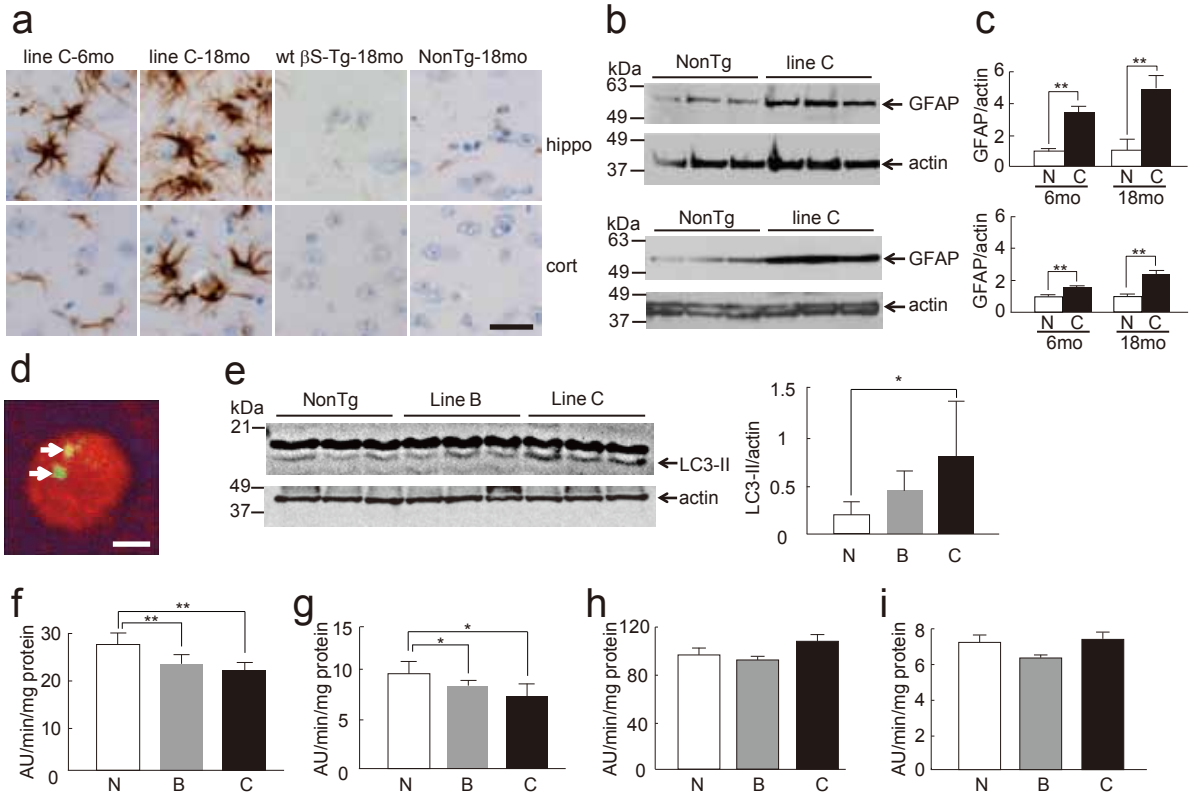
A β-synuclein mutation linked to dementia produces neurodegeneration when expressed in mouse brain

Masayo Fujita[1], Shuei Sugama[1], Kazunari Sekiyama[1], Akio Sekigawa[1], Tohru Tsukui[2], Masaaki Nakai[1], Masaaki Waragai[1], Takato Takenouchi[1,3], Yoshiki Takamatsu[1], Jianshe Wei[1], Edward Rockenstein[4], Albert R. LaSpada[5-9], Eliezer Masliah[4], Satoshi Inoue[2,10], Makoto Hashimoto[1]

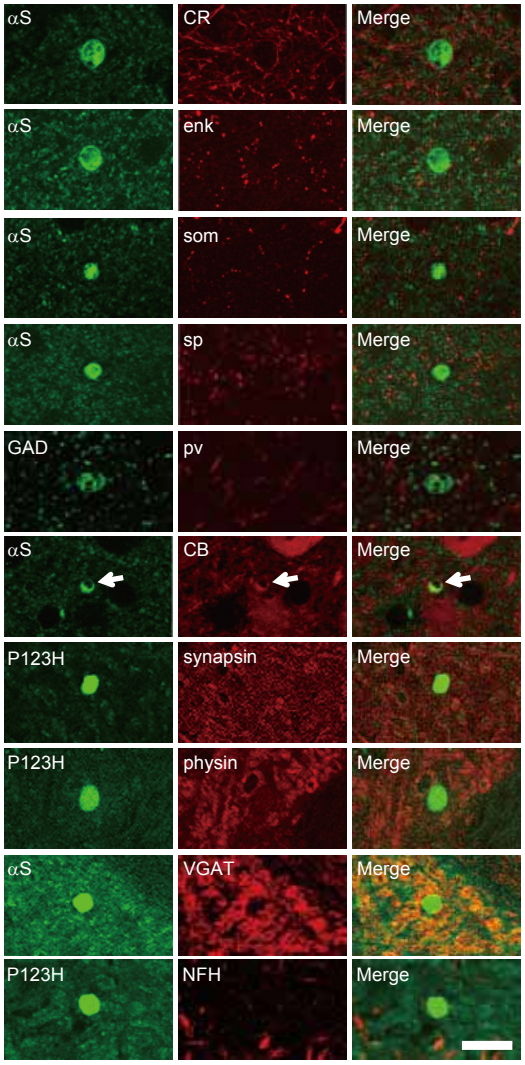
The Supplementary information contains:

- Supplementary Figures (S1, S2, S3, S4, S5, S6, S7)
- Supplementary Tables (S1, S2)
- Supplementary Methods
- Supplementary References



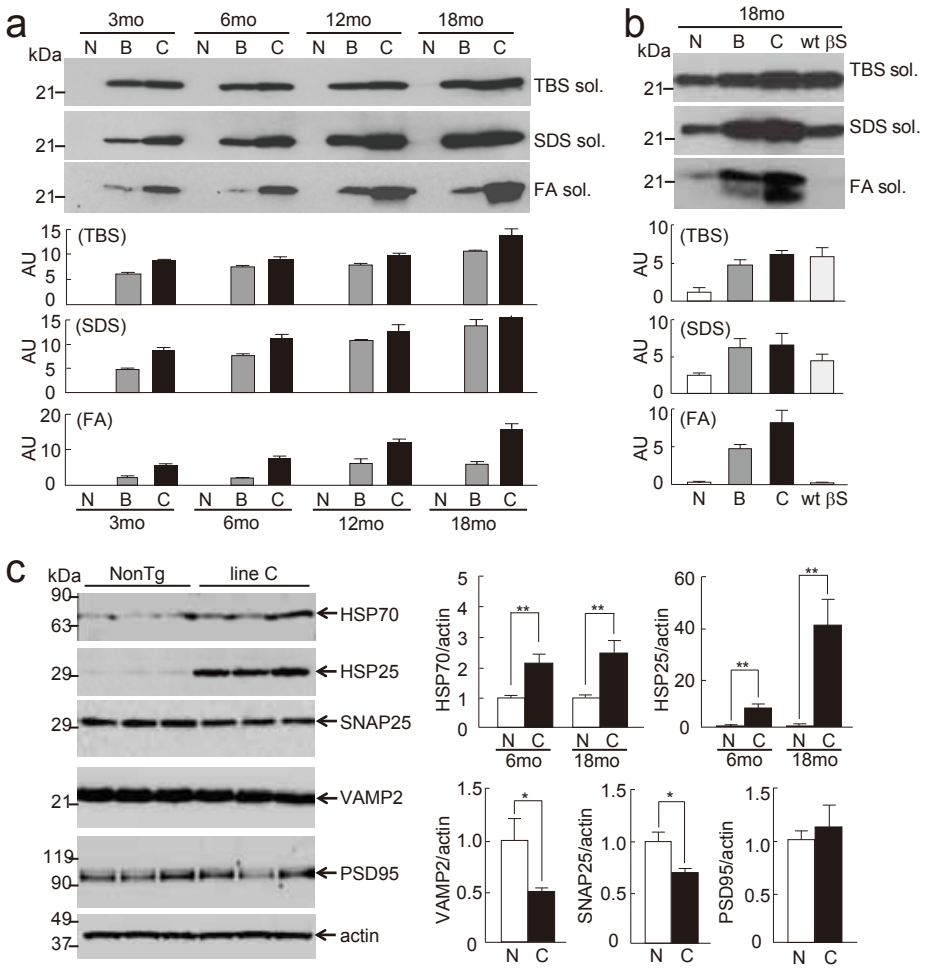
Supplementary Figure S1. Characterization of astrogliosis and lysosomal dysfunction of P123H βS tg mice.

(a) Immunohistochemistry of GFAP shows many activated astrocytes in the hippocampus (hippo) and cortex (cort) of P123H βS tg mice (line C) at 6 and 18 mo, but not in the same area of both wt βS tg mice (wt βS-Tg) and non-tg littermates (NonTg) at 18 mo. Scale bar=10 μm. (b, c) Immunoblot analysis of GFAP for 1% triton-insoluble fractions of brain extracts prepared from hippocampus and cortex of P123H βS tg mice (line C) and non-tg littermates (NonTg). In b, representative blots for the hippocampus extracts of mice at 6 mo (upper) and 18 mo (lower) are shown. In c, quantification of the band intensities of GFAP versus actin was performed for the hippocampus (upper) and cortex (lower) extracts of mice (line C: C, NonTg: N) at 6 mo and 18 mo. Data are shown as the mean ± SEM (n=8). **: $p<0.01$. (d, e) Evaluation of LC3 immunoreactivity. In d, P123H βS-immunopositive (red) globules in the basal ganglia of 18 mo P123H βS tg mice (line C) were double-stained with anti-LC3 antibody (green). Arrows indicate LC3 positive dots. Scale bar = 2 μm. In e, immunoblot analysis shows that LC3-II expression in the basal ganglia of P123H βS tg mice (line C) at 18 mo was higher than those in non-tg littermates. A representative blot and quantification of the band intensities of LC3-II versus actin are shown (line B: B, line C: C, NonTg: N). Data are shown as the mean ± SEM (n=6). *: $p<0.05$. (f-i) Lysosomal activities are decreased in P123H βS tg mice. Cathepsin and proteasome activities were measured as described in Supplementary Methods. Activities of cathepsin B (f) and D (g) in the striatum of P123H βS tg mice (line B: B, line C: C) at 18 mo were significantly lower than those in non-tg littermates (N), whereas no significant difference of proteasome activities, including peptidylglutamyl-peptide hydrolyzing activity (h) or chymotrypsin-like activity (i), were observed between two groups. Data are shown as the mean ± SEM (n=5-7). *: $p<0.05$, **: $p<0.01$.

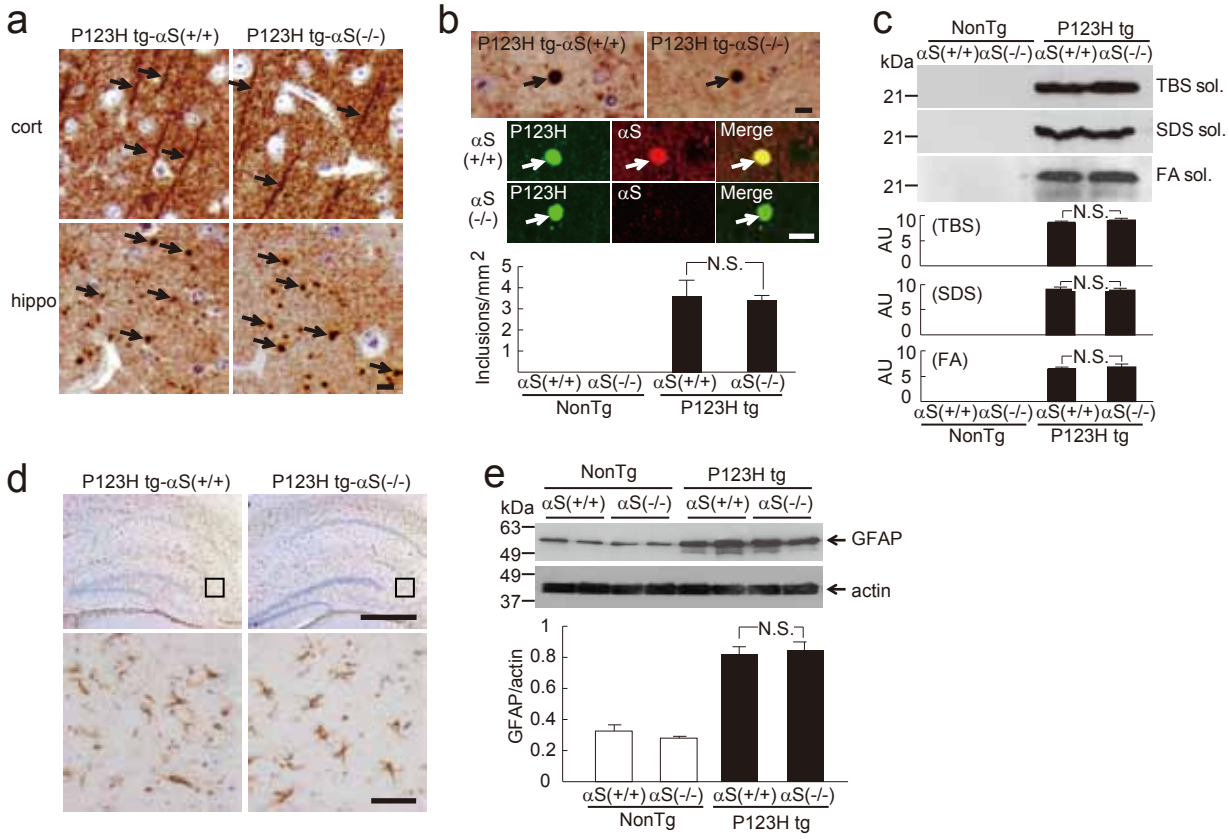


Supplementary Figure S2. Double immunofluoresence analysis of globules in P123H βS tg mice.

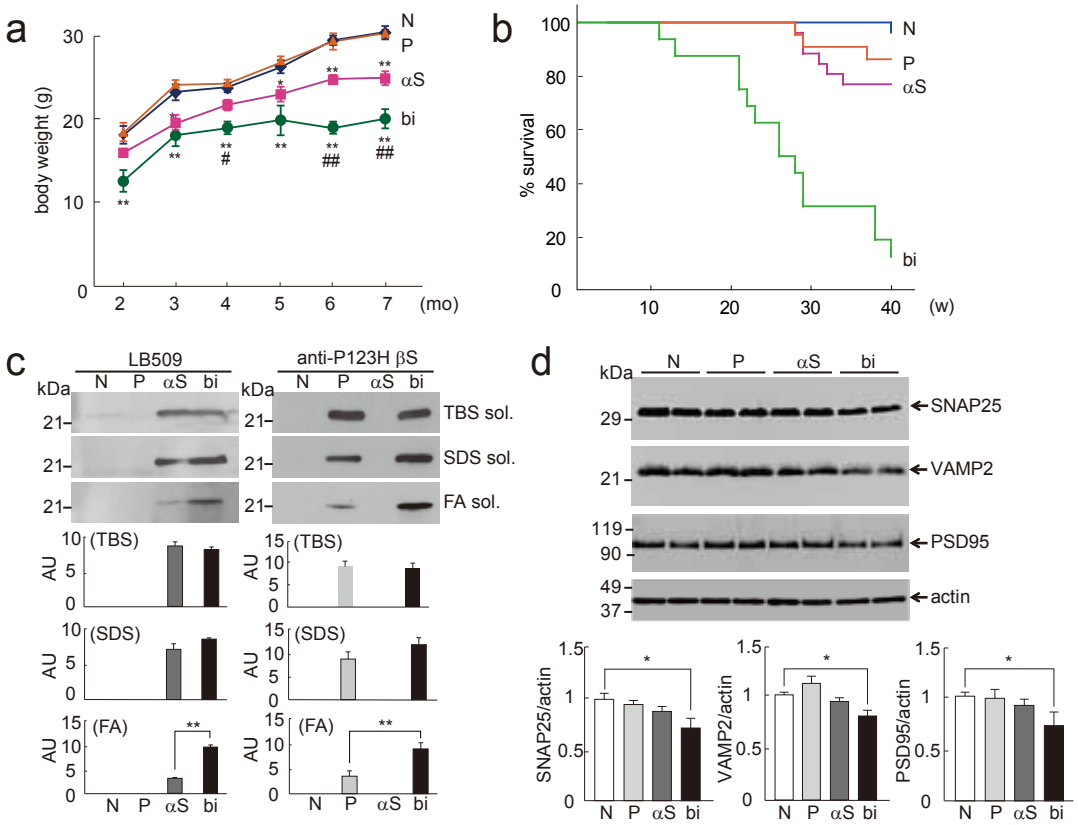
P123H βS-immunopositive globules in the basal ganglia of 18 mo P123H βS tg mice (line C) were consistently immunopositive for αS and GAD (~100%) (Fig. 2e). Double immunofluoresence was performed using P123H βS, αS, or GAD as a positive control. Vesicular GABA/glycine transporter (VGAT) was negative, but immunoreactivity for calbindin (CB) was weakly but consistently observed (arrows), suggesting that the globules were derived from projection type GABAergic neurons. Further results showed that calretinin (CR), enkephalin (enk), somatostatin (som), substance-P (sp), and parvalbumin (pv) were negative. Neither synaptic markers, such as synaptophysin (physin) and synapsin I (synapsin), nor axonal markers, including neurofilaments (NFH), were detectable. Scale bar=10 μm.



Supplementary Figure S3. Biochemical analysis of neurodegeneration in P123H βS tg mice. (a, b) Age-dependent accumulation of P123H βS. In a, whole brain homogenates from mice (line B: B, line C: C, and non-tg littermates: N) at different ages (3, 6, 12 and 18 mo) were extracted into TBS SDS and FA fractions as described in Supplementary Methods. A representative immunoblot using anti-P123H βS is shown (upper panels) and the quantified band intensities of P123H βS (lower panel) are displayed in arbitrary units (AU). Data are shown as the mean ± SEM (n=5). In b, whole brain homogenates prepared from P123H βS tg mice (line B: B, line C: C), wt βS tg mice (wt βS), and age-matched non-tg mice (N) at 18 mo were sequentially extracted into TBS, SDS and FA fractions. Each fraction was analyzed by immunoblot with anti-pan-βS antibody (upper panels). The quantified band intensities of βS are displayed in arbitrary units (AU) (lower panels). Data are shown as the mean ± SEM (n=5). (c) Immunoblot analysis of HSPs (HSP70 and HSP25) and synaptic proteins (VAMP2, SNAP25 and PSD95). Brain extracts were prepared from the cortex of P123H βS tg mice (line C) and non-tg littermates at 6 and 18 mo. Representative blots at 18 mo are shown (left panels). The band intensities of both HSPs and synaptic proteins were quantified against the actin bands (line C: C, NonTg: N) (right panels). Data are shown as the mean ± SEM (n=8). *: $p<0.05$, **: $p<0.01$.

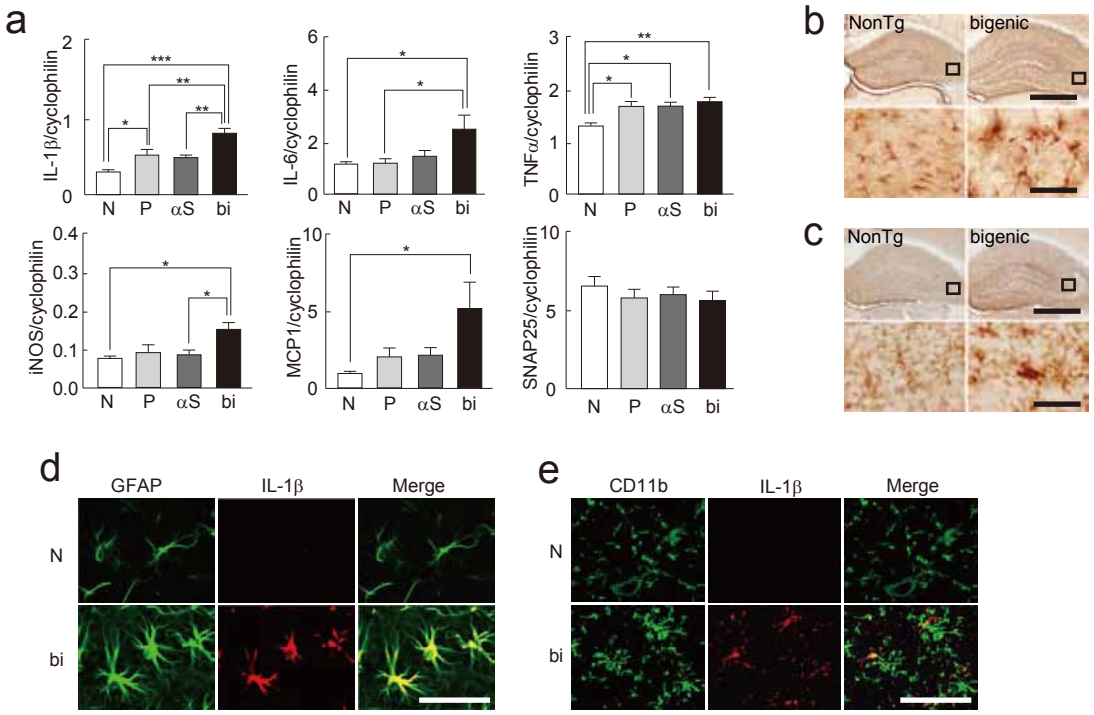


Supplementary Figure S4. Cross-breeding of P123H βS tg mice with αS KO mice.
A cross-breeding of P123H βS tg mice with αS KO mice was conducted as described in Supplementary Methods. Histological and biochemical analyses were performed to compare various neuropathological features between P123H βS tg-αS(-/-) and P123H βS tg-αS(+/+) mice. (a) Immunohistochemistry of P123H βS shows P123H βS accumulated in apical dendrites in the cortex (cort) and axonal dots in the hippocampus (hippo) were detected to similar extents between P123H βS tg-αS(-/-) and P123H βS tg-αS(+/+) mice (6 mo) (arrows). Scale bar=10 μm. (b) Immunohistochemistry and immunofluoresence studies for the evaluation of globule formation in basal ganglia shows P123H βS-positive/αS-negative inclusions in P123H βS tg-αS(-/-) mice (12 mo) and P123H βS-positive/αS-positive inclusions in P123H βS tg-αS(+/+) mice. Scale bar=10 μm. A histogram showing the number of inclusions is shown. Data are shown as the mean ± SEM (n=4). N.S.: not significant. (c) Immunoblot analysis of P123H βS shows immunoreactivities of P123H βS in all the brain fractions (TBS, SDS and FA fractions) did not differ significantly between P123H βS tg-αS(-/-) and P123H βS tg-αS(+/+) mice. Representative blots are shown (upper) and quantitative data (lower) are displayed in arbitrary units (AU) (mean ± SEM, n=4). N.S.: not significant. (d) Immunohistochemistry of GFAP. Similar levels of activated GFAP-positive astrocytes were observed in the hippocampus of P123H βS tg-αS(-/-) and P123H βS tg-αS(+/+) mice (6 mo). The lower panels are the insets from the upper panels. Scale bars=200 μm (upper) and 20 μm (lower). (e) Immunoblot analysis of GFAP shows immunoreactivity of GFAP did not differ significantly between P123H βS tg-αS(-/-) and P123H βS tg-αS(+/+) mice at 6 mo. A representative blot (upper) and quantification of the band intensities of GFAP against actin (lower) are shown. Data are shown as the mean ± SEM (n=4). N.S.: not significant.

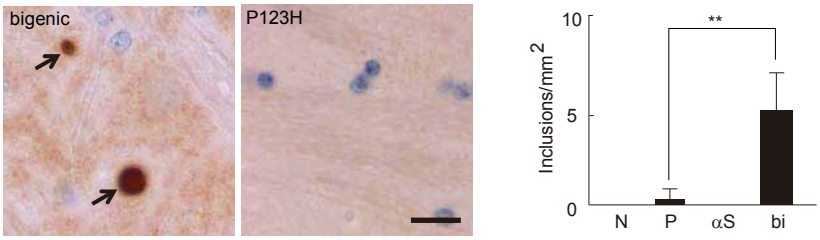


Supplementary Figure S5. Characterization of various neurodegenerative features in bigenic mice overexpressing αS and P123H βS.

(a, b) Body weights and survival of bigenic (bi) mice and littermates (αS tg: αS, P123H βS tg: P, and non-tg: N) were analyzed. In a, bigenic mice had lower body weights compared to αS tg mice. αS tg mice but not P123H βS tg mice had lower body weights compared to non-tg littermates. Data are shown as the mean ± SEM (n=18~24). *: p<0.05, **: p<0.01 versus non-tg mice. #:p<0.05, ##:p<0.01 versus αS tg mice. In b, Kaplan-Meier survival curve analysis was completed using a log rank test (n=16~28). Bigenic mice showed lower survival (median lifespan of 27 weeks) compared to littermates. p<0.001. (c) Immunoblot analyses of human αS and P123H βS. Brain homogenates from bigenic (bi) mice and littermates at 7 mo were sequentially extracted into TBS, SDS and FA fractions and analyzed using anti-human αS specific LB509 (left) or anti-P123H βS antibody (right). Representative blots are shown (upper) and quantitative data (lower) are displayed in arbitrary units (AU) (mean ± SEM, n=8). **: p<0.01. (d) Decreased expression of synaptic proteins in bigenic mice. Homogenates of cortex were prepared from bigenic (bi) mice and littermates at 7 mo, and analyzed by immunoblot using anti-VAMP2, anti-SNAP25 and anti-PSD95 antibodies. Representative blots (upper) and data quantified against actin bands (lower) are shown (mean ± SEM, n=8). *: p<0.05.



Supplementary Figure S6. Enhanced neuroinflammation in bigenic mice.
Neuroinflammation conditions were analyzed for bigenic (bi) mice and littermates (αS tg: αS, P123H βS tg: P, and non-tg: N) at 6 mo. (a) Quantitative-PCR (qPCR) evaluation of neuroinflammation markers, including IL-1β, IL-6, iNOS, TNFα, MCP-1 and SNAP25. Total RNA was prepared from hippocampal regions of the brains derived from bigenic mice and littermates. qPCR was performed as described in Supplementary Method. Data are shown as the mean ± SEM (n=5-7). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. (b, c) Immunohistochemistry of GFAP (b) and CD11b (c) for hippocampus sections. Representative images of bigenic mice and non-tg (NonTg) littermates are shown. Scale bars=500 μm (for upper panels) and 50 μm (for lower panels). The lower panels are the insets from the upper panels. (d, e) Double immunofluorescence of IL-1β (red) with either GFAP (green) (d) or CD11b (green) (e) for hippocampus sections. Scale bar=50 μm.



Supplementary Figure S7. Globule formation in basal ganglia is accelerated in bigenic mice. Formation of P123H βS-immunopositive inclusions were observed in bigenic mice at 7 mo but not in littermates. Representative figures are shown with a histogram showing the number of inclusions in bigenic mice (bi) and littermates (αS tg: αS, P123H βS tg: P, and non-tg: N). Data are shown as the mean ± SEM (n=8). **: p<0.01. Note that globule formation was significantly accelerated in bigenic mice compared to P123H βS tg mice. Scale bar=10 μm.

Primer	Accession no.	Sequence	Nucleotide position	Product size
genotyping for wt−βS Tg and βsyn(P123H)Tg	—	F: 5'-TGCCCTACCAGCTGGCTGAC-3' R: 5'-GCCCTTCATGAACACGTCCAT-3'	— —	484 bp
genotyping for αS Tg	—	F: 5'-GACGGGTGTGACAGCAGTAGCC-3' R: 5-GATGATGGCATGCAGCACTGG-3'	— —	349 bp
genotyping for αS wild	—	F: 5'-GGCGACGTGAAGGAGCCAGGGA-3' R: 5'-CAGCGAAAGGAAAGCCGAGTGATGTACT-3'	— —	320 bp
genotyping for αS KO	—	F: 5'-CTTGGGTGGAGAGGCTATTC-3' R: 5'-AGGTGAGATGACAGGAGATC-3'	— —	280 bp
mouse IL-1β	NM_008361.3	F: 5'-CACCTCACAAGCAGAGCACAA-3' R: 5'-AGTCCAGCCCATACTTTAGGAAGAC-3'	788-808 867-891	104 bp
mouse IL-6	NM_031168.1	F: 5'-ACAACCACGGCCTTCCCTACT-3' F: 5'-CACGATTTCCCAGAGAACATG-3'	92-112 200-220	129 bp
mouse iNOS	NM_010927.3	F: 5'-CAGCTGGGCTGTACAAACCTT-3' F: 5'-CATTGGAAGTGAAGCGTTTCG-3'	2175-2195 2249-2269	95 bp
mouse TNFα	NM_013693.2	F: 5'-GACCCTCACACTCAGATCATCTTCT-3' F: 5'-CCTCCACTTGGTGGTTTGCT-3'	384-408 444-463	80 bp
mouse MCP1	NM_011333.3	F: 5'-GTGCTGACCCCAAGAAGGAA-3' F: 5'-TGCTGAAGACCTTAGGGCAGAT-3'	309-328 403-424	116 bp
mouse SNAP25	NM_011428.3	F: 5'-TGAGGAAGGGATGGACCAAA-3' F: 5'-TGTTACAGGGACACACACAAAGC-3'	393-412 471-493	101 bp
mouse CD11b	NM_001082960.1	F: 5'-CATCAACACAACCAGAGTGGATTC-3' F: 5'-GCTCCTAAAACCAAGCTTTGGA-3'	1270-1293 1350-1371	102 bp
mouse cyclophilin	NM_008907.1	F: 5'-TCCATGGCAAATGCTGGAC-3' F: 5'-GTCTTGCCATTCCTGGACCC-3'	336-354 477-496	161 bp

Supplementary Table S1. Primers for genotyping and qPCR analysis of neuroinflammation. Primers designed using Primer Express 1.0 software (Applied Biosystems, Foster City, CA) are listed .

Antigen	Maker	Code	IHC	IB
α synuclein	BD biosciences (San Jose, CA)	610787	1:1000	1:5000
β synuclen	BD biosciences (San Jose, CA)	612508	1:2000	1:5000
Tyrosine hydroxilase	BD biosciences (San Jose, CA)	612301	1:5000	1:10000
α synuclein (LB509)	Invitrogen (Carlsbad, CA)	18-0215	—	1:5000
GFAP	Progen (Heidelberg, Germany)	65011	1:50	1:500
GAD	Biomol International (Philadelphia, PA)	GC3008	1:1000	1:5000
GABA	Sigma (Saint Louis, MO)	A2052	1:2000	—
Actin	Sigma (Saint Louis, MO)	AC-15	—	1:10000
HSP70	Cell signaling (Danvers, MA)	4872	—	1:5000
Phospho-c-jun (Ser73)	Cell signaling (Danvers, MA)	9164	1:300	—
PSD95	Cell signaling (Danvers, MA)	3409	—	1:5000
Ubiquitin	Millipore (Billerica, MA)	MAB1510	1:200	—
Synaptophysin	Millipore (Billerica, MA)	MAB5258	1:1000	—
Dopamine transporter	Millipore (Billerica, MA)	MAB369	—	1:5000
NeuN	Millipore (Billerica, MA)	MAB377	1:1000	—
SNAP25	Synaptic Systems (Gotingen, Germany)	111111	—	1:5000
VAMP2	Synaptic Systems (Gotingen, Germany)	104211	—	1:5000
VGAT	Synaptic Systems (Gotingen, Germany)	131004	1:1000	—
Synapsin	Synaptic Systems (Gotingen, Germany)	106001	1:200	—
c-Fos	Santa Cruz (Santa Cruz, CA)	sc-52	1:10000	—
LC3	Novus Biologicals (Littleton, CO)	NB600-1384SS	1:1000	1:5000
Dopa decarbxylase	Aviva Systems Biology (San Diego, CA)	ARP41425_T100	—	1:5000
HSP25	Stressgen (Ann Arbor, MI)	SPA-801	—	1:5000
CD11b	AbD Serotec (Oxford, UK)	MCA711	1:500	—
IL-1β	Thermo Scientific (Rockford, IL)	PR427B	1:1000	—
Dynorphin A	Peninsula Laboratories Inc. (San Carlos, CA)	T-4280	1:2000	—
Parvalbumin	Chemicon (Temecula, CA)	MAB1572	1:2000	—
Calretinin	Swant (Bellinzona, Switzerland)	7699/4	1:2000	—
Calbindin D-28K	Swant (Bellinzona, Switzerland)	Calb	1:5000	—
Neurofilament-H	Covance (Berkeley, CA)	SMR-32R	1:1000	—

Supplementary Table S2. Antibodies for immunohistochemistry, immunofluorescence and immunoblot.

An antibody against P123H βS was custom-made at Sigma-Aldrich, Japan KK, Tokyo Japan. The antibody was generated by immunizing New Zealand white rabbits with the Keyhole limpet hemocyanin-conjugated synthetic peptide (NH-Cys-Glu-Asp-Pro-His-Gln-Glu-COOH). The affinity-purified anti-P123H βS antibody was used for immunohistochemistry (IHC) at 1:50 dilution and immunoblot (IB) analysis 1:500 dilution, respectively. This antibody was not cross-reactive with mouse endogenous wt βS. Anti-somatostatin antibody (IHC, 1:1000), anti-met-enkephalin (IHC, 1:1000) and anti-substance P (IHC, 1:1000) were kind gifts from Dr. Shigeo Daikoku (University of Tokushima)[48], Dr. Shigeru Kobayashi (Shizuoka College of Pharmacy)[49] and Dr. Yoshihiro Tsuruo (University of Tokushima)[50], respectively. Information of other primary antibodies used in the study is described in the list.

Supplementary Methods

Preparation of the fractionated brain extracts for immunoblot analysis of P123H βS and αS proteins

Whole brain samples were homogenized in TBS and centrifuged at 100,000 × g for 30 min. The supernatants were collected as the TBS fraction. The pellets were extracted in 1% SDS and centrifuged at 100,000 × g for 30 min. The supernatants were collected as the SDS fraction. The pellets were re-extracted using 70% FA and centrifuged at 15,000 rpm. The supernatants were evaporated with speed vac, dissolved in SDS sample buffer, and used as the FA fraction. Protein concentrations of the TBS fraction were determined BioRad Protein Assay reagent (BioRad, Hercules, CA). Ten μg of the TBS fraction and the corresponding volume of SDS and FA fractions were used for immunoblot analysis.

Measurement of cathepsin and proteasome activity

Cathepsin B and D activities were measured as previously described[38] with some modifications. Briefly, mice striatum and cortex samples were collected, sonicated in extraction buffer (50 mM HEPES (pH 6.0), 10 mM EDTA, 10 mM NaCl) and centrifuged at 100,000 × g for 30 min. Then, ten μg of the supernatants were incubated in the extraction buffer containing either Z-RR-AMC fluorogenic cathepsin B substrate (40 μM) or Bz-RGFFP-4-MeObNA cathpsin D substrate (40 μM) (Chemicon). For measurement of proteasome activities, 10 μg of the supernatants were incubated in the extraction buffer (50 mM HEPES (pH 7.4), 10 mM EDTA, 10 mM NaCl) containing either Z-LLE-AMC fluorogenic substrate (40 μM) (Chemicon) for peptidylglutamyl-peptide hydrolyzing activity or Z-VKM-AMC substrate (40 μM) (Chemicon) for chymotrypsin-like activity. These enzyme activities were assayed by continuous recording of the fluorescence activity released from fluorogenic substrate using Berthold Mithras LB940 microplate reader (Berthold, Bad Wildbad, Germany) for 1 h at 37 ºC (excitation 380 nm, emission 460 nm for cathepsin B and proteasome and excitation 345 nm, emission 425 nm for cathepsin D) and the reaction rate was analyzed. These enzyme activities were described as arbitrary unit/min/mg protein.

Cross-breeding experiment of P123H βS tg mice with αS KO mice.

αS KO mice were primarily purchased from Jackson laboratory (Bar Harbor, ME)[51,52] and were maintained by successive (more than 7 generations) backcrossing into the C57BL/6 strain. A cross-breeding experiment of P123H βS tg mice with αS KO mice was conducted as previously described[53] with modifications. Briefly, P123H βS tg mice (line C, heterozygote) were first crossed with these αS KO mice. Among their littermates, P123H βS tg/αS heterozygote mice and αS heterozygote mice were used for further cross-breeding. Among the resulting littermates, P123H βS tg/αS KO mice: P123H βS tg-αS(-/-), P123H βS tg mice: P123H βS tg-αS(+/+), αS KO mice: NonTg-αS(-/-), and control mice: NonTg-αS(+/+) were subjected to histological and biochemical analyses. After extraction of genomic DNA from tail biopsies, genotyping of P123H βS and murine endogenous αS was performed by PCR using specific primers (Supplementary Table S1).

qPCR evaluation of neuroinflammation markers

Total RNA was prepared from hippocampal regions of the brains derived from bigenic mice and littermates. cDNA was synthesized from 2.5 μg of total RNA using a Superscript III First-Strand Synthesis System (Invitrogen). qPCR was performed using 2× master mix composed of SYBR Green PCR core reagents (Roche) and 30 nM primers in an ABI Prism 7700 system (Applied Biosystems) using the following sequence: 2 min at 50^{o}C, 10 min at 95^{o}C, 40 cycles of 15 s at 95^{o}C, and 1 min at 60^{o}C. The mRNA expression level of each gene was normalized to that of cyclophilin. The primers used for the evaluation of neuroinflammation are described in Supplementary Table S1.

Supplementary References

48. Daikoku, S., Hisano, S., Kawano, H., Okamura, Y. & Tsuruo, Y. Ontogenetic studies on the topographical heterogeneity of somatostatin-containing neurons in rat hypothalamus. *Cell Tissue Res.* **233**, 347-354 (1983).
49. Kobayashi, S. *et al.* Immunocytochemical demonstration of the co-storage of noradrenaline with Met-enkephalin-Arg6-Phe7 and Met-enkephalin-Arg6-Gly7-Leu8 in the carotid body chief cells of the dog. *Arch. Histol. Jpn.* **46**, 713-722 (1983).
50. Tsuruo, Y., Kawano, H., Nishiyama, T., Hisano, S. & Daikoku, S. Substance P-like immunoreactive neurons in the tuberoinfundibular area of rat hypothalamus. Light and electron microscopy. *Brain Res.* **289**, 1-9 (1983).
51. Abeliovich, A. *et al.* Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron* **25**, 239-252 (2000).
52. Nakai, M. *et al.* Expression of alpha-synuclein, a presynaptic protein implicated in Parkinson's disease, in erythropoietic lineage. *Biochem. Biophys. Res. Commun.* **358**, 104-110 (2007).
53. Chandra, S., Gallardo, G., Fernandez-Chacon, R., Schluter, O.M. & Sudhof, T.C. Alpha-synuclein cooperates with CSPalpha in preventing neurodegeneration. *Cell* **123**, 383-396 (2005).