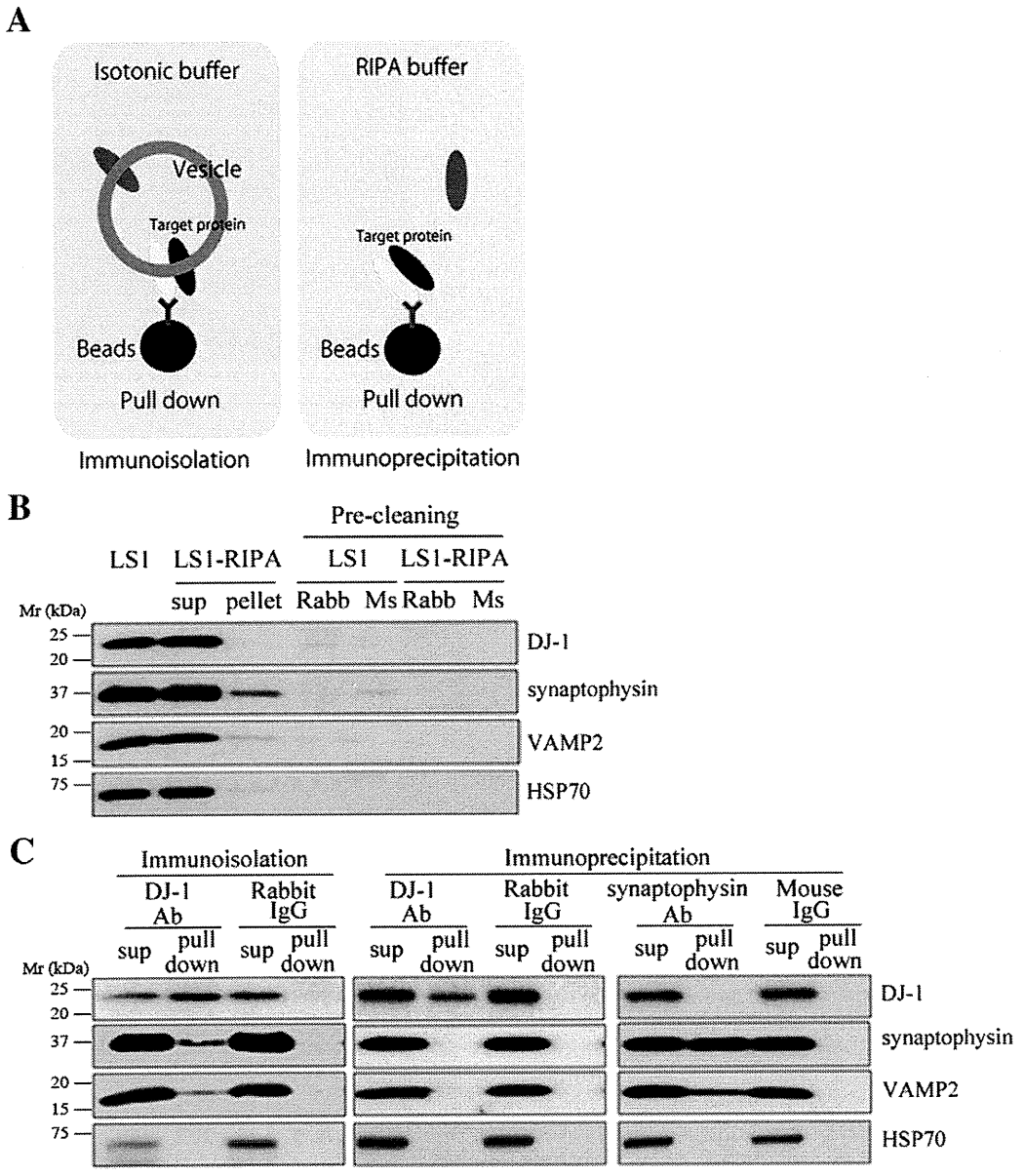


Fig. 4. DJ-1 cannot directly interact with synaptophysin and VAMP2, but associates with the same synaptic vesicles. (A) Image of immunoisolation and immunoprecipitation. Immunoisolation was used to pull down the target protein from the subcellular organelles (synaptic vesicle) from the homogenate, which was reacted with the isotonic buffer. Meanwhile, immunoprecipitation was used to pull down the target protein from the homogenate, which reacted with the buffer containing the detergent in order to examine the direct interactions between proteins. These methods were then used to assess the protein localized on the subcellular organelles with the target protein. (B) The LS1 fraction was first pre-cleaned. To remove nonspecifically-binding material, the LS1 fraction was treated with Dyna-beads cross-linked with normal rabbit or mouse IgG. It was confirmed that the targeting proteins were not lost in this reaction. (C) Sucrose buffer or RIPA buffer extracts of the mouse brain synaptic vesicle fractions were immunoisolated or immunoprecipitated using Dyna-beads coated with each antibody. Immunoisolates, immunoprecipitates and their corresponding supernatants were subjected to SDS–PAGE followed by immunoblotting using antibodies against the indicated proteins. Synaptophysin and VAMP2 were immunoisolated using Dyna-beads coated with the DJ-1 antibody, but they were not immunoprecipitated with the same bead slurry. Sup, supernatant.

Consequently, this proves that DJ-1 cannot directly interact with synaptophysin and VAMP2, but colocalizes with them on the same vesicles.

FRET analyses were performed to examine whether DJ-1 interacts with synaptophysin. We confirmed that FRET occurred between CFP–VAMP2, considered as positive control and synaptophysin-YFP (Pennuto et al., 2002). However, FRET was detected only in a small proportion of HeLa cells expressing CFP–DJ-1 and synaptophysin-YFP (Fig. 5A). $FRET_C$ median values with CFP–VAMP2, CFP–DJ-1, and CFP alone for more than 20 cells, were expressed as 0.363, 0.0413, and 0.0163, respectively (Fig. 5B). 293F cells expressing CFP–VAMP2 or CFP–DJ-1 and synaptophysin-YFP were also subjected to fluorescence lifetime flow cytometry, and fluorescence lifetimes of more than 10,000 cells in each sample were measured. Again, FRET efficiency observed between DJ-1 and synaptophysin was substantially lower than that between VAMP2 and synaptophysin, but significantly higher than that of the control (Fig. 5C). Confocal microscopic analyses revealed that CFP–DJ-1 also merged with synaptophysin-YFP. This pattern is similar to the colocalization between CFP–VAMP2 and synaptophysin-YFP (Fig. 5D, E). These results indicate that DJ-1 is able to localize with synaptophysin-positive vesicles and may interact with synaptophysin in living cells.

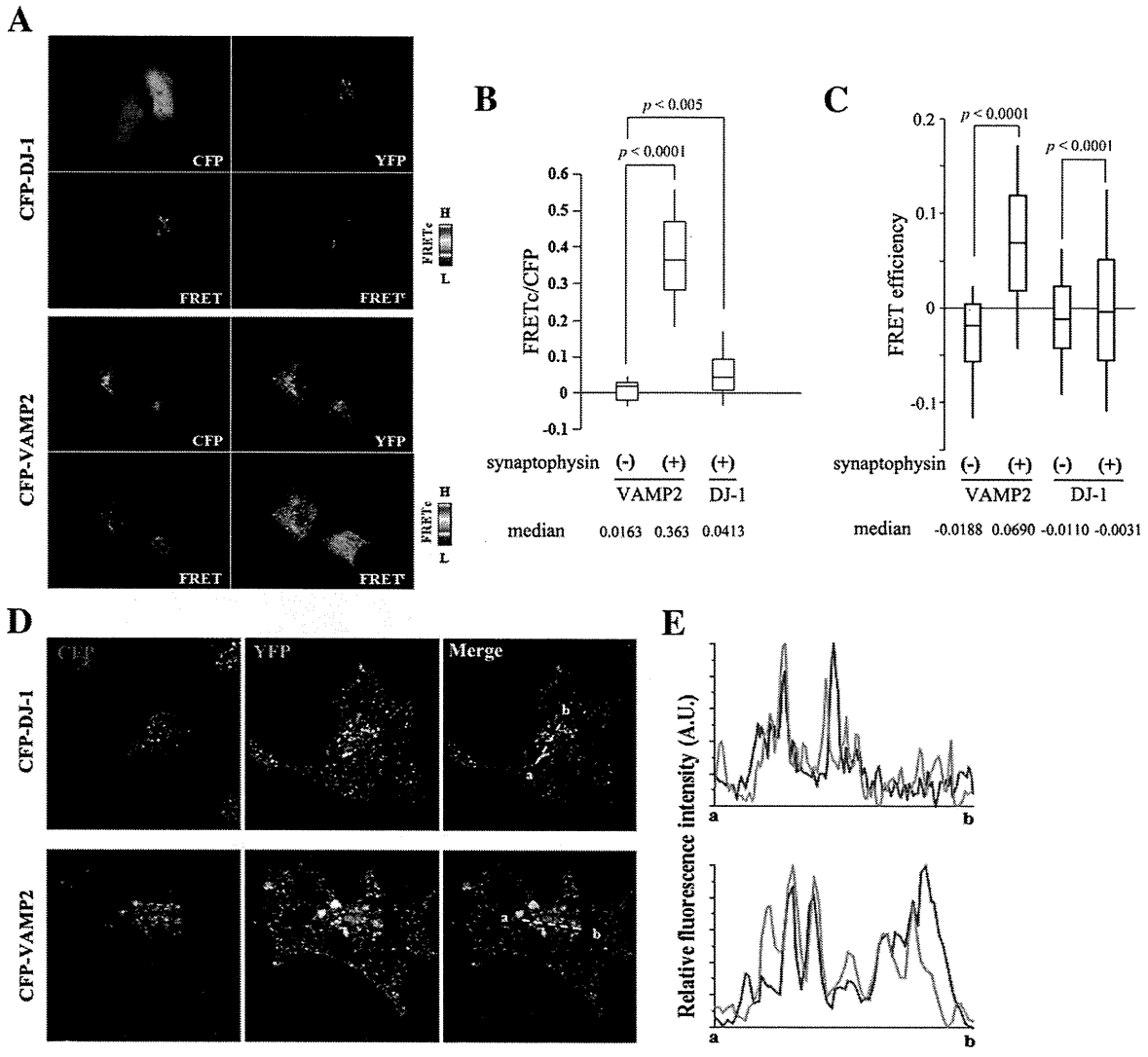


Fig. 5. FRET occurred in HeLa cells expressing CFP–DJ-1 and synaptophysin-YFP. (A) HeLa cells expressing CFP–DJ-1 or CFP–VAMP2, and synaptophysin-YFP were subjected to microscopic analysis as described in Materials and methods, and representative images are shown. FRET was detected in HeLa cells expressing CFP–VAMP2 and synaptophysin-YFP. FRET occurred in a small proportion of HeLa cells expressing CFP–DJ-1 and synaptophysin-YFP. (B) $FRET_C$ values calculated for each cells were plotted in the box and whisker plot. Representative data from three independent experiments are shown. The highest and lowest boundaries of the box represent the 25th and 75th percentiles, respectively, and whiskers above and below the box designate the 10th and 90th percentiles, respectively; the line within the box indicates the median value. (C) 293F cells expressing CFP–DJ-1 or CFP–VAMP2 and synaptophysin-YFP were subjected to fluorescence lifetime flow cytometry as described in Materials and methods. Fluorescence lifetimes of more than 10,000 cells in every sample were plotted in the box and whisker plot, where the highest and lowest boundaries of the box represent the 25th and 75th percentiles, respectively, and whiskers above and below the box designate the 10th and 90th percentiles, respectively; the line within the box indicates the median value. (D) Cells were imaged on a confocal laser microscope and representative images are shown. In a small proportion of cells CFP–DJ-1 merged with synaptophysin-YFP. CFP–VAMP2 colocalized with synaptophysin-YFP. (E) Fluorescence intensities of CFP (red) and YFP (green), along with the line in the merged image in (D), were plotted from a to b. Note that overlapping peaks indicate colocalization.

DJ-1 directly associates with membranes

The results from the immunocytochemical and biochemical experiments indicated that DJ-1 localizes in membranous structures, but it is unclear how DJ-1 associates with membranes. To address this issue, the effect of ionic strength on the association between DJ-1 with membranes was examined. PC12 cells were fractionated by centrifugation at 100,000*g* to pellets and supernatants, corresponding to membrane and cytosolic fractions, respectively. DJ-1 was collected in both the cytosol and the membranes. Although DJ-1 does not shift from the membrane to the cytosol regardless of high salt conditions, non-ionic detergent Triton X-100 solubilizes DJ-1 in a way similar to that of the transferrin receptor (Tfn-R) with a transmembrane domain (Fig. 6A). Meanwhile, parkin, which associates with lipid rafts (Fallon et al., 2002; Kubo et al., 2001), did not dissociate from the membrane by solubilization with Triton X-100 (Fig. 6A).

To characterize membrane-binding of DJ-1 protein, an in vitro binding assay using PC12 cells was employed as previously described (Kubo et al., 2005). In this assay, DJ-1 was found to be bound to purified plasma membranes. Treatment of plasma membranes purified from PC12 cells with Proteinase K (PK) for 60 min at 30 °C did not alter the localization of DJ-1 (Fig. 6C). We confirmed that digestion in PK for 60 min largely eliminated the protein as detected by silver staining (Fig. 6B).

L166P mutation affects membrane-binding ability

To investigate the pathogenicity of the mutant DJ-1 on membrane-binding ability, a membrane-binding assay was performed using the GST recombinant protein of wild type DJ-1 (GST–DJ-1 WT) and various pathogenic mutants. To eliminate the effects of endogenous DJ-1, DJ-1 knockout (KO) mice were used for this experiment. Synaptosomes from

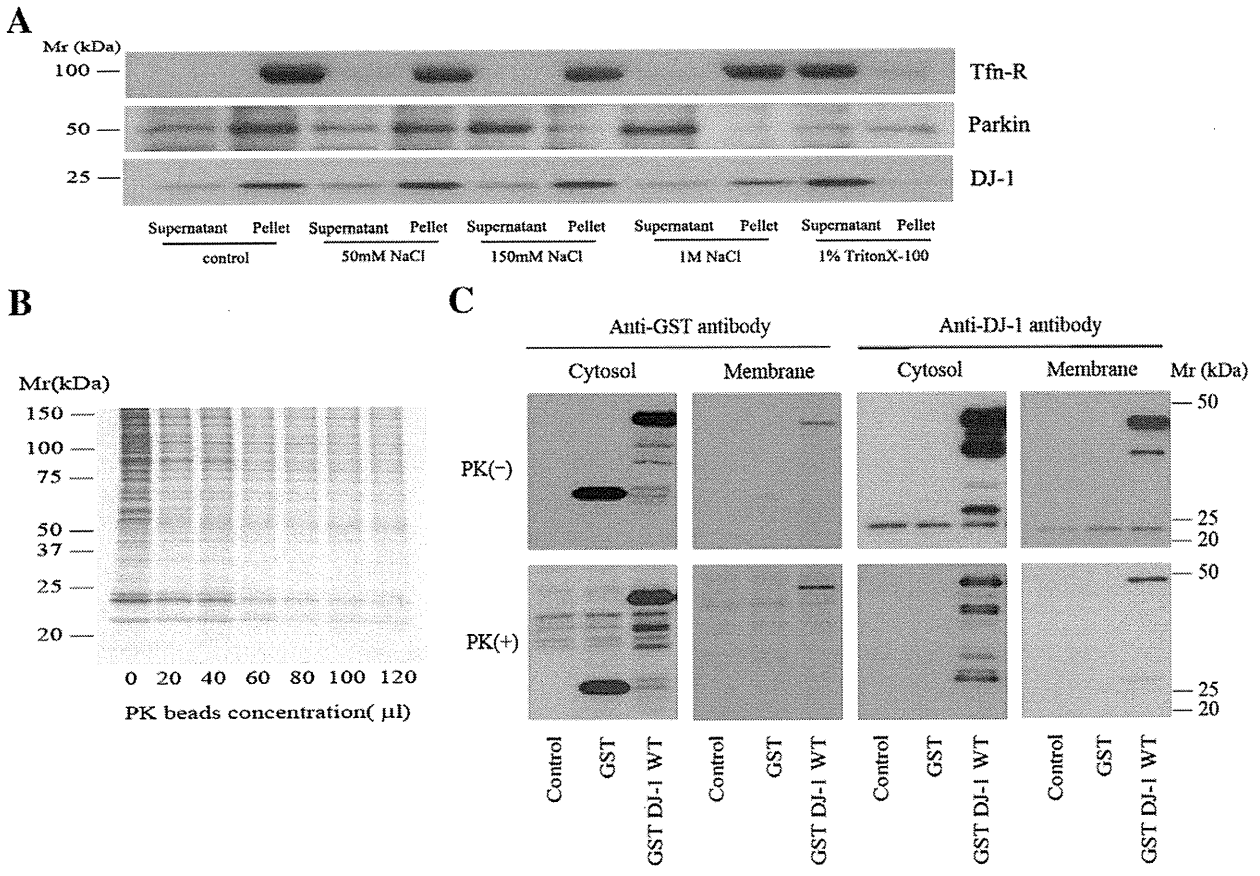


Fig. 6. Endogenous DJ-1 associates with cytosol and plasma membrane in PC12 cells. (A) Effects of various salt concentrations and non-ionic detergent on solubilization of DJ-1, Parkin, and transferrin receptor (Tfn-R). DJ-1 was concentrated in both the cytosol and membrane fractions of PC12 cells in the detergent-free isotonic buffer (control). DJ-1 did not shift from the membrane to the cytosol with increasing salt concentration, whereas Parkin relocalized from the membrane to cytosol, and Tfn-R remained in the pellet. However, DJ-1 did release from the membrane after being subjected to Triton X-100. Tfn-R was readily solubilized in this condition as well. Parkin remained in the pellet. Equal volumes of each of the fractions were loaded, followed by immunoblotting. (B) Silver staining of PC12 membranes treated with Proteinase K (PK) for 60 min at 30 °C showed a progressive loss of detectable membrane proteins with increasing PK concentration. (C) Recombinant DJ-1 wild type (WT), fused at its N terminus to the GST protein, was reacted with PC12 membranes or PK-treated membranes for 60 min at 30 °C. The GST-tagged protein, which served as a negative control, was also reacted. The reacted samples were centrifuged and divided into supernatant and pellet. Both supernatant and pellet were subjected to SDS–PAGE followed by immunoblotting. Anti-GST antibody detected the GST–DJ-1 WT recombinant protein band in the pellet fraction, whereas the GST-tagged protein was not detected in the pellet fraction. GST–DJ-1 WT recombinant protein directly associated with the plasma membrane in the in vitro assay.

DJ-1 KO mice were incubated with the GST–DJ-1 WT recombinant protein, or the GST–DJ-1 mutant recombinant proteins. Bound proteins were separated by centrifugation at 260,000*g* for 2 h. Compared with WT, the L166P mutant exhibited less binding to the synaptic membranes obtained from DJ-1 KO mice. However, there were no apparent differences between other pathogenic mutants and the WT in their membrane-binding property (Fig. 7A, B).

To further analyze the subcellular localization of various pathogenic DJ-1 mutants, HeLa cells were transfected with various DJ-1 mutants, as well as WT DJ-1 as control. M26I, A104T, and D149A showed diffuse and punctate distribution, similar to WT. By comparison, L166P exhibited localization near the plasma membrane (Fig. 7C).

Discussion

The overarching goal of this study was to determine the endogenous localization and membrane binding ability of DJ-1 and to elucidate potential differences in its properties between WT and pathogenic mutants. Immunocytochemistry for endogenous DJ-1 showed that the labeled structures distributed diffusely and displayed punctate staining. In the biochemical experiments, endogenous DJ-1 localized to the Golgi apparatus, cellular membranes, and synaptic vesicles which contain synaptophysin and Rab proteins. The GST–DJ-1 protein was found to be bound to cultured cellular membrane and mouse synaptosomes, as evidenced in the in vitro binding assay. Furthermore, this study shows that compared with WT, the L166P mutant exhibited less binding to the synaptic vesicles from DJ-1 KO mice.

Although several studies have reported on the mitochondrial localization of DJ-1 in cultured cells and mouse brains (Bonifati et al., 2003; Canet-Aviles et al., 2004; Miller et al., 2003; Zhang et al., 2005), Bandopadhyay et al. reported that they could not confirm the mitochondrial localization of endogenous DJ-1 in mouse primary astrocytes and hippocampal neurons (Bandopadhyay et al., 2004). Olzmann et al. described that DJ-1 localizes to the striatal axons and pre-synaptic terminals, suggesting a role for DJ-1 in dopaminergic neurotransmission (Olzmann et al., 2007). Zhang et al. also showed that DJ-1 was found in a synaptic-enriched fraction, however, they did not mention whether DJ-1 is associated with membrane trafficking (Zhang et al., 2005). In our experiments, DJ-1 partly localized to the synaptic cytosol, vesicles and membranes in the synaptic terminals of the mouse brain. However, a small portion of endogenous DJ-1 was located in mitochondria under steady state conditions, consistent with previous reports (Bandopadhyay et al., 2004; Nural et al., 2009). Thus, the present findings of endogenous DJ-1 localization provide evidence that DJ-1 may be associated with synaptic vesicles.

DJ-1 has the same distribution as members of the monomeric GTPases family called Rab proteins, known through biochemical

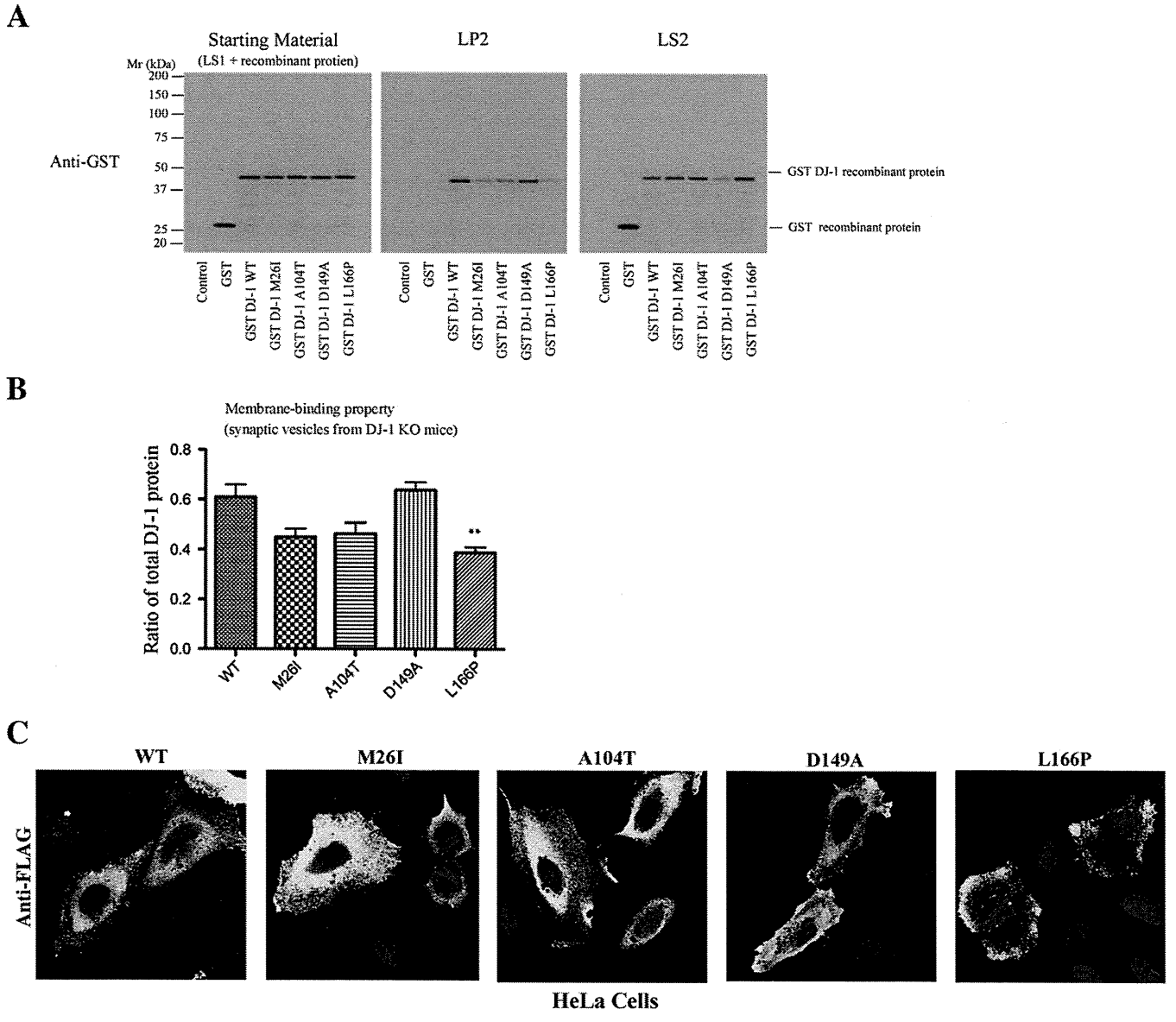


Fig. 7. Pathogenic DJ-1 mutants can bind membranes in the in vitro assay. (A) The LS1 fraction of DJ-1 KO mouse was reacted for 20 min at 30 °C with 500 nM GST recombinant protein, WT, or various mutants. Each of the bound proteins was divided into an LP2 fraction (synaptic vesicles) and an LS2 fraction (synaptic cytosol) by ultra-centrifugation at 260,000g for 2 h at 4 °C. The samples were subjected to SDS–PAGE followed by immunoblotting. The A104T, L166P, and M26I mutations also had lower bands corresponding to synaptic vesicles (LP2 fraction), compared with WT and D149A DJ-1. The GST-tagged protein, which served as a negative control was not detected. (B) Quantitative data from three independent experiments showed that the L166P mutant had reduced binding ability with the synaptic membrane. Immunoreactivity was quantified and expressed as percentage of bound (LP2) to total DJ-1 protein (LP2 + LS2). The data were plotted as the mean ± SEM. $^{**}P<0.001$ vs. WT, one-way ANOVA with Dunnett's Multiple Comparison Test. The other mutations were not statistically significant. The data were analyzed by GraphPad Prism (GraphPad Software, Inc.). (C) HeLa cells were transfected with expression vectors for FLAG-DJ-1 WT, M26I, A104T, D149A, or L166P. After 24 h, immunocytochemistry assay was performed on the cells. WT and the mutants, with the notable exception of the L166P mutant, appeared to have diffused subcellular distribution. WT was localized to the cytosol and in punctate spots. Similar results were obtained for mutants DJ-1, except for L166P. L166P localized near the plasma membrane.

studies as proteins associated with membrane trafficking (Harald Stenmark, 2001). DJ-1 may possibly associate with one and/or some of the Rab proteins. Actually, DJ-1 was found to partly colocalize with Rab3A by double-staining. Rab3A associates with immature secretory granules from the trans-Golgi network and has positive roles in exocytosis (Handley et al., 2007). Considering the colocalization between DJ-1 and Rab3A at synaptic terminals, DJ-1 could be involved in the vesicular trafficking system in such processes as exocytosis. Actually, DJ-1 KO mice exhibited altered synaptic functions, such as less sensitivity to the inhibitory effects of D2 auto receptor stimulation (Goldberg et al., 2005).

How can DJ-1 participate in synaptic vesicle transport? Corresponding with the results of the in vitro immunoisolation and immunoprecipitation assay, DJ-1 was found to not bind synaptophysin and VAMP2 directly, but DJ-1 localized with the synaptophysin and VAMP2-associated vesicles. In the in vivo FRET assay, a small portion of DJ-1 interacted with synaptophysin. Therefore, part of DJ-1 may be fairly close to synaptophysin and VAMP2. Neurotransmitter exocytosis involves sequential association of many synaptic proteins. Vesicular fusion, which is the central process of exocytosis, is mediated by the regulation of soluble N-ethylmaleimide sensitive factor (NSF) attachment protein receptor complexes, including VAMP2, syntaxin and synaptosome-associated protein 25 kDa (SNAP-25). These proteins interact with each other and play a critical role in the step between vesicle docking and fusion (Edelmann et al., 1995). Interestingly, several steps of vesicle fusion are regulated by molecular chaperones such as

NSF, 70 kDa heat-shock cognate protein, and cysteine-string protein (Zinsmaier and Bronk, 2001). DJ-1 is supposed to be a member of the DJ-1/YajL/PfpI superfamily, which function as molecular chaperones, and in RNA binding and hydrolase activity (Wei et al., 2007). Therefore, DJ-1 may participate in the regulation of neurotransmitter release as a molecular chaperone on synaptic vesicles.

From the results of the membrane-binding assay, DJ-1 was found to directly associate with membranes without an intermediary protein. Considering that the membrane binding of DJ-1 was not influenced by high-salt conditions, DJ-1 does not appear to associate with the membrane through electrostatic interactions such as ionic bonds, hydrogen bonds, and van der Waals attraction. Incubation with the non-ionic detergent Triton X resulted in release of DJ-1. This may mean that DJ-1 might prefer not to associate with lipid rafts, which are microdomains on membranes containing GM1 ganglioside, GPI anchor proteins, and several other membrane proteins (Edidin, 2003; Legler et al., 2005). Additionally, DJ-1 has no obvious amino acid sequences that serve as a targeting signal and transmembrane domains based on computer analysis (Kyte, 1982). Therefore, DJ-1 probably attaches to membranes through hydrophobic interactions.

Membrane proteins can bind to the lipid bilayer in various ways (Bruce Alberts, 2002; Lomize et al., 2007). In the proteins, peripheral membrane proteins temporarily adhere to the surface of the membrane. Some of them interact with membranes via an amphipathic α helix in the cytosolic monolayer (Bruce Alberts et al., 2002; Lomize et al., 2007). Based on crystal analyses, DJ-1 consists of a six-stranded parallel β-sheet sandwiched by eight α-helices and with a β-hairpin on one end and a three-stranded anti-parallel β-sheet on the opposite end (Anderson and Daggett, 2008; Wilson et al., 2003). Although the structure of DJ-1 is similar to that of a bacterial protein Pfp1, which is known as a cysteine protease, one major difference is the presence of an additional α-helix (helix αH) at the C terminus of DJ-1. The function of the helix αH is assumed to play a role in dimerization in combination with the helix αG (Honbou et al., 2003; Wilson et al., 2003). L166P is at the middle of the helix αG and is associated with significant structural deformations in this helix (Wilson et al., 2003). Additionally, the L166P mutant influences the membrane-binding property and disrupts the DJ-1 dimer (Anderson and Daggett, 2008). Therefore, we suspect that the α helices at the C terminus of DJ-1 are also able to function in membrane binding.

We also showed that the L166P mutant exhibits less binding to the synaptic vesicles from the DJ-1 KO mice compared with the WT, using the membrane-binding assay with the WT and various pathogenic mutations. Considering that the membrane-binding abilities of other mutations had no statistical difference with WT, it is presumed that the helix αG at the C terminus of DJ-1 associates with membrane binding. Actually, the results of the immunocytochemistry analysis of WT or mutants of DJ-1-overexpressing cells also revealed that the L166P mutant altered intracellular localization.

Based on our experiments, we believe that the association between DJ-1 and synaptic vesicles may contribute to the pathomechanisms in *PARK7*-linked PD. The previous studies have reported that α-synuclein, Parkin, and LRRK2 also localize to synaptic membranes and are associated with membrane trafficking (Abeliovich et al., 2000; Fallon et al., 2002; Hatano et al., 2007; Kahle et al., 2000; Kubo et al., 2001; Shin et al., 2008). Abnormality of membrane trafficking could be considered an important pathomechanism of PD as a common pathway. Further research may elucidate how DJ-1 associates with synaptic vesicles and why the loss of DJ-1 causes dopaminergic neuronal degeneration in PD.

Conclusions

This study is the first report showing the precise localization of endogenous DJ-1. We showed that DJ-1 colocalized with the Golgi apparatus proteins GM130 and the synaptic vesicle proteins synaptophysin and Rab3A. Although wild-type DJ-1 protein directly associated with membranes without an intermediary protein, the pathogenic L166P mutation of DJ-1 exhibited less binding to synaptic vesicles. Our findings indicate that DJ-1 associates with membranous organelles including synaptic membranes for its normal function.

Acknowledgments

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Review

Cell PRESS

Genetic mutations and functions of PINK1

Sumihiro Kawajiri, Shinji Saiki, Shigeto Sato and Nobutaka Hattori

Department of Neurology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan

Parkinson's disease (PD) is the second most common neurodegenerative disease. Mutations in *PINK1* (*PARK6*) are the second most frequent cause of autosomal recessive, young-onset PD, after *parkin* (*PARK2*). PINK1 (a kinase with an N-terminal mitochondrial targeting sequence) provides protection against mitochondrial dysfunction and regulates mitochondrial morphology via fission/fusion machinery. PINK1 also acts upstream of parkin (a cytosolic E3 ubiquitin ligase) in a common pathway. Recent studies have described PINK1/parkin function in the maintenance of mitochondrial quality via autophagy (mitophagy). PINK1/parkin-mediated mitophagy provides new insights into the etiology of PD and could be a suitable target for new treatment of PD. In this review, we discuss the molecular genetics and functions of PINK1, which could be key factors in novel rational therapy for sporadic PD as well as *PINK1*-linked PD.

Parkinson's disease (PD)

PD is the second most common neurodegenerative disease worldwide after Alzheimer's disease. The prevalence of PD increases with age, and is estimated to be ~1% in those aged > 65 years [1]. The major clinical features are: (i) motor symptoms (called 'parkinsonism'), which include resting tremor, rigidity, bradykinesia (slowness in executing movement) and postural instability; and (ii) non-motor symptoms (e.g. cognitive dysfunction, autonomic nervous system dysfunction, sleep disorders). Pathological features include pronounced loss of dopaminergic neurons in the substantia nigra pars compacta and eosinophilic cytoplasmic inclusion containing α-synuclein aggregates (known as Lewy bodies) in the remaining dopaminergic neurons. No treatment is available to suppress the progression of cell death, and the goal of current therapies is only to alleviate symptoms.

The pathogenesis of PD remains unclear, although mitochondrial dysfunction due to oxidative stress has been proposed to play a major part [2]. Most cases of PD are sporadic, but ~5–10% of PD cases are hereditary. Several genes (e.g. *α-synuclein, parkin, PTEN-induced putative kinase 1* (*PINK1*), *DJ-1, leucine rich repeat kinase 2* (*LRRK2*)) have been identified as causative genes for familial Parkinson's disease (FPD) [3]. *PINK1*-linked PD (*PARK6*-linked PD) is the second most common autosomal recessive young-onset PD, after *parkin*-linked PD (*PARK2*-linked PD). Initial studies suggested that PINK1 provided protection against mitochondrial dysfunction [4–6]. However, the exact function of PINK1 remains unclear. Recent evidence also suggests that PINK1 plays a part in mitochondrial quality control via autophagy machinery, in collaboration with parkin (a cytosolic E3 ligase). In this review, we analyze the recent work published on PINK1 function, which can be a key factor in novel rational therapy for sporadic PD as well as *PINK1*-linked PD.

Clinical characteristics of PINK1-linked PD

Clinical features

The clinical features of *PINK1*-linked PD include parkinsonism associated with a good response to levodopa (the precursor to the dopamine), frequent occurrence of levodopa-induced dyskinesias, and infrequent occurrence of dystonia at onset, similar to those of sporadic PD. The only distinctive features are the earlier age of onset and slower progression [7]. The age of onset of *PINK1*-linked PD is around the early thirties [8,9], whereas that of sporadic PD is after the age of 60 years[1]. Unlike *parkin*-linked PD, hyperreflexia and sleep benefit are not common in *PINK1*-linked PD. However, some patients with *PINK1*-linked PD exhibit foot dystonia at onset and sleep benefit, mimicking those with *parkin*-linked PD. Others with *PINK1*-linked PD show atypical clinical features associated with psychiatric problems and dementia, both of which are rare in patients with *parkin*-linked PD.

Pathological features

As mentioned above, accumulation of Lewy bodies is the pathological hallmark of sporadic PD. Lewy bodies were also detected in the brain of a *PINK1*-linked PD patient with compound heterozygous mutations (c.1252_1488 del and c.1488 + 1G > A) [10], whereas they are absent in the brains of *parkin*-linked PD patients [11,12]. Other pathological changes seen in the PD patient with the compound heterozygous *PINK1*-mutations include neuronal loss in the substantia nigra pars compacta accompanied with astrocytic gliosis and moderate microgliosis. No apparent cell loss, Lewy bodies, or abnormal neurites are seen in the locus ceruleus. However, pathological examination has been reported in only one case of *PINK1*-linked PD, so further pathological studies are needed to determine the association between Lewy bodies and the pathogenesis of *PINK1*-linked PD.

PINK1

Molecular structure

The *PINK1* gene contains 8 exons spanning ~1.8 kilobases and encodes a 581–amino acid protein. The transcript is

Corresponding author: Hattori, N. (nhattori@juntendo.ac.jp).

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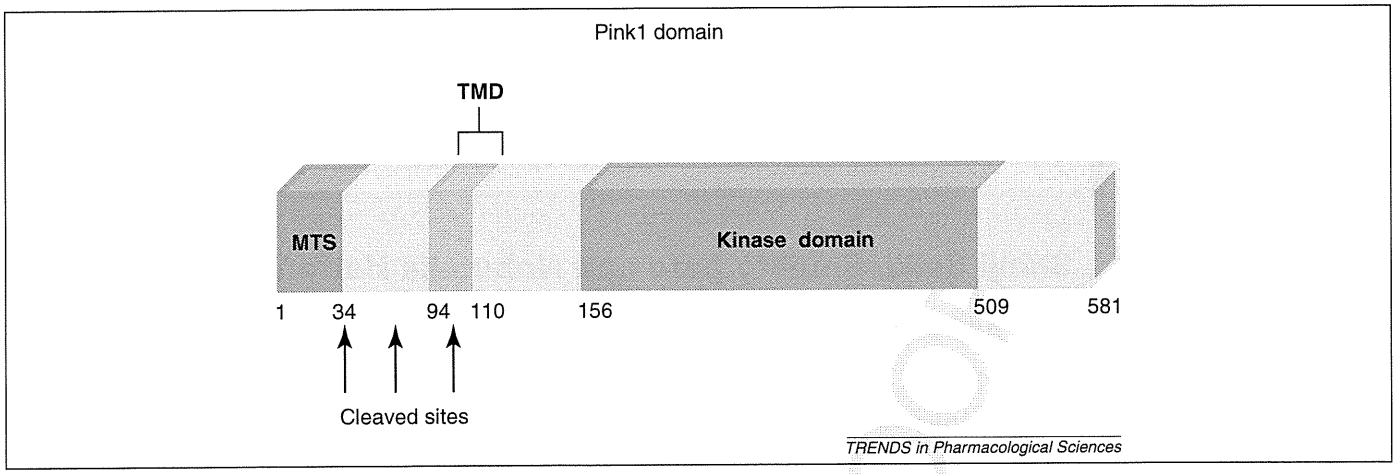

Figure 1. Putative functional domains and motifs of PINK1. Possible cleaved sites consist of the end of mitochondrial targeting sequence (MTS), sites between MTS and the transmembrane domain (TMD), and sites in the TMD.

ubiquitously expressed and predicted to encode an N-terminal 34–amino acid mitochondrial targeting sequence (MTS), a transmembrane domain (TMD) (residues 94–110) and a highly conserved protein kinase domain (residues 156–509) showing a high degree of homology to the serine/threonine kinases of the Ca^{2+}/calmodulin family [13] (Figure 1). It is important to understand the topology and subcellular distribution of PINK1 when considering PINK1 functions. PINK1 is a mitochondrial membrane integral protein whose kinase domain localizes in the outer mitochondrial membrane and is accessible from the cytoplasm. The TMD is crucial for anchoring PINK1 to the mitochondrial membrane and to ensure that the kinase domain faces the cytoplasm [14]. Subcellular fractionation shows that overexpressed PINK1 is localized in the mitochondria and cytoplasm [15], although the localization of endogenous PINK1 is not clear. This is because PINK1 is so rapidly turned over under basal conditions [16,17] that the expression level of PINK1 is very low and no antibodies against endogenous PINK1 are available. A chimeric protein consisting of a fluorescence protein fused to the N-terminus of PINK1 localizes to the mitochondria. Thus, the putative MTS of PINK1 is sufficient for its mitochondrial localization [18]. PINK1 translocated to the mitochondria by MTS is processed at several sites, such as 34 and/or 77 amino acids at the N-terminus and another site in the TMD. PINK1 mainly includes the full-length form (~63 kDa) and the cleaved form (~55 kDa) [15,18–20] (Figure 1).

Human genetics

PINK1 (PARK6) was identified in 2004 as a causative gene of autosomal recessive young-onset PD [13]. Since then, several mutations have been identified in PD patients in Europe and Japan [21]. With regard to the mode of inheritance (e.g. recessive form), loss of PINK1 function is proposed as the mechanism of *PINK1*-linked PD. The estimated prevalence of PINK1 mutations in different ethnicities is 1–8% of familial or young-onset PD [22]. Approximately 50 pathogenic mutations (missense mutations, genomic rearrangements, truncating mutations) have been identified in diverse populations. Most of the mutations are observed in the serine/threonine kinase domain, suggesting that loss of kinase activity plays a crucial part in the pathogenesis of *PINK1*-linked PD. Although the genotype–phenotype correlation has not been confirmed, the mean age at onset in patients with single heterozygous mutations is higher than that in patients with homozygous mutations [9,23]. Homozygous mutations in *PINK1* invariably cause *PINK1*-linked PD, whereas heterozygous mutations have been suggested to be a susceptibility factor for sporadic PD [24].

PINK1 function

Kinase activity

As mentioned above, PINK1 is a serine/threonine kinase protein. Several studies have reported that pathogenic mutations in PINK1, such as p.K219A, p.G309D, p.L347P, p.D362A, p.D384A, p.G386A, p.G409 V, p.E417G, are associated with reduced kinase activity [5,15,18,25]. Furthermore, the C-terminus of PINK1 regulates its kinase activity, although there is controversy over whether it up-regulates or down-regulates [18,25] activity [25]. Mutations in the PINK1 C-terminus cause early-onset parkinsonism [26]. Therefore, we have to consider the effect of the C-terminus on kinase activity to be significant. TNF receptor-associated protein 1 (TRAP1), a mitochondrial chaperone, has been identified as a PINK1 substrate. PINK1 might provide protection against oxidative stress-induced apoptosis by the phosphorylation of TRAP1 [5]. Another candidate substrate of PINK1 is parkin. The linker region of parkin is phosphorylated by PINK1, and parkin phosphorylated by PINK1 promotes its mitochondrial translocation [27]. The activity of parkin E3 ligase functions to catalyze the K63-linked polyubiquitination of IKKγ, which is a critical step in the cytoprotective signaling pathway that activates NF-κB, a ubiquitously expressed transcription of several pro-survival genes [18,28]. PINK1 is thought to modulate the phosphorylation status of another mitochondrial protein, Omi/HtrA2 (a gene product for *PARK13*), possibly through indirect mechanisms [6]. Rictor, a specific component of mammalian target of rapamycin complex 2 (mTORC2), is phosphorylated by overexpression of PINK1. Enhanced Akt through activation of

mTORC2 provides cytoprotection [20,29]. These reports corroborate the fact that kinase activity has crucial roles in the pathogenesis of *PINK1*-linked PD.

Interaction with other PD-associated genes

PINK1-deficient *Drosophila* and *PINK1*-linked PD patients show very similar phenotypes, in contrast to flies and patients whose symptoms are caused by *parkin* mutations. Moreover, overexpression of parkin rescues the phenotype of *PINK1*-deficient *Drosophila*, but not *vice versa* [30,31]. However, Omi/HtrA2 is not an essential component of the PINK1/parkin pathway in *Drosophila* [32]. In cultured cells, PINK1 knockdown phenotypes are also rescued by overexpression of parkin, but not *vice versa* [33]. Parkin stabilizes PINK1 through direct interaction [34]. These results suggest that PINK1 functions upstream of parkin in a common pathway. Furthermore, parkin, PINK1, and DJ-1 form a ubiquitin E3 ligase complex that promotes the degradation of unfolded proteins [35]. In *Drosophila*, DJ-1 can rescue the consequences of PINK1 loss (except for infertility), but not the consequences of parkin loss. Furthermore, parkin cannot rescue DJ-1 loss, suggesting that DJ-1 may not be directly downstream of PINK1 [26,36]. In human neuroblastoma cells, parkin protects against the loss of DJ-1 and, although DJ-1 does not alter *PINK1*-deficient mitochondrial phenotypes, DJ-1 is active against rotenone-induced damage in the absence of PINK1 [27,37]. These findings indicate that DJ-1 works in parallel to the PINK1/parkin pathway to maintain mitochondrial function.

PINK1 knockdown causes proteasome dysfunction, accompanied by increased α-synuclein aggregation [38]. *PINK1*-deficient *Caenorhabditis elegans* exhibits a reduced length of mitochondrial cristae, increased sensitivity to paraquat (a herbicide which causes oxidative stress and parkinsonism) and defective axonal outgrowth of a pair of canal-associated neurons. In the absence of LRRK2, all these phenotypic aspects can be suppressed [39]. Furthermore, in *Drosophila*, overexpression of LRRK2 potentiates the bristle loss phenotype of PINK1 [40]. These results suggest that loss of LRRK2 suppresses the PINK1 phenotype. Taken together, evidence suggests that dysfunction of several causative gene products might contribute to the pathogenesis for PD through a common pathway (Figure 2).

Mitochondrial regulation

Mitochondrial function: In cultured cells, overexpression of PINK1 confers resistance to toxins against mitochondria such as staurosporine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and rotenone, and PINK1 expression provides resistance to MPTP-induced dopaminergic neuronal loss in mice [4,41]. Consistent with these reports, reduction of PINK1 levels by RNA interference (RNAi) in cultured cells resulted in enhanced cell death in the presence of MPTP and rotenone [42]. In this regard, *PINK1*-deficient animal models and model-derived cells have provided important information on the endogenous function of PINK1. *PINK1*-deficient *Drosophila* showed degeneration of flight muscles, male sterility, and dopaminergic neuronal cells accompanied by mitochondrial abnormality, and shared phenotypic similarity with *parkin*-deficient *Drosophila* [30,31]. These models exhibit decreases in mitochondrial membrane potential ($\Delta\Psi m$), mitochondrial DNA, complex I, ATP, and an increased proportion of swollen mitochondria and susceptibility to apoptotic stimuli. Concomitant defects in synaptic function are also observed [43]. These results suggest that

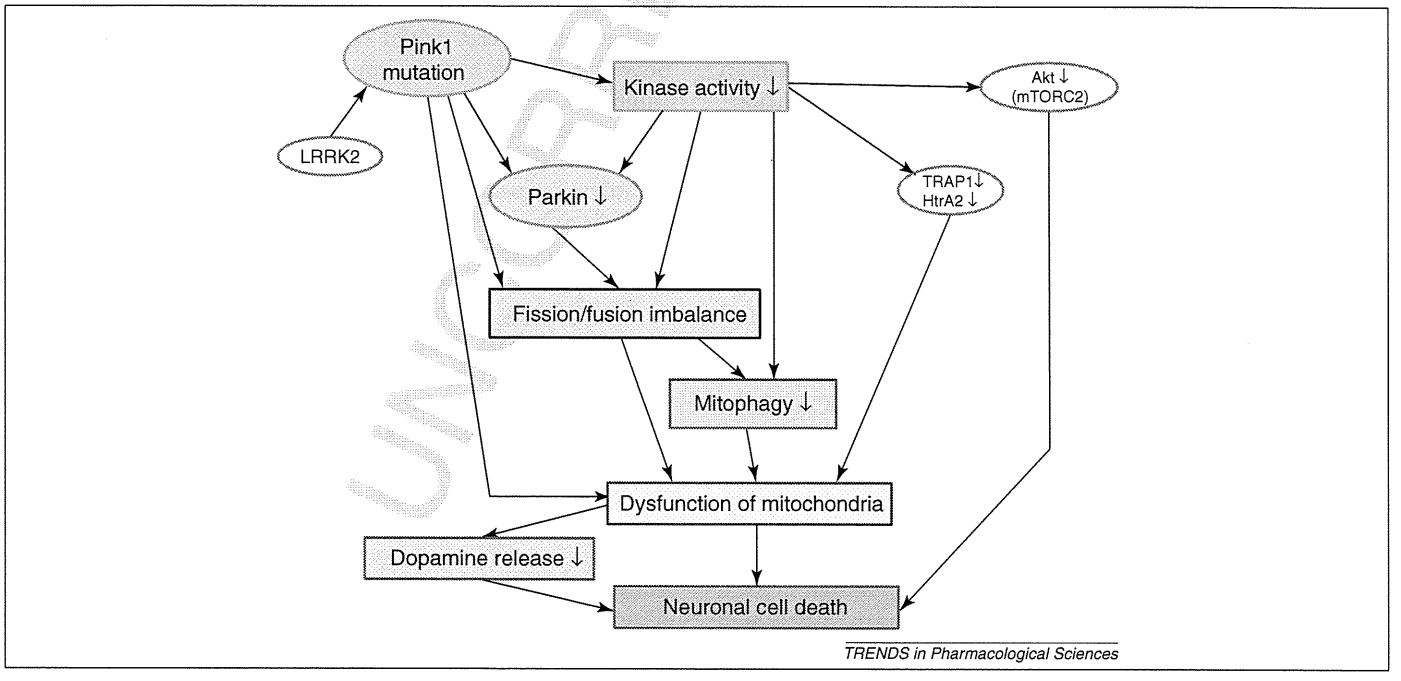

Figure 2. Proposed mechanism of *PINK1*-linked PD. Loss of PINK1 on mitochondria inhibits parkin functions and induces mitochondrial fission/fusion imbalance, which in turn causes impaired mitophagy, resulting in mitochondrial dysfunction. The kinase activity of PINK1 is important for mitophagy. Decreased dopamine release due to mitochondrial dysfunction induces neuronal cell death. Furthermore, TRAP1 and HtrA2 are not activated under reduced PINK1 kinase activity, leading to mitochondrial dysfunction. Reduced PINK1 kinase activity also results in failure of activation of Akt, leading to decreased cytoprotective function. LRRK2 exacerbates loss of PINK1.

loss of PINK1 can cause dopaminergic neuronal cell death due to functional defects in mitochondria.

In contrast, *PINK1*- and *parkin*-deficient mice do not exhibit major abnormalities, whereas absence of PINK1 causes several deficits, including: (i) reduced synaptic dopamine release and plasticity in the striatum [44]; (ii) impaired mitochondrial respiration in the striatum at 3–4 months of age and in the cerebral cortex at 2 years [45]; and (iii) progressive weight loss and selective reduction of locomotor activity for spontaneous movements in old age [46]. These findings indicate that the mitochondria in *PINK1*-deficient models are more vulnerable to aging. Primary cultured neurons derived from *PINK1*-deficient mice showed increased intracellular calcium levels and vulnerability, with subsequent excess production of reactive oxygen species (ROS), decreased glucose availability, loss of $\Delta\Psi m$ and defects of complex I, causing pathological opening of the mitochondrial permeability transition pore [47]. In *PINK1*-deficient mouse embryonic fibroblasts (MEFs), $\Delta\Psi m$ and cellular ATP levels are lower than in wild-type MEFs. However, mitochondrial proton leak (which reduces membrane potential in the absence of ATP synthesis) is not altered by loss of PINK1. Instead, low activity of the respiratory chain (which produces membrane potential oxidizing substrates using oxygen) has been observed [48]. These results suggest that decreased $\Delta\Psi m$ caused by loss of PINK1 is not due to proton leak, but to respiratory chain defects [39].

Similar to *parkin*-deficient mice, *PINK1*-deficient mice do not show prominent phenotypes. However, results from *PINK1*-deficient mice indicate that dysfunction of mitochondrial proteins (e.g. complex I) and aging are important in the pathogenesis of *PINK1*-linked PD as well as in sporadic PD.

Samples obtained from patients with hereditary PD can provide important information regarding the pathogenesis of PD. Skin fibroblasts of patients homozygous for the p.G309D *PINK1* mutation show: (i) a mild decrease in complex I activity and a trend of superoxide elevation [49]; (ii) fragmented mitochondrial morphology [33]; and (iii) low expression of parkin and selective vulnerability to proteasomal stress-triggered caspase activation [41,50].

In samples obtained from mice and PD patients, as well as from cultured cells, loss of PINK1 causes mitochondrial dysfunction and vulnerability to cell death.

Mitochondrial morphology: In mammalian cultured cells (with the exception of COS7 cells), PINK1 knockdown phenotype shows fragmented mitochondria [33,51]. In human neuroblastoma cells transduced with a PINK1 shRNA lentivirus, the activity of dynamin-related protein 1 (Drp1), which is controlled by phosphatase calcineurin, enhances the effects of PINK1 upon mitochondrial morphology [52]. In contrast, transgenic *Drosophila* with PINK1 promotes mitochondrial fission in dopaminergic neurons, whereas complete ablation of PINK1 leads to excessive fusion [53]. Fis1 (mitochondrial fission 1 protein) may act in-between PINK1 and Drp1 in controlling mitochondrial fission [53]. Heterozygous loss-of-function mutations of Drp1 are largely lethal in a *PINK1* or *parkin* mutant background. Conversely, the degeneration of flight muscle and mitochondrial morphological changes which result from mutations in *PINK1* and *parkin* are strongly suppressed by increasing the dosage of the Drp1 gene and by heterozygous loss-of-function mutations in OPA1 and Mfn2. In pseudopupil analyses, an eye phenotype associated with increased activity of the PINK1/parkin pathway is suppressed by perturbations that reduce mitochondrial fission but enhanced by perturbations that reduce mitochondrial fusion [54,55]. These results suggest that the PINK1/parkin pathway promotes mitochondrial fission, and that the loss of mitochondrial and tissue integrity in *PINK1* and *parkin* mutants is due to reduced mitochondrial fission in *Drosophila*. The cortical neurons of *PINK1*-deficient mice show reduced fission and increased aggregation of mitochondria only under stress [46]. Dopaminergic neuronal rat cells with a *PINK1* mutation (p.L347P) show mitochondrial fragmentation and dysfunction, which can be prevented by inhibitors of mitochondrial division [47,56].

There is controversy over whether PINK1 modulates mitochondrial fission or fusion. Fragmented mitochondria in *parkin* and/or *PINK1*-deficient *Drosophila* S2 cells are observed at day 2 after double-stranded RNA (dsRNA) treatment. At days 3 and 4 after dsRNA treatment, a dense network of fine thread-like mitochondria is observed. *PINK1*-deficient primary mouse hippocampal neurons show a decrease in the length of mitochondria and an increase in mitochondrial fragmentation. The mitochondrial phenotype observed in *parkin*- and *PINK1*-deficient cells can be rescued morphologically and functionally by increased expression of a dominant negative mutant of Drp1 [51]. Considering the discrepancy between cellular and fly models with low PINK1 expression, we might observe an acute manifestation of *parkin* or *PINK1* knockdown in cultured cells and the chronic phenotype influenced by compensatory effects in adult *Drosophila* (although the mitochondrial morphological change might be dependent upon cell lines). Otherwise, PINK1 might regulate and maintain a balance between fission and fusion depending upon certain conditions (e.g. phase stress).

Mitochondrial fission and fusion are highly regulated processes that are critical for the maintenance of mitochondria (especially in neurons). Imbalance of mitochondrial fission and fusion machineries has increasingly been linked to neurodegeneration [57]. Based on this concept, PINK1 has a crucial role in the pathogenesis of PD.

Mitophagy: Recent studies have provided new insights about the PINK1/parkin pathway. It has been reported that parkin is translocated to depolarized mitochondria and that the parkin-labeled mitochondria are subsequently eliminated by autophagy (mitophagy) [58]. Several studies on this pathway were subsequently reported. Endogenous PINK1 is not detected under basal conditions because it is rapidly degraded [16]. However, reduction of $\Delta\Psi m$ induced by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler, results in gradual accumulation of endogenous PINK1 (full-length form) on mitochondria [17,59]. Interestingly, clearance of CCCP results in immediate disappearance of the accumulated endogenous PINK1 in the presence and absence of cycloheximide (an inhibitor of protein biosynthesis) by interfering with the translocation step of protein synthesis.

Likewise, CCCP treatment does not alter PINK1 mRNA levels [17]. These results suggest that PINK1 is stabilized by reduced $\Delta\Psi m$ and subsequently accumulates on depolarized mitochondria. By contrast, parkin is not translocated to the mitochondria in *PINK1*-deficient CCCP-treated MEFs, and subsequent mitochondrial clearance is also completely blocked, indicating that PINK1 is indispensable for parkin translocation to depolarized mitochondria. Parkin E3 ligase activity is suppressed under basal conditions, although the activity is increased in mitochondria with low $\Delta\Psi m$. PINK1 accumulation induces recruitment of parkin to the depolarized mitochondria, and subsequently the mitochondria are eliminated by mitophagy. These processes are inhibited by pathogenic mutants of PINK1 or parkin [16,17,59]. Furthermore, elimination of parkin-labeled mitochondria is blocked in *Atg5*- or *Atg7*-deficient MEFs [58,59]. Moreover, MTS, kinase activity of PINK1, and the linker domain of parkin are indispensable for PINK1/parkin-mediated mitophagy [17,52,59].

In cells with normal $\Delta\Psi m$, expression of PINK1 on the outer mitochondrial membrane or overexpression of PINK1 induces translocation of parkin to the mitochondria [60,61]. Furthermore, valinomycin- and hydrogen peroxide (H_2O_2)-induced stress result in mitochondrial localization of parkin in skin fibroblasts of healthy controls but not in those of PD patients with PINK1 mutations (p.Q456X) [56,62]. Valinomycin, but not H_2O_2, reduces $\Delta\Psi m$. Taken together, it seems that PINK1 expression on the mitochondria, rather than low $\Delta\Psi m$, is indispensable for parkin translocation to the mitochondria.

The mitochondrial inner membrane rhomboid protease presenilin-associated rhomboid-like protein (PARL) mediates cleavage of PINK1 in the TMD in a $\Delta\Psi m$-dependent manner. In the absence of PARL, full-length PINK1 accumulates on the outer mitochondrial membrane, where it recruits parkin to the impaired mitochondria. Thus, the role of PARL in the PINK1/parkin pathway appears to include facilitating the rapid degradation of PINK1 by mediating the cleavage of PINK1 [19,20,63].

PINK1/parkin-mediated mitophagy involves the formation of linkage-specific polyubiquitin chains (K27 and K63) and requires the ubiquitin-autophagy adaptor p62/SQSTM1. VDAC1 is a mitochondrial target of parkin-dependent K27 ubiquitination, and VDAC1 ubiquitination in neuronal cells is dependent upon functional parkin. Because VDAC1 is a component of the mitochondrial permeability transition pore (mPTP), which is involved in apoptosis, a parkin-dependent, timely mitophagic clearance may prevent the release of pro-apoptotic factors from damaged mitochondria under physiological conditions [58,64]. By contrast, p62 is reported to be necessary for mitochondrial aggregation but not mitophagy, and mitochondrial-associated proteins other than VDAC1 and VDAC3 are K63-polyubiquitinated in a parkin-dependent manner [65,66]. Another candidate parkin substrate on the mitochondria is mitofusin (MFN), which is involved in mitochondrial fusion. *Drosophila* Marf (a fly MFN ortholog), which is localized on the outer surface of mitochondria, is ubiquitinated by parkin and accumulates in *parkin* mutants [67,68]. Mammalian cells contain two types of MFN: MFN1 and MFN2. Treatment of cultured cells with CCCP results in ubiquitination of MFN1 and MFN2 in a PINK1/parkin-dependent manner [63,69]. Moreover, parkin-ubiquitinated MFN1 and MFN2 are degraded by the proteasome. The hexametric AAA-type ATPase, p97, is required downstream of PINK1 and parkin to promote the proteasomal turnover of ubiquitinated MFN. Parkin-promoted MFN degradation prevents refusion of damaged mitochondria with healthy mitochondria [64,70]. Fission followed by selective fusion segregates dysfunctional mitochondria and permits their removal by autophagy [71]. These reports suggest that mitochondrial fission may be a key factor in PINK1/parkin-mediated mitophagy. However, there is controversy over the substrates of parkin, making further investigations necessary.

PINK1/parkin-mediated mitophagy model

Based on the collective results of the studies mentioned above, we propose the model illustrated in Figure 3 for PINK1/parkin-mediated mitophagy. PINK1 accumulated on the mitochondrial outer membrane due to the low $\Delta\Psi m$ or certain mitochondrial insults recruit parkin to the mitochondria. Parkin E3 ligase activity is subsequently activated and ubiquitinates MFN on the mitochondrial outer membrane. Ubiquitinated MFN assembles p97 and the complex is degraded by proteasome. Mitochondria lacking MFN, which cannot fuse with other healthy mitochondria, can be a target for mitophagy. The model advances our understanding of the pathogenic process of PD and the role of the direct associations between parkin and mitochondria, as well as between the PINK1/parkin pathway and autophagy. Mitochondrial dysfunction is also considered to be one of the main causes of the sporadic form of PD, and impaired autophagy leads to neurodegenerative diseases such as PD. However, to fully understand the molecular mechanisms of this pathway, further details are needed. For example, how does PINK1 recruit parkin to the damaged mitochondria? What is the mechanism responsible for parkin activation in the mitochondria? There is controversy over whether PINK1 phosphorylates parkin in this pathway [16,17,27,28,61,66]. PINK1 kinase activity is essential for parkin translocation to mitochondria [17,59]. Therefore, it is a feasible and attractive hypothesis that parkin is directly phosphorylated by PINK1. However, further investigations are needed.

Other candidate PINK1 substrates that recruit parkin or function as parkin receptors on the outer mitochondrial membrane also need to be investigated. In yeast, Atg32 (which has no known metazoan homolog) is identified as an outer mitochondrial membrane protein necessary for mitophagy [72,73], and contains a conserved WXXI/L/V motif for interaction with Atg8. Recent studies reported that another outer mitochondrial membrane protein, Nix (which has no yeast homolog and contains a WXXL-like motif), is crucial for PINK1/parkin-mediated mitophagy [68,74]. Does endogenous parkin contribute to mitophagy? Most reports on this pathway are conducted by overexpressing parkin. It remains unclear at this stage whether parkin deficiency or parkin partial knockdown down-regulates mitophagy. Furthermore, there is no information on whether $\Delta\Psi m$ of the brain is decreased in patients with

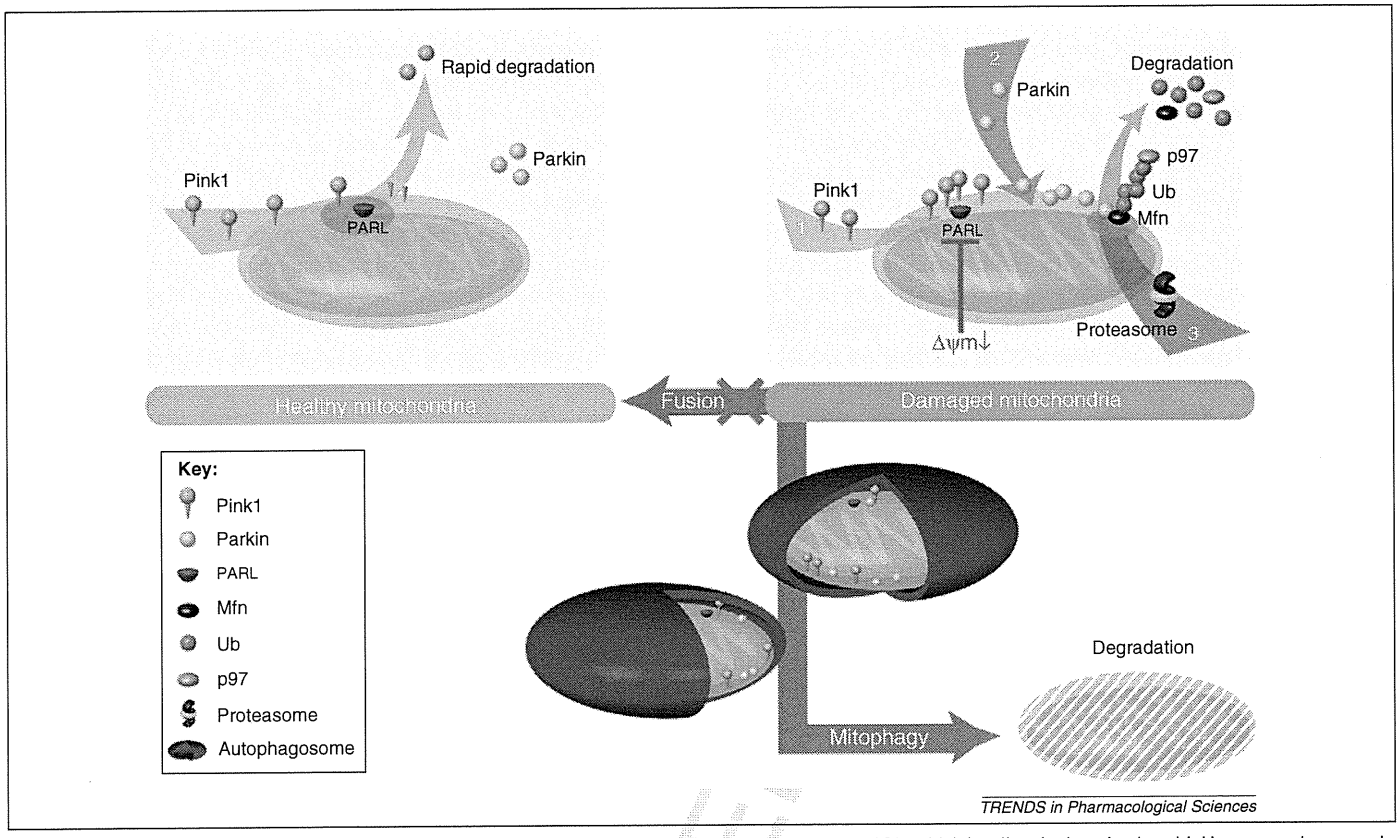

Figure 3. Proposed model of PINK1/parkin-mediated mitophagy. PINK1 on mitochondria is processed by PARL, which localizes in the mitochondrial inner membrane under basal conditions. The processed PINK1 is rapidly degraded by the proteasome. In the presence of a low membrane potential or certain insults to the mitochondria, PINK1 accumulates in the mitochondrial outer membrane. This results in translocation of parkin to the damaged mitochondria and subsequent activation of parkin-E3 ligase. Activated parkin ubiquitinates mitofusin (MFN) on the mitochondrial outer membrane. This leads to the assembly of p97 on ubiquitinated MFN and proteasomal degradation. Mitochondria lacking MFN cannot fuse with other healthy mitochondria and become a target for mitophagy.

sporadic PD. As mentioned above, *PINK1*-deficient mice and MEFs as well as PINK1 knockdown cells show decreased $\Delta\Psi m$. However, skin fibroblasts derived from neither the nonsense (p.Q456X) nor the missense (p.V170G) mutation show significantly low $\Delta\Psi m$ [75]. There is no model of CCCP-induced human parkinsonism, unlike with MPTP-and rotenone-induced parkinsonism. In this regard, there is no evidence for translocation of parkin to mitochondria in cells treated with MPTP or rotenone. CCCP treatment does not reflect a human physiological condition. Therefore, there is a limitation in discussing the pathogenesis of PD using models based on CCCP treatment.

Is there any association between PINK1/parkin-mediated mitophagy and α-synuclein? As discussed above, α-synuclein is a major component of Lewy bodies (the hallmark of sporadic PD) and is considered to be a key protein in the pathogenesis of sporadic PD. There is no direct association between PINK1/parkin-mediated mitophagy and α-synuclein, although wild-type and mutants of α-synuclein are degraded by chaperone-mediated autophagy and macroautophagy, respectively [76,77]. Studies demonstrated that α-synuclein inhibits autophagy [71,78], and that inhibition of mitochondrial fusion by α-synuclein is rescued by PINK1 and parkin [72,73,79]. To develop a novel therapeutic strategy for sporadic PD as well as *PINK1*- and *parkin*-linked PD, it is necessary to investigate the relationship between aggregation of α-synuclein and dysfunction of PINK1/parkin-mediated mitophagy.

Concluding remarks

PD is a progressive neurodegenerative disease for which the frequency increases with age. As 'developing nations' face rapidly aging populations, the prevalence of PD is expected to rise. PD patients in the advanced stage require great support from families and the medical community. However, curative therapies to suppress the progression are lacking. Therefore, it is important to elucidate the pathogenesis and establish novel and rational treatments. PINK1 provides valuable clues regarding the molecular pathogenesis of PD because the pathomechanism for sporadic PD probably has certain common pathways with that of *PINK1*-linked PD. Based on the available information on PINK1, we propose the model shown in Figure 2 for the mechanisms involved in the development of *PINK1*-linked PD. PINK1 mainly protects the mitochondria via mitophagy with parkin, and may subsequently suppress neuronal cell death due to reduced dopamine release. However, PINK1 may have a cytoprotective function by activating Akt in the cytoplasm. We expect that a compound which inhibits this pathway could be established as a novel treatment for not only *PINK1*-linked PD but also sporadic PD. In particular, PINK1/parkin-mediated mitophagy (i.e. mitochondrial quality control via autophagic machinery that includes the combination of PINK1 and parkin) could be viewed as the foundation for the design of novel therapies for PD, although several issues remain unresolved. If we could determine the precise mechanism of mitophagy which eliminates abnormal mitochondria, we

might regulate the clearance of deleterious mitochondria with new chemicals which target this pathway to maintain cell integrity and subsequently suppress cell death. However, further studies are required to elucidate the exact pathomechanism and develop effective therapies for hereditary PD and sporadic PD.

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Comprehensive Predictions of Target Proteins Based on Protein-Chemical Interaction Using Virtual Screening and Experimental Verifications

Hiroki Kobayashi[1]*, Hiroko Harada[1]*, Masaomi Nakamura[1], Yushi Futamura[1], Akihiro Ito[2], Minoru Yoshida[2], Shun-ichiro Iemura[3], Kazuo Shin-ya[3], Takayuki Doi[4], Takashi Takahashi[5], Tohru Natsume[3], Masaya Imoto[1], Yasubumi Sakakibara[1§]

[1]Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University , 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, 223-8522, Japan

[2]Chemical Genetics Laboratory, RIKEN Advanced Science Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan

[3]National Institute of Advanced Industrial Science and Technology (AIST), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan

[4]Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aza-Aoba, Aramaki, Aoba, Sendai 980-8578, Japan

[5]Department of Applied Chemistry, Tokyo Institute of Technology, 2-12-1 Ookayama, Meguro, Tokyo 152-8552, Japan

*These authors contributed equally to this work

[§]Corresponding author

E-mail: yasu@bio.keio.ac.jp

E-mail addresses:

HK: kobayashihiroki@riken.jp

HH: e39976@jp.ibm.com

MN: nakamura@dna.bio.keio.ac.jp

YF: futamuray@riken.jp

AI: akihiro-i@riken.jp

MY: yoshidam@riken.jp

SI: iemura-shun@aist.go.jp

KS: k-shinya@aist.go.jp

TD: doi_taka@mail.pharm.tohoku.ac.jp

TT: ttak@apc.titech.ac.jp

TN: t-natsume@aist.go.jp

MI: imoto@bio.keio.ac.jp