Extracellular α-synuclein induces sphingosine 1-phosphate receptor subtype 1 uncoupled from inhibitory G-protein leaving β-arrestin signal intact

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Parkinson’s disease (PD) is the second most common progressive neurodegenerative disorder after Alzheimer’s disease. The pathological hallmarks of PD are selective loss of the dopaminergic neurons in the substantia nigra pars compacta and the presence of α-synuclein (α-Syn)-positive intracytoplasmic inclusions, known as Lewy bodies, which develop in both idiopathic1–3 and hereditary PD, i.e., missense mutations, α-Syn(A53T)4, α-Syn(A30P)5, and α-Syn(E46K)6 as well as multiplication in the α-Syn gene7,8.

α-Syn with 140 amino acids is highly expressed in neurons and enriched in synaptic terminals suggesting a role in synaptic function and plasticity9,10. α-Syn is a natively unfolded molecule that can self-aggregate to form oligomers and fibrillar intermediates11,12. Subsequently, it has been suggested that oligomers rather than fibrillar structures might actually show toxicity to cells through binding to membrane lipids and causing membrane perturbations13,14. It has also been suggested that α-Syn has an ability to interact with gangliosides in the cholesterol and sphingolipid-rich membrane microdomains known as lipid rafts, and that it has a potency to alter the functions of several signalling molecules at the raft regions15. Recent studies suggest that α-Syn is detected in cerebrospinal fluid and plasma16,17, and cell-to-cell transmission of α-Syn plays a role in the progression of PD, i.e., α-Syn pathology is initiated in the peripheral nervous system and olfactory bulb, ascends toward the brainstem and into the midbrain such as substantia nigra, and then eventually spreads to the forebrain as suggested by "Braak’s hypothesis"18. It remains elusive as to how extracellular α-Syn participates in the pathogenesis of PD.

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Our laboratory has recently demonstrated that extracellular α-Syn causes inhibition of platelet-derived growth factor (PDGF)-induced chemotaxis in human neuroblastoma-derived SH-SY5Y cells through selective suppression of rac1 activation which is required for cell migration. It is known that the ability of growth factors such as PDGF to enhance cell migration utilizes the transactivation of sphingosine 1-phosphate (SIP) receptors in a variety of cell types. SIP is a phosphorylated product of sphingosine catalysed by sphingosine kinase (SphK) and has been implicated in an important lipid mediator acting both inside and outside the cell. SIP binds to members of GTP-binding protein (G-protein)-coupled SIP receptor family (SIP1–4) and triggers diverse cellular processes, including cell angiogenesis, cardiac development, immunity, cell motility, neurotransmitter release and endosome maturation.

From this background we have studied the extracellular effects of α-Syn on SIP signalling-mediated cell motility using both functional and fluorescence resonance energy transfer (FRET)-based structural analyses. Here we show that extracellular α-Syn causes SIP1 receptor–selective uncoupling from inhibitory G-protein (Gi) while other signalling events including SIP1 receptor-mediated signalling remain intact. A possible interpretation of α-Syn-induced changes in cellular signalling in conjunction with pathophysiological relevance is discussed herein.

**Results**

**PDGF utilises transactivation of SIP1 receptor for maximal chemotaxis.** It has recently been reported from this laboratory that extracellular α-Syn inhibits PDGF-induced chemotaxis. To identify signalling pathway, which is important in PDGF-induced chemotaxis and that is sensitive to extracellular α-Syn, we first examined the involvement of SIP1 signalling in this phenomenon. An SIP1 receptor-specific blocker, W146, inhibited PDGF-induced chemotaxis to an extent similar to α-Syn(A53T) treatment (Fig. 1a). Since SIP1 receptor is known to couple exclusively with Gi, it is reasonable to assume that pertussis toxin (PTX) inhibits the cell motility. In contrast, a selective SIP2 receptor antagonist JTE-013 showed no effect on PDGF-induced chemotaxis. Involvement of SIP1 signalling in PDGF-induced chemotaxis was further confirmed by downregulating one of the subtypes of SphK, SphK1 expression using small interfering RNAs (siRNAs). SphK1-siRNA caused inhibition of PDGF-induced chemotaxis by 30% as compared with the control siRNA (Fig. 1b). Similarly, knockdown of SIP1 receptor by SIP1 receptor-siRNA caused 40% inhibition of PDGF-induced chemotaxis. These results indicate that SIP1 signal is involved in PDGF-induced chemotaxis, consistent with a previous report. As described in a previous study showing a successful detection of conformational changes in α2A-adrenergic receptor using a fluorescence resonance energy transfer (FRET)-based technique, we have similarly constructed a probe to detect conformational changes in SIP1 receptor using a FRET technique, where the cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were separately fused in the same SIP1 receptor molecule (Fig. 1c). Under a resting state these fluorophores are closely situated, which enables FRET to occur, whereas the specific agonist (SIP) stimulation causes conformational changes in the receptor, resulting in FRET decrease. Upon stimulation by SIP1, cells expressing this FRET probe resulted in FRET changes in a W146-sensitive manner (Fig. 1d), validating this probe. Noticeably, when the cells were stimulated with PDGF instead of SIP, there was a rapid change in the CFP/FRET ratio, which was inhibited in SphK1-siRNA-treated cells (Fig. 1e), strengthening that PDGF utilises SIP1 receptor transactivation for maximal chemotaxis. Our previous findings that extracellular α-Syn suppressed PDGF-induced chemotaxis in SH-SY5Y cells facilitated an investigation of whether α-Syn has any effects on SIP signal. To assess this issue, SIP1 in place of PDGF was used as an agonist to activate SIP1 receptor to simplify the system thereafter.

**α-Syn causes uncoupling of SIP1 receptor from Gi.** To investigate the effect of extracellular α-Syn on SIP1 signalling, SIP1 receptor-mediated downstream events, i.e., G-protein subunit dissociation, were monitored by FRET-based conformational changes. Since SH-SY5Y cells express mainly SIP1 and SIP2 receptors as judged by real-time quantitative reverse transcription–PCR (Fig. 2a), we focused on these two subtypes of the SIP receptors for SIP1 signalling. To see the effect of α-Syn on each SIP receptor-mediated signalling, we carried out a FRET analysis using each SIP receptor-CFP and Gγ–YFP as a FRET pair. Under basal conditions heterotrimeric G-protein subunits are associated (Gαβγ form, low FRET). Upon stimulation by SIP1, these subunits dissociate, and SIP1 receptor-CFP and Gγ–YFP become associated (high FRET). In control cells expressing SIP1 receptor-CFP and Gγ–YFP, SIP1 caused a rapid increase in FRET efficiency, demonstrating a successful detection of SIP1 receptor–mediated G-protein subunit dissociation (Fig. 2b). Surprisingly, α-Syn treatment made the receptor refractory toward SIP1 receptor–mediated G-protein subunit dissociation. It is notable that α-Syn(A53T), a mutant α-Syn found in familial PD, caused more potent effects than a wild-type α-Syn with the same concentration (1 μM). On the other hand, when the cells expressing SIP1 receptor-CFP and Gγ–YFP were stimulated with SIP1, the agonist-induced G-protein dissociation occurred. However, wild-type α-Syn and α-Syn(A53T) treatment showed no significant effects on SIP1 receptor–mediated G-protein subunit dissociation (Fig. 2c). These results suggest that α-Syn causes a selective SIP1 receptor uncoupling from the G-protein but not SIP2 receptor. α-Syn effects were not from cytotoxic ones since SIP1 receptor-mediated signalling occurred normally in α-Syn-treated cells (Fig. 2c). Furthermore, two days incubation of the cells with 1 μM α-Syn(A53T) did not cause apoptosis as judged by chromatin condensation assay (Table I). Since the effect of α-Syn(A53T) is clearer than the wild type with the same concentration, subsequent analysis was mainly carried out using α-Syn(A53T).

To demonstrate that α-Syn(A53T) causes SIP1 receptor uncoupling from Gi more directly, we have carried out the experiments using another FRET pair with CFP–CFP and Gγ–YFP. Under basal conditions these subunits were associated (high FRET) and SIP1 receptor stimulation resulted in the dissociation of the Gαγ from the Gβγ subunits (low FRET) (Fig. 2d, closed bars). As expected, α-Syn(A53T) treatment abolished the SIP1-induced FRET changes (Fig. 2d, hatched bars), indicating that α-Syn(A53T) causes SIP1 receptor uncoupling from Gi.
Next, to address whether agonist stimulation facilitates S1P1 receptor coupling with Gi, the association of S1P1 receptor with Gi$\alpha$ before and after agonist stimulation was assessed by FRET analysis. Under basal conditions S1P1 receptor-YFP was already associated with Gi$\alpha$-CFP as detected by a high FRET efficiency (Fig. 2e, closed bar). S1P and PDGF stimulation caused a significant (light grey bar) and a mild decrease (dark grey bar) in FRET efficiency, respectively. These changes may reflect agonist-induced Gi subunit dissociation. Critically, α-Syn(A53T) caused a significant decrease in FRET efficiency (hatched bar). Taken together with the findings that α-Syn(A53T) impairs the S1P-induced Gi subunit dissociation (Fig. 2d), extracellular α-Syn(A53T) may segregate S1P1 receptor from Gi.

Abrogation of endogenous Gi function by α-Syn(A53T). The effect of α-Syn(A53T) on endogenous Gi function was assessed next. When cells were treated with S1P, forskolin-stimulated adenylate cyclase activity was potently inhibited (a phenomenon known as a classical Gi$\alpha$ function) in a S1P1 receptor antagonist W146-sensitive manner (Fig. 3a), evaluating the S1P1 receptor/Gi$\alpha$ function in an endogenous cell system. Importantly, α-Syn(A53T) abrogated the S1P1 receptor/Gi$\alpha$-caused inhibition of forskolin-stimulated adenylate cyclase, supporting the premise that α-Syn(A53T) caused S1P1 receptor to uncouple from Gi protein. α-Syn(A53T) itself had no inhibitory effect on forskolin-stimulated adenylate cyclase activity. In contrast, S1P-induced increase in cellular Ca$^{2+}$ through S1P1 receptor, which was inhibited by JTE-013 (Fig. 3b), was insensitive to α-Syn(A53T) (Fig. 3c). These results indicate that α-Syn(A53T) causes selective impairment of S1P1
Figure 2. α-Syn-induced uncoupling of S1P₁ receptor from G-protein, while leaving S1P₂ receptor unchanged. (a) Expression level of S1P₁, S1P₂, S1P₃, S1P₄ and S1P₅ receptor mRNAs in SH-SY5Y cells were quantitated by real-time quantitative reverse transcription PCR. Values of mRNA amounts were normalised to GAPDH expression. (b) Cells transiently expressing the S1P₁-receptor-CFP, Gβ and Gγ-YFP were pretreated without (closed bars) or with 1μM wild-type α-Syn (grey bars) or α-Syn(A53T) (hatched bars) for 18 hr and then stimulated with 100 nM S1P for 1 min, fixed and analysed for FRET efficiencies. Values represent means ± s.e.m. (n ≥ 50). Statistical significance was analysed by Student’s t-test (*P < 0.05 versus S1P control). (c) SH-SY5Y cells transiently expressing the S1P₂ receptor-CFP, Gβ and Gγ-YFP were treated with either buffer (control) or 1μM wild-type α-Syn (grey bars) or α-Syn(A53T) (hatched bars), stimulated with S1P and analysed for FRET efficiencies as in (b). Values represent means ± s.e.m. (n ≥ 50). (d) Cells transiently expressing the Gγ-CFP, Gβ and Gγ-YFP were pretreated without (closed bars) or with 1μM α-Syn(A53T) (hatched bars) for 18 hr and then stimulated with 100 nM S1P for 1 min, fixed and analysed for FRET efficiencies. Values represent means ± s.e.m. (n ≥ 50). Statistical significance was analysed by Student’s t-test (*P < 0.05). (e) Cells transiently expressing the S1P₁-YFP and Gα-CFP were pretreated without or with 1μM α-Syn(A53T) for 18 hr and then stimulated with 100 nM S1P or 20 ng/ml PDGF for 1 min, fixed and analysed for FRET efficiencies. Values represent means ± s.e.m. (n ≥ 50; *P < 0.05, **P < 0.01 versus control).
receptor/Gi signalling leaving S1P2 receptor signalling intact in an endogenous protein system as well as transient expression system (Figs 2 and 3).

Figure 3. α-Syn-induced impairment of S1P1 receptor- but not S1P2 receptor-mediated signalling assessed in an endogenous protein system. (a) SH-SY5Y cells transiently expressing the cAMP biosensor Epac1-camps were treated with 0.5 mM cAMP phosphodiesterase inhibitor, isobutylmethylxanthine, 20 μM forskolin with or without 100 nM S1P, or 10 μM W146 as indicated. Alternatively, cells transiently expressing the cAMP biosensor, which had been treated with 1 μM α-Syn(A53T) for 18 hr, were stimulated with each agonist as indicated (hatched bars). The FRET efficiency was estimated using acceptor photobleaching. Values represent means ± s.e.m. of 3 independent experiments carried out in triplicate. Statistical significance was analysed by Student's t-test (*P < 0.05). (b) SH-SY5Y cells were serum starved for 18 hr and loaded with 2 μM Fluo-4 AM for 20 min. Cells were washed and pretreated with 10 μM W146 or 10 μM JTE-013 or without (control) for 10 min. The Fluo-4 emission signal for each cell was acquired at a frequency of 1 Hz by fluorescence microscope. After taking basal level signal 100 nM S1P was added (arrow) and the change in fluorescence was monitored. One of the representative quantification results of fluorescence changes in 40 control, 40 W146- and 40 JTE-013-treated cells from 3 independent experiments is shown. (c) SH-SY5Y cells were serum starved for 18 hr with or without 1 μM α-Syn(A53T). Cells were then loaded with Fluo-4 AM and stimulated with 100 nM S1P (arrow) in the absence or presence of 1 μM α-Syn(A53T). One of the representative quantification results of fluorescence changes in 40 control and 40 α-Syn(A53T)-treated cells from 3 independent experiments is shown.
Mechanism underlying α-Syn(A53T)-induced uncoupling of S1P1 receptor from Gi. It is well known that PTX causes G-protein-coupled receptor uncoupling from Gi by ADP-ribosylation of the Giα subunit. The general feature of PTX is to eliminate widely the Gi-dependent phenomena including chemotactic formyl-Met-Leu-Phe (fMLP)-induced respiratory burst. We therefore undertook a study to compare α-Syn(A53T) with PTX for the ability to cause uncoupling of fMLP receptor. As reported previously, PTX treatment inhibited almost completely fMLP-induced respiratory burst in differentiated HL-60 cells (Fig. 4). In contrast, α-Syn(A53T) had no effects on the phenomena, suggesting that the site of action of α-Syn(A53T) may be distinct from that of PTX. Next, the effect of α-Syn(A53T) on S1P1 receptor was assessed using an S1P1 receptor FRET tool. S1P-induced conformational changes in the receptor were not influenced by α-Syn(A53T) treatment (Fig. 5). Importantly, α-Syn(A53T) treatment had little or no effects on the S1P1-induced β-arrestin association with the receptor (Fig. 6b, hatched bars). Next, the effect of α-Syn(A53T) on S1P1-induced internalisation of the S1P1 receptor, one of the well known outcomes of β-arrestin signal, was studied. The cell

Figure 4. Differential mode of action between α-Syn(A53T) and PTX toward Gi-coupled receptors. Differentiated HL-60 cells were pretreated with 1 μM α-Syn(A53T) or 100 ng/ml PTX for 18 hr. Upon stimulation by 0.1 μM fMLP, superoxide anion production was measured. Values represent means ± s.e.m. of 3 independent experiments carried out in triplicate. Statistical significance was analysed by Student’s t-test (n = 9; **P < 0.01 versus control cells treated with fMLP).

Figure 5. Ineffectiveness of α-Syn(A53T) in S1P-induced conformational changes in S1P1 receptor. SH-SY5Y cells transiently expressing the FRET probe as in Fig. 1c were serum-starved for 18 hr in the absence (open circles) or presence (closed circles) of 1 μM α-Syn(A53T). Cells were stimulated with 100 nM S1P (arrow) and analysed for FRET analysis in living cells. A representative emission ratio of the 2 fluorophores from 3 independent experiments is shown.
surface proteins were biotinylated from outside the cells expressing S1P1 receptor-YFP before and after S1P stimulation, and then biotinylated S1P1 receptor was quantitated. α-Syn(A53T)-treated cells showed a decrease in a cell surface S1P1 receptor after S1P stimulation to an extent similar to that in the control cells (Fig. 7a, compare hatched bars with closed bars). This observation was further confirmed by detecting cell surface endogenous S1P1 receptor using an antibody to detect extracellular epitope of the receptor. Stimulation of cells with S1P caused a decrease in endogenous cell surface S1P1 receptor both in control and α-Syn(A53T)-treated cells to a similar extent (Fig. 7b and c, hatched bars versus closed bars). Furthermore, S1P1 receptor internalisation was demonstrated directly in an immunocytochemical study. Upon stimulation of cells with S1P, S1P1 receptor-YFP became localised in small punctate structures suggesting S1P1 receptor internalisation (Fig. 7d). It should be noted that α-Syn(A53T) had little or no effects on S1P-induced formation of S1P1 receptor-positive dot-like structures. Indeed, these results indicate that α-Syn(A53T) causes uncoupling between S1P1 receptor and Gi protein, leaving β-arrestin signal unchanged.

Discussion

Recent studies have revealed that α-Syn can be released from cultured cells by exocytosis32 or by exosomes33 and that α-Syn is detected in cerebrospinal fluid and plasma16,17. In addition, cell-to-cell transmission of α-Syn was shown experimentally to induce an inclusion formation and neuronal cell death34. From this background we have reasoned that studies on changes in cellular functions induced by exogenous α-Syn may help understand the pathophysiology of α-Syn.

In the present studies we have shown that extracellular α-Syn causes S1P1 receptor-selective uncoupling from Gi as determined by both functional (Fig. 3) and FRET-based structural analyses (Fig. 2b,c,d). It has previously been reported that in 1-methyl-4-phenylpyridinium (MPP+) treatment of SH-SY5Y cells, an in vitro PD model, there was a significant decrease in SphK1 gene expression and that Sphk1 inhibition plays an important role in caspase-dependent apoptotic neuronal death35. However, the mRNA levels of S1P signalling molecules such as SphK1, SphK2, S1P1, and S1P2 receptors were unchanged under the experimental condition used in the present studies (1 μM α-Syn treatment for 18 hr) although in the proapoptotic conditions such as a higher dose (10 μM) of α-Syn or prolonged time (42 hr) treatment the mRNA level of SphK1 decreased, while that of SphK2 increased (data not shown).

As for the site of action of α-Syn(A53T), it may not act on Gi protein but on S1P1 receptor because fMLP-induced respiratory burst was insensitive to α-Syn(A53T) in differentiated HL-60 cells, although it was completely inhibited by PTX, which acts on Gia subunit directly (Fig. 4). α-Syn(A53T) had little or no effect on S1P1 receptor as judged by a FRET-based analysis (Fig. 2c) and cellular Ca2+ rise in an endogenous protein system (Fig. 3c). Taken together, it may be plausible to assume that α-Syn/α-Syn(A53T) works specifically to
S1P₁ receptor. However, a pull-down of S1P₁ receptor was not able to detect α-Syn(A53T) association (data not shown). This suggests that α-Syn(A53T) may exert its action indirectly or with the aid of other molecules. In this context it has been shown that the translocation of S1P₁ receptor to caveolin-enriched microdomains is necessary for the subsequent efficient signalling. In their studies oxidised 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine-mediated rapid recruitment to caveolin-enriched microdomains of signalling molecules including the S1P₁ receptor and Akt is important in endothelial barrier enhancement in human pulmonary endothelial cells. It has been suggested that α-Syn has an ability to interact with gangliosides in the cholesterol and sphingolipid-rich membrane microdomains known as lipid rafts, and has a potency to alter the functions of several signalling molecules at the raft regions. In these lines it has been shown that disruption of lipid raft by methyl-β-cyclodextrin caused impairment of G-protein effector signalling but not α₁a-adrenergic receptor internalisation. To support this we have recently observed that ganglioside binding-deficient mutant of α-Syn(A53T), α-Syn(A53T)-AAA, lost its ability to suppress PDGF-induced chemotaxis in SH-SYSY cells. Consequently, it may be possible that α-Syn/α-Syn(A53T) causes changes in the membrane microdomain environment, which in turn alters S1P₁ receptor function.

The present results also show that another downstream signalling of S1P₁ receptor, β-arrestin-involved signalling was insensitive to α-Syn(A53T) (Fig. 6) and its physiological effect—S1P-induced internalisation of the
S1P₁ receptor was unaffected by α-Syn(A53T) (Fig. 7). It has been shown that β-arrestin binding requires the ligand-induced conformational changes of the G-protein-coupled receptor and the subsequent receptor phosphorylation⁴⁰. We have shown that α-Syn(A53T) caused no effects on the S1P-induced conformational changes in the S1P₁ receptor as judged by FRET-based studies (Fig. 5). These conformational changes may trigger phosphorylation of the receptor necessary for subsequent β-arrestin binding. The demonstration here indicates that exogenous α-Syn modulates S1P₁, receptor-mediated signalling from both Gi and β-arrestin signals into β-arrestin-biased one by uncoupling of the receptor from Gi.

Although the discovery of hereditary forms of PD has contributed greatly to understand the pathogenesis of the disease, that of sporadic PD, the majority forms of PD, is still elusive. It has recently been revealed that mutations in the GBA and SMPD1 genes are risk factors for PD. GBA encodes the lysosomal enzyme glucocerebrosidase that catalyses the breakdown of the glycolipid glucosylceramide to ceramide and glucose. GBA mutations, when homozygous, lead to Gaucher’s disease, while predispose to PD when heterozygous⁴⁹. SMPD1 mutations that cause Niemann-Pick type A were significantly higher in patients with PD compared to young controls⁴⁰. SMPD1 encodes sphingomyelin phosphodiesterase 1 (acid sphingomyelinase), a lysosomal enzyme that hydrolyzes sphingomyelin to generate phosphorylcholine and ceramide. Interestingly, both gene products share a common feature, i.e., their enzymatic products are ceramide. Ceramide is shown to be involved in exosomal vesicle formation in multivesicular endosomes (MVEs)⁴². Furthermore, we have recently reported that continuous activation of S1P₁ receptor by S1P, a further metabolite of ceramide, on MVEs has an essential role in the cargo sorting into exosomes⁴⁹. Along with the evidence that α-Syn is secreted in the form of exosomes⁴³, it is tempting to speculate that uncoupling of S1P₁ receptor from Gi caused by extracellular α-Syn may inhibit exosomal release of α-Syn, which results in the accumulation of α-Syn inside the cells, a pathological hallmark of PD. Further studies on the mechanism underlying extracellular α-Syn-caused uncoupling of S1P₁ receptor from Gi will be the keys to understand the pathogenesis of sporadic PD.

**Methods**

**Reagents.** S1P was purchased from Enzo Life Sciences; PTX, forskolin and dibutyryl cAMP from Wako Pure Chemical Industries; fMLP and cytochrome c were from Sigma Aldrich. W146 and JTE-013 were from Cayman Chemical Company. Other reagents and chemicals were of analytical grade.

**Plasmids and mutations.** Human α-Syn was amplified and subcloned into the bacterial expression vector pET3a. For α-Syn(A53T), alanine 53 was mutated to a threonine using a QuikChange site-directed mutagenesis protocol. mS1P₁ receptor-CFP, Gin-CFP, Gβ3 and Gγ-YFP plasmid constructs were prepared as described previously⁴⁶. Murine S1P₁ (mS1P₁) receptor (GenBank accession number NM_010333.4) cDNA was amplified from mouse brain cDNA, which had been reverse transcribed from fetal mouse brain mRNA (Invitrogen) by PCR (sense primer, 5′-CGGAATTTGCCACCATGGGGGCTTATACTCAGAG-3′; antisense primer, 5′-CGGAATTCGACACTGTGTACCCCTCGAG-3′) to make a C-terminally CFP-fused construct in pECFP-N1. A one-molecular FRET probe for detection of cAMP, Epac1-camps, was constructed as reported previously ⁴². For one-molecule FRET probe for the detection of conformational changes of S1P₁ receptor, EFPP was inserted in the third intracellular loop of murine S1P₁ receptor between Lys243 and Ala244. Briefly, the cDNA encoding Met1 to Lys243, Ala244 to Ser382 of mS1P₁ or EFPP was amplified by PCR using 5′-CGGAATTCATGGTGAGCAAGGGCGAG-3′ and 5′-ATAGATTGTTCAGGTGTCCATGACCATC-3′ and 5′-GGGCAAGGCCUUGCAGCUCdTdT-3′ and 5′-GCTCTAGACTTGTACAGCTCGTCCATG-3′, respectively. Each cDNA fragment was treated with EcoRI and BamHI, XbaI and EcoRI or BamHI and XbaI, respectively, and these three fragments were inserted into ECOI-cut pECFP-N1. All the constructs were verified by sequencing.

**siRNA.** For RNA interference following oligonucleotides (Japan Bio Services, Saitama, Japan) were used: Sense 5′-GGGAACAGCAAUAAACUGdTdT-3′ and antisense 5′-CAGUUUAAGCUCUCUCCdTdT-3′ for human S1P₁ receptor; sense 5′-GGGGCAAGGCCCUUGCAGCUCdTdT-3′ and antisense 5′-GGCUGCAAGGCCUCUCCGCDdTdT-3′ for human S1P₁ receptor and antisense 5′-UUCUCGGAAGCUGUCAGCdtddT-3′ and antisense 5′-AGUGUGACCGUUCGGAGAAdtddT-3′ for control.

SH-SY5Y cells were transfected with the siRNAs using Lipofectamine RNAiMAX according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA).

**Bacterial expression and purification of recombinant α-Syn and α-Syn(A53T).** Recombinant α-Syn and α-Syn(A53T) cDNAs subcloned into pET3a was transformed in E. coli. BL21 (DE3) and protein expression was induced by 0.1 mM IPTG for 3 hr. Bacterial pellets were resuspended in TE buffer (10 mM Tris-HCl, pH 7.5 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 dialysed against TE-20 mM NaCl, filtered by 0.22 μm 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(A53T) (Fig. 7). It has been shown that α-Syn(A53T) caused no effects on the S1P-induced conformational changes in the S1P₁ receptor as judged by FRET-based studies (Fig. 5). These conformational changes may trigger phosphorylation of the receptor necessary for subsequent β-arrestin binding. The demonstration here indicates that exogenous α-Syn modulates S1P₁, receptor-mediated signalling from both Gi and β-arrestin signals into β-arrestin-biased one by uncoupling of the receptor from Gi.

**Cell cultures and transfections.** SH-SY5Y cells (American Type Culture Collection, CRL-2266) were maintained in DMEM/F-12 medium (Wako Pure Chemical Industries) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in 5% CO₂. HL-60 cells were grown in RPMI 1640 medium (Wako Pure...
and analysed by flow cytometry (FACScalibur, BD Biosciences). and antisense) were as follows: for human S1P1 receptor, 5′-GCCATCAATGACCCCTTCATT-3′ and 5′-GGAGGGACATCACAAGAGATA-3′ for human GAPDH, 5′-AGTGCTGAGTTGAGATGATAA-3′ and 5′-GAGAAGCAATAGCAGGCACTC-3′ for human S1P5 receptor, 5′-GAGGAGAACTGCCTGTGCGCCTTTGAC-3′ and 5′-TAGTGTCGCCAGCTTCTGAGATGTCG-3′ for human S1P4 receptor, 5′-AAGTTCCTGGGCGCTTGTGAC-3′ and 5′-ATCAAGAGAGAACAGAGATGTGAC-3′ for human S1P3 receptor, 5′-TTCACTGGCTGTCTGTGGCAGC-3′ and 5′-TTCAGAGTTGTTGCGCTGAGC-3′ for human GAPDH, 5′-GCCATCAATGACCCCTTCATT-3′ and 5′-TCTCGCTCCTGGAGAATGG-3′. Differences were statistically evaluated using one-way ANOVA followed by the Bonferroni post hoc test. Data are mean ± s.e.m. of 3 independent experiments. Values represent means ± s.e.m. of 3 independent experiments carried out in triplicate. To detect endogenous S1P1 receptor internalisation, SH-SY5Y cells (1 × 10^6 cells) were serum-starved in the absence or presence of 1 μM α-Syn(AS3T) for 18 hr. After cell stimulation with S1P, cells were chilled on ice and harvested by using Cell Dissociation Buffer (Life Technologies, Inc.). Cells were washed with PBS containing 1% sodium azide and 1% BSA and incubated with 1 μg/ml of anti-S1P1 receptor antibody conjugated with Alexa 488 (Novus Biologicals), which detects endogenous cell surface S1P1 receptor from outside of the cells, at 4 °C for 1 hr, followed by washing and analysed by flow cytometry (FACSCalibur, BD Biosciences).

**Measurement of superoxide anion production in differentiated HL-60 cells.** HL-60 cells were cultured for 48 hr with 0.2 mM dibutyryl cAMP to induce differentiation followed by serum starvation in the absence or presence of 1 μM α-Syn(AS3T) or 100 ng/ml PTX for 18 hr. Differentiated HL-60 cells were suspended in the HEPES-buffered medium consisting of 10 mM HEPES/NaOH (pH 7.4), 130 mM NaCl, 4.7 mM KCl, 1 mM CaCl_2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 10 mM glucose and 0.2% BSA with or without 1 μM α-Syn(AS3T). Cell suspension (10^6 cells/tube) was treated with 0.1 μM IMLP and 50 μM cytochrome c at 37 °C for 10 min followed by rapid centrifugation. The cell supernatant production was estimated by measuring the reduction of cytochrome c as the increase in absorbance at 550 nm using a spectrophotometer.
Ca\textsuperscript{2+} measurements. SH-SY5Y cells seeded on glass bottom dish were incubated with or without 1 \mu M \alpha-Syn(A53T) for 18 hr in serum-free DMEM/F-12. Cells were loaded with 2 \mu M Fluo-4 AM (Dojindo Laboratories) in HEPES-buffered medium consisting of 10 mM HEPES/NaOH (pH 7.4), 130 mM NaCl, 4.7 mM KCl, 1 mM CaCl\textsubscript{2}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 1.2 mM MgSO\textsubscript{4}·7H\textsubscript{2}O, 10 mM glucose with or without 1 \mu M \alpha-Syn(A53T) at 37 °C for 20 min and washed. Cells were pretreated with 10 \mu M W146 or 10 \mu M JTE-001 or without (control) for 10 min. In some experiments cells were treated with 1 \mu M \alpha-Syn(A53T) for 10 min. The glass bottom dish was mounted on IX70 fluorescence microscope (Olympus) and Fluo-4 fluorescence was recorded. Excitation wavelength was set to 490 nm and emission was recorded at 535 nm. After taking basal level fluorescence for 30 s, 100 nM S1P was added and the change of fluorescence was monitored at a frequency of 1 Hz. The acquired Fluo-4 emission signal for each cell was normalised to the basal signal and plotted against time.

Co-immunoprecipitation assay between S1P\textsubscript{3} receptor-FLAG and \beta-arrestin 2-YFP. SH-SY5Y cells expressing S1P\textsubscript{3} receptor-FLAG and \beta-arrestin 2-YFP were serum-starved for 18 hr with or without 1 \mu M \alpha-Syn(A53T) and stimulated with 100 nM S1P for the indicated time periods. Cells were then treated with 2 mM Dl(N-succinimidyl) 3,3',Dithiodipropionate (DSP) (Tokyo Chemical Industry Co., LTD.) for 20 min at room temperature, followed by solubilisation with lysis buffer (40 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10 mM NaF, 0.2% n-dodecyl \beta-D-maltoside (DDM) and protease inhibitor cocktail (Nacalai tesque)). Cell lysates were cleared by centrifugation at 13,000 × g for 15 min and immunoprecipitated by anti-FLAG beads (Wako Pure Chemical Industries). The immunoprecipitates were separated by SDS-PAGE and co-immunoprecipitated \beta-arrestin 2-YFP was detected by rabbit monoclonal anti-\beta-arrestin 2 antibody (Cell Signaling Technology) in immunoblotting.

Apoptosis assay. SH-SY5Y cells were serum-starved in the absence or presence of 1 \mu M wild-type \alpha-Syn or \alpha-Syn (A53T) for the indicated time periods. Cells were stained with 4,6-diamidino-2-phenylindole-2-HCl (DAP) and chromatin condensation during apoptosis was visualised by using LSM510 confocal microscope.

Statistical analysis. Results are expressed as means ± s.e.m. Data were analysed by t-test. P-values < 0.05 were considered significant.

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Author Contributions

T.O. and L.Z. conducted most of the experiments equally. T.O. performed FRET analysis. C.H., S.M.M.B. and T.K. performed biochemical and immunocytochemical analysis. T.O. and S.N. designed the study and S.N. wrote the manuscript together with contributions from T.O. and T.K.

Additional Information

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Erratum: Extracellular α-synuclein induces sphingosine 1-phosphate receptor subtype 1 uncoupled from inhibitory G-protein leaving β-arrestin signal intact

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The original version of this Article contained an error in the indexing of the author Shaymaa Mohamed Mohamed Badawy. This error has now been corrected.

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