

Accumulation of α -Synuclein Triggered by Presynaptic Dysfunction

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Pathological examination of dementia with Lewy bodies patients identified the presence of abnormal α -synuclein (α Syn) aggregates in the presynaptic terminals. α Syn is involved in the regulation of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. Importantly, α Syn-transgenic mouse and postmortem examination of patients with Parkinson's disease have demonstrated the abnormal distribution of SNARE protein in presynaptic terminals. In this study, we investigated the effects of SNARE dysfunction on endogenous α Syn using *Snap25*^{S187A/S187A} mutant mice. These mice have homozygous knock-in gene encoding unphosphorylatable S187A-substituted synaptosomal-associated protein of 25 kDa (SNAP-25). The mice displayed a significant age-dependent change in the distribution of α Syn and its Ser¹²⁹-phosphorylated form in abnormally hypertrophied glutamatergic nerve terminals in the striatum. Electron-microscopic analysis revealed the abnormally condensed synaptic vesicles with concomitant mislocalization of α Syn protein to the periactive zone in the glutamatergic nerve terminals. However, the *Snap25*^{S187A/S187A} mutant mouse harbored no abnormalities in the nigrostriatal dopaminergic neurons. Our present results suggest that SNARE dysfunction is the initial trigger of mislocalization and accumulation of α Syn, and probably is an important pathomechanism of α -synucleinopathies.

Introduction

α -Synucleinopathies are a subgroup of neurodegenerative diseases including dementia with Lewy bodies (DLB), multiple system atrophy (MSA), and Parkinson's disease (PD). The pathological hallmark of the disorders is the formation of intracellular aggregates composed mainly of α -synuclein (α Syn), which are called Lewy bodies and Lewy neurites (Spillantini and Goedert, 2000; Galvin et al., 2001; Yasuda et al., 2012). It was reported recently that >90% of α Syn aggregates are present in presynaptic terminals in the form of very small deposits of the affected neurons in DLB (Neumann et al., 2002; Kramer and Schulz-Schaeffer, 2007; Schulz-Schaeffer, 2010). However, the mechanisms responsible for presynaptic accumulation of abnormal α Syn remain elusive.

Generally, α Syn is abundantly localized in the presynaptic nerve terminals (Maroteaux et al., 1988; Iwai et al., 1995). While the physiological functions of α Syn have yet to be defined, several lines of evidence implicated this protein in the modulation of neurotransmitter release through the regulation of formation of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (Chandra et al., 2005; Burré et al., 2010; Darios et al., 2010) and the size of synaptic vesicle pool (Murphy et al., 2000; Cabin et al., 2002; Larsen et al., 2006; Nemani et al., 2010). Vesicle-associated membrane protein-2 (VAMP-2) present in the synaptic vesicles, and syntaxin and synaptosomal-associated protein of 25 kDa (SNAP-25) in the presynaptic plasma membrane form the core SNARE complex, which regulate the docking and fusion of synaptic vesicles to the presynaptic membrane (Südhof, 2004). A recent study showed the physical interaction of α Syn with VAMP-2 to promote SNARE assembly (Burré et al., 2010). Cysteine-string protein- α (CSP α) also participates in SNARE assembly, and mutant mice lacking CSP α displayed impaired SNARE formation and premature death, but both of these phenotypes are counteracted by transgenic expression of α Syn (Chandra et al., 2005; Sharma et al., 2011). However, overexpression of α Syn with no overt toxicity inhibits neurotransmitter release, due to a defective reclustering of synaptic vesicles after endocytosis (Nemani et al., 2010). Additionally, overexpressed α Syn indirectly inhibited SNARE-mediated exocytosis by sequestering arachidonic acid, which upregulates syntaxin and enhances its engagement with SNARE complex (Darios et al., 2010). Importantly, abnormal redistribu-

Received May 8, 2012; revised Aug. 28, 2012; accepted Oct. 5, 2012.

Author contributions: Y.N., T.Y., and H.M. designed research; Y.N., T.Y., M.F., S.Y., M.I., T.N., H.H., and A.K. performed research; Y.N., T.Y., M.F., S.Y., M.I., T.N., H.H., A.K., M.K., M.N., H.S., M.T., Y.M., and H.M. analyzed data; Y.N. and T.Y. wrote the paper.

This work was supported by Grants from the Japan Science and Technology Agency, Core Research for Evolutional Science and Technology (H.M.); Grants-in-Aid from the Research Committee of CNS Degenerative Diseases, the Ministry of Health, Labour and Welfare of Japan (H.M.); Grant-in-Aid for Research on Applying Health Technology (H23–015) from the Ministry of Health, Labour and Welfare of Japan (H.M.); Ministry of Education, Culture, Sports, Science and Technology of Japan Grant S0801035 (H.M.); and Grant-in-Aid for Scientific Research on Innovative Areas (Brain Environment) from the Ministry of Education, Science, Sports and Culture of Japan (H.M.).

The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.2220-12.2012

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tion of SNARE proteins has been observed in human PD patients and mice overexpressing a truncated form of human α Syn, which showed decreased release of dopamine (DA) in the striatum (Garcia-Reitböck et al., 2010). Therefore, presynaptic SNARE dysfunction is considered an initial pathogenic event in α -synucleinopathies.

Based on the above background, the present study was designed to investigate the effects of impaired SNARE assembly on the distribution of naive α Syn. We exploited *Snap25^{S187A/S187A}* mutant mice. This strain is resistant to the protein kinase C-mediated phosphorylation of SNAP-25 at Ser¹⁸⁷ and represents a concomitant reduction of neurotransmitter release, including serotonin and DA, from the amygdala, and develops convulsive seizures and anxiety-related behavior in general activity and light-and-dark preference tests (Kataoka et al., 2011). The results indicated that dysfunction of SNARE proteins could trigger abnormal localization and accumulation of presynaptic α Syn, possibly representing one of the pathogenic events in DLB or MSA.

Materials and Methods

Mice. All experimental protocols described in this study were approved by the Animal Experimentation and Ethics Committee of the Kitasato University School of Medicine. Homozygous SNAP-25 knock-in mutant mice bearing S187A mutation and wild-type littermate control mice were generated based on the procedures described in detail by Kataoka et al. (2011). Briefly, embryonic stem cells with the heterozygous *Snap25^{S187A}* mutant allele were generated and microinjected into C57BL/6N blastocysts to obtain heterozygous mutant mice. Male chimeras were bred with C57BL/6N female mice. After the line was backcrossed 13 times, homozygous mice (designated as *Snap25^{S187A/S187A}* mice) were obtained by *in vitro* fertilization. The genotype was checked by PCR. In this experiment, wild-type and *Snap25^{S187A/S187A}* mice were used at age 11 (females), 54 (females), 60 (males), and 61 (females) weeks.

Tissue processing. Deeply anesthetized mice (with 250 mg/kg, i.p., sodium pentobarbital) were perfused transcardially with PBS or with 2% paraformaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer (PB). The brains were removed en bloc from the skull and cut sagittally into two brain hemispheres for confocal microscopy, cell counting, electron microscopy, Western blotting, and catecholamine analysis. Tissue processing for conventional electron microscopy is described below (see Conventional electron microscopy). For confocal microscopy and cell counting, hemisphere brain blocks of mice perfused transcardially with PBS or with 2% paraformaldehyde/2% glutaraldehyde in 0.1 M PB were fixed overnight in 4% paraformaldehyde in PBS and immersed in PBS containing 30% sucrose until sinking. Coronal sections of the striatum and the entire rostrocaudal extent of the substantia nigra (SN) were cut serially at 20 μ m thickness using a cryostat (CM1850; Leica Microsystems). For Western blotting and catecholamine measurement by HPLC, brain blocks of mice perfused transcardially with PBS were dissected using the method described previously by our group, to yield the striatal tissues and ventral parts of midbrain tissues including the SN (Yasuda et al., 2011). After completing the dissection, the sections were immediately frozen on dry ice and stored at -80°C until analysis.

Antibodies. The primary antibodies used for confocal microscopy were mouse anti- α Syn (clone 42; diluted at 1:100; BD Biosciences) (Tapiá-González et al., 2011), rabbit anti- α Syn (1:500; Millipore Bioscience Research Reagents) (Chung et al., 2003; Herzig et al., 2011), rabbit anti-tyrosine hydroxylase (TH) (1:5000; Calbiochem) (Yasuda et al., 2011), mouse anti-synaptophysin (clone SY38; 1:500; Millipore) (Masliyah et al., 2001; Tabuchi et al., 2007; Gimbel et al., 2010), rat anti-dopamine transporter (DAT) (clone MAB369; 1:500; Millipore) (Herzig et al., 2011; Kaushal et al., 2011), rabbit or goat anti-vesicular glutamate transporter-1 (VGLUT1) (1:300; Frontier Institute) (Miyazaki et al., 2003; Kawamura et al., 2006), goat anti-VGLUT2 (1:300; Frontier Institute) (Miyazaki et al., 2003; Kawamura et al., 2006), and rabbit anti-Ser¹²⁹-phosphorylated α Syn (ab59264; 1:100; Abcam) (Pan-Montojo et al., 2010; Yasuda et al., 2011). For Western blotting, primary antibodies

used were mouse anti- α Syn (clone 42; 1:150; BD Biosciences), rabbit anti-Ser¹²⁹-phosphorylated α Syn (ab59264; 1:500; Abcam), rabbit anti-SNAP25ct (1:1000) (Yamamori et al., 2011), mouse anti-syntaxin 1 (10H5) (1.4 μ g/ml) (Yamamori et al., 2011), rabbit anti-VAMP-2 (1.4 μ g/ml) (Kataoka et al., 2011), mouse anti-synaptophysin (clone SY38; 1:500; Millipore), rabbit anti-TH (1:500; Calbiochem), and rabbit anti- β -tubulin (1:500; Abcam). For immunoelectron microscopy, the primary antibodies used were mouse anti- α Syn (clone 42; 1 μ g/ml; BD Biosciences), guinea pig anti-DAT (1 μ g/ml; Frontier Institute), and rabbit anti-VGLUT1 (1 μ g/ml; Frontier Institute).

Confocal microscopy. Free-floating sections were washed in PBS medium containing 0.05% Triton X-100 (PBS-T). As to the sections prepared from mice perfused transcardially with 2% paraformaldehyde/2% glutaraldehyde in 0.1 M PB, they were incubated with Liberate Antibody Binding (L.A.B.) solution (Polysciences) followed by washing in PBS medium and then processed in the same way as other sections. The sections were soaked with blocking agents and then incubated with the primary antibody dissolved in dilution reagent at 4°C for 48 h. Vector M.O.M. Immunodetection Kit (Vector Laboratories) was used for blocking and antibody dilution according to the instructions provided by the manufacturer. Subsequently, for fluorescent visualization of the antigens, the sections were incubated for 2 h in fresh medium containing fluorescein isothiocyanate-conjugated anti-mouse or rabbit IgG, and Cy3-conjugated anti-mouse, rabbit, goat, or rat IgG secondary antibodies (1:200–500; Jackson ImmunoResearch Laboratories). The sections were mounted on slide glass and coverslipped with Vectashield Mounting Medium with or without DAPI (Vector Laboratories). Images were captured using a confocal laser-scanning microscope (model LSM510; Zeiss) equipped with ZEN 2009 software (Zeiss) with identical settings. The software was used for image processing (brightness and contrast adjustment) applied equally to the images of wild-type and mutant mice.

Conventional electron microscopy. For conventional electron microscopy, the hemisphere brains prepared from mice perfused transcardially with 2% paraformaldehyde/2% glutaraldehyde in 0.1 M PB were post-fixed in 2% OsO₄ for 1 h. After washing in water, the samples were incubated in 2% uranyl acetate for 30 min. After dehydration using 50, 70, 90, and 100% ethanol solutions and 100% propylene oxide, the samples were embedded in Epon 812 resin (Nissin EM). Ultrathin sections were obtained using a Leica Ultracut UCT and stained with 2% uranyl acetate and lead citrate. Electron micrographs were taken with an H-7650 electron microscope (Hitachi).

Immunoelectron microscopy. For preembedding immunoelectron microscopy, parasagittal brain sections (60 μ m in thickness) were incubated successively with 5% normal goat serum, primary antibodies, and peroxidase- or colloidal gold (1.4 nm)-conjugated secondary antibodies (Invitrogen). Immunoreaction was visualized with 3,3'-diaminobenzidine or silver enhancement kit (HQ silver; Nanoprobes). When combining the two methods for double immunoelectron microscopy, the brain tissue was subjected first to immunoperoxidase and then to silver-enhanced immunogold. Sections labeled by immunoperoxidase and silver-enhanced immunogold were subjected to electron microscopy as described above. The size of the nerve terminal was quantitated by using the ImageJ 1.43 software (Wayne Rasband, NIH, Bethesda, MD; <http://rsb.info.nih.gov/ij>).

Western blotting. Frozen striatal and ventral midbrain tissues were sonicated in chilled CellLytic-MT mammalian tissue lysis/extraction reagent (Sigma-Aldrich) mixed with protease inhibitor mixture set I (Calbiochem) and phosphatase inhibitor mixture set V (Calbiochem). The samples were centrifuged (20,000 \times g for 10 min at 4°C), and the resulting supernatants were collected and used for Western blotting. The protein concentration in the lysate of all samples was determined using BCA protein assay kit (Pierce). Each protein sample (5–15 μ g) was resolved by SDS-PAGE by means of Compact-PAGE-twin (ATTO) and then electrotransferred to Clear Blot Membrane-P (ATTO) using powered BLOT-mini (ATTO). The membrane was washed in PBS-T, incubated for 1 h in PBS-T containing 50% ChemiBLOCKER (Millipore), and then incubated for 24 h with the primary antibody in the same fresh medium. Subsequently, the membrane was incubated for 2 h in fresh medium containing horseradish peroxidase-linked anti-mouse or rabbit IgG secondary antibody (1:10,000–20,000; GE Healthcare), followed by devel-

opment of chemiluminescence using GE Healthcare ECL Plus Western Blotting Detection System (GE Healthcare). The image was captured using LAS-4000 (Fujifilm), and the signal intensity was quantitated using the ImageJ 1.43 software.

Assay for SNARE complex assembly. SNARE complex assembly was assessed by measuring the levels of the high-molecular-weight SDS-resistant complex in Western blotting performed without boiling of the samples before gel electrophoresis (Hu et al., 2002; Asuni et al., 2008; Sakisaka et al., 2008). Striatal lysate samples (15 μ g of protein/sample) of wild-type and *Snap25*^{S187A/S187A} mice were resolved in the SDS sample buffer. Samples were incubated for 20 min at room temperature and were further incubated at room temperature or boiled at 100°C for 3 min. Subsequently, they were analyzed by SDS-PAGE followed by the immunoblotting using the rabbit anti-SNAP25ct primary antibody (1:1000) (Yamamori et al., 2011) and horseradish peroxidase-linked anti-rabbit IgG secondary antibody (1:10,000; GE Healthcare). Except for the sample preparation before gel electrophoresis, all procedures were the same as described above (see Western blotting).

Determination of striatal levels of DA and its metabolites by HPLC. Frozen striatal tissues were sonicated in 50 mM sodium acetate. The samples were centrifuged (20,000 \times g for 10 min at 4°C), and the resulting supernatants were mixed with equal volumes of 0.2N perchloric acid. The samples were centrifuged (20,000 \times g for 10 min at 4°C), and the resulting supernatants were subjected to measurement of DA, homovanillic acid (HVA), and 2-(3,4-dihydroxyphenyl)-acetic acid (DOPAC) concentrations by HPLC, using an HPLC system equipped with electrochemical detection (ECD-100; EICOM; applied voltage, 500 mV) and reverse-phase column (TSK gel ODS-80TM; Tosoh). The HPLC system consisted of a pump (PU-980; Jasco; flow rate, 1.0 ml/min), autosampler (AS-1550; Jasco), column oven (860-CO; Jasco), and degasser (DG-2080–53 Jasco). The mobile phase consisted of a solution, pH 3.72, containing the following: 0.085 M NaH₂PO₄ · 2H₂O, 2.5 mM 1-octanesulfonic acid sodium salt, 20 μ M EDTA-2Na, and 15% methanol. The concentrations of DA, HVA, and DOPAC were determined in nanomoles per gram of tissue weight, and the results were expressed relative to the values of wild-type mice.

Cell count. For DA cell counting in the SN pars compacta (SNpc), every fourth 20- μ m-thick serial section of the brain was immunostained for TH and counterstained with cresyl violet (Nissl staining) (Furuya et al., 2004; Yasuda et al., 2011). The numbers of TH- and Nissl-double-positive cells in the SNpc were counted both in wild-type and *Snap25*^{S187A/S187A} mice in a blind manner. The SNpc cells that have nuclei optimally visible by TH immunostaining, and nuclei, cytoplasm, and nucleoli prominently stained by Nissl staining were counted. To avoid double counting of neurons with unusual shapes, TH- and Nissl-double-positive cells were counted only when their nuclei and nucleoli were optimally visualized. The rostral end of the SNpc was determined to the level where TH- and Nissl-double-positive cells began to appear (~2.60 mm caudal to the level of the bregma) (Franklin and Paxinos, 2008), and the caudal end of the SNpc was defined to the level where TH- and Nissl-double-positive cells and oculomotor nerves can be observed (~3.80 mm caudal to the level of the bregma) (Yasuda et al., 2011). In the rostral half region of the SN (~2.60–3.16 mm caudal to the level of the bregma), the SNpc was distinguished from medial ventral tegmental area (VTA) by the vertical line extended from the medial end of the cerebral peduncle. In the caudal region of the SN where the medial lemniscus can be observed (~3.16–3.80 mm caudal to the level of the bregma), the SNpc and medial VTA were divided along the medial lemniscus.

Statistical analysis. All data are expressed as mean \pm SEM, excluding those of VGLUT1-positive nerve area, which are expressed as mean \pm SD. The two-tailed Student's *t* test (for two groups) was applied. A value of *p* < 0.05 denoted the presence of statistically significant difference.

Results

Abnormal distribution of α Syn protein at presynaptic terminals in the striatum of *Snap25*^{S187A/S187A} mice

Using immunohistochemistry, we investigated the distribution of endogenous α Syn in the striatum of *Snap25*^{S187A/S187A} mice,

which have unphosphorylatable Ala at position Ser¹⁸⁷ in the SNAP-25 protein. A significant decrease of neurotransmitter release was noted in serotonergic and dopaminergic systems in the amygdala, which was associated with behavioral abnormalities, including spontaneous convulsive seizures (Kataoka et al., 2011). Confocal microscopic analysis of the striatal sections of the *Snap25*^{S187A/S187A} mice showed altered distribution of endogenous α Syn, which resembled coarse granular deposits, at the age of 11 weeks, compared with age-matched wild-type control. These changes were prominent in 60-week-old (54-week-old or more was designated as aged, hereafter) *Snap25*^{S187A/S187A} mice (Fig. 1*A,B*), indicating an age-dependent progressive manner of redistribution.

Next, we examined the localization of α Syn in the presynaptic terminals by using anti-synaptophysin antibody. Aged *Snap25*^{S187A/S187A} mice showed similar alternation of the immunoreactivity for synaptophysin as observed for α Syn, compared with the wild-type (Fig. 1*C,D*). Moreover, α Syn-immunopositive granular structures were largely overlapped with synaptophysin. These results indicate that α Syn accumulates mainly in the presynaptic terminals in the striatum. We also analyzed the immunostaining pattern for Ser¹²⁹-phosphorylated α Syn (p- α Syn), which was found specifically in human α -synucleinopathies (Fujiiwara et al., 2002). Majority of α Syn-positive granular deposits were immunopositive for p- α Syn, which were detected at negligible amounts in wild-type control mice (Fig. 1*F,G*). These results indicate that the abnormal accumulation of α Syn in the striatum of *Snap25*^{S187A/S187A} mice represents in part the pathological changes in human DLB brains.

Abnormal accumulation of α Syn and p- α Syn proteins in the striatum of *Snap25*^{S187A/S187A} mice

Next, we measured α Syn and p- α Syn protein levels in the striatal tissues of aged *Snap25*^{S187A/S187A} mice. A significant decrease of SNAP-25 protein was found in *Snap25*^{S187A/S187A} mice (*p* < 0.0001) compared with age-matched wild-type mice, confirming our previous findings (Kataoka et al., 2011). Importantly, α Syn (~1.5-fold; *p* = 0.0047) and p- α Syn (~1.7-fold; *p* = 0.0080) protein levels were higher than age-matched wild-type mice (Fig. 2*A,B*). These results indicate that the abnormal distribution of α Syn and p- α Syn in the presynaptic terminals (Fig. 1*F*) was reflected by increased amount of these proteins. In addition, high levels of VAMP-2 (~1.3-fold; *p* = 0.0011), but neither syntaxin nor synaptophysin, were found in the striatum of aged *Snap25*^{S187A/S187A} mice (Fig. 2*A,B*), compared with whole-brain lysate prepared from young *Snap25*^{S187A/S187A} mice (Kataoka et al., 2011).

Ultrastructural analysis of α Syn distribution in presynaptic terminals

To investigate in detail the morphological changes in the presynaptic terminals, we used electron microscopy to analyze the striatum of the aged mice. In *Snap25*^{S187A/S187A} mice, condensed synaptic vesicles were abundantly present in large excitatory nerve terminals, compared with the normal uniform distribution of the vesicles in wild-type mice (Fig. 3*A,B*). These results suggest possible decrease of presynaptic neurotransmitter release in *Snap25*^{S187A/S187A} mice. Subsequent immunoelectron-microscopic examination of α Syn in the synapses showed predominant localization of α Syn proteins in the periaxial zone of enlarged excitatory presynaptic nerve terminals in the aged *Snap25*^{S187A/S187A} mice, compared with the wild type (Fig. 3*C,D*).

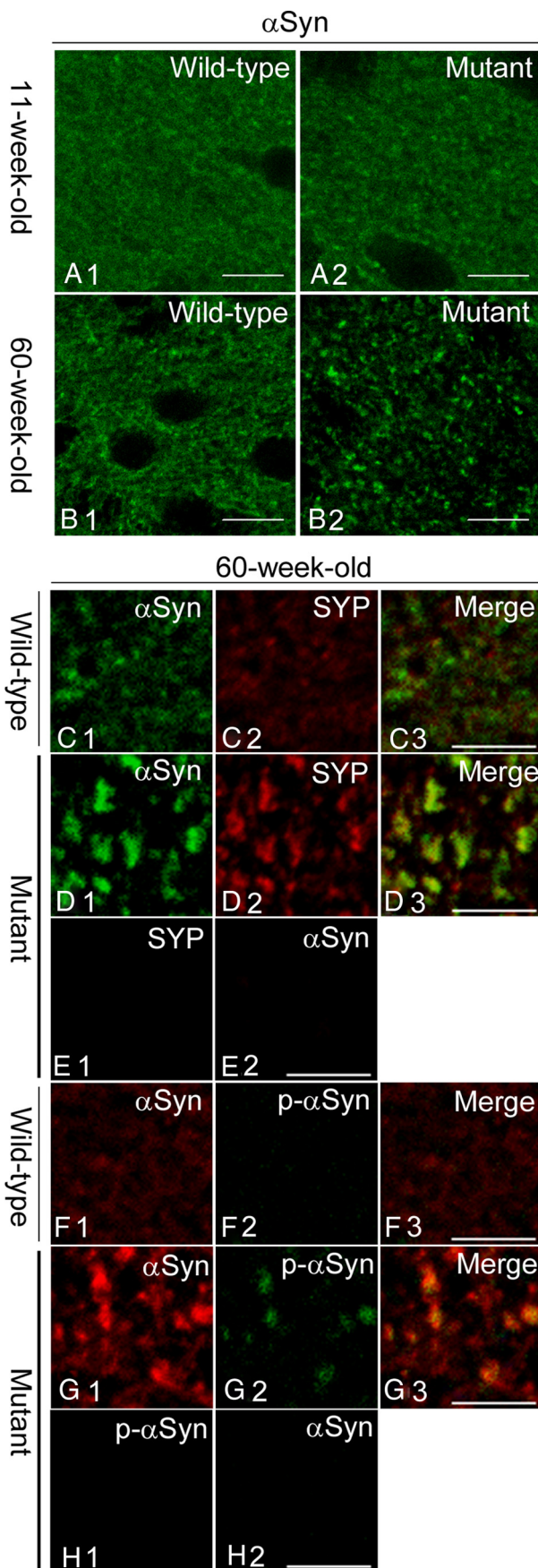


Figure 1. Altered localization of α Syn and its accumulation in presynaptic terminals in the striatum of *Snap25^{S187A/S187A}* mice. **A, B**, Striatal sections of wild-type and *Snap25^{S187A/S187A}*

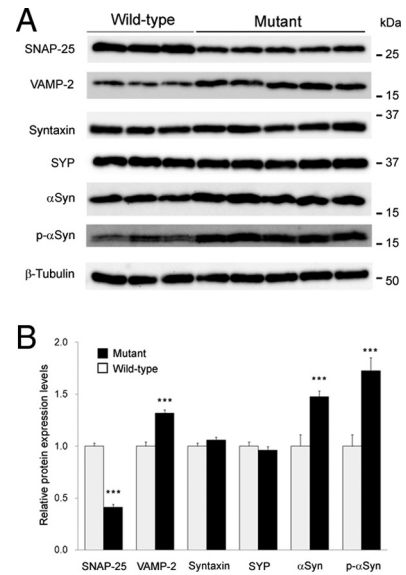


Figure 2. Increase in endogenous α Syn and p- α Syn in the striatum of *Snap25^{S187A/S187A}* mice. **A, B**, Western blotting was performed for synaptic marker proteins using the striatal tissues at postnatal week 61. **A**, Representative Western blotting images of two to five independent experiments, each involving three and five mice for wild-type and *Snap25^{S187A/S187A}* (mutant), respectively. Mutant mice showed increased band intensity of α Syn, p- α Syn, and VAMP-2 compared with the wild type, while that of SNAP-25 was decreased. **B**, Relative protein expression levels expressed relative to the loading control (β -tubulin). The band intensity was quantitated using the ImageJ 1.43 software and expressed as the relative protein expression level. Significant increases in α Syn, p- α Syn, and VAMP-2, and significant decrease in SNAP-25 were observed in mutant mice. No significant differences were noted in other synaptic protein markers. Data are mean \pm SEM of two to five independent experiments, each involving three and five mice for wild-type and mutant, respectively. *** $p < 0.01$ (two-tailed t test).

Decreased ability of SNARE complex assembly in *Snap25^{S187A/S187A}* mice

Given that *Snap25^{S187A/S187A}* mice clearly displayed the significant decrease in the SNAP-25 protein expression and abnormal distribution of the synaptic vesicle in the presynaptic terminals (Figs. 2, 3B), we evaluated the functional ability to produce the SNARE complex in *Snap25^{S187A/S187A}* mice. SNARE complex assembly was assessed by measuring the levels of the high-molecular-weight heat-sensitive SDS-resistant complex in Western blotting. As shown in Figure 4A, compared with boiled sample of the wild type, SNAP-25-immunoreactive bands were shifted to the higher molecular masses (~75 to ~250 kDa) in unboiled samples of wild-type and *Snap25^{S187A/S187A}* mice. However, the relative signal intensity of higher molecular masses normalized by β -tubulin (loading control) was significantly reduced in *Snap25^{S187A/S187A}* mice, compared with the wild-type mice ($p = 0.00463$) (Fig. 4B). These observations indi-

(mutant) mice at postnatal weeks 11 and 60 were immunostained for α Syn ($n = 3$ for each genotype). Compared with the wild-type mice (A₁, B₁), α Syn was localized and formed coarse granular deposits in mutant mice at postnatal weeks 11 and 60 (A₂, B₂). The severity of the change was more prominent at postnatal week 60. Scale bars, 10 μ m. C–H, Sections were coimmunostained for α Syn, and synaptophysin (SYP) or p- α Syn. Confocal images showed altered localization of SYP (D₂) and p- α Syn (G₂) and colocalization with α Syn-positive coarse granular deposits (D₁, G₁) in mutant mice (D₃, G₃). No cross-reactivity was observed for the sections of the mutant mice exposed to a single primary antibody followed by the treatment of both secondary antibodies were observed (E₁, E₂; H₁, H₂) [i.e., negligible green (E₁) and red fluorescence (E₂) for the sections treated with anti-SYP and α Syn antibody, respectively]. Scale bars, 5 μ m.

cated the decreased ability of the SNARE complex assembly in *Snap25*^{S187A/S187A} mice compared with the wild-type mice. This is considered to represent the functional deficit possibly attributable to the decreased level of the SNAP-25 and the insensitivity of protein kinase C-mediated phosphorylation at Ser¹⁸⁷ in *Snap25*^{S187A/S187A} mice.

α Syn accumulation and enlargement of corticostriatal nerve terminals

The striatal spiny interneurons receive excitatory outputs from cortical and thalamic tracts, and these projections use glutamate as the neurotransmitter (Gerfen and Surmeier, 2011). VGLUT1 and VGLUT2 are localized to the excitatory nerve terminals projecting from the cortical and the thalamic area, respectively, in the striatum. Then, we investigated the distribution of α Syn-immunopositive deposits with VGLUT1 or VGLUT2 to verify the abnormal accumulation of α Syn in the corticostriatal or the thalamostriatal neuronal terminals. As shown in Figure 5B₂, we found similar alteration of the immunoreactivity for VGLUT1 as observed for α Syn in the aged *Snap25*^{S187A/S187A} mice compared with wild-type normal ones (Fig. 5A₂). Importantly, such immunoreactivity for VGLUT1, but not for VGLUT2, was exclusively colocalized with α Syn (Fig. 5B₃,E₃). We also performed double immunostaining with anti-pSyn and anti-VGLUT1 antibodies and found the VGLUT1-immunopositive structures to be immunopositive for p- α Syn (Fig. 5G₃). These observations suggest accumulation of α Syn and p- α Syn mainly in the enlarged VGLUT1-positive nerve terminals.

Immunoelectron-microscopic examination showed massive enlargement of the VGLUT1-positive nerve terminals in the aged *Snap25*^{S187A/S187A} mice (Fig. 5I,J). Quantitative analysis of the size of the VGLUT1-positive nerve terminals based on the cumulative probability curve confirmed a significantly larger size in *Snap25*^{S187A/S187A} mice compared with wild-type mice (Fig. 5K). The size of the VGLUT1-positive nerve terminals reached a plateau at 0.8 μ m² in wild-type mice, while that of *Snap25*^{S187A/S187A} mice reached a plateau at 3.6 μ m². The size of ~40% of the VGLUT1-positive nerve terminals in the mutant mice was larger than the maximum size measured in wild-type mice. Specifically, the size of the VGLUT1-positive nerve terminals in the mutant mice was approximately fourfold larger than the wild type ($p = 0.0030$) (Fig. 5L).

No significant changes in nigrostriatal dopaminergic system

Finally, we examined the effect of SNARE dysfunction on the nigrostriatal dopaminergic system, which is affected in parkinsonian brains. The striatal sections of aged *Snap25*^{S187A/S187A} mice were co-

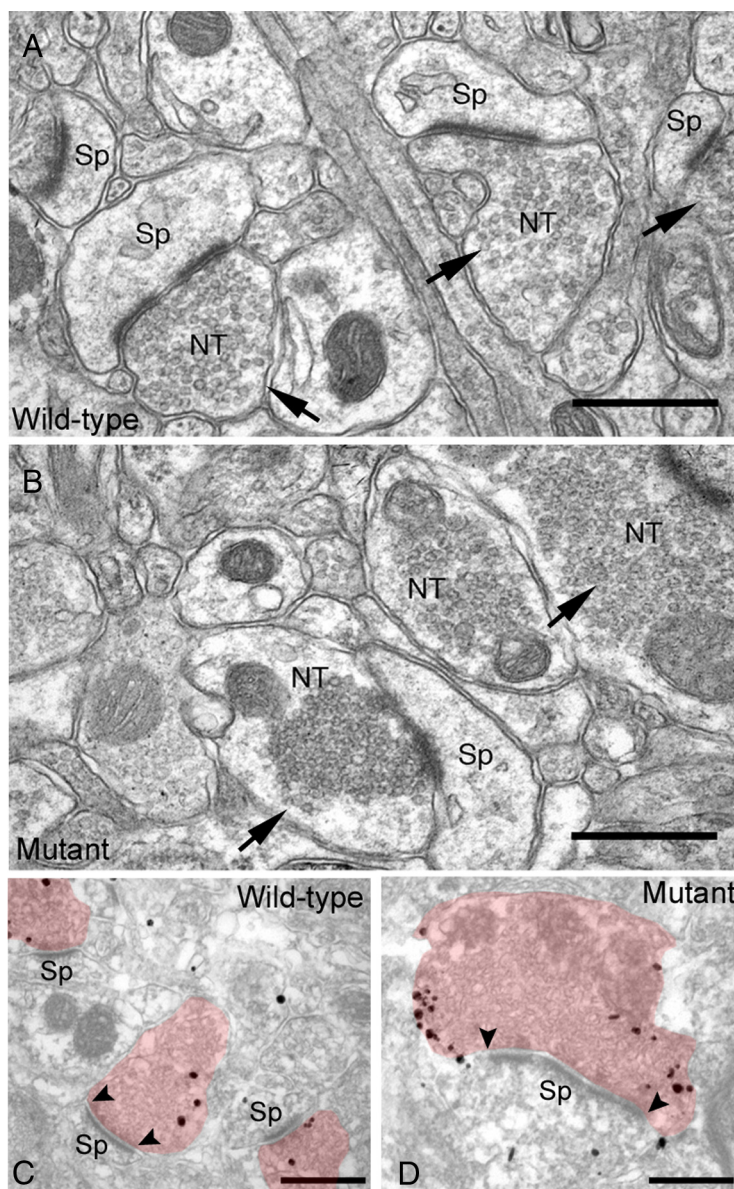


Figure 3. Abnormal excitatory nerve terminals in the striatum of *Snap25*^{S187A/S187A} (mutant) mice. Representative electron micrographs of wild-type and mutant mice at postnatal week 60 ($n = 3$ for each genotype). Synaptic vesicles were homogeneously distributed in the cytoplasm of the excitatory nerve terminals in wild-type mice (A), while they were tightly assembled in mutant mice (B). Note the alteration of subcellular localization of α -Syn (immunogold particles) and increased number of α Syn aggregates deposited in association with the cytoplasmic face of the plasma membrane at the periaxial zone of the enlarged nerve terminals in mutant mice (D), but not in wild-type mice (C). Arrows, excitatory nerve terminals; arrowheads, edges of active zone. NT, Nerve terminal; Sp, dendritic spine. Scale bars, 500 nm.

immunostained for α Syn and dopaminergic neuronal markers (TH or DAT). The α Syn-immunopositive deposits appeared adjacent to, but hardly colocalized with both DAT and TH in *Snap25*^{S187A/S187A} mice. The distribution patterns of DAT- and TH-immunoreactive deposits in the striatum were not significantly different between wild-type and mutant mice (Fig. 6A–D). These results imply that the nigrostriatal dopaminergic neurons are not affected in *Snap25*^{S187A/S187A} mice.

To clarify this point further, we conducted immunoelectron-microscopic examination of DAT-positive dopaminergic nerve terminals. There were no significant differences in any structural alternations including condensation of synaptic vesicles (Fig. 6E,F). Furthermore, we found no significant differences in the subcellular localization of α Syn protein between the aged wild-type and *Snap25*^{S187A/S187A} mice (Fig. 6G,H).

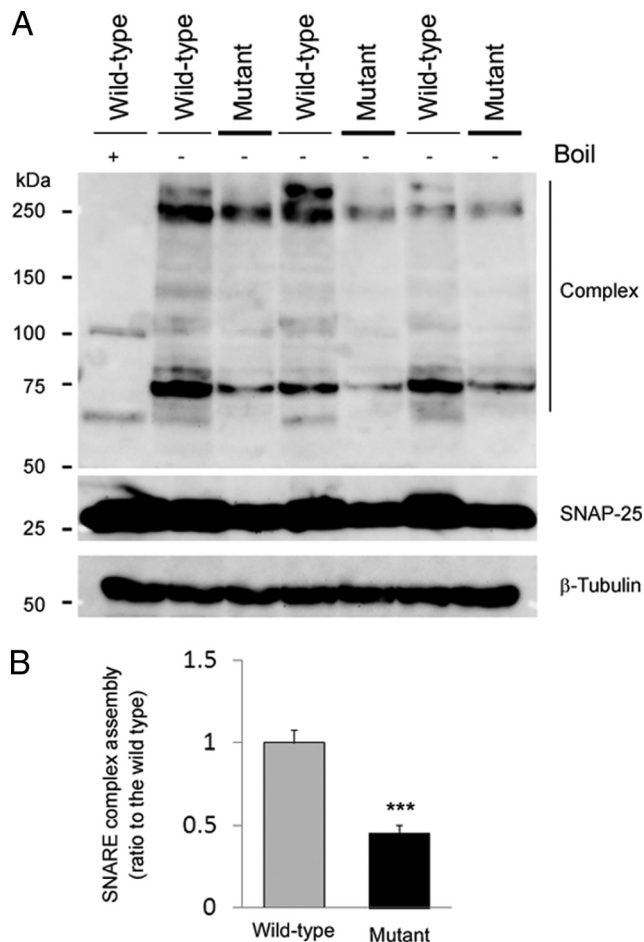


Figure 4. Decreased ability of the SNARE complex assembly in *Snap25^{S187A/S187A}* (mutant) mice. **A, B**, Western blotting was performed for unboiled or boiled samples of wild-type and mutant mice (at postnatal week 61) before electrophoresis followed by the immunoblotting using SNAP-25ct antibody. **A**, Representative Western blotting image of two to three independent experiments was provided. Heat-sensitive SDS-resistant SNAP-25ct-immunoreactive bands were detected at high molecular mass (~75 to ~250 kDa) in wild-type and mutant mice, indicating the presence of the SNARE complex. However, band intensity of the mutant mice, especially at ~75 kDa, was decreased compared with the wild-type mice. **B**, Relative levels of the SNARE complex assembly relative to the loading control (β -tubulin). The band intensity was quantitated using the ImageJ 1.43 software and expressed as the relative protein expression level. Significant decrease in SNARE complex assembly was observed in mutant mice compared with the wild-type mice. Data are mean \pm SEM of two to three independent experiments, each involving three mice for each genotype. *** $p < 0.01$ (two-tailed t test).

We also investigated the functional preservation of the nigrostriatal dopaminergic neurons in the aged *Snap25^{S187A/S187A}* mice. No significant difference was found in the number of TH- and Nissl-double-positive cells in the SNpc between wild-type and *Snap25^{S187A/S187A}* mice (Fig. 6I, J). There were also no significant differences in the levels of TH protein in the midbrain tissue and DA and its metabolites, HVA and DOPAC, in the striatum between the aged wild-type and *Snap25^{S187A/S187A}* mice (Fig. 6K–M). These results suggest that SNARE dysfunction does not seem to be involved in the initiation of PD-related pathologic changes in nigrostriatal dopaminergic neurons.

Discussion

In this study, we found that SNARE dysfunction leads to presynaptic accumulation of endogenous α Syn, a process that probably represents the initial pathological event in DLB. Previous studies using neural preparations showed that the neurotransmitter release is regulated by protein kinase C, which phosphorylates

Ser¹⁸⁷ residue in SNAP-25, augmenting exocytosis of synaptic vesicles (Majewski and Iannazzo, 1998; Morgan et al., 2005). Patch-clamp analysis of chromaffin cells that overexpress the S187A form of SNAP-25 inhibited the rate of refilling of presynaptic vesicle pool (Nagy et al., 2002). Recently, we reported that *Snap25^{S187A/S187A}* mice show reduced DA and serotonin release in amygdala (Kataoka et al., 2011). In human DLB brains, >90% of α Syn aggregates are located in the presynaptic terminals in the form of small deposits (Neumann et al., 2002; Kramer and Schulz-Schaeffer, 2007; Schulz-Schaeffer, 2010). This is consistent with the present findings of abnormal accumulation of α Syn in presynapses, suggesting that this process is the initial pathological event in DLB, eventually leading to the death and degeneration of neuronal cells (Orimo et al., 2008). Another finding that lends support to the role of α Syn aggregates in the presynaptic terminals in DLB was the lack of histopathological changes in the dopaminergic terminals in the present study.

In experiments on glutamate release conducted in hippocampal slices prepared from α Syn knock-out mice (Gureviciene et al., 2007), paired-pulse facilitation was significantly weaker, and high-frequency-induced long-term potentiation and frequency facilitation were not observed. These findings suggest that α Syn contributes to mobilization of glutamate-containing vesicles from the reserve pool (Gureviciene et al., 2007). Thus, α Syn may act as a positive regulator of neurotransmitter release at presynaptic terminals. Therefore, presynaptic accumulation of α Syn observed in *Snap25^{S187A/S187A}* mice might reflect a compensatory response to a possible SNARE dysfunction-related chronic shortage of neurotransmitter release in the VGLUT1-positive nerve terminals.

In the striatum, the medium spiny neurons, which constitute >90% of all striatal neurons, receive output from glutamatergic axons that contact the spine head and dopaminergic axons that synapse with the dendritic spine neck. DA released from dopaminergic axons regulates the release of glutamate via D₂-like receptors on the corticostriatal nerve terminals (Bamford et al., 2004; Wickens and Arbuthnott, 2005). In the present study, we found no significant changes in the striatal tissue levels of DA and its metabolites. These findings confirmed the results reported in our previous study using *Snap25^{S187A/S187A}* mice, whereas microdialysis analysis study revealed marked reduction of DA release from the amygdala (not measured in other brain regions) (Kataoka et al., 2011). In addition, in another *in vitro* study using PC12 cells, phosphorylation of SNAP-25 at S187 potentiated calcium-dependent DA release and recruitment of synaptic vesicles containing DA (Shimazaki et al., 1996; Iwasaki et al., 2000; Shoji-Kasai et al., 2002). These observations suggest decreased striatal DA release in *Snap25^{S187A/S187A}* mice, resulting in increased demand for neurotransmitter release at glutamatergic nerve terminals. Thus, presynaptic accumulation of α Syn might reflect a possible compensatory response to low DA inhibitory control over cortical glutamatergic drive.

Increased expression of VAMP-2 protein accompanied increased α Syn expression in the striatum of *Snap25^{S187A/S187A}* mice. Binding of the C terminus of α Syn to the N terminus of VAMP-2 primes the subsequent SNARE complex assembly (Burgoyne and Morgan, 2011). Therefore, the increased VAMP-2 level might also reflect a compensatory response to the impaired synaptic vesicle release by enhancing SNARE complex formation in concert with the increased α Syn.

Presynaptic neurotransmitter release is mediated by the synaptic vesicle cycle, consisting of exocytosis followed by en-

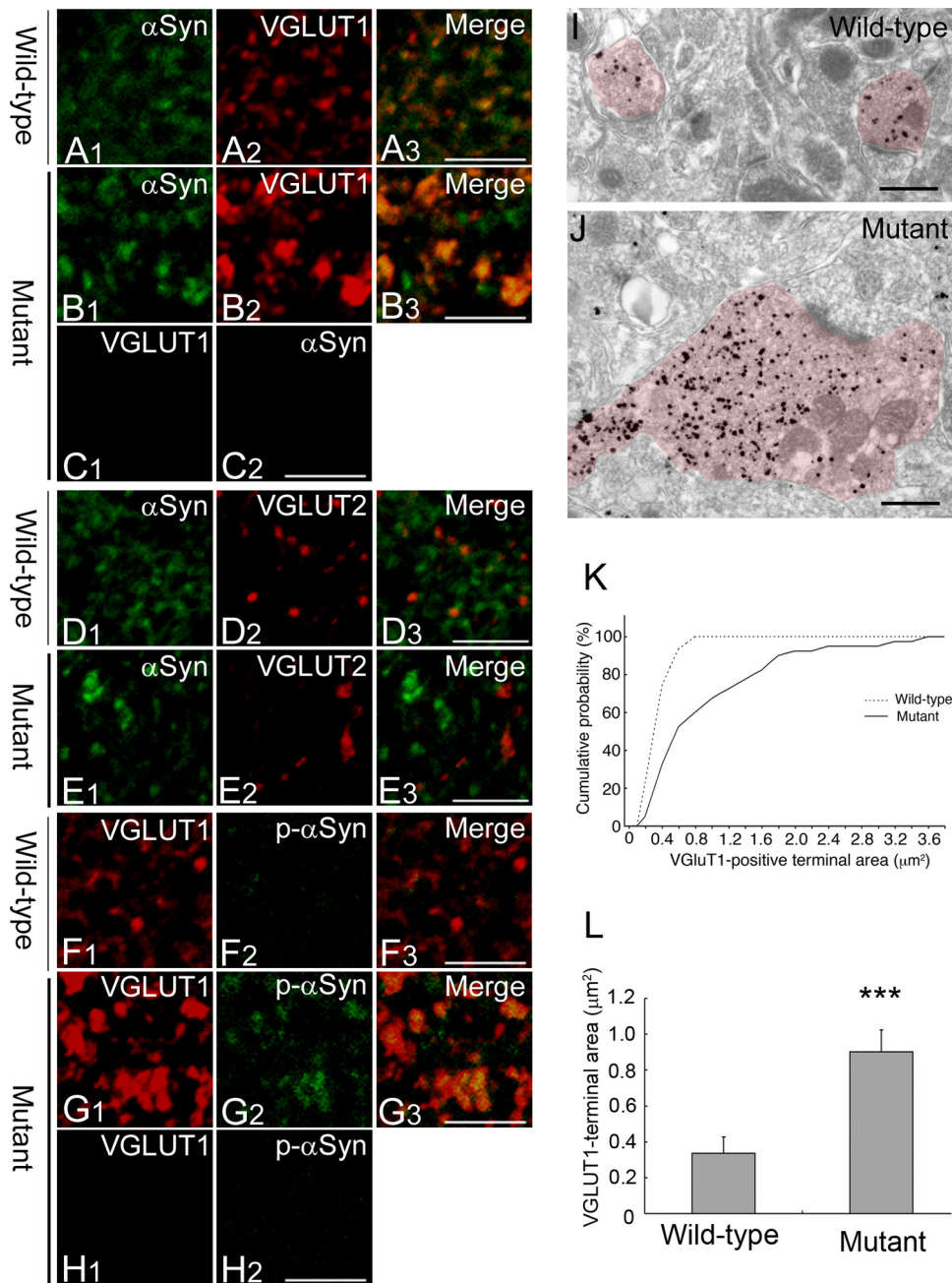


Figure 5. Accumulation of α Syn in the hypertrophied VGLUT1-positive nerve terminals. **A–E**, Striatal sections of wild-type and *Snap25^{S187A/S187A}* (mutant) mice at postnatal week 60 were coimmunostained for α Syn (green) and VGLUT1 or VGLUT2 (red) ($n = 3$ for each genotype). Confocal images showed altered localization of VGLUT1 with more granular appearance in mutant mice (**B₂**) compared with wild-type mice (**A₂**). Immunopositive structures for VGLUT1, but not for VGLUT2, marked colocalization with α Syn-positive granular deposits (**B₃**, **E₃**). No cross-reactivity was observed for the sections of the mutant mice treated with a single antibody followed by both the secondary antibodies, as evidenced by negligible green (**C₁**) and red fluorescence (**C₂**) for the sections treated with anti-VGLUT1 and α Syn antibody, respectively. Scale bars, 5 μm . **F–H**, Sections were coimmunostained for VGLUT1 (red) and p- α Syn (green) ($n = 3$ for each genotype). Confocal images showed marked colocalization of VGLUT1 and p- α Syn in mutant mice (**G₃**). Similarly, no cross-reactivity was observed as evidenced by negligible red (**H₁**) and green fluorescence (**H₂**) for the sections treated with anti-VGLUT1 and α Syn antibody, respectively. Scale bars, 5 μm . **I, J**, Representative electron micrographs showing VGLUT1-positive nerve terminals (labeled with immunogold particles). Note the hypertrophy of VGLUT1-positive nerve terminals in mutant mice (**J**), but not in wild-type mice (**I**). Scale bars, 500 nm. **K, L**, Quantitative analysis of VGLUT1-positive nerve terminal area in three mice per group. The size was measured in 100 nerve terminals per animal. Cumulative probability (**K**) and group mean value (**L**) were calculated and showed a larger nerve terminal area in mutant mice than the wild type. Data are mean \pm SD. *** $p < 0.01$ (two-tailed t test).

docytosis and recycling. Exocytosis incorporates synaptic vesicles into the presynaptic terminal membranes and increases the surface area, while endocytosis retrieves the excess plasma membrane components followed by recycling to form other synaptic vesicles. Under normal conditions, the dynamics of the balance between exocytosis and endocytosis is well preserved to maintain the correct surface area of the presynaptic

terminal (Hayes and Baines, 1996; Haucke et al., 2011). However, excessive accumulation of presynaptic vesicles and enlargement of the VGLUT1-positive nerve terminals was observed in *Snap25^{S187A/S187A}* mice. Taking into consideration the synaptic vesicle cycle, our findings suggest that the balance of the cycle was biased toward decreased exocytosis concomitant with possible decreased endocytosis.

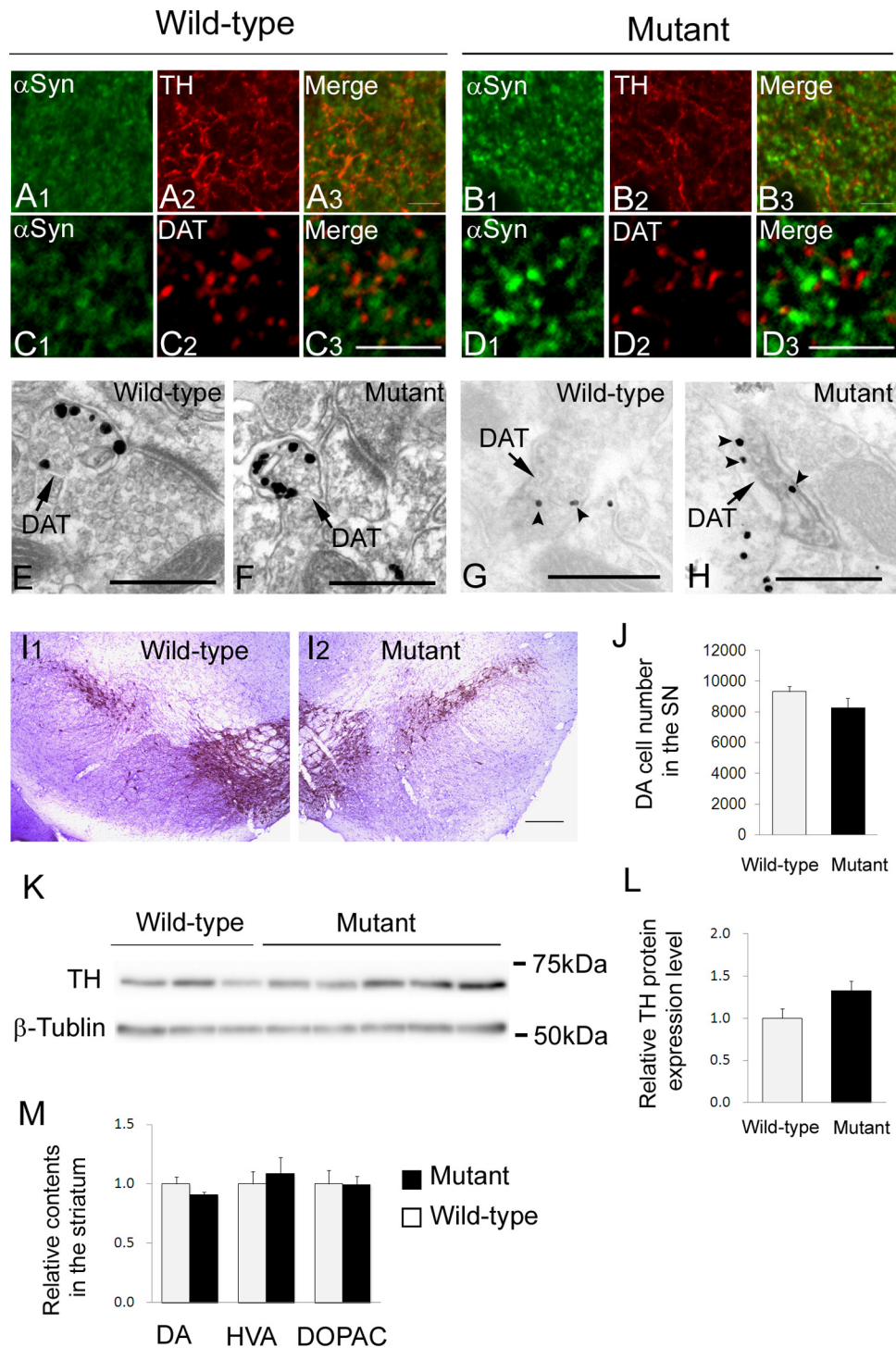


Figure 6. Lack of neurodegenerative changes in nigrostriatal dopaminergic neurons. **A–D**, Striatal sections of wild-type and *Snap25^{S187A/S187A}* (mutant) mice at postnatal week 60 were coimmunostained for α -Syn (green) and TH or DAT (red) ($n = 3$ for each genotype). There were no significant differences in the distribution pattern of TH (**A₂, B₂**) and DAT (**C₂, D₂**) between wild-type and mutant mice. α -Syn-positive granular deposits (**B₁, D₁**) were rarely colocalized with TH or DAT (**B₃, D₃**). Scale bars, 5 μ m. **E–H**, Representative electron micrographs of dopaminergic nerve terminals at postnatal week 60 ($n = 3$ for each genotype). They were identified by silver particles (**E, F**) or DAB labeling (**G, H**) for DAT (arrows). There were no significant differences in subcellular localization of synaptic vesicles (**E, F**) or α -Syn (arrowheads, silver particles) (**G, H**) between wild-type and mutant mice. Scale bars, 500 nm. **I, J**, Representative micrographs of the substantia nigra of wild-type and mutant mice at postnatal week 60 immunostained for TH and counterstained with cresyl violet. Scale bars: **I₁, I₂**, 200 μ m. Number of DA cell bodies counted in three mice of each genotype. Data are mean \pm SEM (**J**). **K, L**, Western blotting was performed using substantia nigral tissues at postnatal week 61. Representative images of Western blotting from two independent experiments, each involving three and five mice of wild-type and mutant mice, respectively (**K**). Relative TH protein expression levels (relative to the expression of β -tubulin) were expressed relative to the value of wild-type mice (**L**). Data are mean \pm SEM of two independent experiments, each involving three and five mice of wild-type and mutant, respectively. **M**, Quantification of DA, HVA, and DOPAC levels in the striatum of mice at postnatal week 54. Data were expressed relative to those of wild-type mice and represent the mean \pm SEM of duplicate measurement of one experiment involving four mice of each genotype. There were no significant differences between wild-type and mutant mice (**J, L, M**) (two-tailed *t* test).

The enlarged VGLUT1-positive nerve terminals of *Snap25^{S187A/S187A}* mice showed concomitant accumulation of α Syn and p- α Syn. Kramer and Schulz-Schaeffer (2007) have previously reported that 90% or even more of α Syn aggregates in DLB cases were located at the presynapses in the form of very small deposits. In parallel, dendritic spines were retracted, whereas the presynapses were relatively preserved, suggesting that a neurotransmitter deprivation may explain the cognitive impairment in DLB (Kramer and Schulz-Schaeffer, 2007; Schulz-Schaeffer, 2010). While the presynaptic aggregates did not contain much p- α Syn in their examination (Kramer and Schulz-Schaeffer, 2007; Schulz-Schaeffer, 2010), widespread varicosities and dot-like structures containing p- α Syn are commonly observed in mouse model and human DLB brains (Saito et al., 2003; Scott et al., 2010). This may represent axonal transport defects and presynaptic dysfunctions (Saito et al., 2003; Scott et al., 2010). Recent study showed that mutant α Syn (A53T) diminished levels of various motor proteins in neurons (Chung et al., 2009), supporting this scenario. Alternatively, excessive amounts of misfolded α Syn and p- α Syn may aggregate at synapses, physically preventing the targeting of other presynaptic proteins (Kramer and Schulz-Schaeffer, 2007). In experiments using *Caenorhabditis elegans* overexpressing the human α Syn, four genes related to the endocytosis process were identified as genetic modifiers for α Syn toxicity (Kuwahara et al., 2008). They included the two subunits of the adaptor protein (AP) complex 2, which interacts with clathrin and promotes presynaptic clathrin-mediated vesicle recycling (Morgan et al., 2000). Furthermore, proteomics analysis revealed that p- α Syn also preferentially interacted with the proteins involved in endocytosis, including clathrin heavy chain and subunit of AP-2 and AP-1 complexes, over the nonphosphorylated α Syn (McFarland et al., 2008). Clathrin-mediated recycling of exocytosed synaptic vesicles occurs in the periaxonal zone, a region adjacent to the active zone where synaptic vesicle is endocytosed (Hauke et al., 2011). Similarly, in *Snap25^{S187A/S187A}* mice, immunoelectron microscopy showed preferential localization of α Syn at the periaxonal zone of the excitatory presynaptic nerve terminals (Fig. 3D). This might reflect the interaction of α Syn and p- α Syn with the proteins involved in clathrin-mediated endocytosis. Taking these findings together, presynaptic accumulation of α Syn and p- α Syn could disturb the endocytosis process and consequently contribute to the development of VGLUT1-positive terminal enlargement.

Presynaptic accumulation of α Syn is considered an early event in the pathogenesis of α -synucleinopathies (Neumann et al., 2002; Kramer and Schulz-Schaeffer, 2007; Schulz-Schaeffer, 2010). Mice overexpressing human α Syn showed presynaptic accumulation of α Syn and low DA release in the striatum. These findings were associated with abnormal distribution of SNARE proteins, which colocalized with α Syn aggregates. Similarly, accumulation of SNARE proteins and α Syn were reported in the striatum of PD patients (Garcia-Reitböck et al., 2010). These observations suggest that SNARE dysfunction likely occurs at an early stage of pathogenesis in nigrostriatal dysfunction observed in PD. By considering the findings observed in the VGLUT1-positive nerve terminals, we expected that SNARE dysfunction might have induced presynaptic accumulation of α Syn, which consequently results in the development of neurodegenerative changes in the nigrostriatal system. However, contrary to our expectation, *Snap25^{S187A/S187A}* mice showed no significant neurodegenerative changes in nigrostriatal dopaminergic neurons,

suggesting that SNARE dysfunction alone was insufficient to cause nigrostriatal degeneration as observed in PD, and appeared to be a downstream event associated with abnormal accumulation of α Syn.

In conclusion, the present study demonstrated that SNARE dysfunction leads to accumulation of endogenous α Syn in the corticostriatal nerve terminals. Presynaptic accumulation of α Syn is considered to be a key early event in the pathogenesis of α -synucleinopathies. Although the “prion-like” propagation hypothesis of α Syn, including tau and TAR DNA-binding protein 43 kDa, is currently receiving considerable attention worldwide, our findings provide an insight to our understanding of the possible mechanisms that lead to presynaptic accumulation of endogenous α Syn. Moreover, given that SNAP-25 is reduced in the striatum of MSA brains (Tong et al., 2010), we speculate that a discontinuous pattern of α Syn pathologies usually found in MSA [i.e., glial cytoplasmic inclusions in the putamen oligodendrocytes, and neuronal cytoplasmic inclusions and neuronal nuclear inclusions in the cortex (Yoshida, 2007; Ubhi et al., 2011)] might be potentially linked through the presynaptic accumulation of α Syn in the corticostriatal neurons. Further investigations on the *Snap25* mutant mice with genetic ablation of α Syn would contribute to understanding the essential role of redistributed α Syn and should be a central issue in the following studies.

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