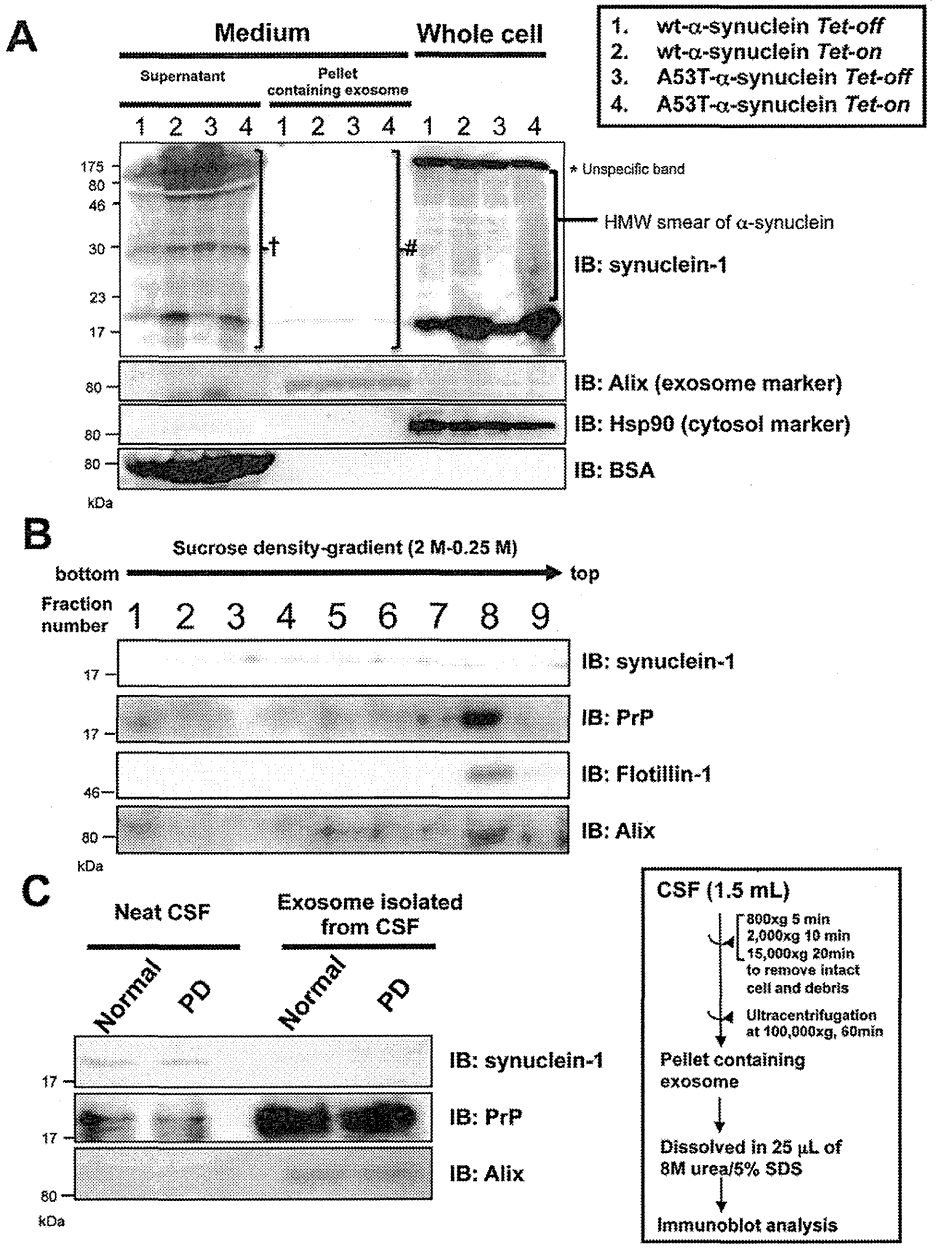

Figure 2. α-synuclein is detected in supernatant but not in the exosome-containing pellets from neuronal culture medium and CSF by standard immunoblot analysis. *A.* wt and A53T mutant αSYN were inducibly expressed in SH-SY5Y cells for 48 hours. Culture medium as well as whole cell lysates (50 μg protein per lane) were subjected to Western blot analysis. The collected media were further separated into the supernatant and exosome-containing pellets before loading onto gels. Alix, Hsp90, and BSA were used as markers for exosome, cytosol, and culture medium, respectively. In the neuronal culture medium, both monomeric/oligomeric wt and mutant αSYN were recovered in the supernatant (*dagger*) rather than exosome-containing pellets (*hash*). *Asterisk* indicates unspecific band. *B.* The resuspended exosome-containing pellets from the culture medium were further separated by sucrose-density gradient followed by Western blot analysis. Immunoblot probed with synuclein-1 anti-αSYN, anti-PrP Abs and the successful separation of exosome was confirmed by exosomal markers, Flotillin-1 and Alix. As shown in the blot, PrP migrated near the top of the density gradient (fraction #8) with concomitant enrichment of exosome-associated proteins. By contrast, no exosomal enrichment was observed with αSYN. *C.* CSF (1.5 mL) from 5 PD patients together with age-matched controls was pooled and exosome-containing pellets were isolated by successive centrifugation indicated. Equal concentrations (50 μg per lane) of total CSF samples were loaded alongside CSF-derived exosomes and then probed with anti-αSYN and PrP antibodies. PrP detected in CSF-derived exosomes was enriched compared to neat CSF. αSYN was weakly but specifically detected in neat CSF, whereas no αSYN-positive signal could be detected in CSF-derived exosomes. No significant difference was observed in the expression levels of CSF αSYN between PD patients and normal controls. Representative Western blots from three independent experiments are presented.

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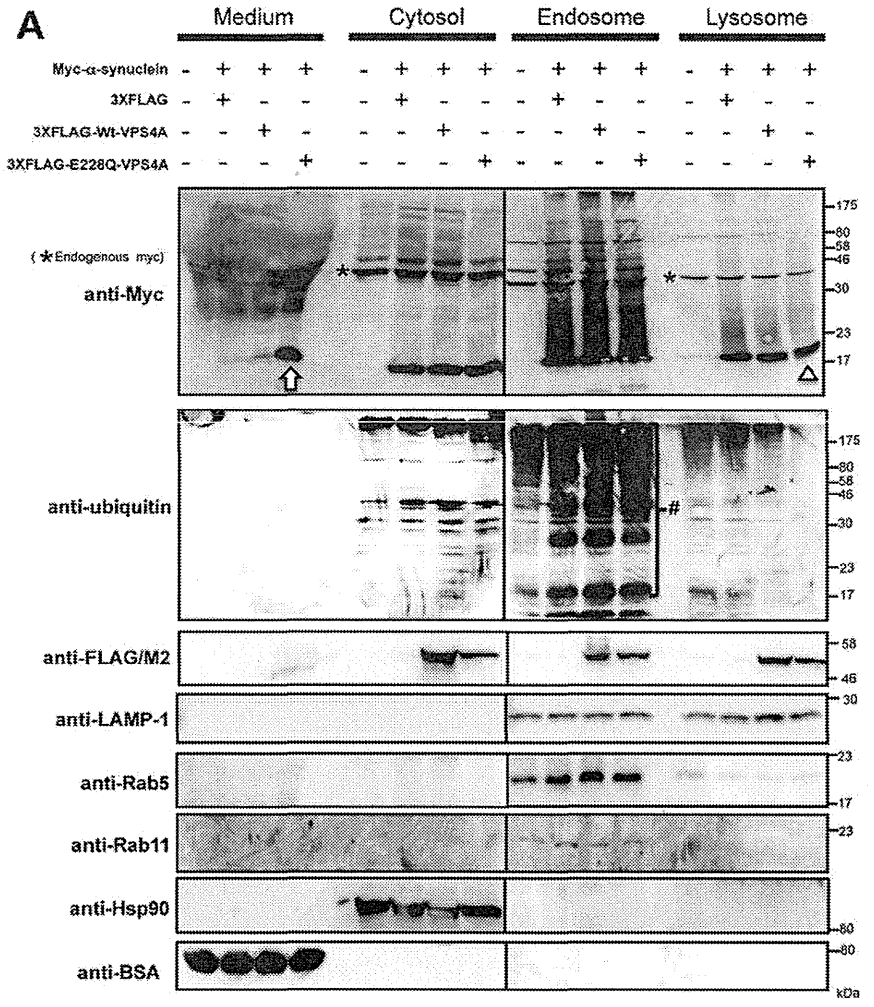
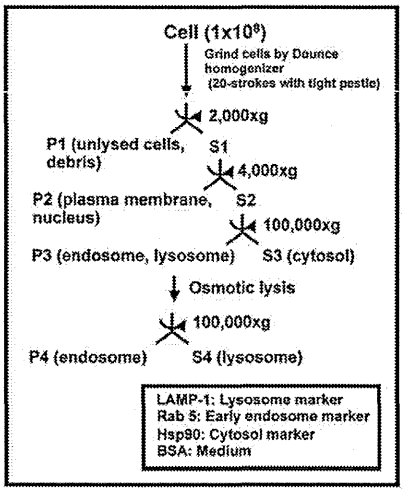
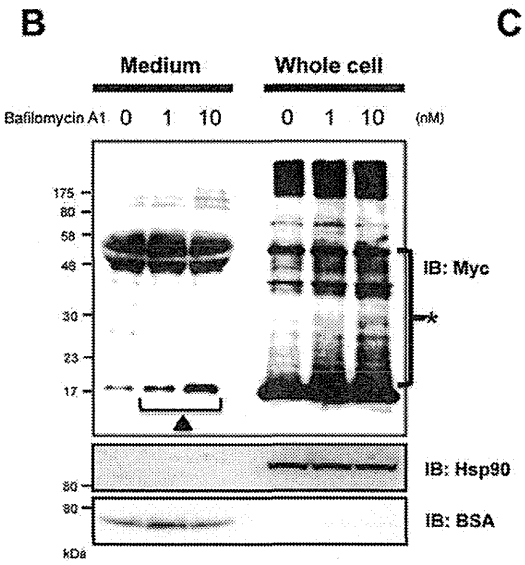

A
Medium
Cytosol
Endosome
Lysosome
Myc-α-synuclein
3XFLAG
3XFLAG-Wt-VPS4A
3XFLAG-E228Q-VPS4A
(*Endogenous myc)
anti-Myc
anti-ubiquitin
anti-FLAG/M2
anti-LAMP-1
anti-Rab5
anti-Rab11
anti-Hsp90
anti-BSA
kDa


Cell (1x10⁸)
Grind cells by Dounce homogenizer (20-strokes with tight pestle)
2,000xg
P1 (unlysed cells, debris)
S1
4,000xg
P2 (plasma membrane, nucleus)
S2
100,000xg
P3 (endosome, lysosome)
S3 (cytosol)
Osmotic lysis
100,000xg
P4 (endosome)
S4 (lysosome)
LAMP-1: Lysosome marker
Rab 5: Early endosome marker
Hsp90: Cytosol marker
BSA: Medium


B
Medium
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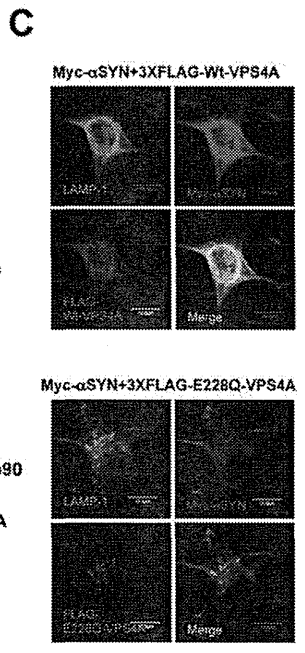
C
Myc-αSYN+3XFLAG-Wt-VPS4A
Myc-αSYN+3XFLAG-E228Q-VPS4A
LAMP-1
Merge

Figure 3. Over-expression of DN VPS4 in HEK293T cells leads to increased extracellular α-synuclein and parallel decrease in lysosome. *A.* αSYN-expressing HEK293T cells were co-transfected either with 3XFLAG-tagged wt-VPS4A or DN mutant (E228Q) VPS4A. Forty-eight hours after transfection, HEK293T cells were fractionated into the cytosol (S3), endosome (P4), and lysosome (S4). Fractionated cell lysates as well as protein extracts from the culture medium (50 μg protein per lane) were subjected to Western immunoblot analysis using anti-Myc, anti-ubiquitin, anti-FLAG/M2 Abs. Each fraction was verified by the presence of a specific marker protein: LAMP-1 (late endosome and lysosome), Rab5 (early endosome), Rab11 (recycling endosome), Hsp90 (cytosol), and BSA (culture medium). As shown in the blot, marked increase of extracellular αSYN monomer and multimers (*white arrow*) concomitant with slightly decreased lysosomal αSYN-immunopositive smear (*open triangle*) were observed by over-expression of DN VPS4A. Note that endosomal proteins including αSYN seemed to be heavily ubiquitylated (*hash*). *Asterisk* indicates endogenous myc band. *B.* Treatment with lysosomal inhibitor bafilomycin A1 (0–10 nM) for 24 hours induced the buildup of cellular αSYN oligomers (*asterisk*) in parallel with the increase of extracellular αSYN monomer (*closed triangle*). *C.* Subcellular localization of Myc-αSYN (red) in HEK293T cells expressing wt or DN VPS4A (magenta). LAMP-1 (green) was used as a marker for late endosome and lysosome. DN VPS4 distibuted as aberrant cytoplasmic punctate structures, showing a marked contrast to wt-VPS4A with diffuse perinuclear distribution. Representative Western blots from three separate experiments are shown. Scale bar: 10 μm.
doi:10.1371/journal.pone.0029460.g003

of aberrant cytoplasmic punctate structures, providing a distinct contrast to the diffuse perinuclear distribution of wt-VPS4A (Fig. 3C) [43]. We confirmed that the aberrant secretion of αSYN by DN-VPS4A expression was not a cell-type-specific phenomenon in HEK293T cells since we observed an identical result in SH-SY5Y neuronal cells, namely, wt as well as A53T mutant αSYN secretion was significantly increased by the nucleofection of wt- and DN-VPS4A (Fig. 4A). Note that the extracellular secretion of monomeric wt-αSYN was much higher than that of A53T mutant αSYN in mock-transfected cells as well as in DN-VPS4A engineered cells (Fig. 4B). Nucleofection of SH-SY5Y cells using the Nucleofector device provided a technique for introducing constructs into SH-SY5Y cells with ~70% efficiency as estimated from the EGFP fluorescence at 48 hours post-transfection (our unpublished data).

VPS4 is found in the core structures of Lewy bodies

As shown in Fig. 3A, we found that αSYN in endosome and lysosome is more prone to aggregate than in cytosol. This result implies that endosomal/lyosomal organelles containing αSYN aggregates might be the potential source of Lewy bodies. To prove this, the substantia nigra and the temporal lobes from four patients with PD and four age-matched controls dying from known, non-neurological causes were subjected to immunohistochemical analysis using anti-human VPS4 Ab. In all brain tissues from PD patients, the core structures of Lewy bodies showed VPS4 immunoreactivity (Fig. 5), whereas only weak background staining was observed in control brain sections (data not shown). The percentage of VPS4-immunoreactive Lewy bodies in the substantia nigra (A and B) and the temporal lobes (C and D) of four PD brains are 90% and 10%, respectively.

Increased Secretion of α-Synuclein by DN-VPS4A Is Restored by DN-Rab11a

It was shown that αSYN incorporated from the extracellular space was able to be resecreted out of neurons via a process modulated by recycling endosome regulator Rab11a [50]. To test the possible implication of the Rab11a-dependent recycling pathway in the secretion of αSYN *in vivo*, αSYN-expressing HEK293T cells were co-transfected with EGFP, EGFP-tagged wt-Rab11a, Q70L constitutively active (CA)-Rab11a, or S25N DN-Rab11a construct, respectively (Fig. 6). The S25N point mutation in Rab11a has been known to increase its activity for GDP, thereby locking the Rab GTPase in an inactive, non-membrane-associated state [51]. In comparison with EGFP, wt-Rab11a, and CA-Rab11a expressing cells, the cells expressing DN-Rab11a showed a slight decrease of the extracellular oligomeric αSYN in CM as well as the appearance of αSYN-immunopositive HMW smear in the endosome and, to a lesser extent, cytosolic and lysosomal fractions. This finding indicated that a part of endogenous αSYN was trafficked via a recycling endosome pathway for extracellular secretion, and the reduced recycling efficiency by DN-Rab11a expression would probably yield the aberrant retention of αSYN both in endosomes and lysosomes. Given the role of Rab11a in regulating the secretion of cellular αSYN, we speculated that the Rab11a-regulated recycling pathway could also be involved in the hypersecretion of αSYN from HEK293T cells transfected with DN-VPS4A. To test this, HEK293T cells doubly expressing αSYN and DN-VPS4A or SH-SY5Y neuronal cells expressing DN-VPS4A were further co-transfected with DN-Rab11a that lacks GTP-binding activity, then whole cell lysates as well as CM were subjected to immunoblot analysis (Fig. 7A and B, respectively). As shown in the blots, the augmented secretion of over-expressed and endogenous αSYN induced by VPS4 malfunction were effectively restored by the co-expression of GDP-locked DN-Rab11a, whereas the total cellular levels of αSYN remained unchanged.

Discussion

Until recently, αSYN has been considered to exert its physiological as well as pathogenic effects intracellularly. However, accumulating evidence suggests that both monomeric and oligomeric αSYN can be secreted into the extracellular environment, thereby affecting the normal physiological state of neighboring neuronal and glial cells [17]. In the case of prion protein, cell-to-cell transmission by means of exosome shuttle, caveolae-mediated endosomal pathway, and tunneling nanotubes has been suggested [23,25,52,53]. Therefore, it is tempting to speculate that similar mechanisms could be involved in the transmission of other amyloidogenic proteins. Given that the prion enrichment and infectivity were confirmed in the cell culture media of infected cells as well as body fluids from suffering animals, prion transfer could occur by a process other than through direct cell contact [25,41,43]. In addition to prion protein, several reports suggested that exosomes may serve as vehicles for the transcellular spread of amyloidogenic proteins in neurodegenerative diseases including PD [17,54,55,56]. As reported previously [23,24,25], we found a striking condensation of prion in exosomes in CM and human CSF, whereas such enrichment was not observed with αSYN (Fig. 2A, 2B and 2C). The marked discrepancy in terms of the exosomal localization implies that the secretory mechanism of αSYN might be different from that of prion protein. This idea is also supported by our findings showing that, in contrast to prion protein, the suppression of MVB-exosome biogenesis by DN VPS4A significantly increased the extracellular αSYN in non-neuronal and neuronal cells (Fig.3A and 4A). It is true that our results would seem to conflict with previous reports demonstrating that αSYN is secreted from

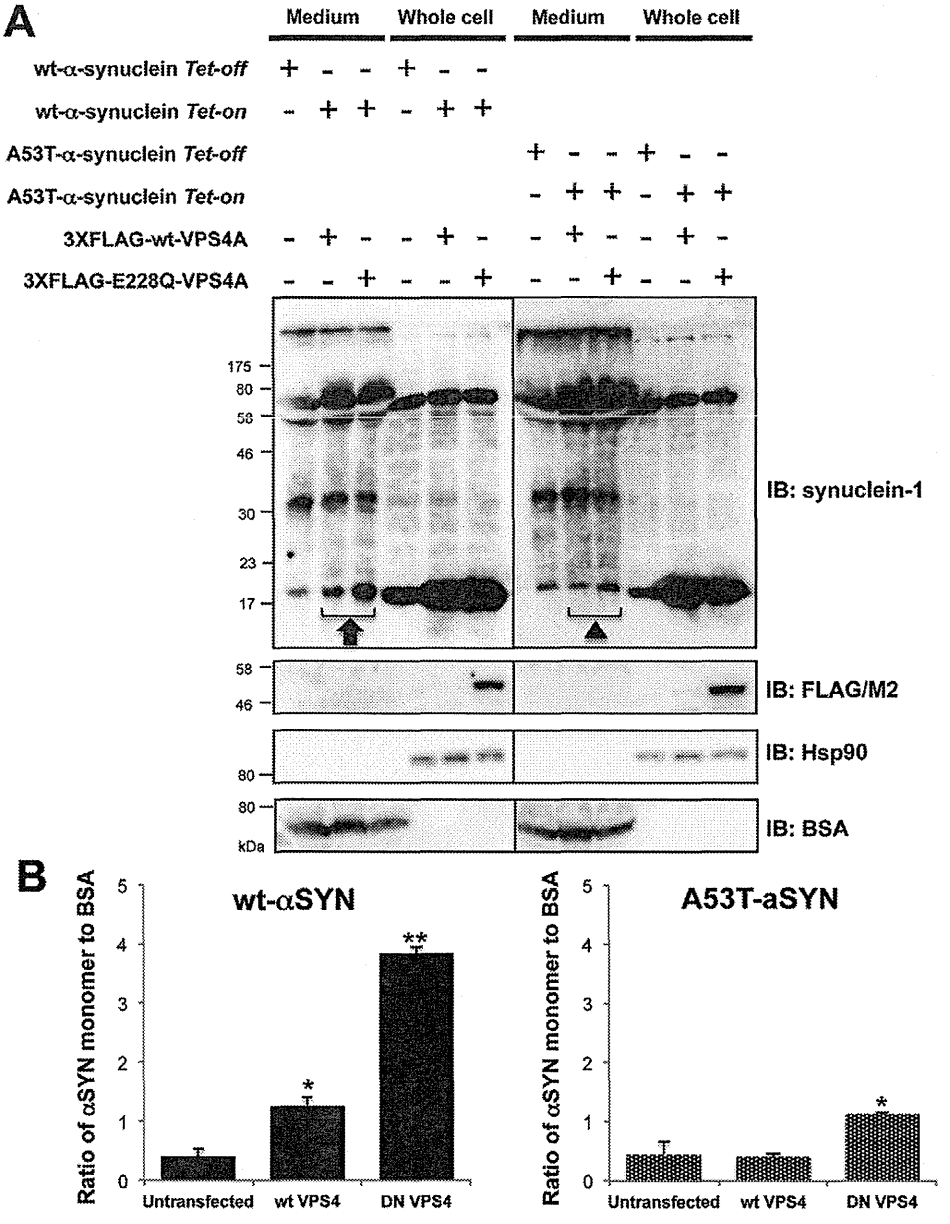

Figure 4. Expression of DN VPS4A increases wt as well as A53T mutant α-synuclein in SH-SY5Y neuronal cells. *A.* SH-SY5Y cells inducibly expressing wt or A53T mutant αSYN were further co-transfected with 3XFLAG-tagged wt or DN E228Q mutant VPS4A plasmids. After 48 hours of αSYN induction with doxycycline, whole cell and proteins from culture media (50 μg protein per lane) were subjected to immunoblot analysis using anti- synuclein-1 and anti-FLAG/M2 antibody. Hsp90 and BSA were used as markers for cytosol and culture medium, respectively. Increased extracellular secretion of wt and A53T mutant αSYN were observed by DN VPS4A. Note that the extracellular secretion of monomeric wt-αSYN (*black arrow*) was higher than that of A53T mutant αSYN (*closed triangle*) in mock-transfected cells as well as in DN-VPS4A engineered cells. Representative immunoblots from three independent experiments are shown. *B.* Densitometric measurement of monomeric αSYN secreted into culture media. Values indicate the ratio of αSYN monomer to BSA. Significant increase of wt as well as A53T αSYN in culture media was observed by co-expression of wt and or DN VPS4A (*$p<0.05$, **$p<0.005$).
doi:10.1371/journal.pone.0029460.g004

neuronal cells by exosomes under both physiological and pathological conditions [27,28]. However, it remains possible that αSYN might be secreted through different secretory pathways depending on the size of the aggregates or cellular condition. Indeed, part of the newly synthesized αSYN was rapidly secreted from MES cells via unconventional, endoplasmic reticulum/Golgi-independent exocytosis [49]. Another study has demonstrated that the internalized extracellular αSYN was resecreted out of neurons via a process modulated by the recycling endosome regulator Rab11a [50]. The functional importance of the recycling pathway was also verified in the cellular trafficking of amyloid-β precursor protein [57]. Our result showing that DN-Rab11a restored the aberrant αSYN secretion triggered by impaired MVB genesis also supports the functional relevance of the recycling pathway in αSYN secretion. Supposedly, under the physiological state, endosomal αSYN is destined for lysosomal degradation (Fig. 8A) or introduced into the extracellular milieu through the Rab11a-dependent recycling endosomal pathway (Fig. 8B) and, to

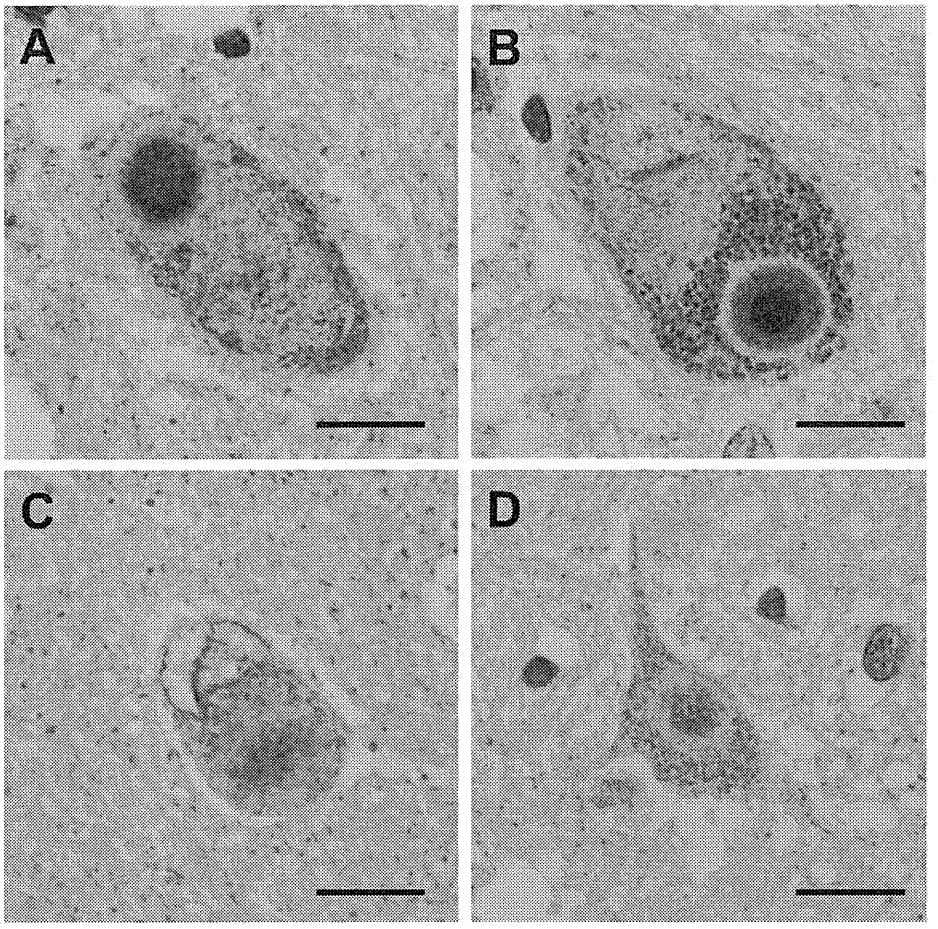

Figure 5. VPS4 was found to be a component of Lewy body. Paraffin embedded sections including the substantia nigra and the temporal lobes from four patients with PD with a mean age of 77.5 years and the controls with a mean age of 77.3 years were subjected to immunohistochemical analysis using anti-human VPS4 Ab. Diaminobenzidine were used to visualize the staining and nuclei were counterstained with hematoxylin. In all brain tissues from patients with PD, the core structures of Lewy bodies showed VPS4 immunoreactivity (Fig. 5). Only weak background staining was observed in control brain sections (data not shown). The percentage of VPS4-immunoreactive Lewy bodies in the substantia nigra (A and B) and the temporal lobes (C and D) of PD brains were 90% and 10%, respectively. Scale bar: 20 μm.
doi:10.1371/journal.pone.0029460.g005

a lesser degree, MVB-exosome pathway (Fig. 8C). However, if the intracellular αSYN reaches a toxic level or the MVB sorting pathway is dammed up for any reason, a torrent of endocytic αSYN may flow out mainly through the recycling endosome pathway. Perhaps the recycling pathway might serve as a "vent" to discharge excess αSYN that would be potentially harmful to cells. Another important finding observed in this study is that the extracellular secretion of wt-αSYN was constitutively higher than A53T mutant αSYN in mock-transfected cells as well as in DN-VPS4A engineered cells. This finding is interesting when considering the cytotoxic property of mutant αSYN, which might be liable to be entrapped inside the cells and eventually lead to cell-autonomous degeneration. It should be noted that we used cell lines over-expressing αSYN in some experiments of this study. Therefore, we cannot completely exclude the possibility that over-expressed αSYN itself might somehow affect its subcellular distribution since over-expression of αSYN hinders vesicle trafficking and recycling as a result of interaction with prenylated Rab acceptor protein 1 [58].

Since αSYN does not contain a predicted transmembrane domain or known lipid anchor, there remains a fundamental question on how it associates with endosomal vesicles. It is known that the amino-terminal amphipathic α-helical domain of αSYN is quite similar to the class A2 α-helix found in the lipid-binding motif of several apolipoproteins [59]. In fact, αSYN binds artificial liposomes containing phospholipid vesicles with acidic head groups, lipid droplets, and lipid rafts [49]. It has been shown that the portion of αSYN stably cofractionated with vesicles from brain tissues and cultured neuronal cells was not only bound to the outer membrane but certainly localized in the vesicle lumen [49]. Therefore, αSYN might be integrated into vesicles in at least two different ways. Namely, some are loosely bound to the surface of vesicles where the interaction is controlled in the balance of the free cytosolic αSYN. The others are incorporated and sequestrated into the lumen of vesicles. The mechanism by which cytosolic αSYN moves into the endosomal vesicle is poorly understood; however, apart from the vesicle permeabilization by protofibrillar αSYN [60,61], intracellular αSYN exocytosed into the extracellular space could be internalized and directly packaged into the endosomal vesicles [15,49,62]. Intriguingly, it is known that the aggregation of αSYN was faster and more robust in the vesicles than in the cytosol [49,63]. We also observed a noticeable

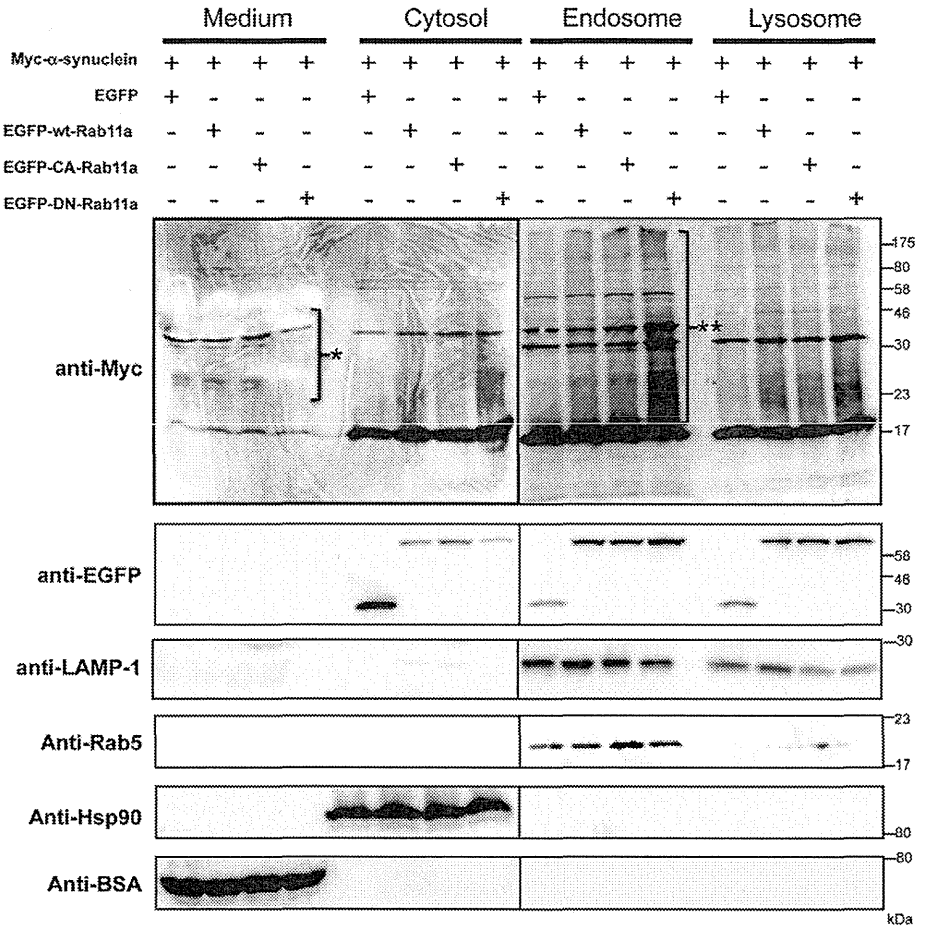

Figure 6. Part of the cellular α-synuclein was trafficked via a recycling endosome pathway for extracellular secretion. HEK293T cells expressing Myc-αSYN were co-transfected with mock (EGFP), EGFP-wt-Rab11a, EGFP-CA-Rab11a, or EGFP-DN-Rab11a expression plasmids. At 48 hours following transfection, the cells were harvested and fractionated into cytosol, endosome, and lysosome. Fractionated samples as well as total proteins from the culture media (50 μg per lane) were subjected to immunoblot analysis using anti-Myc, anti-EGFP Abs. A successful fraction was verified by the presence of a specific marker proteins. As shown in the blot, secretion of αSYN oligomer in culture medium was partly reduced by the over-expression of GDP-locked DN-Rab11a (*asterisk*), accompanied by the extensive retention of HMW αSYN species in the endosome (*double asterisk*). Representative blots from three separate experiments are shown.
doi:10.1371/journal.pone.0029460.g006

aggregation tendency in endosomal/lysosomal αSYN and the core structures of Lewy bodies showed immnoreactivity with VPS4 Ab. These findings are interesting when considering the biogenesis of Lewy bodies, because the pale body, an early cytoplasmic change before Lewy body maturation, often contains ubiquitinated proteins as well as lysosomes and vacuolar structures [64,65]. It is uncertain why intravesicular αSYN has a high propensity to form aggregates. However, specific environments inside the vesicle such as a high calcium concentration and low pH as well as the molecularly crowded milieu might synergistically promote αSYN fibrillization [66,67,68,69]. In addition, the extensive ubiquitination of endosomal αSYN found in this study may indicate a role for ubiquitin in αSYN sorting along the endosomal pathway, since multiple monoubiquitylation and Lys-63-linked polyubiquitylation have been recognized as important sorting signals for cargo proteins in the endosome membrane [33,70].

In summary, we found that impaired MVB-exosome biogenesis by DN VPS4A strikingly increased extracellular αSYN, which was correlated with the decreased lysosome-resident αSYN. The inhibited recycling efficiency by DN-Rab11a can not only cause a decrease of the extracellular αSYN oligomer but also restore the hypersecretion of αSYN by DN-VPS4A. Furthermore, VPS4 was found to be a component of the nigral as well as the cortical Lewy bodies. Our results demonstrate how failure of the MVB sorting machinery contributes to the extracellular secretion as well as lysosomal targeting of αSYN and may thus be involved in the propagation of Lewy pathology in PD. The importance of the endosomal/lysosomal transport system in the pathogenesis of PD is also highlighted by very recent findings that a mutation in *VPS35* gene encoding a retromer complex involved in the retrograde transport of proteins from the endosome to the trans-Golgi network causes late-onset familial PD [71,72]. Furthermore, in a manner similar to vaccination therapy, a reduction of the extracellular αSYN brain burden by regulating the MVB sorting could be a novel therapeutic strategy for PD and other synucleinopathies. Although the concept of prion-like propagation has been recognized as a common phenomenon in many neurodegenerative diseases, it is likely that the molecular mechanisms underlying the spreading of protein-misfolding may differ depending on the biochemical nature of the protein

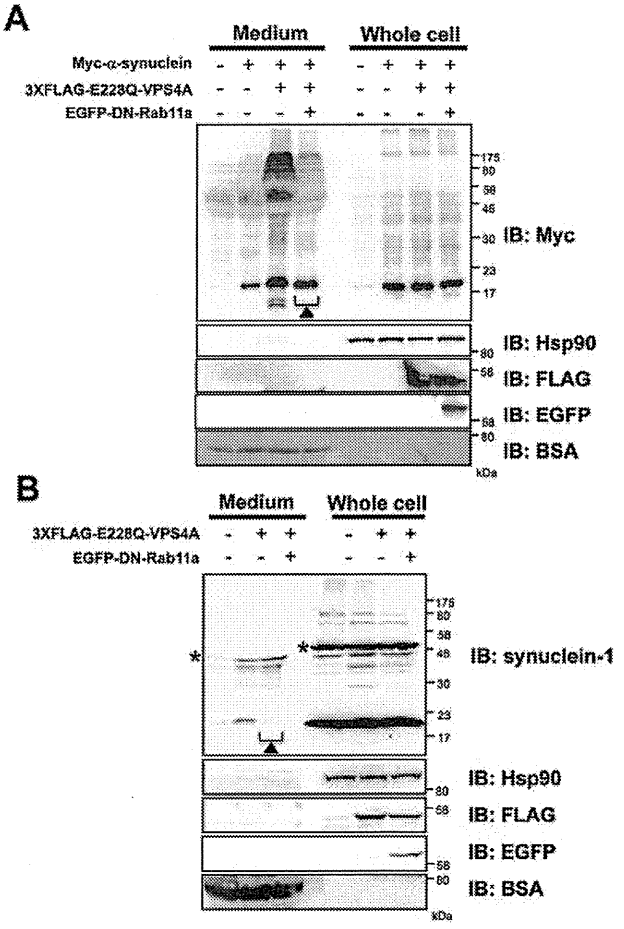

Figure 7. Increased secretion of α-synuclein by DN-VPS4A is restored by DN-Rab11a. GDP-locked DN-Rab11a strikingly restored the hypersecretion of αSYN triggered by the impaired MVB sorting pathway (*closed triangle*). HEK293T cells co-expressing Myc-αSYN and 3XFLAG-DN-VPS4A (*A*) and SH-SY5Y neuronal cells expressing 3XFLAG-DN-VPS4A (*B*) were further transfected with EGFP-DN-Rab11a. Forty eight hours post transfection, the cells were harvested and solubilized in RIPA buffer. Whole cell lysates as well as total proteins from culture media (50 μg per lane) were then subjected to immunoblot analysis using anti-Myc, anti-synuclein-1, anti-FLAG, and anti-EGFP Abs. Hsp90 and BSA were used as markers for the cytosol and culture medium, respectively. *Asterisk* indicates unspecific band. Representative blots from three independent experiments are presented.
doi:10.1371/journal.pone.0029460.g007

aggregate, level of cellular stress, or the cell-type. Further studies will be needed to gain insight into the cellular mechanisms of disease progression and to identify molecular targets for therapeutic intervention in PD and other neurodegenerative diseases.

Materials and Methods

Plasmid Construction and Preparation

N-terminal Myc-tagged wild-type (wt) αSYN was subcloned into the *Bgl*II and *Not*I sites of pCMV mammalian expression vector (Invitrogen, Carlsbad, CA). For inducible expression, human wt and A53T mutant αSYN cDNAs were introduced into pcDNA4/TO doxycycline (Dox)-inducible expression vector (Invitrogen) using the restriction enzymes *Kpn*I and *Not*I. The plasmid pcDNA6/TR encoding tetracycline repressor protein was purchased as a part of the T-REx tetracycline-regulated mammalian expression system (Invitrogen). Triple FLAG (3xFLAG)-tagged human wt- and DN E228Q VPS4A were subcloned into the *Eco*RI and *Bam*HI sites of pCMV vector. The pEGFP-C1 plasmids encoding EGFP-tagged human wt-Rab5a, wt-Rab7, wt-Rab11a, CA-Q70L-Rab11a, DN-S25N-Rab11a were kindly provided by Dr. Mitsunori Fukuda (Laboratory of Membrane Trafficking Mechanisms, Department of Developmental Biology and Neurosciences, Tohoku University Biological Institute, Sendai, Japan). Plasmid DNAs were isolated and purified using the GenoPure Plasmid Maxi Kit (Roche, Indianapolis, IN). The fidelity and orientation of the expression constructs were confirmed by restriction enzyme digestion and/or nucleotide sequence analyses.

Cell Culture and Transfection

HEK293T human embryonic kidney cells (kindly gifted by Dr. Taeko Miyagi, Institute of Molecular Biomembrane and Glycobiology, Tohoku Pharmaceutical University, Sendai, Japan) and SH-SY5Y human dopaminergic neuroblastoma cells (CRL-2266; American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen/GIBCO) containing 4.5g/l glucose, 2mM L-glutamine (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Thermo Scientific/HyClone, Rockford, IL) at 37°C under humidified 5% CO_2/air. The SH-SY5Y cell lines in which wt or A53T mutant αSYN can be induced were established using the T-REx expression system which consists of two key expression vectors, pcDNA4/TO and pcDNA6/TR [73,74]. Stably transfected Dox-inducible SH-SY5Y cells were maintained in DMEM containing 4.5g/l glucose, 2mM L-glutamine supplemented with 10% FBS under selective pressure by 5 μg/ml Blasticidin and 300 μg/ml Zeocin (both from InvivoGen, San Diego, CA). HEK293T cells seeded 24 hours prior to transfection were transiently transfected using FuGENE 6 transfection reagent (Roche) at FuGENE 6 (μl)/DNA (μg) ratio of 3:1. SH-SY5Y cells were nucleofected using the Nucleofector I device (LONZA AG, Cologne, Germany) with program A-023. Cells were harvested 48 hours post transfection unless otherwise stated. To evaluate αSYN decay in the presence of lysosomal inhibitor, cells were treated with bafilomycin A1 (0–10 nM dissolved in DMSO; purchased from Sigma) for 24 hours.

Immunofluorescence Confocal Microscopy and Immunohistochemistry

Cells seeded onto UV-sterilized coverslips coated with self-made rat-tail collagen were fixed in 4% (w/v) paraformaldehyde in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 5min, and blocked with 3% normal goat serum (Wako Pure Chemical Industries, Osaka, Japan) in PBS for 30min. Primary antibodies (rat monoclonal antibody (mAb) anti-DYKDDDDK (FLAG peptide)-tag (1:500; Agilent Technologies, Foster City CA), mouse mAb anti-cMyc (clone 9E10, 1:1000; DSHB, Iowa City, IA), rabbit pAb anti-αSYN (1:1000, CST, Danvers, MA) and mouse mAb anti-LAMP-1 (clone H4A3, 1:1000; DSHB)) were applied for 2 hours followed by anti-mouse IgG Alexa 488 conjugates, anti-rabbit IgG Alexa 568 conjugates, or anti-rat IgG Alexa 647 conjugates (1:2000; Invitrogen/Molecular Probes) for 1 hour. Nuclei were counterstained with TO-PRO3 iodide and pseudo-colored as blue (Invitrogen/Molecular Probes). After immunostaining, coverslips were placed upside down on a drop of PermaFluor antifade mounting medium (Thermo Scientific). Fluorescent images were analyzed with a FV300 confocal laser scanning microscope system equipped with HeNe-Green (543 nm), HeNe-Red (633 nm) and Ar (488 nm) laser units (Olympus Corporation, Tokyo, Japan). In the multiple labeling

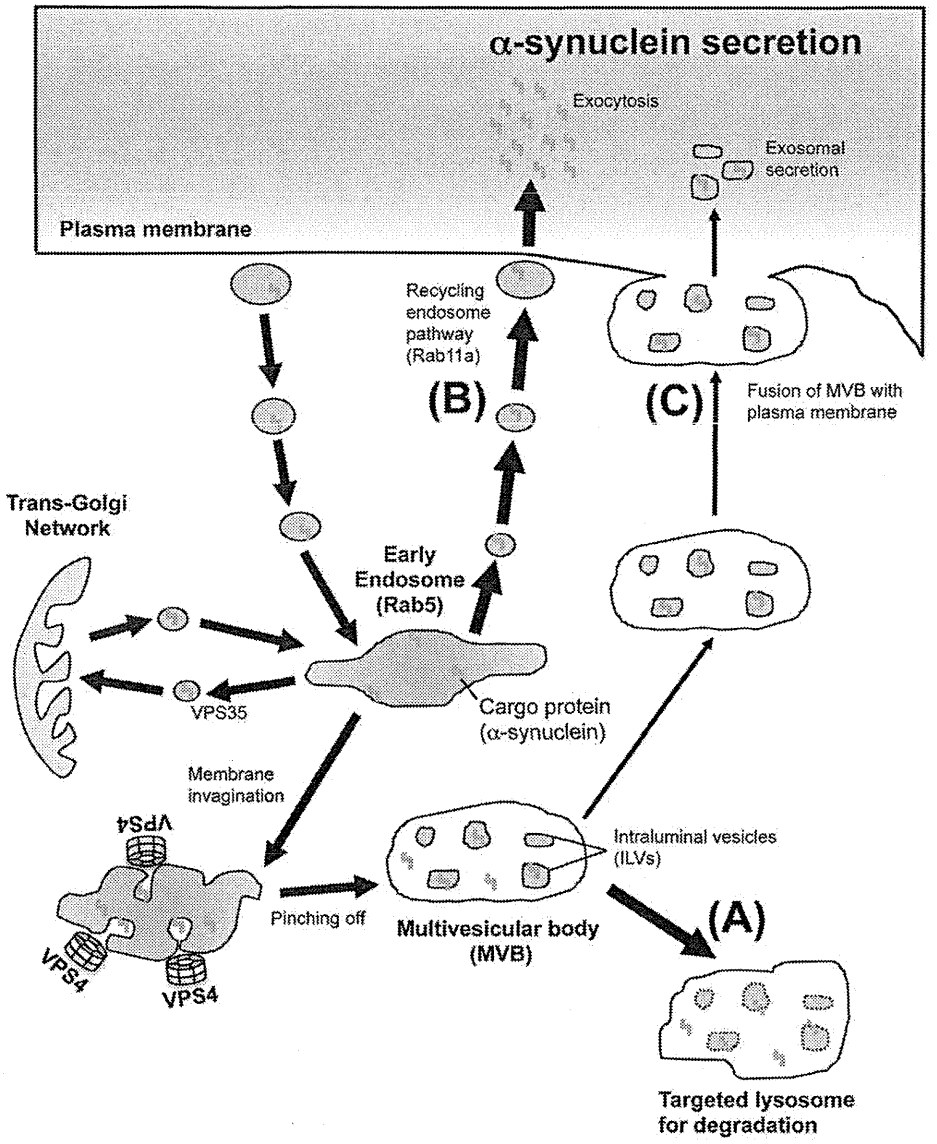

Figure 8. Schematic presentation of endosomal pathways and the functional relevance of MVB sorting machinery and Rab11a-mediated recycling pathway in the secretion as well as lysosomal targeting of α-synuclein. Membrane-associated cargo proteins including αSYN are translocated to early endosomes, which also receive cargoes from the trans-Golgi network. Some cargoes are recycled back to the plasma membrane. Others are sequestered in intraluminal vesicles of MVB. Individual ESCRT proteins and VPS4 contribute to MVB formation through the induction of invagination and final scission of the endosomal membrane. MVB directs either for lysosomal degradation or for secretion as exosomes by exocytic fusion with the plasma membrane. Under the physiolosical condition, αSYN in the early endosome may be transferred to MVB then targeted for lysosomal degradation (A). Alternatively, part of the endosomal αSYN may be cast into the extracellular milieu through the Rab11a-dependent recycling endosome (B) and, to a lesser degree, MVB-exosome pathway (C). If the intracellular αSYN reaches a toxic level or the MVB sorting is dammed up, excessive amounts of endocytic αSYN will flow out mainly through the recycling endosome pathway.
doi:10.1371/journal.pone.0029460.g008

experiments, images were collected using a single excitation for each wavelength separately and then merged using Fluoview image analyzing software (version 4.3, Olympus). For immunohistochemistry, 4-μm-thick sections of formalin fixed paraffin embedded samples including the substantia nigra and the temporal lobes from patients with PD with a mean age of 77.5 years (n = 4, range 67 to 84 years) and the controls with a mean age of 77.3 y (n = 4, range 67 to 87 years) were subjected to immunohistochemical investigations using the avidin-biotin-peroxidase complex (ABC) method with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Polyclonal Ab against human VPS4 (SAB4200025, 1:100; Sigma) was used as primary Ab. Diaminobenzidine was used as the chromogen. The sections were counterstained with hematoxylin. No pretreatment of sample before Ab incubation was required.

Subcellular Fractionation by Sequential Centrifugation

For the subcellular fractionation of cultured cells, we adopted an established protocol with slight modifications [75]. All steps of the fractionation scheme were carried out at 0–4°C with ice-cold reagents. Cells (1×10^7) were resuspended with 2 ml ice-cold

fractionation buffer (10 mM Tris/acetic acid pH 7.0, 250 mM sucrose) and homogenized using 20 strokes in a 2-ml Dounce tissue grinder with a tight pestle (GPE, Bedfordshire, England). The cell homogenate was initially cleared by three successive centrifugation steps (500×*g* for 2 min, 1,000×*g* for 2 min, 2,000×*g* for 2 min) to remove debris and undestroyed cells. The supernatant was transferred to a new tube and centrifuged at 4,000×*g* for 2 min to pellet the plasma membrane and nuclei. The supernatant was ultracentrifuged at 100,000×*g* (P50S2 swing rotor, Hitachi Koki Co., Ltd., Tokyo, Japan) for 2 min to pellet the mitochondria, endosomes, and lysosomes (fraction EL). Lysosomes were isolated from the fraction EL by 10-min osmotic lysis using five times the pellet volume of distilled water. After another centrifugation step with 100,000×*g* for 2min, lysosomes remained in the supernatant, while mitochondria and endosomes were in the pellet.

TCA/acetone Protein Extraction from Culture Medium and CSF

Total protein in CM and CSF was extracted by trichloroacetic acid (TCA)/acetone precipitation protocol. Briefly, freshly collected samples were cleared by three successive centrifugation steps (800×*g* for 5 min, 2,000×*g* for 10 min, and 10,000×*g* for 20 min at 4°C) to pellet the debris and intact cells. The supernatant was transferred to a new tube and added with an equal volume of ice-cold 20% TCA/acetone, followed by incubation at −20°C for 3 hours. After adding 3 additional volumes of ice-cold acetone, proteins were allowed to precipitate overnight at −20°C. The protein was pelleted by centrifugation at 5,000×*g* for 60min, dissolved in 8M urea/5% SDS with sonication, and subjected to Western immunoblot analyses.

Exosome Isolation from Culture Medium and CSF

To isolate exosomes, CM or pooled CSF was collected and subjected to a multi-step differential centrifugation process. In brief, freshly collected samples were subjected to three successive centrifugations at 800×*g* for 5 min, 2,000×*g* for 10 min, and 15,000×*g* for 20 min at 4°C to remove debris and intact cells. After filtration through a 0.22 μm Millipore syringe filter, exosomes were pelleted by ultracentrifugation at 100,000×*g* (P40ST swing rotor, Hitachi Koki, Co., Ltd.) for 1 hour at 4°C. In some experiments, the exosome-containing pellet was resuspended in ice-cold PBS and further purified by continuous linear sucrose-density gradient centrifugation (2.0–0.25M sucrose, 20 mM HEPES, pH 7.2) according to the method described previously. The exosomal proteins Alix and flotillin-1 were used as markers for the exosome-containing fraction [27].

SDS-Polyacrylamide Gel Electrophoresis and Western Immunoblot Analysis

After preparing the cell lysates using radio-immunoprecipitation assay (RIPA) buffer (1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1mM EDTA, 10mM sodium pyrophosphate, 50mM sodium fluoride, 1mM sodium orthovanadate, 150mM sodium chloride, 50mM Tris-HCl (pH 8.0) plus 1x Complete protease inhibitor cocktail; Roche), the protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (BioRad, Hercules, CA). Lysates containing 50 μg total protein were boiled in Laemmli loading buffer and then electrophoresed on denaturing 12.5% SDS-polyacrylamide gels using the Mini-PROTEAN 3 cell system (BioRad). Electroblotting onto polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, MA) was performed at 100V for 2 hours. After a blocking step with Tris-Buffer Saline (TBS: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) with 0.05% Tween 20 (TBST) supplemented with 5% nonfat dry milk, membranes were incubated with anti-cMyc mouse mAb (clone 9E10, 1:1000; DSHB), M2 anti-FLAG/M2 (1:1000; Sigma) mouse mAb, anti-GFP mouse mAb (1:4000; MBL, Nagoya, Japan) anti-synuclein-1 mouse mAb (1:1000; BD Bioscience, San Jose, CA), anti-Alix mouse mAb (clone 3A9, 1:1000; CST), anti-flotillin-1 mouse mAb (1:500; BD Transduction laboratories, Franklin Lakes, NJ), anti-Hsp90 mouse mAb (1:4000; Stressgen, Victoria, BC, Canada), anti-BSA rabbit polyclonal antibody (pAb) (clone B-140, 1:4000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-prion protein mouse mAb (1:1000; Sigma), anti-ubiquitin Ab (clone P4D1, 1:1000; Santa Cruz), anti-LAMP-1 mouse mAb (clone H4A3, 1:1000, DSHB), anti-Rab5 rabbit pAb (1:4000, Santa Cruz), and anti-Rab11 rabbit pAb (1:1000; CST, Danvers, MA). Primary antibodies were followed by horseradish peroxidase-conjugated secondary Ab (1:10000; Jackson ImmunoResearch Laboratories, West Grove, PA). Bands were visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore) and images were captured by the LAS-3000mini lumino image analyzer (Fujifilm, Tokyo, Japan). Quantification of the band intensity was performed using the Image J version 1.44 software for Mac (developed at the National Institutes of Health, Bethesda, MD) [76]. All experiments were performed at least three times with identical results.

Author Contributions

Conceived and designed the experiments: TH AT. Performed the experiments: TH M. Konno TB NS AK M. Kobayashi EM FM KW. Analyzed the data: TH M. Konno TB. Contributed reagents/materials/analysis tools: NT KT KF HA KW MA YI. Wrote the paper: TH.

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Syndrome Handbook

症候群ハンドブック

◉総編集◉
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◉編集◉
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レット症候群 Rett syndrome

■疫学 約12,000～15,000人の女児に1人，通常すべて女性．

■病因と発症に関わる遺伝子 *MECP2*（メチル-CpG-結合蛋白2〈methy1-CpG-binding protein 2〉Xq28）が病因遺伝子である．

■診断 これまでいくつかの診断基準が提唱されている．それらは典型例，非典型例として臨床症状をまとめたものである．病因遺伝子が解明され，ほぼ80～90%にその変異が見つかるが，見つからない例もあり，基本的には臨床診断である．

本症は，特徴的な神経精神症状が年齢依存性に出現する特異な神経精神発達障害である．その臨床症状の概要は次のとおりである．

当初，本症の発症につき乳児期後半または幼児期早期までは全く正常とされたが，詳細な病歴聴取により，軽微であるが，乳児期早期から抗重力筋の筋緊張低下，早期の自閉傾向（おとなしくて泣かない，睡眠覚醒リズムの形成遅滞）がみられ，その後，定頸は正常に獲得されるが多くの場合，寝返り以降マイルストーンが遅滞する．特にロコモーションの障害（はいはいの異常および獲得遅滞）は特徴的で，屈曲姿勢，はいはい不能例が多い．乳児期後期にそれまで獲得された手の機能が消失し，前後して特徴的な手の常同運動が出現する．これは多くの場合両手を握りしめる，打ち合わせるなどである．歯ぎしり，舌を出し，指をなめるなど口周辺の常同運動もみられる．

その後，下肢から始まる筋緊張の亢進（ジストニア）が出現し，内反尖足または外反足を呈する．年齢とともに上肢のジストニアが出現し，上記の常同運動のベースとなる．したがって，左右の手の位置関係は固定する．体幹のジストニアは小児期以降，前彎，側彎を呈する．当初，自閉傾向をみるが，その後重度の精神遅滞を呈する．頭囲の発達は乳児期後半から停滞し，多くの場合，幼児期早期には小頭を呈する．てんかんをみることも多い．また，覚醒時の息つめ，過呼吸などの呼吸パターンの異常，冷たい足など自律神経系の異常も特徴的である．通常，側彎，呼吸パターンの異常など一部の症状は進行性の経過をとることがあるが，10歳代で症状は固定することが多い．

■治療 病因遺伝子異常の解明以後，それに基づいた基本的な治療に関する研究がなされてきているが，現時点では対症療法である．すなわち，理学療法，作業療法，療育支援などである．脳波の異常は，ほぼ必発であるが，てんかんの程度には個人差がある．臨床的にてんかんをみる場合は抗てんかん薬を投与する．異常な呼吸など発作的な症状をみることが多く，それらとてんかんとの鑑別が困難なことがあり，注意を要する．

■関連語・同義語 Rett症候群は一つの神経精神発達障害である．本症はDSM-Ⅳでは自閉性障害のなかに区分されており，いわゆる"折れ線型の経過をとる女児の自閉症"の一部にRett症候群が含まれていたことが知られている．しかし，本症は自閉症と混同されるべきではない．

■EBM・診療ガイドライン これまでいくつかの診断基準が提唱されているが，上記のように本症は特徴的な臨床症状が年齢依存性に出現することに基づく臨床診断である．*MECP2*の変異は典型例には80～90%にみられるが，非典型例ではその率は低下する．

■大規模臨床研究 治療に関して病態に基づいた臨床研究の試みはなされてきているが，大規模のものはない．疫学研究は世界各地においてなされてきているが，それぞれ比較的限局した地域に基づくものが多い．*MECP2*の解明以降，臨床症状と*MECP2*の変異との関連につき，世界的な規模でデータベースの作成がいくつかのセンターで行われている．

■関連団体・学会 世界各地で患者の会がつくられ，活発な活動がなされてきている．わが国では，全国的な規模の「日本レット症候群協会」，九州地区レット症候群親の会「さくらんぼ会」がある．

学会に関しては，1983年以降，国際学会が行われてきている．わが国ではレット症候群国際会議が2000年に開催された．

■解説　本症は，1966年オーストリアのAndreas Rettにより初めて記載され，1983年，Bengt Hagbergらの報告により世界的に注目されるところとなった．以後，世界的な共同研究により，詳細な臨床研究がなされてきた．病態に関してはモノアミン神経系が重要な役割をもつ発達障害であることが提唱された．病因遺伝子*MECP2*の解明以後，その機能に関する研究がなされてきている．それらは，単に本症の理解だけでなく，他の神経精神発達障害の理解に重要である．

■所見　前記の診断の項参照．　（野村芳子）

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改訂第2版

よくわかるパーキンソン病のすべて

編集 水野美邦 近藤智善

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よくわかる パーキンソン病 のすべて

編集

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CLINICAL TEXTBOOK OF PARKINSON'S DISEASE

永井書店

12 Pantothenate kinase 2欠損症

1 概　念

▶ Hallervorden-Spatz syndrome (HSS)

▶ neurodegeneration with brain iron accumulation (NBIA)

Hallervorden-Spatz syndrome (HSS) は多くの場合小児期に発症し、脳に鉄の沈着をみる稀な常染色体劣性遺伝性の神経変性疾患 (neurodegeneration with brain iron accumulation ; NBIA) である[1]。

歴史的には Hallervorden と Spatz による1922年の報告に始まる[2]。すなわち、12人の同胞のうち、5人の姉妹が構音障害、固縮、不随意運動、知能低下を呈し、剖検にて淡蒼球と黒質に鉄の沈着をみた症例で、その後同様の症例の報告が積み重ねられ Hallervorden-Spatz syndrome と呼ばれるようになった。

▶ pantothenate kinase 2 (PANK2)

▶ pantothenate kinase-associated neurodegeneration (PKAN)

2001年、HSS/NBIA の臨床症状を呈する患者の多くに pantothenate kinase 2 (PANK2) をコードする遺伝子に変異を呈することが報告され[3]、近年これらの患者は pantothenate kinase-associated neurodegeneration (PKAN) と呼ばれるようになった。

2 病　因

Pantothenate kinase 2 (PANK2) をコードする遺伝子 *PANK2* は染色体20p12.3-p13[4]にあり、その変異が多くの HSS/NBIA の病因である。

Pantothenate kinase 2はミトコンドリア内に存在し[5]、エネルギー、脂肪酸、神経伝達物質の代謝などに重要な coenzyme A (CoA) の生合成にかかわる。

ヒトでは pantothenate kinase はほかに、関連蛋白 PANK1、PANK3、PANK4 をコードする3つの遺伝子があるが、ミトコンドリア内に局在するのは PANK2 のみである。

3 病　理

特徴的な病理所見は淡蒼球と黒質網様体の鉄沈着と軸索にスフェロイドがみられることである[6]-[9]。スフェロイドは軸索輸送の障害のため軸索が腫脹したものと考えられ、鉄の沈着の多いところに検出される。神経細胞の内部、細胞外で血管の周囲、また、ミクログリア、マクロファージに貪食されていることもある。タウ、α-シヌクレイン

の蓄積を伴うLewy小体、神経原線維変化をみる場合もある[10)-12)]。

4 臨床症候[1),8),9),13)]

▶固縮
▶ジストニア
▶歩行障害
▶視野障害
▶発達遅滞
▶構音障害
▶錐体路症状

典型例の臨床症状は小児期早期、通常6歳前(平均3.4歳)に発症する。

初発症状は下肢の固縮、ジストニアによる歩行障害が主である。網膜変性のため視野障害を呈することもある。発達遅滞を呈することもあり、この場合運動発達遅滞が多いが、全般性の遅滞のこともある。

経過中みられる症状は、錐体外路系の症状、すなわち、ジストニア、構音障害、固縮などで、ジストニアは常にみられ、通常早期から出現する。頭部、顔面、四肢のジストニアから全身に及ぶジストニアである。強いジストニアのために舌の損傷、骨折を生ずることもある。筋痙直、腱反射亢進、バビンスキー反応など錐体路症状を呈することもある。痙攣は稀である。知的退行は主症状の1つである。網膜色素変性は典型例の約2/3にみられる。網膜変性は夜盲から始まり、辺縁視野障害を呈し、盲に進行する場合がある。視神経萎縮は稀である。

経過は進行性で、その速度は発症年齢による。すなわち、早期の発症は進行の速度が速い。進行するとジストニア、痙直のため歩行が不能となり、10代半ばまでに車いすとなることが多い。進行の仕方は一定でなく、1～2ヵ月のうちに急速に進行し、その後安定することもある。

生命予後は一定しない。成人まで生存することも多いが、早期の死亡もある。これは多くの場合、顔面口部のジストニアのため嚥下、食事摂取の障害から栄養状態の問題、嚥下性肺炎などの合併症によることが多い。

非典型例の症状は多様である。発症年齢は多くの場合20歳台までであり(平均13.6歳)、進行は典型例に比し緩徐である。初発症状は言語障害が多い。その内容はpalilalia(繰り返し単語、文節をいう)、tachylalia(急速に単語、文節をいう)、dysarthria(構音障害)などである。運動チック、音声チックを呈する例もある。衝動的、乱暴などの性格変化、うつ状態、情緒不安定などはしばしば遅い発症例にみられる。早期発症例と同様に認知障害をみることもあるがその場合軽度である。運動症状を伴う場合は、その出現は通常遅れるが、小児期から不器用であったとされることが多い。筋痙性、腱反射亢進、バビンスキー反射など錐体路症状はしばしばみられ、歩行障害を呈する。PDを思わせるようなfreezing gaitを呈することもある。本態性振戦に似た症状の報告もある[14)]。非定型例では網膜障害は稀であり、視神経萎縮はみない。

5 診断、鑑別診断

はじめに述べた如く、HSSは当初、臨床病理学的な診断であった。したがって、当初その病因は異種のものが含まれていた。その後、脳内、特に錐体外路系に鉄の沈着をみる遺伝性の神経変性疾患(NBIA)としての概念が出てきた。NBIAの診断基準は初めDoolingによりなされ、その後Swaimanにより見直された。NIBAはかつてHSSとされたいくつかの異なった疾患を含んでいた。すなわち、表5に挙げたものであるが、これらが鑑別診断となる。

その後PKANの特徴として次の点が挙げられた。

①MRIの特徴的な所見が報告された。すなわち、*PANK2*の変異では、T2強調画像にて淡蒼球内節に低信号の中に高信号を呈するものである。これは壊死、または浮腫による組織損傷を反映するといえる。これは"eye-of-tiger sign"といわれ、本症に特異的である[1]。T1強調画像では等信号となる。

▶ eye-of-tiger sign

これに比し、non-PKAN NBIAは、多くの場合T2強調画像にて淡蒼球は均一に低信号である。すなわち、鉄の沈着を示唆する。

赤核、歯状核の鉄の沈着、小脳萎縮はNBIAにはしばしばみられる所見である。

②PKANには通常てんかんはみないが、non-PKAN NBIAにはてんかんは顕著である。

③骨髄でのsea-blue histiocytes：歴史的にはHSSの特徴とされたが、PKANにはみられず、他のNBIAにみられることがある。

▶ sea-blue histiocytes

遺伝子検査より*PANK2*の変異が証明されれば、確定診断といえる。

鑑別疾患としては、表5に示したnon-PKAN NBIAのほかに、典型的なPKANでは、X-linked mental retardation with Dandy-Walker malformation、alfa fucosidosisなどがある。また非典型的なPKANは、early-onset Parkinson disease

表5. NBIAの鑑別疾患

1. 早期発症、急速な進行を呈するNBIA：10歳前に発症するもので、次のものがある。
   ①PKANの典型例
   ②Infantile neuroaxonal dystrophy
   ③最近明らかにされた*PLA2G6*の変異による疾患[15]
2. 発症の遅い、緩徐進行性のNBIA：10歳以降に発症するもので、次のものがある。
   ①PKANの非典型例
   ②Neuroferritinopathy：ferritinのlight chainをコードする*FTL*の変異によるもの
   ③Aceruloplasminemia：ceruloplasminをコードする遺伝子の変異によるもの
   ④非典型的なneuroaxonal dystrophy：infantile neuroaxonal dystrophyより慢性の経過をとり、*PLA2G6*の変異によるもの
   ⑤本態性のNBIA

(PARK2)、progressive supranuclear palsy、primary psychiatric illnessesがある。

6 治療、予後

治療は対症療法である。特に主となるのはジストニアの対症療法である。経口投与薬としてtrihexyphenidylおよびbaclofen、ボトックス®注射、intrathecal baclofen、定位脳手術(淡蒼球または視床をターゲットとする)などがなされてきているが、

脳深部刺激術

近年淡蒼球の脳深部刺激術がよい効果を呈したという報告がある[16][17]。

特に口、舌のジストニアに対してのサポートおよび治療、重度のジストニアのために経口摂取ができない場合、胃瘻も検討される必要がある。網膜の症状がある場合は盲に対するサポートも必要である。移動に関する歩行器、車いすなどのサポート、また、社会的なサポートも必要である。

パントテン酸の大量療法、docosahexanoic acid(DHA)の可能性が検討されている。前者は残存酵素が想定される場合、PANK2酵素の基質であるパントテン酸を投与することである。その効果は不明であるが、副作用は知られていない。後者は、脂肪酸の合成、分解におけるCoAの役割において、DHAが重要であることからその効果が推測されているが、治験はなされていない。

予後は通常進行性である。

(野村芳子)

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