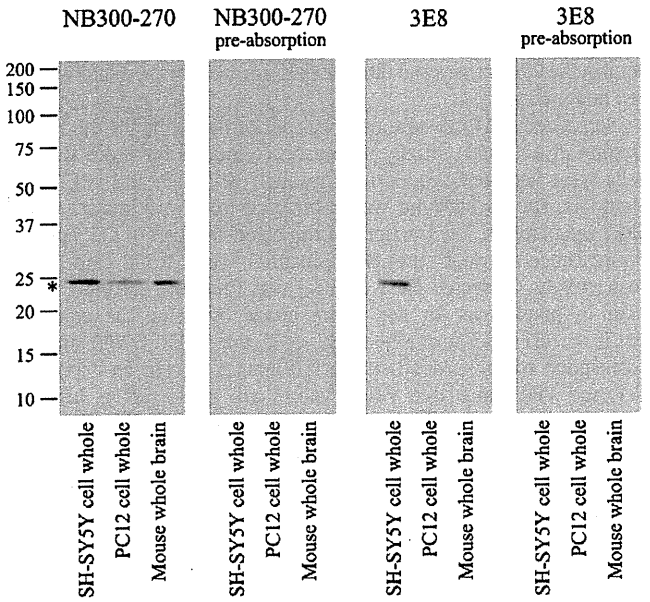


Fig. 1. Characterization of anti-DJ-1 antibodies. (A) Immunoblot of lysates from SH-SY5Y cells, PC12 cells, and mouse whole brain. Commercially available DJ-1 rabbit polyclonal antibody and mouse monoclonal antibody were used, as mentioned in Materials and methods. Specificities of these antibodies were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

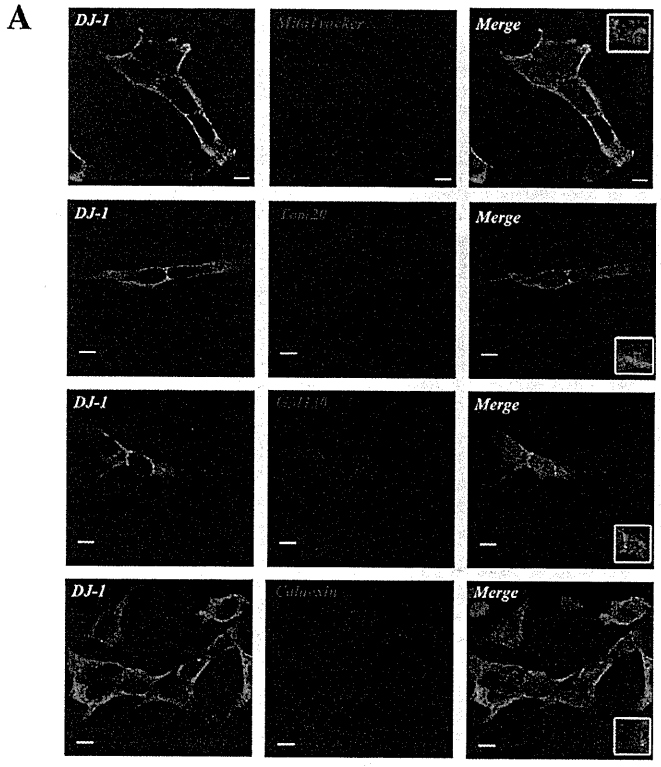
To determine the endogenous localization of DJ-1, we employed the use of the commercially available mouse monoclonal and rabbit polyclonal DJ-1 antibodies. Immunoblot analyses using NB300-270 antibody (a rabbit polyclonal antibody) revealed a single band around 22 kDa corresponding to endogenous DJ-1 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.

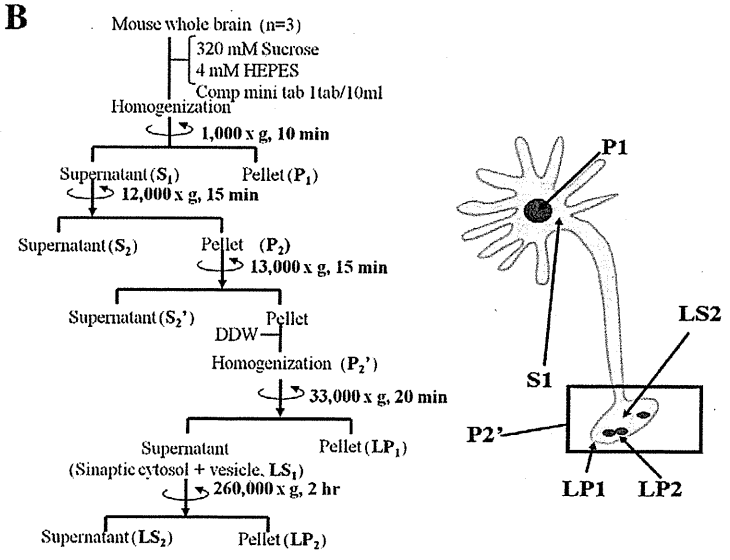
DJ-1 diffusely distributes with main membranous organelles

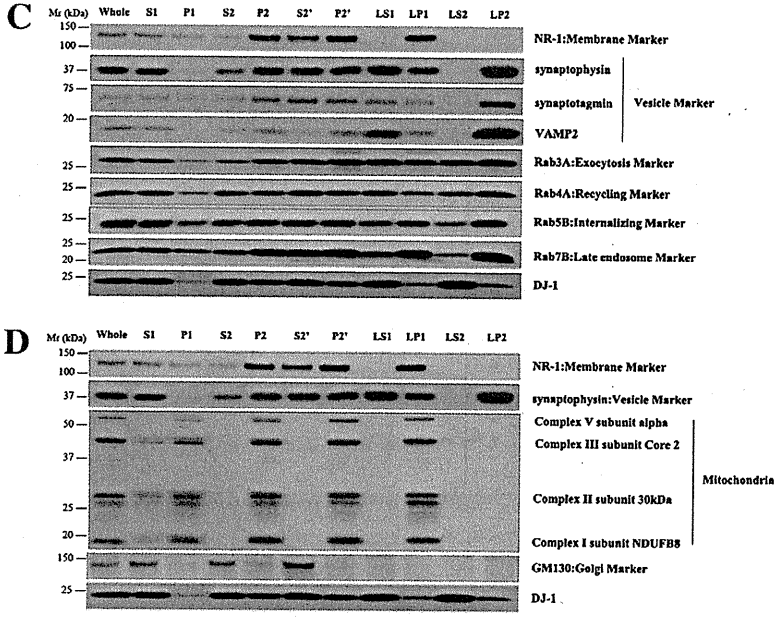
To examine the subcellular localization of DJ-1, SH-SY5Y cells were double-stained with the DJ-1 antibody and organelle-specific antibodies. Microscopic observation revealed diffuse DJ-1 immunostaining and the protein partly colocalized with GM130, a marker for Golgi apparatus. A small portion of DJ-1 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.


A
DJ-1
Merge
DJ-1
Merge
DJ-1
Merge
DJ-1
Merge


B
Mouse whole brain (n=3)
320 mM Sucrose
4 mM HEPES
Comp mini tab 1tab/10ml
Homogenization
1,000 x g, 10 min
Supernatant (S1)
Pellet (P1)
12,000 x g, 15 min
Supernatant (S2)
Pellet (P2)
13,000 x g, 15 min
Supernatant (S2')
Pellet
DDW
Homogenization (P2')
33,000 x g, 20 min
Supernatant
(Sinaptic cytosol + vesicle, LS1)
Pellet (LP1)
260,000 x g, 2 hr
Supernatant (LS2)
Pellet (LP2)
P1
LS2
S1
P2'
LP1
LP2


C
Mr (kDa)
Whole
S1
P1
S2
P2
S2'
P2'
LS1
LP1
LS2
LP2
NR-1:Membrane Marker
synaptophysin
synaptotagmin
VAMP2
Vesicle Marker
Rab3A:Exocytosis Marker
Rab4A:Recycling Marker
Rab5B:Internalizing Marker
Rab7B:Late endosome Marker
DJ-1
D
NR-1:Membrane Marker
synaptophysin:Vesicle Marker
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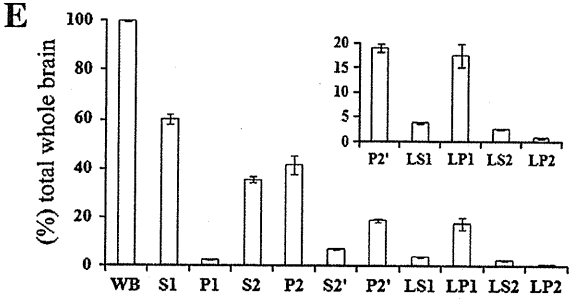
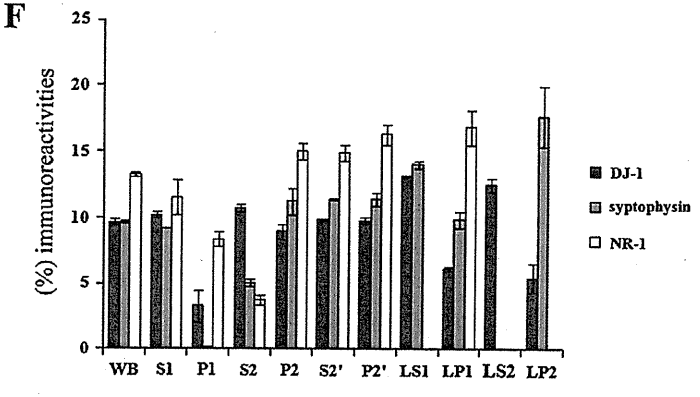
Complex II subunit 30kDa
Complex I subunit NDUFB8
Mitochondria
GM130:Golgi Marker
DJ-1


E
(%) total whole brain
WB
S1
P1
S2
P2
S2'
P2'
LS1
LP1
LS2
LP2


F
(%) immunoreactivities
DJ-1
syptophysin
NR-1
WB
S1
P1
S2
P2
S2'
P2'
LS1
LP1
LS2
LP2

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

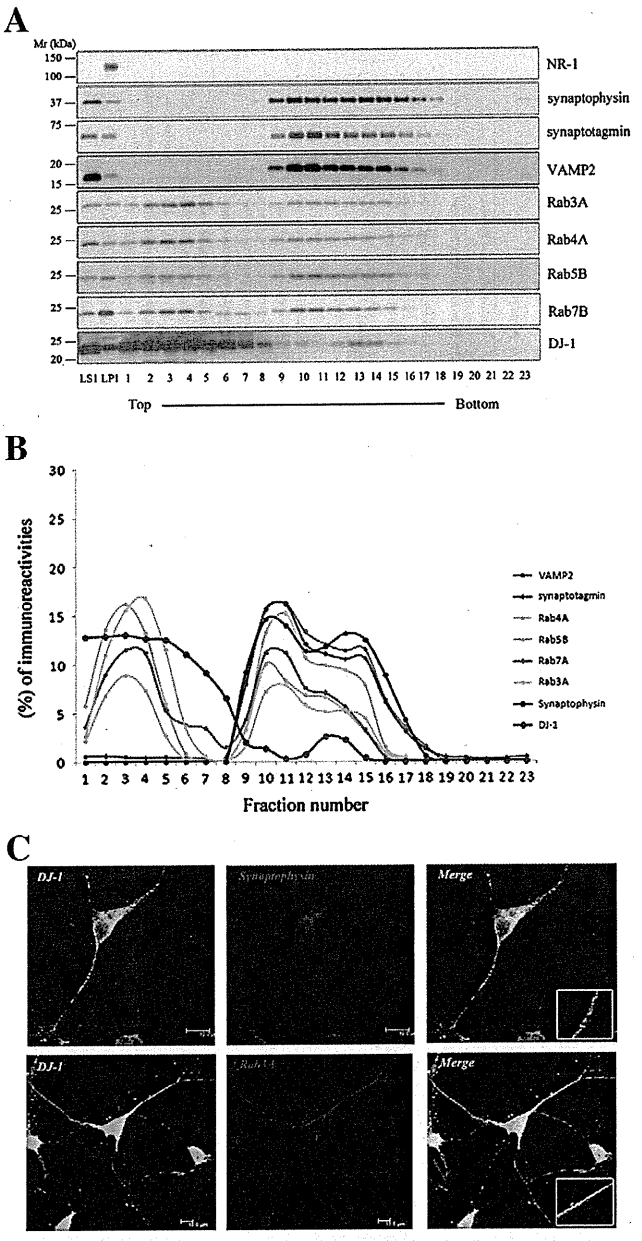


Fig. 3. DJ-1 associates with synaptic vesicles and colocalizes with synaptophysin and Rab3A. (A) The LS1 fraction was layered on top of a linear sucrose density gradient ranging from 0.2–2.0 M sucrose dissolved in HEPES buffer. Fractions were collected and 15 μl of each fraction were subjected to SDS–PAGE 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.

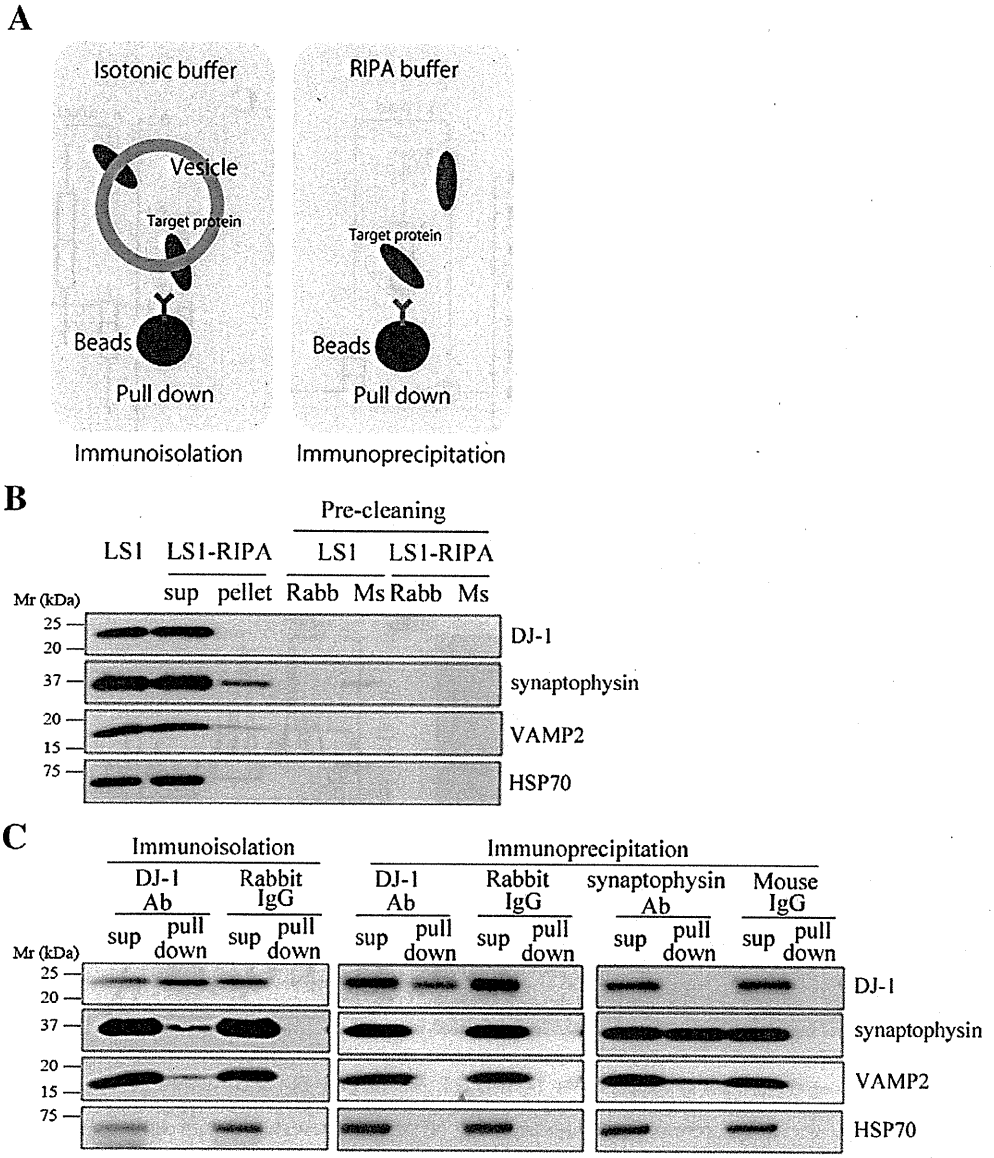


Fig. 4. DJ-1 cannot directly interact with synaptophysin and VAMP2, but associates with the same synaptic vesicles. (A) Image of immunoisolation and immunoprecipitation. Immunoisolation was used to pull down the target protein from the subcellular organelles (synaptic vesicle) from the homogenate, which 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.

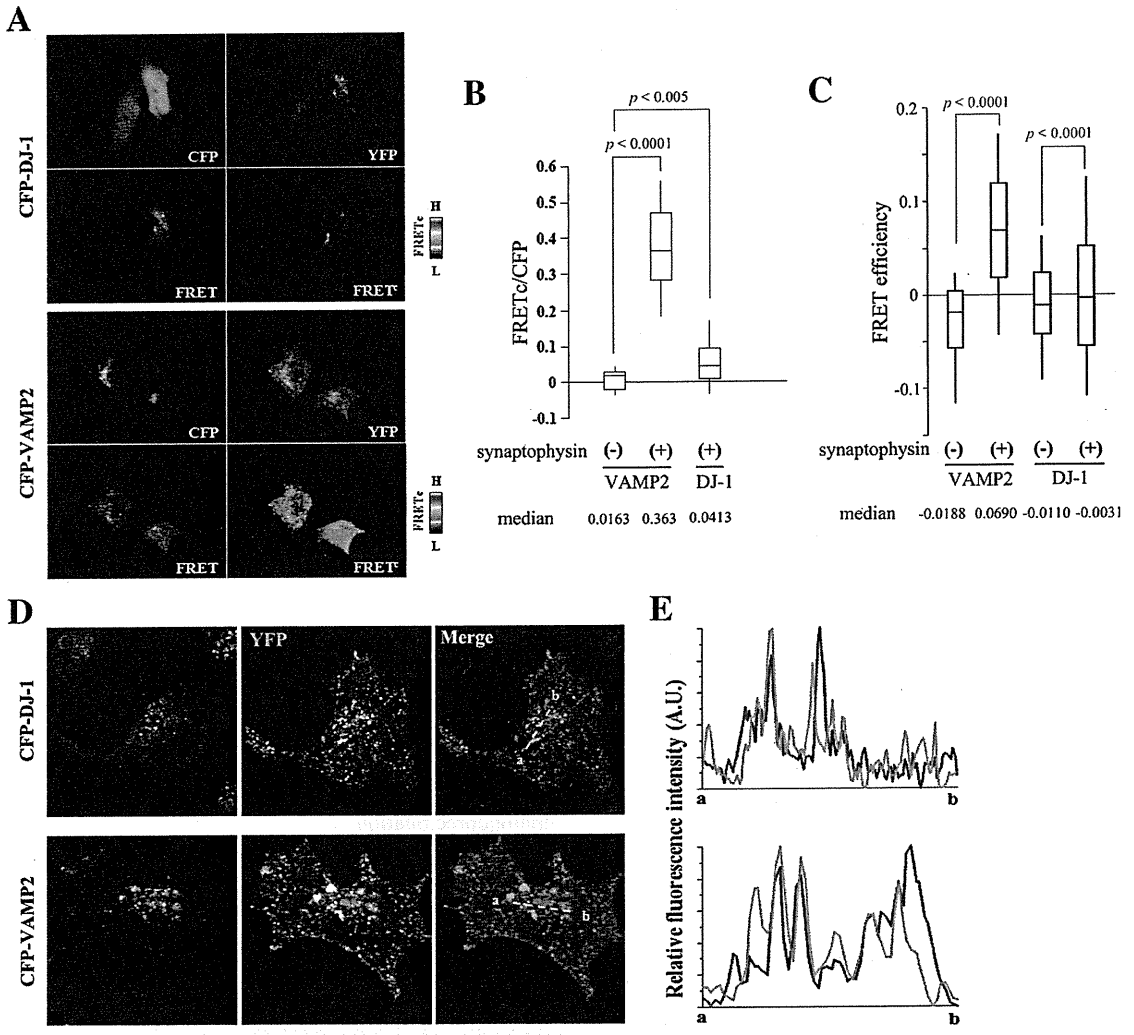


Fig. 5. FRET occurred in HeLa cells expressing CFP–DJ-1 and synaptophysin-YFP. (A) HeLa cells expressing CFP–DJ-1 or CFP–VAMP2, and synaptophysin-YFP were subjected to microscopic analysis as described in Materials and methods, and representative images are shown. FRET was detected in HeLa cells expressing 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

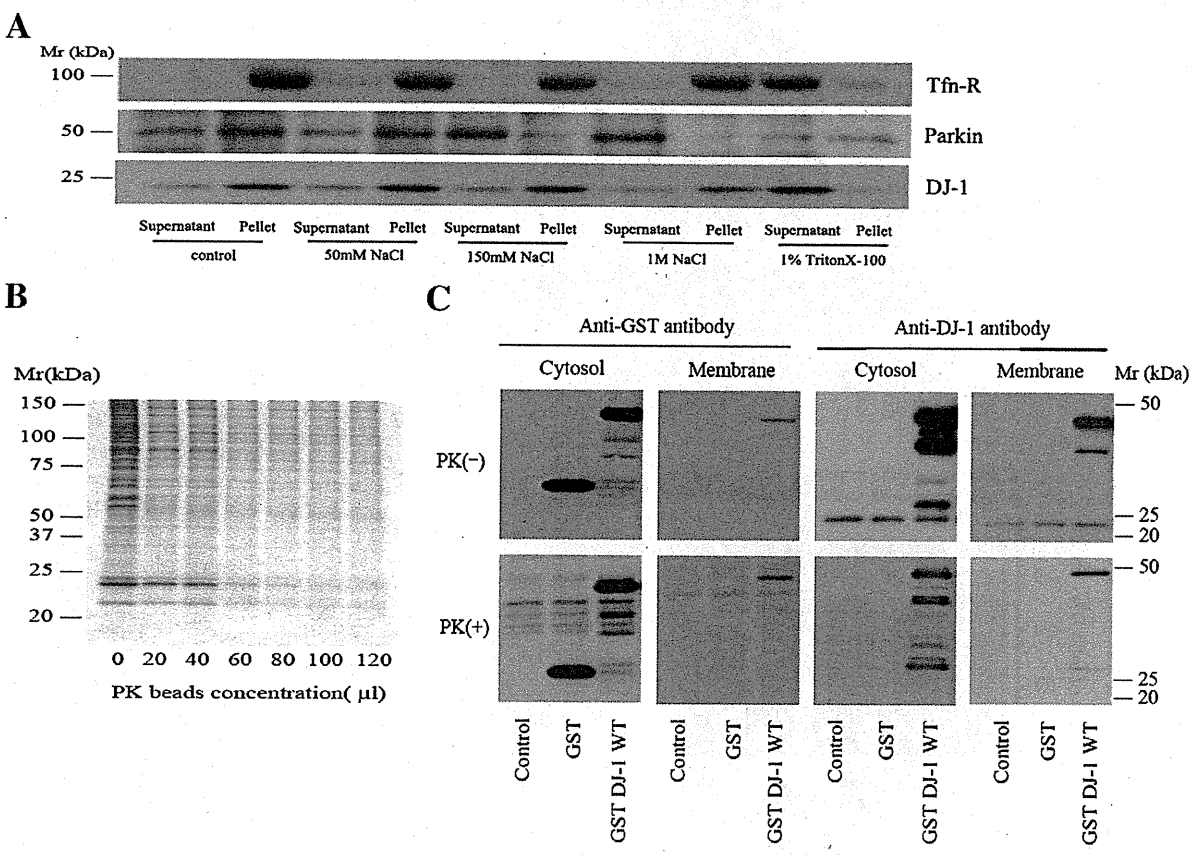


Fig. 6. Endogenous DJ-1 associates with cytosol and plasma membrane in PC12 cells. (A) Effects of various salt concentrations and non-ionic detergent on solubilization of DJ-1, Parkin, and transferrin receptor (Tfn-R). DJ-1 was concentrated in both the cytosol and membrane fractions of PC12 cells in 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 260,000g 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

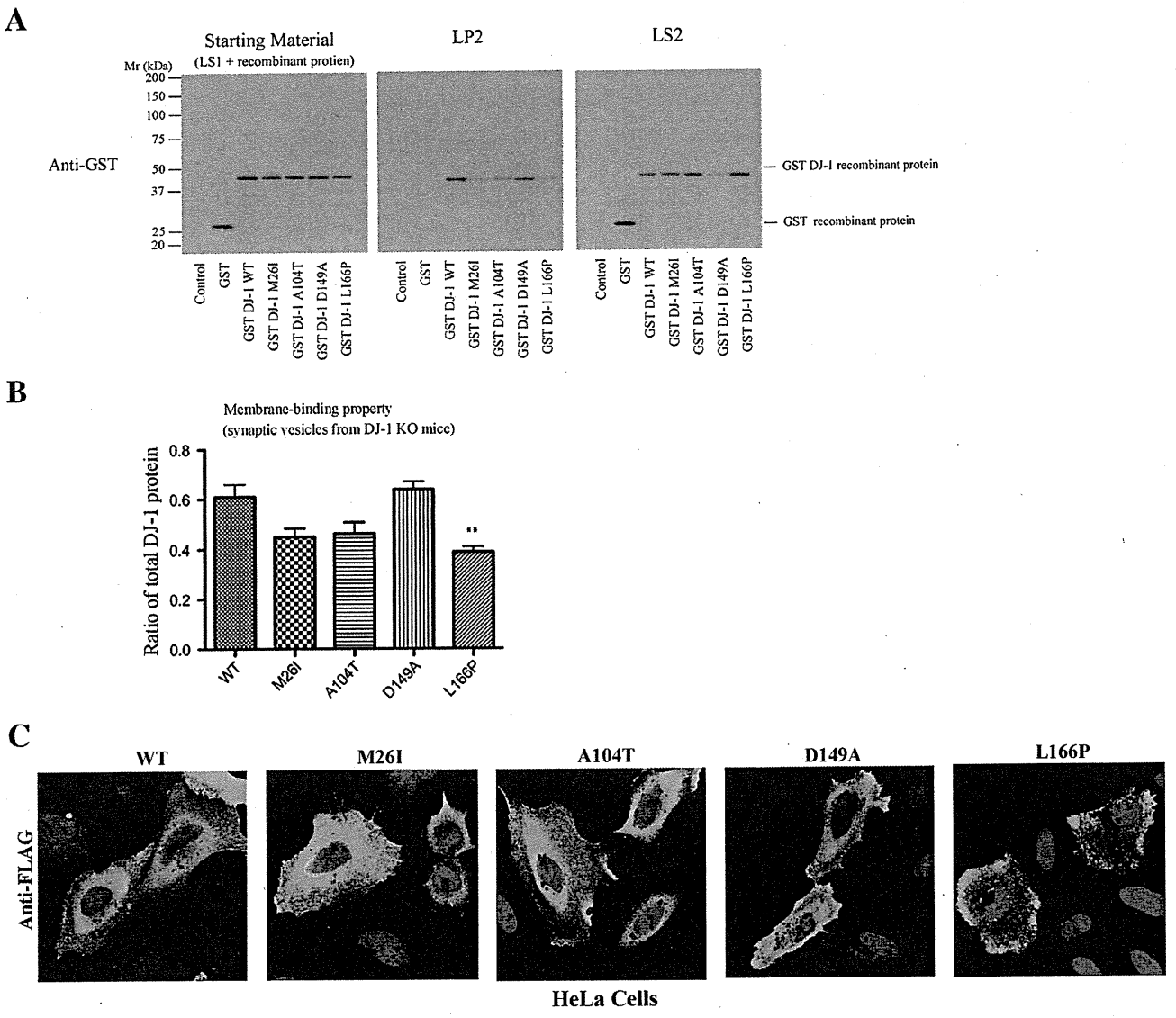


Fig. 7. Pathogenic DJ-1 mutants can bind membranes in the in vitro assay. (A) The LS1 fraction of DJ-1 KO mouse was reacted for 20 min at 30 °C with 500 nM GST recombinant protein, WT, or various mutants. Each of the bound proteins was divided into an LP2 fraction (synaptic vesicles) and an LS2 fraction (synaptic cytosol) by ultra-centrifugation at 260,000*g* 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.

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Clinical Study

Nonmotor Symptoms in Patients with *PARK2* Mutations

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Decreased ^{123}I-meta-iodobenzylguanidine (MIBG) uptake in MIBG myocardial scintigraphy, olfactory dysfunction, and rapid eye movement (REM) sleep behavior disorder (RBD) are considered useful early indicators of Parkinson disease. We investigated whether patients with *PARK2* mutations exhibited myocardial sympathetic abnormalities using MIBG scintigraphy, olfactory dysfunction using the Sniffin' Sticks olfactory test, and RBD using polysomnography. None of the examined patients had RBD, and all except 1 patient exhibited an increase in the olfactory threshold. Moreover, one of the oldest patients exhibited impairment in identification and discrimination. Of 12 patients with *PARK2* mutations, 4 patients, who were older than patients without abnormal uptake, exhibited decreased MIBG uptake. The results obtained in this study suggest that some patients with *PARK2* mutations have increased thresholds of olfactory function and myocardial sympathetic dysfunction as nonmotor symptoms.

1. Introduction

Mutations in the *parkin* gene (*PARK2*) are considered to be the predominant cause of early-onset Parkinson disease particularly when the family history is compatible with autosomal recessive inheritance [1]. This condition is characterized by early onset of disease, usually before the age of 40 years, dystonia, sleep benefit, early complications from levodopa treatment, and slow progression. *Parkin*-associated tremor-dominant parkinsonism includes a spectrum of late-onset disorders without manifestations of foot dystonia, hyperreflexia, diurnal fluctuations, sleep benefit, or early susceptibility to levodopa-induced dyskinesia [2]. Therefore, patients with *PARK2* mutations are often clinically indistinguishable from those with sporadic Parkinson's disease (PD).

PD patients exhibit decreased myocardial uptake of meta-iodobenzylguanidine (MIBG) during ^{123}I-MIBG myocardial scintigraphy—a finding indicative of cardiac sympathetic denervation [3]. Olfactory impairment, an early symptom of PD, occurs in more than 70% of patients with PD [4]. Rapid eye movement (REM) sleep behavior disorder (RBD) is characterized by a loss of normal skeletal muscle atonia and complex motor activity, specifically during REM sleep associated with dream mentation. Thirty-eight percent of RBD patients aged ≥ 50 years were eventually diagnosed with PD [5]; therefore, RBD may serve as an early indicator of PD.

Here, we examined nonmotor symptoms in patients with *PARK2* mutations.

2. Methods

Mutation of the parkin gene was confirmed by gene analysis [1]. Eight women and 7 men possessed mutations in the *PARK2* gene: cases 1, 2, 3, 8, and 13 carried homozygous-deletions, and the remaining carried heterozygous mutations or deletions (Table 1). Clinical findings and medications are shown in Table 1.

The MIBG study involved 6 women and 7 men (mean (SD) age, 58.5 (11.4) years) with *PARK2* mutations: 5 subjects had homozygous deletions, and 8 had heterozygous mutations or deletions. Patients had parkinsonism for a mean (SD) period of 22.0 (11.59) years (range, 10–44 years). When MIBG scintigraphy was performed, the patients were not medicated with monoamine oxidase B (MAOB) inhibitors, selective serotonin reuptake inhibitors, or antidepressant drugs. Data was collected by E CAM at 30 minutes and 3 hours after injection of ^{123}I-MIBG

Table 1: Clinical findings and medication of patients with PARK2 mutation.

Case	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Age sex	71 F	55 M	46 M	41 F	38 M	36 F	76 M	70 M	63 M	61 F	61 F	60 F	57 M	44 F
Parkin	exon 2–4 homo deletion	exon 5 homo deletion	exon 6, 7 homo deletion	exon 6 hetero deletion	exon 4, intron 4 accepter site, A → G	exon 10 hetero mutation	exon 10 hetero mutation	exon 2 homo-deletion	exon 2, 3, 4 hetero deletion	exon 2, 3 hetero deletion	exin 4 hetero deletion	exon 3, 4 hetero deletion	exon 2, 3, 4 homo-deletion	exon 5 hetero deletion
On set	61	28	28	27	18	20	65	45	33	29	47	16	45	34
Disease duration	10	27	18	14	20	16	11	28	36	34	14	44	12	10
Family history	–	+	+	–	–	+	+	–	–	+	–	+	+	+
Hoehn & Yahr stage on	2	2	2	1	1.5	2	2	3	3	3	3	3	1	1
Rigidity*	1	1	0	1	2	0	1	0	0	1	0	0	0	0
Tremor*	1	0	0	1	0	0	1	1	0	0	0	0	0	0
Hesitation*	0	1	1	0	0	2	0	1	1	2	2	1	0	0
Wearing-off	+	+	+	–	+	+	–	+	+	+	+	–	+	–
Dementia	–	–	–	–	–	+**	–	–	–	–	–	–	–	–
Hallucination	–	–	–	–	–	+	–	–	–	–	+	–	–	–
Sleep violent behavior	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Constipation	+***	–	–	–	–	–	–	–	–	–	+	–	–	–
Levodopa	700 mg	600 mg	600 mg	300 mg	400 mg	300 mg	–	300 mg	500 mg	995 mg	800 mg	200 mg	450 mg	–
Agonist non-ergot	prami-pexole 1.5 mg	prami-pexole 4.5 mg	prami-pexole 3 mg	ropinirole 9 mg	prami-pexole 1.5 mg	ropinirole 12 mg	prami-pexole 4.5 mg	–	prami-pexole 0.75 mg	prami-pexole 1.5 mg	prami-pexole 2.25 mg	ropinirole 16 mg	–	pram-ipexole 1.5 mg
Agonist ergot	–	pergolide 2.25 mg	–	–	caber-goline 4 mg	–	–	–	–	–	–	–	caber-goline 2 mg	–
Selegiline	–	5 mg	–	–	10 mg	5 mg	–	–	–	–	5 mg	–	2.5 mg	–
Entacapone	–	400 mg	600 mg	–	300 mg	600 mg	–	–	400 mg	–	–	–	–	–
Trihexyphenidyl	–	–	–	–	3 mg	–	–	5 mg	–	–	–	–	–	–
Amantadine	300 mg	150 mg	300 mg	–	300 mg	–	–	–	150 mg	200 mg	100 mg	300 mg	–	–

DID: dopa induced dyskinesia.
*UPDRS mean score **Thalamotomy ***no medication.

(MyoMIBG-I 123 injection, 111 MBq; FUJIFILM RI Pharma Co. Ltd.). The cut-off ratio of the heart to mediastinum (H/M) uptake ratio of ^{123}I-MIBG in our hospital was set at 1.45.

The olfactory function and polysomnography (PSG) study involved 3 women and 3 men (mean (SD) age, 47.8 (13.2) years) with *PARK2* mutations (Table 1).

The mean olfactory function scores of the PD patients and 10 age-matched Japanese controls, who were evaluated for comparison with patients with *PARK2* mutations, were determined by the Sniffin' Sticks test. Mean age of the controls without neurological disease or dementia was 46.0 (15.3) years (range, 39–79 years). The PD patients (mean age, 69.6 (6.6) years; range, 60–89 years; not age matched to patients with *PARK2* mutations) fulfilled the UK Brain Bank criteria for possible or probable clinical PD, with Hoehn-Yahr stages II and III without dementia.

Olfactory testing was examined by following 3 components. Olfactory threshold and odor discrimination and identification were investigated in 3 separate substrates using standardized Sniffin' Sticks [6]. Sniffin' Sticks are commercially available felt-tip pens.

Odor Thresholds. The olfactory threshold subset consisted of 16 Sniffin' Stick triplets with different concentrations of *n*-butanol. Three sticks were presented to the subject in randomized order. Two contained only the solvent and the third the odorant at a particular dilution. The subjects were tasked to identify the stick with the odorant.

Odor Discrimination. In the odor discrimination subset, 16 Sniffin' Stick triplets were presented in randomized order. Two pens contained the same odorant and the third a different odorant. The task was to identify the stick that had the different smell.

Odor Identification. The third subtest consisted of 16 single sticks and assessed the ability to identify an odor. Using a multiple-choice task, identification of individual odorants was performed from a list of 4 descriptors.

RBD was confirmed by studying the patients' clinical history and video-PSG findings (International Classification of Sleep Disorders, 2nd edition) [7].

Informed consent was obtained from patients with *PARK2* mutations, and patients with PD, and normal volunteers.

The data was statistically analyzed using SPSS ver.11 for Windows.

3. Results

The mean H/M uptake ratio of ^{123}I-MIBG scintigraphy in *PARK2* patients was 1.79 (0.31) in the early phase and 1.75 (0.51) in the delayed phase (Table 2). However, a 58-year-old woman, with a 10-year disease duration and orthostatic hypotension and constipation without myocardial damage, exhibited accelerated MIBG elimination (H/M ratio: early, 1.23; delayed, 1.15). Three patients (cases 2, 7, and 12) had exhibited slightly decreased uptake in the delayed phase.

The Sniffin' Sticks test revealed a slight olfactory dysfunction with the following mean scores in examined *PARK2* patients (Table 3): threshold score, 6.1 (1.6) ($P < .05$ when compared with controls); odor discrimination score, 10.0 (2.4); odor identification score, 10.1 (4.8) (no significant differences when compared to controls). Odor discrimination and identification functions were not impaired in any of the patients with *PARK2* mutations, except in patient 1. In the Japanese examined normative controls, the mean olfactory function scores were as follows: threshold score, 8.0 (1.3); discrimination score, 11.9 (2.4); identification score, 10.9 (2.0); in PD patients, these mean scores were 2.2 (6.6), 6.1 (2.5), and 5.1 (1.8), respectively.

PSG did not reveal tonic responses in the mentalis and tibialis muscles during REM (Table 3). Twitching of the tibialis muscle was observed in 2 patients. None of the patients with *PARK2* mutations met the ICSD-II criteria for RBD.

4. Discussion

Decreased ^{123}I-MIBG uptake was observed clearly in 1 patient with *PARK2* mutations who had autonomic dysfunction. Early phase myocardial uptake of MIBG in all of the other patients showed no decrease, and patients had no autonomic dysfunction. Similar to our study, in a previous study [8], 1 of 4 patients with *PARK2* mutations with a 12-year disease duration and unclear autonomic dysfunction exhibited decreased uptake of ^{123}I-MIBG. Additionally, 3 patients in our study who showed decreased ^{123}I-MIBG uptake were slightly older than the other patients, although a significance in mean age (63.0 (9.1) versus 55.9 (10.8); $P > .05$) did not exist. Estorch et al. and Tsuchimochi et al. reported that the uptake of ^{123}I-MIBG decreased with age, suggesting that aging could affect patients with *PARK2* mutations [9, 10]. Decreased myocardial uptake of MIBG is considered to indicate the presence of alpha-synuclein aggregates in the axons in PD [11]. In MIBG-myocardial scintigraphy, the H/M ratio of patients with *PARK2* mutations was reported to be within the range of the normal controls [12]. Moreover, postmortem examination of patients with *PARK2* mutations revealed that tyrosine hydroxylase immunoreactive nerve fibers in the epicardium were well preserved [13]. These findings might reflect normal functioning myocardial sympathetic nerve terminals in patients with *PARK2* mutations. MIBG scintigraphy might be a marker for alpha-synuclein in patients with *PARK2* mutations; however, there are no pathological reports on the presence of Lewy bodies in patients with *PARK2* mutations exhibiting decreased MIBG uptake.

Olfactory impairment is a nonmotor symptom of PD. We found that the olfactory threshold was slightly higher in patients with *PARK2* mutations than in controls. The oldest woman in our study, who did not have dementia, exhibited the highest degree of olfactory impairment. Although in self-completed questionnaire study, 3 of 16 patients with *PARK2* mutation had loss of taste/smell [14]. However, in previous

Table 2: The findings of ^{123}IMIBG myocardial scintigraphy in PARK2 patients.

Case	1	2	3	5	7	8	9	10	11	12	13	14	Average ± SD
Examined age	65	55	46	41	76	70	63	61	61	60	57	44	58.3 ± 10.5
Early H/M	2.27	1.64	1.91	1.75	1.52	2.05	1.75	1.66	1.23	1.75	1.62	2.35	1.79 ± 0.31
Delay H/M	2.14	1.33	1.67	1.93	1.34	2.93	1.65	1.54	1.15	1.40	1.60	2.35	1.75 ± 0.51

H/M: the heart to mediastinum uptake ratio of ^{123}IMIBG.

Table 3: Olfactory function by Sniffin' sticks and PSG study in patients with PARK2 mutation, controls, and Parkinson's disease.

Case	1	2	3	4	5	6	*PARK2* total ($n = 6$)	Control ($n = 10$)	Parkinson's disease ($n = 15$)
Age	71	55	46	41	38	36	47.8 ± 13.2	46.0 ± 15.3	69.6 ± 6.6
Sniffin' sticks Test									
Threshold test	4.5	6.3	6.3	5.8	5.0	9.0	6.1 ± 1.6	8.0 ± 1.3*	2.2 ± 2.3**
Discrimination test	8.0	9.0	12.0	14.0	9.0	8.0	10.0 ± 2.4	11.9 ± 2.4	6.1 ± 2.5
Identification test	1.0	13.0	10.0	14.0	13.0	10.0	10.1 ± 4.8	10.9 ± 2.0	5.1 ± 1.8
PSG findings									
Apnea index (times/H)	10.1	22.5	1.2	0.4	1.0	2.1			
Hypopnea index (times/H)	2.2	14.0	4.7	0.3	1.9	9.4			
Apnea Hypopnea index (times/H)	12.3	36.5	5.9	0.8	3.0	11.4			
Arousal index (times/H)	16.0	39.8	37.7	13.4	14.3	11.1			
Respiratory arousal index (times/H)	5.5	27.3	4.6	0.2	1.6	3.3			
PLM index(times/H)	7.8	0.0	7.7	0.0	29.5	0.0			
PLM arousal index(times/H)	0.0	0.0	5.3	0.0	2.8	0.0			
REM sleep twitching on TA muscle	−	+	−	+	−	−			

H/M: theheart to mediastinum uptake ratio, NE: not examined.
PLM: periodic limb movements, TA: tibialis anterior.
*t-test: compared with *PARK2* patients $P < .05$.
**t-test: compared with *PARK2* patients and control $P < .01$.

studies, individuals with *PARK2* mutations were found to have normal olfactory function [15, 16]. The discrepancy between our results and previous ones may be because previous studies used the Pennsylvania Smell Identification Test, which does not include the threshold test. In Kahn's study [15], the odor identification score did not significantly differ between patients with *PARK2* mutations and controls, although this did not necessarily imply that the threshold score was normal in the patients.

PSG did not reveal RBD in any of our patients. However, in a study by Kumru et al., 6 of 10 patients had RBD [17]. We cannot explain this discrepancy, but we hypothesize that it may be due to the differences in patient mean age between the 2 studies. Some of our patients with *PARK2* mutations have twitching in the tibialis muscle; therefore, the possibility that they will eventually develop RBD cannot be ruled out. Neuropathological studies on patients with *PARK2* mutations have revealed neuronal loss and gliosis in the pars compacta of the substantia nigra and in the locus coeruleus [18]. However, these studies have not described the state of the subcoeruleus nucleus, which is considered the primary site affected in RBD.

The results obtained in this study suggest that some patients with *PARK2* mutations have increased thresholds of olfactory function and myocardial sympathetic dysfunction as nonmotor symptoms. We might show that the nonmotor symptoms of *PARK2* were impaired heterogeneously.

Author Contributions'

A. Yoritaka was responsible for conception, execution of research projects, statistical analysis, writing of first draft and review and critique; Yasushi Shimo was responsible for execution of research project; Yumi Shimo and Y. Inoue were responsible for execution of research project (PSG study); H. Yoshino was responsible for gene analysis; N. Hattori was responsible for conception and organization of research project.

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Mild parkinsonian signs in a community-dwelling elderly population sample in Japan

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ABSTRACT

Mild parkinsonian signs (MPS) may represent the mild end of a disease spectrum that spans from normal aging to neurodegenerative diseases. We conducted a population-based study in a rural island town in western Japan, Ama-cho. Participants included 1129 subjects, aged 60 years and older, residing in the town. Participants were classified according to a modified Unified Parkinson's Disease Rating Scale (mUPDRS) score. MPS was determined to be present if any of the following conditions were met: (1) two or more mUPDRS ratings = 1 [MPS-mild]; (2) one mUPDRS rating ≥ 2; or (3) mUPDRS rest tremor rating ≥ 1; [(2) and (3): MPS-severe]. Subjects wore a uniaxial accelerometer (Actiwatch), resulting in the measurement of actigraphic activity counts (AC).

Of the 804 participants with complete data, 178 subjects (22.1%) were classified as demonstrating MPS. AC was significantly lower in the MPS-severe group compared with both the CTL and the MPS-mild groups. Diagnostic sensitivity for MPS-severe became 100% when we adopted a cutoff point of low physical activity, as measured by actigraphy, combined with the presence of subjective depression.

We established the prevalence of MPS in a community-dwelling elderly population sample in Japan. Actigraphy may be a useful objective tool for screening MPS-severe.

1. Introduction

Mild parkinsonian signs (MPS), including bradykinesia, rigidity, gait disturbance and resting tremor, may represent the mild end of a disease spectrum that spans from normal aging [1] to neurodegenerative diseases [2], including Parkinson's disease (PD). MPS has also been reported to be the result of nigrostriatal Alzheimer's disease (AD)-type pathology [3], associated with increased risk of dementia [4], associated with vascular lesions of basal ganglia and white matter [5,6], and a significant predictor of mortality [7]. However, the clinical significance of MPS is not yet fully understood. The prevalence of MPS in sample populations in East Boston, England [8], New York, USA [9] and Jiangsu, China [7] has been reported, but inconsistencies exist across reports because of differences in MPS definition, study methodology, age structure, and cognitive status [10]. The prevalence of MPS in Japan has hitherto not been reported.

We have conducted the first epidemiological study to suggest the prevalence of MPS in Japan. Furthermore, we examined the usefulness of actigraphy as an objective indicator for MPS through a population-based study in order to establish screening methods for MPS in association with questionnaires about motor and nonmotor symptoms of Parkinson's disease (PD).

2. Methods

2.1. Subjects

This study was conducted in the municipality of Ama-cho, a rural island town located 70 km from Yonago city, in the northwestern part of Japan [11]. To be included in the study, subjects were required to be living and to be legally residing in the town on March 31, 2008. The total population of Ama-cho on this day was 2402 (1124 men). The number of elderly people aged 60 years and older was 1129 (479 men, mean age ± SD 74.6 ± 9.1 years old). Board certified neurologists of the Japanese Society of Neurology (neurologists) belonging to our department have visited this town twice a year since 1980, and diagnosed patients having neurological disorders. Before this study, 11 patients with PD were recognized through these visits.

The study was approved by the committee for medical research ethics at Tottori University following the principles outlined in the "Declaration of Helsinki", and all participants provided written informed consent to participate in the study.

2.2. Questionnaire survey

We administered a questionnaire survey in May 2008. First, we mailed the questionnaires to residents aged 60 years or older. To assess motor symptoms, we included the Tanner questionnaire, [12], which is validated as a PD patient screening form. To evaluate depressive symptoms, we included the Japanese version of the

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Geriatric Depression Scale with 15 questions (GDS-15). [13]. It has been validated for the diagnosis of depression, and the recommended cutoff points are ≥6 as mild depression and ≥10 as severe depression [13,14]. We included the Pittsburgh Sleep Quality Index (PSQI) [15] and the REM Sleep Behavior Disorder Screening Questionnaire (RBDSQ) [16] to assess sleep disturbances. The cutoff value of the PSQI for a poor sleeper was 5/6 points, [15], and the RBDSQ to detect REM sleep behavior disorder (RBD) was 5/6 points. [17]. Demographic data, including age, gender, duration of education, and present smoking and drinking habits, were collected using the questionnaire. In order to evaluate nonmotor symptoms, we assessed the presence of constipation, hallucinations, hyposmia, and orthostatic hypotension with the questionnaire.

2.3. Neurological examination

Each participant underwent a structured medical interview including a past history of hypertension, diabetes mellitus, and hyperlipidemia. A standardized neurological examination was conducted by one of four neurologists, including an abbreviated (10-item) version of the motor portion of the Unified PD Rating Scale (UPDRS) in 2008–2009. The 10 items screened for speech, facial expression, tremor at rest, rigidity (rated separately in the neck, right arm, left arm, right leg, and left leg), posture, and body (axial) bradykinesia, with each item rated from 0 to 4. A rating of 1 indicated a mild abnormality and a rating of ≥2 indicated an abnormality of moderate or greater severity [9]. Subjects with a total UPDRS score of 0 were classified as being normal controls (CTL). We assigned a diagnosis of PD based on research criteria [18] and participants were considered to have PD if (1) they had previously received a diagnosis of PD by neurologists and responded to L-dopa or (2) their symptoms fulfilled the UK PD brain bank criteria, [19], or both. Those who had two or more cardinal signs (UPDRS rating≥2) on the standardized neurologic examination were classified as having parkinsonism. These cardinal signs include bradykinesia, rigidity, postural instability, and resting tremor.

MPS were defined as present when any one of the following conditions was met: (1) two or more UPDRS ratings=1; (2) one UPDRS rating≥2; or (3) a UPDRS resting tremor rating≥1 [10]. MPS was further stratified into subtypes according to symptom types and severity as shown in Table 1 [4, 20, 21].

2.4. Measurement of physical activity using actigraphy

In order to obtain participants for the actigraphy study, we gathered subjects in five districts, randomly selected from the fourteen districts in the town (participation rate: 65.0%).

Physical activity was quantified using wrist-worn uniaxial accelerometers (Actiwatch-16, Mini Mitter-Philips Respironics, Bend, OR) [22]. Physical activity was monitored in the participant's own homes, and participants were instructed to continue their normal daily routine. Participants wore Actiwatches on their nondominant wrist for 1 week collecting data in 1-minute epochs. Those subjects with unilateral PD wore monitors on their least affected side. This placement has been shown to better represent whole-body movement [25] and was intended to reduce artifacts such as low level constant activity when writing with the dominant hand or dyskinesias in the most affected arm. At the same time, all participants completed a sleep log for 7 days. All actigraphic data were validated in accordance with entries in sleep logs. Automatic activity analysis using dedicated software (Actiware, Mini Mitter-Philips Respironics) was conducted. The measures analyzed were Total AC (the sum of all valid physical activity counts for all awake epochs), Avg AC (the average of all valid physical activity counts for all awake epochs divided by the epoch length in minutes), and Max AC (the largest of any valid physical activity count for all awake epochs).

Table 1
Classification of mild parkinsonian signs.

Classification according to symptoms	
Axial dysfunction	(1) UPDRS ratings=1 in two or more of the four items of axial function (changes in speech, facial expression, posture, and axial bradykinesia), or (2) one UPDRS rating≥2 in one of the four items
Abnormality in rigidity	Either (1) UPDRS ratings=1 in two or more of the five items of rigidity, or (2) one UPDRS rating≥2 in one of the five items
Tremor	A UPDRS resting tremor rating≥1
Unclassified	Could not be classified into any of the above-mentioned categories
Classification according to severity of UPDRS score	
MPS-mild	A UPDRS rating of 1
MPS-severe	A UPDRS rating of 2 or higher, or presence of resting tremor

MPS: mild parkinsonian signs, UPDRS: Unified PD Rating Scale.

2.5. Statistical analyses

The adjusted prevalence was calculated for all types of MPS and PD using the Japanese population on March 1, 2008. Paired t tests and analysis of variance (ANOVA) were used for comparison of medians for continuous variables, and categorical variables were analyzed using a chi-square test. Pearson's test was used for correlation analyses. Differences in the total physical activities between groups were evaluated with an analysis of covariance (ANCOVA), adjusting for age. Analyses of the relationship between the background of the nonmotor symptoms and MPS-severe were performed by multivariate logistic regression analysis. Significance was defined as $p<0.05$, and all analyses were conducted using the Statistical Package for the Social Sciences version 17.0 software (SPSS17.0, 2008, Tokyo, Japan).

3. Results

3.1. Questionnaire survey

Nine hundred sixty-eight (85.7%) of 1129 residents returned their questionnaire. As compared to survey nonrespondents, respondents were similar in age (mean=74.7 years vs. 75.1 years) and gender (47.1% male vs. 43.5% male).

3.2. Prevalence of PD and MPS in a community-dwelling elderly population sample

Eight hundred four of 1129 subjects received a neurological examination (71.2%). We diagnosed 69 subjects as having parkinsonism (24 men, 82.9±7.1 years). Of the parkinsonism patients, 14 were diagnosed as having PD (4 men, 79.6±7.6 years). The crude prevalence of PD and the age-adjusted prevalence when calculated using the Japanese population in 2008 were 1.5% and 1.3% for PD in those over the age of 65.

Of the examined subjects, 178 were diagnosed as having MPS (62 men, 78.1±8.1 years). The crude prevalence of MPS was 22.1% (95% CI: 19.3–25.0) in participants over 60 years of age, and 23.7% (95% CI: 20.6–26.9) in participants over 65 years of age. The age-adjusted prevalence of MPS was 13.8% in the over 60 population, and 16.8% in the over 65 population. We showed the classification of MPS according to its type and severity in Table 2.

3.3. Physical activity measured by actigraphy

Using actigraphy, we evaluated 265 subjects (121 men; age: 74.2±7.9 years), including 174 control (CTL) subjects (75 men; 72.2±7.2 years), 53 subjects with MPS-mild (22 men; 78.3±7.2 years), 19 subjects with MPS-severe (5 men; 78.4±6.6 years), and 19 subjects with parkinsonism (7 men; 81.0±7.5 years) including 7 PD patients

Table 2
Age- and sex-specific prevalence of MPS.

Age (years)	Residents	Population at risk	MPS																Parkinsonism	
			Total		Type										Severity					
					Axial dysfunction		Rigidity		Mixed		Tremor		Unclassified		MPS-mild		MPS-severe			
			Cases	Prevalence	Cases	Prevalence	Cases	Prevalence	Cases	Prevalence	Cases	Prevalence	Cases	Prevalence	Cases	Prevalence	Cases	Prevalence	Cases	Prevalence
Both sexes																				
60–64	183	88	8	9.1%	1	1.1%	7	8.0%	–	–	–	–	–	–	7	8.0%	1	1.1%	–	–
65–69	180	135	18	13.3%	2	1.5%	12	8.9%	2	1.5%	2	1.5%	–	–	15	11.1%	3	2.2%	1	0.7%
70–74	198	164	28	17.1%	5	3.0%	18	11.0%	3	1.8%	2	1.2%	–	–	23	14.0%	5	3.0%	9	5.5%
75–79	227	183	49	26.8%	12	6.6%	25	13.7%	11	6.0%	1	0.5%	–	–	34	18.6%	15	8.2%	13	7.1%
80–84	158	121	43	35.5%	14	11.6%	16	13.2%	7	5.8%	2	1.7%	4	3.3%	26	21.5%	17	14.0%	11	9.1%
85–	183	113	32	28.3%	9	8.0%	18	15.9%	4	3.5%	–	–	1	0.9%	23	20.4%	9	8.0%	35	31.0%
Total	1129	804	178	22.1%	43	5.3%	96	11.9%	27	3.4%	7	0.9%	5	0.6%	128	15.9%	50	6.2%	69	8.6%
Men																			–	–
60–64	94	38	3	7.9%	1	2.6%	2	5.3%	–	–	–	–	–	–	2	5.3%	1	2.6%	–	–
65–69	84	63	8	12.7%	1	1.6%	5	7.9%	1	1.6%	1	1.6%	–	–	8	12.7%	–	–	1	1.6%
70–74	89	67	11	16.4%	1	1.5%	7	10.4%	2	3.0%	1	1.5%	–	–	9	13.4%	2	3.0%	5	7.5%
75–79	97	72	17	23.6%	4	5.6%	7	9.7%	6	8.3%	–	–	–	–	11	15.3%	6	8.3%	3	4.2%
80–84	53	38	10	26.3%	2	5.3%	3	7.9%	2	5.3%	1	2.6%	2	5.3%	6	15.8%	4	10.5%	2	5.3%
85–	62	44	13	29.5%	2	4.5%	10	22.7%	–	–	–	–	1	2.3%	11	25.0%	2	4.5%	13	29.5%
Total	479	322	62	19.3%	11	3.4%	34	10.6%	11	3.4%	3	0.9%	3	0.9%	47	14.6%	15	4.7%	24	7.5%
Women																			–	–
60–64	89	50	5	10.0%	–	–	5	10.0%	–	–	–	–	–	–	5	10.0%	–	–	–	–
65–69	96	72	10	13.9%	1	1.4%	7	9.7%	1	1.4%	1	1.4%	–	–	7	9.7%	3	4.2%	–	–
70–74	109	97	17	17.5%	4	4.1%	11	11.3%	1	1.0%	1	1.0%	–	–	14	14.4%	3	3.1%	4	4.1%
75–79	130	111	32	28.8%	8	7.2%	18	16.2%	5	4.5%	1	0.9%	–	–	23	20.7%	9	8.1%	10	9.0%
80–84	105	83	33	39.8%	12	14.5%	13	15.7%	5	6.0%	1	1.2%	2	2.4%	20	24.1%	13	15.7%	9	10.8%
85–	121	69	19	27.5%	7	10.1%	8	11.6%	4	5.8%	–	–	–	–	12	17.4%	7	10.1%	22	31.9%
Total	650	482	116	24.1%	32	6.6%	62	12.9%	16	3.3%	4	0.8%	2	0.4%	81	16.8%	35	7.3%	45	9.3%

(2 men; 77.8 ± 7.2 years). Ruling out a selection bias, there were no significant differences between activity measurement participants and non-participants with regard to age (74.3 ± 8.0 vs. 75.0 ± 9.4 years, respectively, p = 0.253), gender (43.3% male vs. 42.1% male, respectively, p = 0.390), or UPDRS score (1.4 ± 2.3 vs. 1.2 ± 2.4, respectively, p = 0.239).

While there was no significant difference in Total AC between the CTL and MPS-mild groups, Total AC in the MPS-severe group was significantly lower than that in the CTL and MPS-mild groups (Fig. 1). Our measure of Avg AC showed the same tendency as Total AC. However, our measure of Max AC was not significantly different among the groups. These three indices of physical activity were significantly associated with age (Total AC: $r = -0.358$, $p<0.001$, Avg AC: $r = -0.330$, $p<0.001$, Max AC: $r = -0.258$, $p<0.001$). ANCOVA analysis, adjusted for the age of subjects, revealed that Total AC in the MPS-severe group was significantly lower than that in the CTL group.

We divided the MPS group according to axial dysfunction scores into three subgroups: non-axial dysfunction (axial dysfunction score = 0, n = 34), mild axial dysfunction (axial dysfunction score = 1 or 2, n = 28), and moderate/severe axial dysfunction (axial dysfunction score = 3 or more, n = 10). Total AC, Avg AC and Max AC in the non-axial dysfunction group were 323,834.6 ± 21,927.8, 383.9 ± 25.0, and 2507.9 ± 151.5, those in the mild axial dysfunction group were 240,077.7 ± 22,175.5, 300.8 ± 25.9, and 2149.2 ± 124.9, and those in the moderate/severe axial dysfunction group were 193,873.6 ± 20,551.1, 245.7 ± 25.6, and 1755.9 ± 174.4, respectively. Total AC and Avg AC of the moderate/severe axial dysfunction group were significantly lower than those of the non-axial dysfunction group. In addition, Total AC, Avg AC, and Max AC of the mild axial dysfunction group were significantly lower than those of the non-axial dysfunction group. However, there were no significant differences in the three activity parameters between the mild axial dysfunction group and the moderate/severe axial dysfunction group.

We also divided the MPS group according to rigidity scores into three subgroups: non-rigidity (maximum rigidity score = 0, n = 17), mild rigidity (maximum rigidity score = 1, n = 53), and moderate/severe rigidity (maximum rigidity score = 2, n = 2). There were no significant differences in the three activity parameters among these groups.

Finally, we also divided the MPS group according to tremor scores into three subgroups: non-tremor (tremor score = 0, n = 67), mild tremor (tremor score = 1, n = 5), and moderate/severe tremor (tremor score = 2, n = 0). There were no significant differences in activity between these groups.

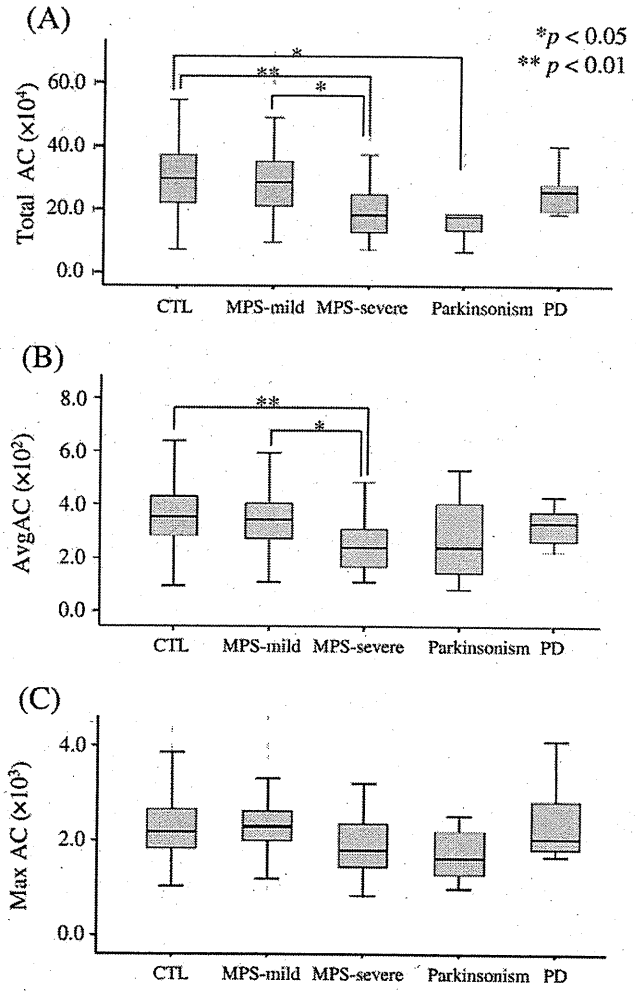

Fig. 1. Comparison of physical activity. The box plots show the median values (thick lines), 25th percentile (lower line of box), and 75th percentile (upper line of box). T bars indicate the 10th and 90th percentiles. Statistical differences were calculated using an ANOVA followed by Tukey tests. CTL: normal controls, MPS: mild parkinsonian signs. PD: Parkinson's disease. (A) Total AC: the sum of all valid physical activity counts for all epochs from the start time to the end time of the given awake interval, (B) Avg AC: the average of all valid physical activity counts for all awake epochs divided by the epoch length in minutes, (C) Max AC: the largest of any valid physical activity count for all awake epochs. *$p<0.05$, **$p<0.01$.

3.4. Association of nonmotor PD symptoms with MPS

There were no significant differences between the CTL group and both the MPS-mild and MPS-severe groups for habitual history, past history, nonmotor PD symptoms, or RBDSQ scores (Table 3). There was a significantly lower proportion of 'sleep disturbance' on the PSQI in the MPS-mild group, but not in the MPS-severe group, as compared with the CTL group.

The GDS scores of the MPS group were significantly higher than those of the CTL group (4.3 ± 3.4 vs. 3.2 ± 3.1, $p = 0.01$) and there was a significantly higher proportion of subjects with 'mild depression' on the GDS in the MPS group as compared with the CTL group (41.3% vs. 27.0%, $p<0.001$), indicating a strong association of subjective depression with MPS.

The proportion of subjects with 'mild depression' on the GDS was significantly higher in the MPS-mild group than in the CTL group. The proportion of subjects with 'severe depression' was significantly higher in the MPS-severe group than in the CTL group.

3.5. Screening for MPS

In the present study, when one point was assumed to be a cutoff in the Tanner questionnaire, the sensitivity for detecting PD was 100%. However, it was only 71.9% for detecting MPS (both MPS-mild and MPS-severe) and 73.3% for detecting MPS-severe. When predictors of MPS-severe were examined by multivariate logistic analysis, GDS and

Table 3
Demographic characteristics of participants stratified by MPS.

	CTL	MPS-mild	MPS-severe
Present smoking, n (%)	33 (7.5%)	4 (3.9%)	3 (7.1%)
Present drinking, n (%)	116 (26.6%)	19 (18.4%)	4 (9.3%)
Constipation, n (%)	97 (22.9%)	37 (37.0%)	15 (38.5%)
Hallucination, n (%)	30 (7.0%)	8 (8.3%)	7 (17.1%)
Hyposmia, n (%)	49 (11.4%)	17 (17.0%)	7 (17.1%)
Orthostatic hypertension, n (%)	79 (18.3%)	24 (24.2%)	14 (32.6%)
GDS ≥ 6, n (%)	123 (27.4%)	42 (40.4%)**	17 (39.5%)
GDS ≥ 10, n (%)	23 (5.1%)	7 (6.7%)	7 (17.1%)**
RBDSQ ≥ 5, n (%)	37 (8.2%)	17 (16.3%)	5 (11.6%)
PSQI ≥ 6, n (%)	107 (23.8%)	18 (17.3%)*	8 (18.6%)

GDS: Geriatric Depression Scale, PSQI: Pittsburgh Sleep Quality Index, RBDSQ: REM Sleep Behavior Disorder Screening Questionnaire. *$p<0.05$, **$p<0.01$ vs. CTL.

Table 4
Predictors of MPS-severe status by multivariate logistic regression analysis.

Variable	Pearson's rank correlation	Univariate logistic regression analysis	Multivariate logistic regression analysis
		Odds ratio (95% CI)	Odds ratio (95% CI)
Age	0.231**	1.129** (1.081–1.181)	–
Education	−0.114*	0.793* (0.665–0.946)	–
Tanner	0.261**	1.435** (1.274–1.616)	–
GDS	0.155**	1.172** (1.074–1.279)	1.4* (1.1–1.8)
PSQI	−0.021	–	–
RBDSQ	−0.010	–	–
Total AC	−0.267**	0.694** (0.553–0.870)	0.5** (0.3–0.8)

Education: duration of education, GDS: Geriatric Depression Scale, PSQI: Pittsburgh Sleep Quality Index, RBDSQ: REM Sleep Behavior Disorder Screening Questionnaire. *$p<0.05$, **$p<0.01$.

Total AC were shown to be independent predictive factors (Table 4). Based on this finding, diagnostic sensitivities, specificity, and positive predictive value (PPV) became 100%, 83.5%, and 62.2% (respectively) for MPS-severe when we adopted a cutoff point of more than 6 points for GDS or less than 40×10^4 for Total AC. When we used the same screening method, diagnostic sensitivities, specificity, and PPV became 85.7%, 83.5%, and 68.2% for the entire MPS group, 94.4%, 83.5%, and 37.8% for the parkinsonism group including PD, and 87.5%, 83.5%, and 73.3% for a combination of all the groups (MPS and parkinsonism including PD), respectively.

4. Discussion

Only a few reports have documented the prevalence of MPS, indicating a prevalence of 15.8% in retired military officers aged 75 years or older in Nanjing [7]; 14.9% (age 65–74), 29.5% (age 75–84), and 52.5% (age 85 and older) in East Boston [8]; and 40.1% in residents aged 65 years or older in New York [9]. Our study revealed that the crude prevalence of MPS was 22.1% in the population over 60 years of age, and 23.7% in the population over 65 years of age. These data are in agreement with earlier cohort studies reporting similar findings.

One of the difficulties in studying the prevalence of MPS is the definition of MPS. Several studies have defined MPS liberally (any one UPDRS rating of 1 or higher [9,21]), while others have defined it more rigorously (two or more such signs or one sign of moderate severity (UPDRS rating≥2) [10]). One motivation for using more rigorous criteria is to try to separate MPS from the signs of normal aging. The more rigorous criteria are also considered to avoid the influence of other chronic illnesses and the aging process, and thus more likely to reflect pathological brain changes resulting in MPS [10]. However, a clear distinction between MPS and normal aging has not been established.

In the present study, we classified MPS into two subgroups according to the severity of the UPDRS rating. In order to investigate differences in physical activity between these two subgroups, we measured physical activity using actigraphy. Previous studies have reported the usefulness of standard actigraphy to assess fluctuation of akinesia [23], tremor, motor fluctuation [24], and sleep in PD patients [25,26]. In the present study, measured activity counts in the PD group were higher compared to the MPS-severe group. We noted that the PD patients who showed higher activity counts on actigraphy measures tended to receive higher Levodopa equivalent doses, had a shorter PD duration, and/or displayed a resting tremor (data not shown). These factors might account for higher activity counts in the PD group compared to the MPS group or parkinsonism group. In particular, the PD patient who generated the highest activity counts in the PD group displayed excessive overactivity due to the side effects of anti-parkinsonian drugs when he wore the Actiwatch. When we excluded this patient from the analysis, the activity counts of the PD group were significantly lower than those of both the CTL group ($p=0.036$) and the MPS-mild group ($p=0.044$). Unfortunately, the number of PD patients present in this study might be too small to confidently analyze their activity counts.

On the other hand, our measure of Total AC in the MPS-severe group was significantly lower than that measured in both the CTL and MPS-mild groups. Levels of physical activity were significantly associated with age, as participants with MPS were significantly older than those in the CTL group. However, an ANCOVA analysis revealed that the Total AC of subjects in the MPS-severe group, even after adjusting for age, was significantly lower compared to the CTL group.

To further clarify the clinical meaning of our actigraphic data, we divided the MPS group according to axial dysfunction scores, rigidity scores, and tremor scores. There were no significant differences between the mild axial dysfunction group and the moderate/severe axial dysfunction group. However, there was a significant difference between the non-axial dysfunction group and the moderate/severe axial dysfunction group in both Total AC and Avg AC, and between the non-axial dysfunction group and the mild axial dysfunction group in Total AC, Avg AC, and Max AC. Among the rigidity groups, there were no significant differences, although the activity counts of the mild tremor group were higher compared to the non-tremor group.

Therefore, we believe that our actigraphic data primarily relates to axial dysfunction.

These data suggest that a UPDRS rating of 2 may be more appropriate than a rating of 1 for distinguishing between MPS and normal aging. Future longitudinal studies evaluating the condition of MPS subjects after several years should be conducted in order to assess the suitability of the distinction between MPS-mild and MPS-severe classifications.

While the sensitivity of the Tanner questionnaire for detecting PD was 100%, its sensitivity for detecting MPS-severe was only 73.3% in our sample, indicating that the Tanner questionnaire is not suitable for screening MPS. Moreover, nonmotor symptoms such as constipation, hallucination, hyposmia, and orthostatic hypotension, which have been considered to be suggestive diagnostic markers for PD, were also not suitable for screening MPS in our sample. Sleep disturbance was also inadequate as a screening marker for MPS. We had a large number of subjects with sleep disturbance in our CTL group. In contrast, GDS scores and our measure of Total AC were independent predictive factors for MPS-severe status when we entered age, duration of education, Tanner questionnaire, GDS, PSQI, and RBDSQ scores, and Total AC as predictors of MPS-severe. Interestingly, when we adopted a cutoff point of more than 6 points on the GDS or less than 40×10^4 of Total AC, diagnostic sensitivities became 100%.

Finally, although depression was associated with the presence of MPS, the presence of depression is not unique to MPS. Depression is a common and disabling disorder in later life [13,27], and while subjects with depression have been reported to have significantly lower scores for activity of daily living (ADL) and quality of life (QOL) than those without depression [28], depression in the elderly has also been reported to be associated with poor cognitive function [29], dementia [30], developing AD [31], premotor symptoms in PD [32], and cerebrovascular disease [33]. Viewing these findings together with the organic pathological changes of the brain, leads us to believe that such brain changes may influence both the mood as well as motor function of the elderly who only have mild symptoms of neurodegenerative disease.

This study has several strengths, including the assessment of a well-characterized cohort of community-dwelling elderly subjects. In addition, our findings are based on validated actigraphy. Limitations include the use of a volunteer cohort and the cross-sectional nature of our study design. An accurate evaluation of sleep disturbances and RBD was not made because we screened subjects based on subjective symptoms without polysomnography. Future longitudinal studies are necessary to clarify the prognosis of MPS and the use of UPDRS rating of 2 to distinguish between MPS and normal aging.

5. Conclusions

Here we report the prevalence of MPS in Japan for the first time. Measuring physical activity using actigraphy and evaluating depression using GDS enabled us to detect MPS, which may lead to the early intervention of neurodegenerative disorders in aging populations.

Authors' roles

Yusuke Uemura: Research project Conception, Organization, Execution, Statistical Analysis Design, Execution, Review and Critique, Manuscript Writing of the first draft, Review and Critique.

Kenji Wada-Isoe: Research project Conception, Organization, Execution, Statistical Analysis Design, Execution, Manuscript Review and Critique.

Satoko Nakashita: Research project Execution.

Kenji Nakashima: Research project Conception, Organization, Execution, Manuscript Review and Critique.

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