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 α -Synuclein (α -Syn)-positive intracytoplasmic inclusions, known as Lewy bodies, are thought to be involved in the pathogenesis of Lewy body diseases, such as Parkinson's disease (PD). Although growing evidence suggests that cell-to-cell transmission of α -Syn is associated with the progression of PD and that extracellular α -Syn promotes formation of inclusion bodies, its precise mechanism of action in the extracellular space remains unclear. Here, as indicated by both conventional fractionation techniques and FRET-based protein-protein interaction analysis, we demonstrate that extracellular α -Syn causes expulsion of sphingosine 1-phosphate receptor subtype 1 (S1P₁R) from the lipid raft fractions. S1P₁R regulates vesicular trafficking, and its expulsion involved α -Syn binding to membrane-surface gangliosides. Consequently, the S1P₁R became refractory to S1P stimulation required for activating inhibitory G-protein (G_i) in the plasma membranes. Moreover, the extracellular α -Syn also induced uncoupling of the S1P₁R on internal vesicles, resulting in the reduced amount of CD63 molecule (CD63) in the lumen of multivesicular endosomes, together with a decrease in CD63 in the released exosomes from α -Syn-treated cells. Furthermore, cholesterol-depleting agent-induced S1P₁R expulsion from the rafts also resulted in S1P₁R uncoupling. Taken together, these results suggest that extracellular α -Syn-induced expulsion of S1P₁R from lipid rafts promotes the uncoupling of S1P₁R from G_i, thereby blocking subsequent G_i signals, such as inhibition of cargo sorting into exosomal vesicles in multivesicular endosomes. These findings help shed additional light on PD pathogenesis.

Parkinson's disease (PD)² is the second most common progressive neurodegenerative disorder after Alzheimer's disease. α -Synuclein (α -Syn) is an acidic protein with 140 amino acids and is highly expressed in neurons and enriched in presynaptic terminals suggesting a role in synaptic function and plasticity (1, 2). α -Syn has been identified as a major component of intracellular fibrillar protein deposits known as Lewy bodies in the cell body of affected neurons in both idiopathic (3–5) and hereditary PD, *i.e.* missense mutations, α -Syn(A53T) (6), α -Syn(A30P) (7), and α -Syn(E46K) (8) as well as multiplication in the α -Syn gene (9, 10).

Although α -Syn is viewed as a cytoplasmic protein, recent studies suggest that α -Syn can be released from cultured cells by unconventional exocytosis (11) or by exosomes (12) and that α -Syn is detected in cerebrospinal fluid and plasma (13, 14). In addition, α -Syn has been reported to penetrate into many cells, such as neurons, platelets, and fibroblast (15–17). These findings may suggest that extracellular α -Syn plays a role in cell-tocell transmission and the progression of PD. In this context, extracellular α -Syn has been reported to affect astrocytes or microglia, possibly causing neuronal cell death (18, 19) or to influence microglial phagocytosis (20).

Sphingosine 1-phosphate (S1P), a phosphorylated product of sphingosine catalyzed by sphingosine kinase, has emerged as a potent lipid mediator with diverse effects on multiple biological processes including angiogenesis (21), cardiac development (22), immunity (23), neurotransmitter release (24), and maturation of multivesicular endosomes (MVEs) (25). Most of these processes are mediated by five S1P-specific G-protein– coupled receptors (S1P₁₋₅R) and show distinct expression in tissues and cells, and also unique G-protein– coupling patterns suggesting distinctive functions (26).

We have recently demonstrated that extracellular α -Syn causes impairment of platelet-derived growth factor (PDGF)– induced chemotaxis through selective inhibition of Rac1 activation, which is important in actin fiber remodeling, in SH-SY5Y cells (27). Subsequent analysis has revealed that extracellular α -Syn induces S1P₁R uncoupled from inhibitory G-protein (G_i) in the plasma membranes. This uncoupling of



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² The abbreviations used are: PD, Parkinson's disease; α-Syn, α-Synuclein; α-Syn(A53T), α-Syn found in hereditary PD where alanine residue at 53 was mutated to threonine; PDGF, platelet-derived growth factor; α-Syn(A53T)-AAA, lysine residues at 34 and 45, and tyrosine residue at 39 were all mutated to alanine; S1P, sphingosine 1-phosphate; S1P₁₋₅R, S1P-specific

G-protein-coupled receptors; G_i, inhibitory G-protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; MBCD, methyl- β -cyclodextrin; MVEs, multivesicular endosomes; ILVs, intralumenal vesicles; IPTG, isopropyl 1-thio- β -D-galactopyranoside; DiD, 1,1'-dioctadecyl-3,3,3',3'tetramethylindodicarbocyanine perchlorate; GM1, Gal β 3GalNAc β 4-(NeuAc α 3)Gal β 4Glc β 1Cer; GM3, NeuAc α 3Gal β 4Glc β 1Cer.



Figure 1. Expulsion of S1P₁R from the lipid raft fractions by extracellular α -**Syn(A53T).** *A*, SH-SY5Y cells expressing S1P₁R-YFP were incubated without (closed black bars) or with 1 μ m α -Syn(A53T) for 18 h (hatched bars) or 0.2 mm MBCD (closed gray bars) for 2 h. Cells were lysed and the lipid raft fractions were separated as described under "Experimental procedures." The amount of S1P₁R in each fraction was measured using a fluorescence spectrophotometer. Values represent mean \pm S.E. of three independent experiments carried out in triplicate. *B*, SH-SY5Y cells expressing S1P₂R-GFP were incubated with or without 1 μ m α -Syn(A53T) for 18 h or 0.2 mm MBCD for 2 h, followed by fractionation and measurement of fluorescence as in (*A*). Values represent mean \pm S.E. of three independent experiments c., SH-SY5Y cells were incubated without (closed black bars) or with 1 μ m α -Syn(A53T) (hatched bars) for 18 h or 0.2 mm MBCD for 2 h, followed by fractionation. The amount of GM1 in each fraction was measured by dot blot assay using HRP-conjugated CTB (*inset*) as described under "Experimental procedures." Values represent mean \pm S.E. of three independent experimental procedures." Values represent mean \pm S.E. of three independent experiments carried out in triplicate. Statistical significance was analyzed by Student's *t* test (*, p < 0.05; **, p < 0.01 versus vehicle control).

S1P₁R results in the impairment of PDGF-induced chemotaxis, whereas leaving β -arrestin signals intact, *e.g.* ligand-induced internalization of S1P₁R (28). Because G_i signaling on plasma membranes and internal vesicles is essential to cell migration (28, 29) and exosomal vesicle maturation (25), it is particularly important to identify downstream signaling events after extracellular α -Syn treatment leading to uncoupling of S1P₁R. In the present study, we showed evidence that extracellular α -Syn induces expulsion of S1P₁R out of a "signaling station" lipid rafts, involving α -Syn binding to ganglioside, resulting in the uncoupling of S1P₁R from G_i-protein. Pathophysiological relevance of these phenomena to PD pathology are discussed herein.

Results

Expulsion of S1P₁R from lipid rafts

Because many signaling molecules are concentrated in cholesterol and sphingolipid-rich membrane microdomains known as lipid rafts for their action, the effect of α -Syn(A53T), found in hereditary PD, on the distribution of S1P₁R in the lipid rafts was assessed by a conventional fractionation analysis. After cell lysis with Triton X-100, the majority of the S1P₁R was recovered in detergent-insoluble lipid raft fractions under control conditions (Fig. 1A, fractions #2, solid black bars) as verified by a raft marker, ganglioside GM1 (Fig. 1C). Disruption of lipid rafts by pretreatment of cells with a cholesterol-depleting agent, methyl- β -cyclodextrin (MBCD), resulted in a robust loss of GM1 in the same fractions (Fig. 1C, solid gray bars in fractions 1 and 2), showing the validity of the fractionation. Distribution of S1P₁R was also suppressed by MBCD treatment (Fig. 1A, solid gray bars in fraction 2). Surprisingly, α -Syn(A53T) treatment caused a marked reduction of S1P₁R, whereas preserving the raft structures as judged by the amount of GM1 unchanged (Fig. 1, A and C, hatched bars in fractions 1 and 2). The amount of S1P₂R, another subtype of the receptor known to be expressed in this cell line (28), in the raft fractions was not changed by α -Syn(A53T) treatment (Fig. 1B, hatched bars in fraction 2). These results suggest that α -Syn(A53T) selectively drives S1P₁R out of the lipid rafts whereas preserving raft structures.

Detection of raft localization of S1P₁R by FRET technique

Flotillin 2, a protein well known to be localized in the lipid rafts, was analyzed for its susceptibility of raft localization to α -Syn(A53T). In contrast to S1P₁R, the raft localization of flotillin 2 was not influenced by α -Syn(A53T) as demonstrated by a conventional fractionation analysis (Fig. 2A, compare solid bars with hatched bars in the raft fractions). This fact facilitated us to develop a new tool to detect raft localization of S1P₁R using a FRET-based protein-protein interaction analysis without conventional laborious procedures like a density gradient centrifugation. When SH-SY5Y cells transiently expressing S1P₁R-cyan fluorescent protein (CFP) and flotillin 2-yellow fluorescent protein (YFP) were measured for FRET efficiency at the plasma membranes, it showed a relatively higher value under control conditions, suggesting a close association of these proteins at the lipid rafts (Fig. 2B). Upon treatment with α -Syn(A53T), the FRET efficiency decreased significantly compared with untreated conditions, whereas WT α -Syn also lowered the efficiency. This suggests that both WT and the mutant α -Syn drove S1P₁R out of the lipid rafts with the potency of its activity being stronger in the mutant protein. The decrease in FRET efficiency proceeded in a time-dependent fashion, which was detectable in 40 min and reached 70% reduction in 60 min (Fig. 3A). Because α -Syn has a propensity to aggregate in oligomeric and polymeric forms upon binding to lipid membranes (30), aggregation states of α -Syn(A53T) during the experiments were tested. Compared with α -Syn(A53T) in the medium, the protein associated with cells showed an increase in a dimer form over the time periods (Fig. 3B). To test the effect of oligomerization of α -Syn(A53T) on the ability of expulsion of S1P₁R from the rafts, α -Syn(A53T) solution was incubated for 24 h at 37 °C to allow self-aggregation (Fig. 3C) and was subjected to FRET analysis and compared with nonpreincubated samples. Addition of self-aggregated forms of α -Syn(A53T) to the culture medium showed no significant differences in the ability of expulsion of the receptor from the lipid rafts as compared with the nonaggregated forms (Fig. 3D, p = 0.3). α -Syn(A53T) and the WT protein showed a dose-dependent inhibition of the FRET efficiency showing sigmoidal curves (Fig. 3E), which sug-





Figure 2. Detection of S1P₁R in the lipid raft fractions by a FRET technique. *A*, SH-SY5Y cells expressing flotillin 2-YFP were incubated without (vehicle control, *closed bars*) or with 1 μ M α -Syn(A53T) (*hatched bars*) for 18 h, followed by lipid raft separation and quantification of the fluorescence in each fraction as in Fig. 1A. Values represent mean \pm S.E. of three independent experiments carried out in triplicate. *B*, SH-SY5Y cells expressing both S1P₁R-CFP and flotillin 2-YFP were incubated without (vehicle control) or with either 1 μ M α -Syn(A53T) or WT α -Syn for 18 h. Cells were fixed and analyzed for FRET efficiency in the plasma membrane areas using the acceptor photobleaching method. Results are expressed as median on scatter-dot plots ($n \ge 50$). Statistical significance was analyzed by Student's *t* test (**, p < 0.01 versus vehicle control).

gests the existence of target molecule(s) that react with α -Syn at the plasma membranes.

Role of gangliosides for extracellular action of α -Syn

Because α -Syn is known to possess the ability to interact with gangliosides in the lipid rafts and has a potency to alter the functions of several signaling molecules at the raft domains (31), the next experiments were sought to clarify the role of gangliosides in the α -Syn(A53T)-induced displacement of S1P₁R from the rafts. After treatment of cells with neuraminidase, which cleaves sialic acid residues of gangliosides at cell surface, the effect of α -Syn(A53T) on S1P₁R distribution was tested. Importantly, neuraminidase treatment resulted in the abrogation of the ability of α -Syn(A53T) to drive S1P₁R out of the raft fractions (Fig. 4, hatched green bars in the raft fractions), indicating gangliosides as a receptor or binding partner of α -Syn(A53T) to elicit pathophysiological responses including expulsion of S1P₁R from the raft fractions. Consistent with this notion, an α -Syn mutant devoid of ganglioside-binding ability, α -Syn(K34A/Y39A/K45A)-derived (32) mutant, α -Syn(A53T/ K34A/Y39A/K45A), and α -Syn(A53T)-AAA (27), lost the capacity to expel S1P₁R from the lipid rafts (Fig. 4). These results indicate that gangliosides in the lipid rafts are necessary in the extracellular action of α -Syn presumably functioning as a potential receptor to the protein. To substantiate this notion, addition of ganglioside mixture (GD1a > GT1b > GM1) rescued extracellular α -Syn(A53T)-induced expulsion of S1P₁R from the lipid raft fractions in a dose-dependent manner (Fig. 5).

Role of extracellular α -Syn as an uncoupler for S1P $_1$ R from G_i on MVEs

We have recently found that extracellular α -Syn makes S1P₁R uncouple from the G_i protein in the plasma membranes (28). It is important to determine whether S1P₁R is also uncoupled from the G_i protein on MVEs after α -Syn treatment, because continuous activation of S1P₁R and subsequent transmission of the G_i protein signal on MVEs has been proven to be critical for cargo sorting into exosomal intralumenal vesicles

(ILVs) (25, 33). SH-SY5Y cells expressing S1P₁R-CFP and $G\gamma$ -YFP were analyzed for S1P₁R activation and subsequent G_i subunit dissociation using FRET analysis. Under unstimulated conditions G_i-protein subunits are associated (S1P₁R/G $\alpha\beta\gamma$ form, low FRET). Upon stimulation by S1P, these subunits dissociate, and the S1P receptor-CFP and $G\gamma$ -YFP become associated $(S1P_1R/G\beta\gamma + G\alpha \text{ form high FRET})$ (25). Under both control and α -Syn(A53T)-treated conditions, these fluoroprobe-fused proteins were distributed both in plasma membranes and CD63-positive MVEs (data not shown). Under unstimulated conditions in control cells, the FRET efficiency in the plasma membranes was low and became high upon stimulation by S1P (Fig. 6A), demonstrating a successful detection of S1P₁R-mediated G-protein subunit dissociation, whereas the value was constantly high on MVEs under unstimulated conditions in control, indicating ongoing activation of S1P₁R on MVEs as previously reported (25). α -Syn treatment made S1P₁R insensitive to FRET changes in the plasma membranes suggesting that S1P₁R became uncoupled from G_i, inconsistent with the previous observation (28). Importantly, FRET efficiency was low on MVEs in α -Syn(A53T)-treated cells, suggesting that S1P₁R is uncoupled from G_i also on MVEs. To study further the causal relationship between expulsion of S1P₁R from the rafts and its uncoupling, the effect of raft disruption by MBCD on S1P₁R coupling with G_i was tested. MBCD made $S1P_1R$ uncoupled from the G_i (Fig. 6B). Together with the result that MBCD drives S1P₁R out of the rafts (Fig. 1A), these results suggest that expulsion of $S1P_1R$ from rafts may suffice to render the receptor uncoupled from G_i.

Because the receptor-mediated S1P signal on MVEs is indispensable to cargo sorting into exosomal ILVs, the effect of extracellular α -Syn(A53T) on this phenomenon was studied. The ability of cargo sorting into the exosomal MVEs was evaluated by measuring the extent of the exosomal cargo marker, CD63-mCherry, expressed in SH-SY5Y cells. Compared with control cells, α -Syn(A53T) treatment caused a remarkable decrease in the content of CD63-mCherry in MVEs (Fig. 7*A*), suggesting that α -Syn(A53T) treatment resulted in the inhibi-



Extracellular α -Syn drives S1P₁ receptor out of lipid rafts



Figure 3. Analysis of extracellular α -Syn-induced expulsion of S1P₁R from the lipid raft fractions as a function of incubation time and α -Syn doses. A_{i} SH-SY5Y cells expressing both S1P₁R-CFP and flotillin 2-YFP were incubated with 1 μM α-Syn(A53T) for various time intervals. FRET efficiency in the plasma membrane areas was measured as described in the legend to Fig. 2*B*. Values represent mean \pm S.E. ($n \ge 50$). Statistical significance was analyzed by Student's t test (**, p < 0.01 versus vehicle control). B, cells were incubated with 1 µM α -Syn(A53T) for various time intervals as in A. After removal of the medium, the cells were scraped and disrupted by sonication. After removal of cell debris by low-speed centrifugation, the lysates were subsequently centrifuged at 100,000 × g for 30 min. The clarified medium and the pellets after high-speed centrifugation (cell membranes) were subjected to immunoblot analysis using anti- α -Syn antibody. Note that dimeric forms of the protein increased during incubation. The results are the representative of 3 independent experiments. C, 1 μ M a-Syn(A53T) was incubated in Dulbecco's modified Eagle's medium/F-12 medium for 0 h (control) or 24 h at 37 °C (incubated). Samples were then subjected to immunoblot analysis using anti- α -Syn antibody. Note that dimeric and oligomeric forms of the protein increased during incubation. The results are the representative of 3 independent experiments. D, SH-SY5Y cells expressing both S1P₁R-CFP and flotillin 2-YFP were incubated for 40 min without (vehicle control) or with either 1 μ M α -Syn(A53T) or preincubated α -Syn(A53T) as described in C. Cells were fixed and analyzed for FRET efficiency in the plasma membrane areas using an acceptor photobleaching method. Results are expressed as median on scatter-dot plots ($n \ge 50$). Statistical analysis between nonincubated and preincubated samples showed nonsignificant (Student's t test, p = 0.3 versus nonincubated). E, SH-SYSY cells expressing both S1P₁R-CFP and flotillin 2-YFP were incubated with various concentrations of either α -Syn(A53T) or WT α -Syn for 18 h. Cells were fixed and analyzed for FRET efficiency in the plasma membrane areas as in Fig. 2B. Values represent mean \pm S.E. ($n \ge 50$). Statistical significance was analyzed by Student's t test (**, p < 0.01; *, p < 0.05versus vehicle control).

tion of this cargo sorting into MVEs, which corresponds to $S1P_1R$ uncoupling from G_i on MVEs (Fig. 6A). To strengthen this notion, exosomes prepared from cultured media in control or α -Syn(A53T)-treated cells were analyzed for exosomal cargo content. Because any marker proteins may also be subjected to sorting conditions, surface lipids of exosomes were labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD) and used for normalization of each exosome size (25). Exosomes prepared from α -

Syn(A53T)–treated cells contained reduced CD63-mCherry as compared with untreated cells (Fig. 8), confirming the notion that extracellular α -Syn(A53T) causes S1P₁R uncoupled from G_i on MVEs and inhibits cargo sorting into ILVs of exosomal MVEs.

Discussion

Growing lines of evidence support that α -Syn has an intrinsic property to interact with gangliosides: interaction of GM1 with





Figure 4. Role of gangliosides in the action of extracellular α -**Syn(A53T).** *A*, SH-SY5Y cells expressing S1P₁R-YFP were incubated without (*closed black bars*) or with 1 μ M α -Syn(A53T) (*hatched orange bars*) or α -Syn(A53T)-AAA (*closed blue bars*) for 18 h. In some experiments cells were pretreated with 3 milliunits/ml of neuraminidase (neu) for 1 h before treatment of 1 μ M α -Syn(A53T) (*hatched green bars*). Cells were subjected to lipid raft separation and fluorescence intensity was measured in each fraction as described in the legend to Fig. 1A. Values represent mean \pm S.E. of three independent experiments carried out in triplicate. Statistical significance was analyzed by Student's *t* test (*, *p* < 0.01). *B*, SH-SY5Y cells expressing both S1P₁R-CFP and flotillin 2-YFP were incubated with 3 milliunits/ml of neuraminidase (neu) for 1 h before treatment of 1 μ M α -Syn(A53T) or α -Syn(A53T). Cells were subjected to lipid raft separation and fluorescence intensity was measured in each fraction as described in the legend to Fig. 1A. Values represent mean \pm S.E. of three independent experiments carried out in triplicate. Statistical significance was analyzed by Student's *t* test (*, *p* < 0.01). *B*, SH-SY5Y cells expressing both S1P₁R-CFP and flotillin 2-YFP were incubated with 3 milliunits/ml of neuraminidase (neu) for 1 h before treatment of 1 μ M α -Syn(A53T). Cells were fixed and analyzed for FRET efficiency in the plasma membrane areas using acceptor photobleaching method. Results are expressed as median on scatter-dot plots ($n \ge$ 50). Statistical significance was analyzed by Student's *t* test (*, *p* < 0.05 *versus* α -Syn(A53T)).



Figure 5. Rescue by gangliosides of extracellular α -Syn-induced expulsion of S1P₁R from the lipid raft fractions. SH-SY5Y cells expressing both S1P₁R-CFP and flotillin 2-YFP were incubated without (vehicle control, *open circles*) or with 1 μ M α -Syn(A53T) in the presence of various concentrations of gangliosides for 18 h. Cells were fixed and analyzed for FRET efficiency in the plasma membrane areas as described in the legend to Fig. 2*B*. Results are expressed as median on scatter-dot plots ($n \ge 20$). Statistical significance was analyzed by Student's t test (**, p < 0.01; *, p < 0.05).

 α -Syn, and inhibition of its fibrillation (31) or contrary to this, acceleration of α -Syn aggregation with vesicles containing GM1 or GM3 (34), determination of ganglioside-binding specificity of α -Syn showing the importance of tyrosine 39 residue of the protein (32), and importance of GM1 for the internalization of extracellular α -Syn in microglia (35). A recent report (27) from our laboratory has shown that extracellular α -Syn causes impairment of PDGF-induced chemotaxis through selective inhibition of Rac1 activation. Subsequently, after dissection of the signaling pathway upstream of the Rac1 activation, we have revealed that extracellular α -Syn induces S1P₁R uncoupled from G_i (28). However, how extracellular α -Syn behaves like an "uncoupler" was still a mystery. In the present study we have shown that extracellular α -Syn(A53T) drives $S1P_1R$ out from lipid rafts, via α -Syn binding to ganglioside. The importance of ganglioside for extracellular α -Syn(A53T) action was ascertained by the fact that the effect of extracellular α -Syn(A53T) was masked by neuraminidase treatment and that a α -Syn(A53T)–derived mutant, α -Syn(A53T)-AAA devoid of ganglioside binding, failed to drive S1P₁R out of the raft fractions (Fig. 4). Furthermore, extracellular α -Syn-induced expulsion of S1P₁R from the lipid rafts was rescued by the addition of ganglioside mixtures (Fig. 5). The neuraminidase from *Arthrobacter ureafaciens* used in the present study hardly hydrolyzes the sialic acid residue of GM1 under conditions without detergent due to the steric hindrance exerted by the neighboring Gal-GalNAc residues (36). Based on neuraminidase sensitivity along with the high affinity of α -Syn for GM3 (32), GM3 may be a potential target molecule for extracellular α -Syn. Further studies are necessary to elucidate a molecular basis of extracellular α -Syn action.

Disruption of lipid rafts by pretreatment of cells with MBCD resulted in expulsion of S1P₁R from the detergent-resistant buoyant fractions (Fig. 1A, fraction 2), with a concomitant uncoupling of $S1P_1R$ from G_i (Fig. 6B). These results suggest that expulsion of S1P₁R from the lipid rafts may suffice to make the receptor uncoupled from G_i . To support this notion, it has previously been reported that disruption of lipid raft by MBCD caused the α_{1a} -adrenergic receptor to be impaired of G-protein signaling (37). As for the mechanism of the expulsion from the rafts, competition between S1P₁R and α -Syn(A53T) for gangliosides may be unlikely, because treatment of cells with neuraminidase had little or no effect of S1P1R in raft localization (Fig. 4A and data not shown). It may be possible that additional posttranslational modifications such as palmitoylation of S1P₁R contributes to raft binding (38, 39), because the lipidation confers hydrophobicity to the protein. Further studies are necessary to clarify the mechanism underlying extracellular α -Syn(A53T)–induced expulsion of S1P₁R from the lipid rafts.

We have shown evidence that extracellular α -Syn(A53T) induces uncoupling of S1P₁R (Fig. 6*A*), shutting down G_i pro-





Figure 6. α -**Syn(A53T)-induced uncoupling of S1P₁R from G**_i **both in the plasma membranes and MVEs.** *A*, SH-SY5Y cells expressing S1P₁R-CFP, G β , G γ -YFP, FLAG-Rab5(Q97L), and CD63-mCherry were pretreated without (vehicle control) or with 1 μ M α -Syn(A53T) for 18 h and then stimulated with 100 nm S1P for 1 min, fixed, and analyzed for FRET efficiencies in the plasma membrane or MVE areas. Results are expressed as median on scatter-dot plots ($n \ge 20$). Statistical significance was analyzed by Student's *t* test (**, p < 0.01; *, p < 0.05). *B*, cells expressing S1P₁R-CFP, G β , and G γ -YFP were pretreated without (vehicle control) on m S1P for 1 min, fixed, and analyzed for FRET efficiencies in the plasma membrane areas. Results are expressed as median on scatter-dot plots ($n \ge 20$). Statistical significance was analyzed by Student's *t* test (**, p < 0.01; *, p < 0.05). *B*, cells expressing S1P₁R-CFP, G β , and G γ -YFP were pretreated without (vehicle control) or with 0.2 mM MBCD for 2 h and then stimulated with 100 nm S1P for 1 min, fixed, and analyzed for FRET efficiencies in the plasma membrane areas. Results are expressed as median on scatter-dot plots ($n \ge 20$). Statistical significance was analyzed by Student's *t* test (**, p < 0.01).



Figure 7. α -**Syn(A53T)-induced inhibition of exosomal cargo sorting into MVEs.** *A*, SH-SY5Y cells expressing both CD63-mCherry and GFP-Rab5(Q79L) were incubated without (*vehicle control*) or with 1 μ M α -Syn(A53T) for 18 h, fixed, and followed by MVE cargo sorting analysis as illustrated in *B*. Results are expressed as median on scatter-dot plots ($n \ge 33$ endosomes; **, p < 0.01 versus control; Student's *t* test). *B*, schematic representation of MVE cargo sorting analysis.

tein signaling, i.e. inhibition of cargo sorting into exosomal ILVs (Fig. 7A) and cargo release in exosomes (Fig. 8). One of the important physiological functions of S1P₁R signaling during vesicular trafficking is continuously transmitting S1P₁R-mediated G_i protein signals on MVEs, which permits cargo sorting into exosomal ILVs of MVEs (25, 33). Idiopathic Parkinson's disease is characterized by the accumulation and aggregation of α -Syn in the cytoplasm of the affected cells. A recent line of evidence suggests that α -Syn aggregates are transmitted from cell-to-cell through a cycle involving uptake of external aggregates, co-aggregation with endogenous α -Syn, and exocytosis of the co-aggregates, contributing to the propagation of PD pathology (40). In the present study self-aggregated α -Syn(A53T) showed an ability similar to nonaggregated α -Syn(A53T) for S1P₁R expulsion from the lipid rafts (Fig. 3D). However, it has previously been shown that self-aggregated α -Syn(A53T) gains a stronger ability to inhibit platelet-derived growth factor-induced chemotaxis in SH-SY5Y cells (27). Che-

motaxis involves a chain of events including growth factor receptor activation, transactivation of S1P₁R and subsequent G_i signals, actin filament remodeling, etc. and takes longer (at least several hours). On the other hand, extracellular α -Syn-induced expulsion of S1P₁R from the lipid rafts may be one of the earliest events triggered by extracellular α -Syn and takes a shorter time (almost 1 h, Fig. 3A), although the precise mechanism of the expulsion needs to be clarified. In this context the aggregationfunction relationship of extracellular α -Syn needs careful evaluation. The causal relationship between extracellular α -Syninduced uncoupling of S1P1R and pathogenesis of PD is still unclear at present, however, it is likely that the S1P signal may participate in regulation of the α -Syn content in the cells. Along with the recent report that α -Syn can be released from cultured cells by exosomes (12), inhibition of exosomal cargo release (including α -Syn), as a result of extracellular α -Syn-induced uncoupling of S1P₁R from G_i protein, may lead to an increase in cellular content of α -Syn, which may facilitate aggregation of α -Syn. Further studies are necessary to clarify pathophysiological relevance of extracellular α -Syn-induced uncoupling of $S1P_1R$ in the development of α -synucleinopathies including PD.

Experimental procedures

Reagent

S1P was purchased from Enzo Life Sciences; MBCD from (Sigma), ganglioside mixture purified from calf brain (IsoSep, Sweden). Other reagents and chemicals were of analytical grade.

Bacterial expression and purification of recombinant α -Syn and α -Syn(A53T)

Recombinant human α -Syn and α -Syn(A53T) were expressed in *Escherichia coli* and purified as described previously (41). Briefly, α -Syn or α -Syn(A53T) cDNAs subcloned into pET3a were transformed in *E. coli* BL21(DE3) and protein expression was induced by 0.1 mM IPTG for 3 h. Bacterial pellets were resuspended in TE buffer (10 mM Tris-HCl, pH 7.5,





Figure 8. α -**Syn(A53T)-induced reduction of cargo content in purified exosomes.** *A*, SH-SY5Y cells expressing CD63-mCherry were incubated without (*vehicle control*) or with 1 μ M α -Syn(A53T) for 18 h, followed by quantification of cargo content in purified exosomes. Exosomes prepared by a sequential centrifugation from cell culture media (5 × 10⁶ cells) were resuspended in equivolume buffer and labeled with DiD and immobilized on streptavidin-functionalized glass surface (see "Experimental procedures"). Images of CD63-mCherry and DiD fluorescence were acquired with a confocal laser scanning microscope. The fluorescence intensity of DiD and CD63-mCherry in DiD-labeled exosomes obtained from the images were plotted on the correlation diagram for each exosome. Note that the number of exosomes in the control and α -Syn(A53T) treatment were 4482 and 4951, respectively, indicating that α -Syn(A53T) treatment has little or no effect on the total number of exosomes. *B*, an average of cargo (CD63) content per each exosome (coefficient numbers of each *red* numerical formula in *A*) is represented. Statistical significance was analyzed by Student's *t* test (**, *p* < 0.01).

and 1 mM EDTA) containing 750 mM NaCl (TE, 750 mM NaCl) with protease inhibitors, heated at 100 °C for 10 min, and centrifuged at 70,000 × g for 30 min. The supernatant was dialyzed against TE, 20 mM NaCl, filtered by 0.22- μ m filter and applied to a Mono S column (GE Healthcare). The unbound fractions were applied to a Mono Q column (GE Healthcare). α -Syn was eluted with a 0 – 0.5 M NaCl linear gradient. The fractions containing α -Syn were identified by Coomassie Brilliant Blue staining and immunoblot analysis following SDS-PAGE. Protein concentration was determined using Bradford protein assay kit (Bio-Rad).

Plasmids and mutations

Human α -Syn was amplified and subcloned into the bacterial expression vector pET3a or mammalian expression vector pCMV5. For α -Syn(A53T), alanine 53 was mutated to a threonine using a QuikChange site-directed mutagenesis protocol. α -Syn(A53T)–AAA was designed and expressed as reported previously (32) with a slight modification that three point mutations were employed in α -Syn(A53T) instead of α -Syn. Human CD63 was amplified and cloned into pmCherry-N1 (Clontech Laboratories) for making mCherry-tagged CD63. Human Rab5 was amplified and cloned into pEGFP-C1 for making N terminally GFP-tagged Rab5. For GFP-Rab5(Q79L), glutamine 79 was mutated to a leucine using a QuikChange site-directed mutagenesis protocol. DsRed-tagged Rab5(Q79L) wasgeneratedbysubcloningtheRab5(Q79L)intopDsRed-monomer-C1 (Clontech Laboratories). Human flotillin 2 (accession number NM_004475) was amplified using 5'-ACAGCTAG-CATGGGCAATTGCCACACGGTG-3' and 5'-ATAGGTA-CCGTCACCTGCACACCAGTGGCC-3' and cloned into pEYFP-C1 (Clontech Laboratories). All the constructs were verified by sequencing.

Cell cultures and transfections

SH-SY5Y cells obtained from American Type Culture Collection (ATCC, CRL-2266) were maintained in Dulbecco's modified Eagle's medium/F-12 medium (Wako Pure Chemical Industries) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in 5% CO₂. Cells were plated onto glass-bottomed 35-mm culture dishes (MatTek) before transfection. Transient transfection was carried out using FuGENE HD (Promega). All experiments were performed 2 to 3 days after transfection.

Lipid raft separation

SH-SY5Y cells transfected with S1P1R-YFP or S1P2R-GFP were incubated with or without 3 milliunits/ml of neuraminidase (Nacalai Tesque) for 1 h, followed by addition of 1 μ M α -Syn(A53T) and further incubation for 18 h in serum-free medium. Cells were washed twice with ice-cold PBS and lysed in 0.2 ml of pre-chilled isolation medium (25 mM Tris-HCl, pH 7.5, 150 mм NaCl, 1 mм DTT, 1 mм EDTA, 1% Triton X-100 and protease inhibitor mixture). Cells were disrupted by using a syringe with a 23-gauge needle, followed by addition of 0.4 ml of ice-cold Optiprep (Axis-Shield, Oslo, Norway). The lysate was transferred to a centrifugation tube and overlaid with 2.4 ml of 30% and then 0.6 ml of 5% OptiPrep-containing isolation medium. Tubes were centrifuged at 200,000 \times g for 4 h at 4 °C using a Beckman Coulter SW60Ti rotor. Fractions of 0.6 ml were collected from the tops of the gradients. S1P₁R-YFP or S1P₂R-GFP distribution in each fraction was measured by fluorescence spectrophotometer F2500 (Hitachi, Tokyo, Japan). Alternatively 10 μ l of each fraction was spotted on Hybond ECL membrane (GE Healthcare), followed by detection of GM1 using horseradish peroxidase-conjugated cholera toxin subunit B (Thermo Fisher Scientific).



Extracellular α -Syn drives S1P₁ receptor out of lipid rafts

Analysis of cargo sorting into MVEs

SH-SY5Y cells plated on glass-bottomed 35-mm culture dishes were transiently expressed with CD63-mCherry and GFP-Rab5(Q79L), then cells were fixed with 4% paraformalde-hyde in PBS 3 days after transfection. Images were then obtained using a LSM 510 META (Carl Zeiss) with a ×63 oil plan-apochromat objective, optical zooms with ×6, and optical slice <0.9 μ m. Following excitation at 488 or 543 nm, GFP emission with a 505- to 530-nm bandpass barrier filter and mCherry emission with a 560- to 615-nm bandpass barrier filter were collected, respectively. The amount of CD63-mCherry in the endosomal lumen was quantified as fluorescence intensity in the lumen *versus* the limiting membrane of enlarged vesicle (diameter; >1 μ m) using ImageJ software.

Acceptor photobleaching

SH-SY5Y cells were transiently cotransfected with S1P₁R-CFP, G β , and G γ -YFP with a 1:1:1 ratio (25) or with S1P₁R-CFP and Flotillin 2-YFP. Two days after transfection, cells were treated with various reagents. Cells were then fixed and each area of interest was subjected to FRET analysis with an acceptor photobleaching method using LSM 510 META with a \times 63 oil plan-apochromat objective. Following excitation at 458 or 514 nm, CFP emission with a 475- to 525-nm bandpass barrier filter or YFP emission with 530- to 600-nm bandpass barrier filter, respectively, was collected. An area of interest was selected for photobleaching of YFP. An automated acquisition protocol was then used, which recorded pre- and post-bleaching images using 458-nm excitation at 8% laser power to limit photobleaching, with a bleaching of the selected area with 100% and 514-nm laser power with 50 iterations (acceptor photobleaching). FRET was resolved as an increase in the CFP (donor) signal after photobleaching of YFP (acceptor). FRET efficiency (E) can be determined from the relative fluorescence intensity of the energy donor (CFP) before (I_{pre}) and after (I_{post}) photobleaching of the energy acceptor (YFP): $E = 1 - (I_{pre}/I_{post})$.

Quantification of cargo content per each exosome

Quantification of cargo content per each exosome was carried out essentially as reported previously (25). Briefly, purified exosomes from SH-SY5Y cell culture media were incubated with a mixture of 100 mg/liter of 1,2-dioleoyl-*sn*-glycero-3phosphoethanolamine-*N*-(cap biotinyl) (DOPE-biotin) and 10 mg/liter of DiD for 15 min. A glass-bottomed 35-mm culture dish was incubated with a mixture of 0.1 g/liter of BSA/BSAbiotin, 10:1, in distilled water for 10 min. Excess protein was removed by gently flushing the surface with PBS and subsequently incubated for 10 min with 0.025 g/liter of streptavidin. After gently rinsing with PBS, DOPE-biotin- and DiD-labeled exosomes were resuspended in equivolume were added onto the surface of functionalized glass coverslip. The fluorescence of mCherry and DiD are observed under a confocal laser scanning microscope (LSM 510 META).

Statistical analyses

Results are expressed as median on scatter-dot plots. Data were analyzed by t test using the GraphPad Prism 6.0

(GraphPad Software, San Diego, CA). p values < 0.05 were considered significant.

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Extracellular α-synuclein drives sphingosine 1-phosphate receptor subtype 1 out of lipid rafts, leading to impaired inhibitory G-protein signaling

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