Inflammation drives synucleinopathy propagation

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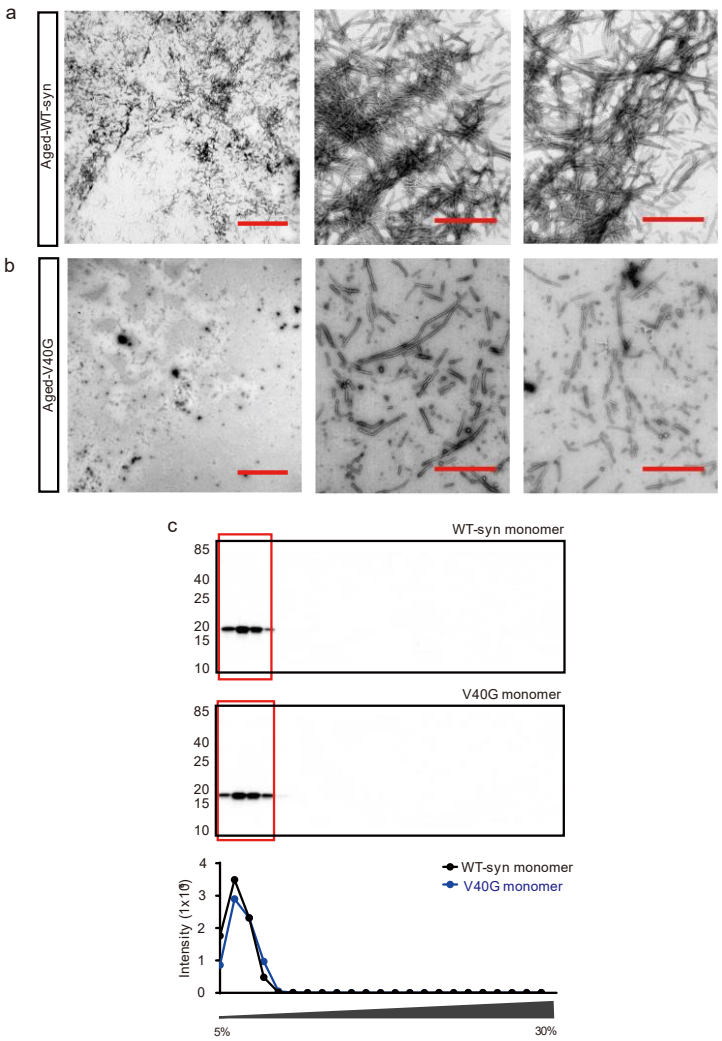
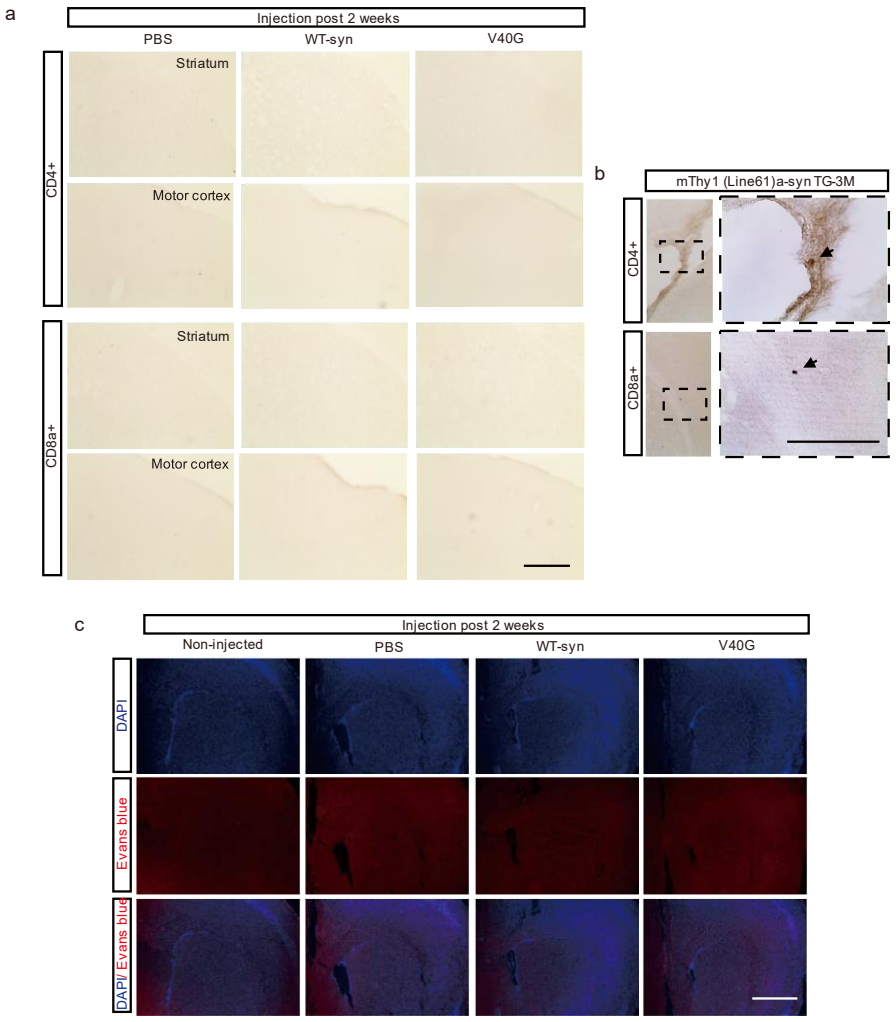
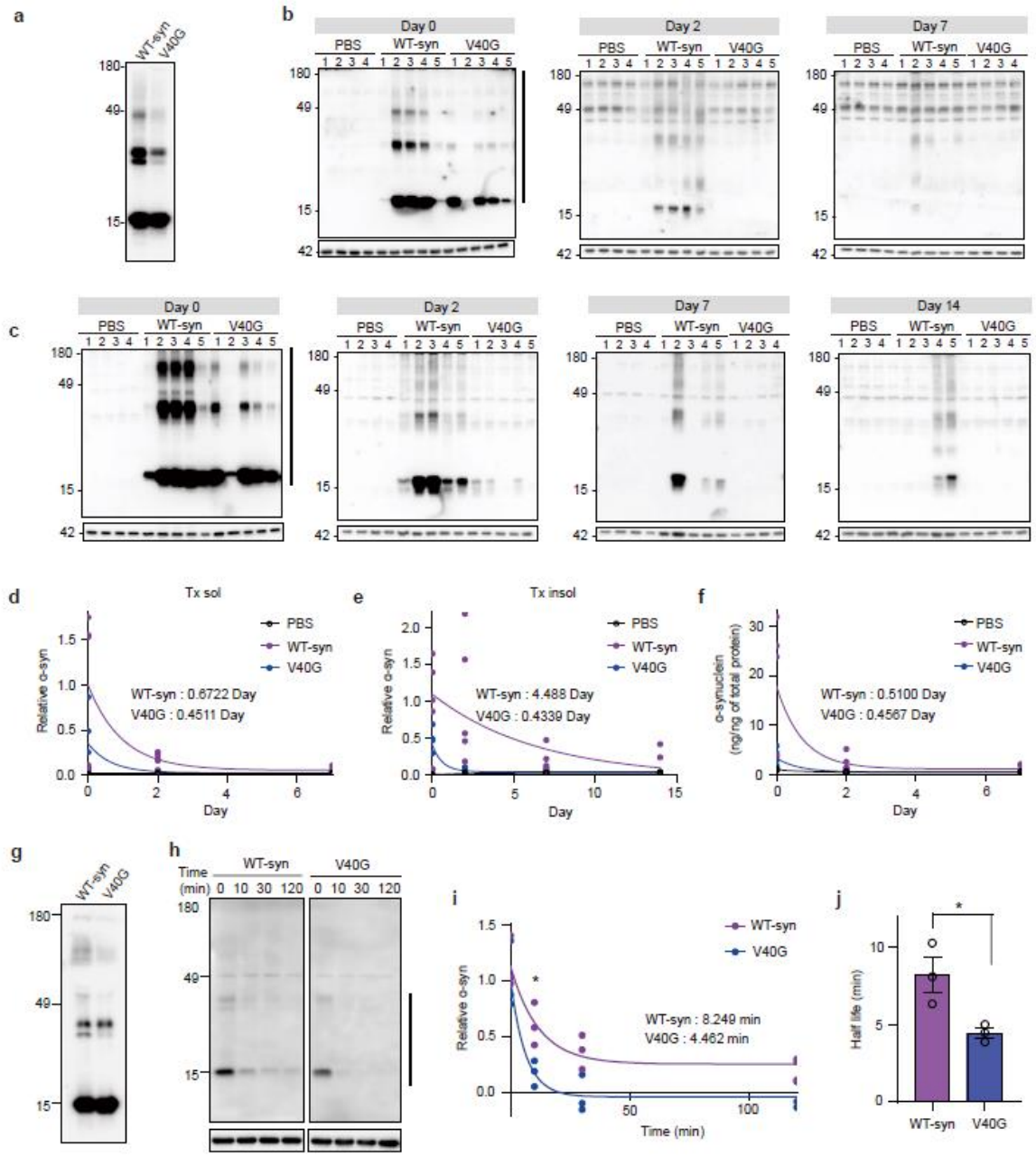
Supplementary information. Supplementary Figures, legends, and Supplementary tables



Supplementary Fig. 1 Characterization of WT-syn and V40G. a, b TEM image of aged WT-syn (**a**) and aged V40G (**b**). Scale bar, low magnification, 5 μm; high magnification, 0.8 μm. **c** Velocity ultracentrifugation of WT-syn and V40G monomers. Western blotting of fractions (top). Quantification of western results (bottom).

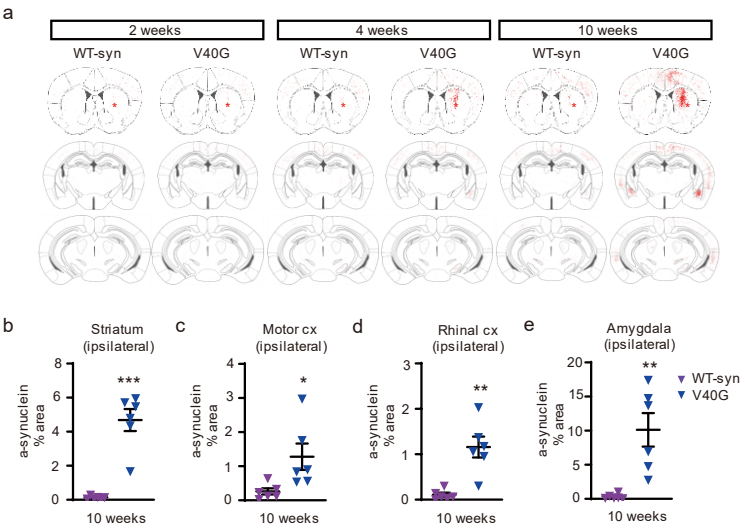


Supplementary Fig. 2 Assessment of BBB impairment after intrastriatal injections. a Representative images of the striatal regions 2 weeks after injection. Sections were stained with CD4 and CD8a antibodies. Scale bar, 200 μm. **b** Representative images of 3-month-old mThy1 α-synuclein transgenic mice stained with CD4 and CD8a antibodies. Scale bar, 200 μm. **c** Representative images of the striatal regions 2 weeks after injection stained with Evans blue (marker for extravasated albumin) and DAPI. Scale bar, 1 mm.

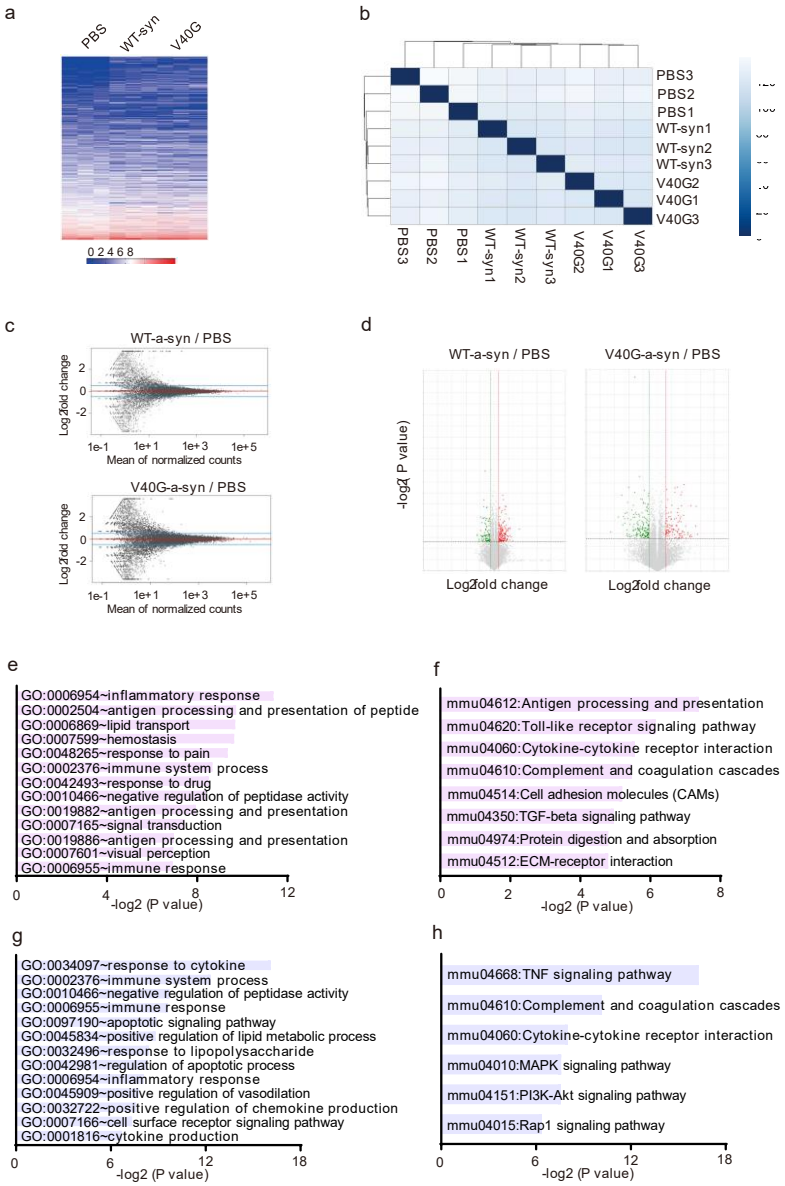


Supplementary Fig. 3 Stability of aged WT-syn and V40G. a Western blotting of the injectants. **b, d** Western blotting and clearance kinetics of aged WT-syn and V40G in the Tx-sol fractions. **c, e** Western blotting and clearance kinetics of aged WT-syn and V40G in the Tx-insol fractions. **f** Degradation kinetics of aged WT-syn and V40G syn in Tx-sol fraction by ELISA. **g-j** Mouse primary microglia cells were treated with 200 nM of aged WT-syn or V40G. After a 30-min incubation, the cells were washed and chased for degradation kinetics of the imported proteins. **g** Western blotting of aged WT-syn and V40G used in the microglia

experiments. **h, i** Degradation kinetics of internalized aged WT-syn and V40G in the Tx-sol fractions. Quantified region was indicated on the right as a bar in (**h**). Relative levels of α-synuclein in (**h**) were quantified in (**i**). n=3. Significance was assessed by two-way ANOVA with Bonferroni's post hoc test, two-sided. **j** Half-life of internalized α-synuclein. n=3. Significance was assessed by a two-tailed paired t-test, $*P<0.05$. Data are expressed as the mean ± SEM.

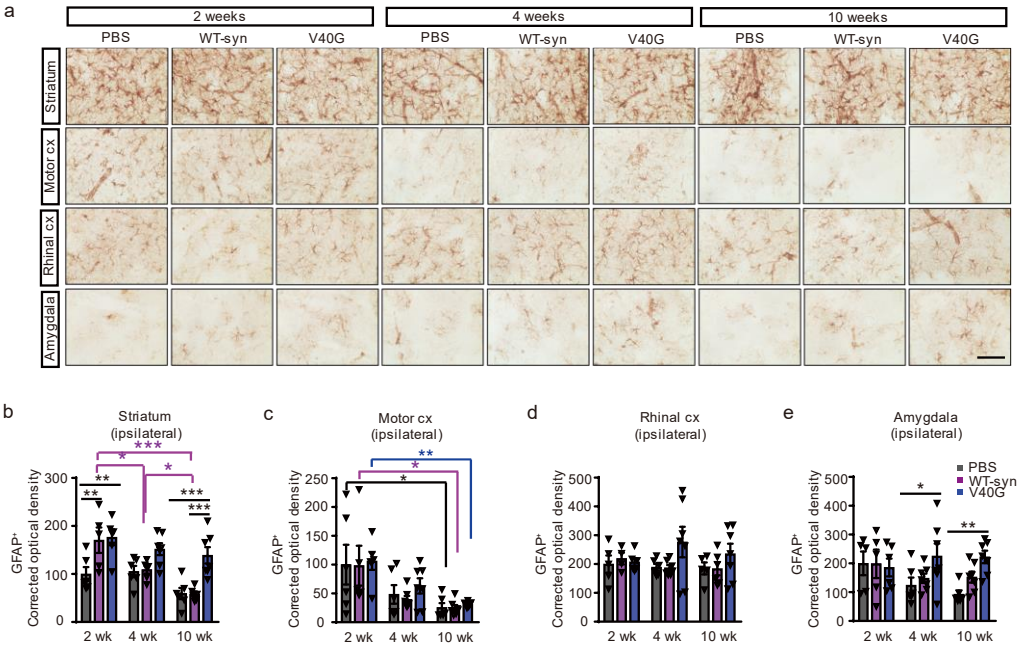


Supplementary Fig. 4 Maps of α-synuclein pathology. a Brain distribution of phospho-α-synuclein accumulations at 2, 4 and 10 weeks after WT-syn fibril and V40G multimer injection (red dots and stippling, respectively; asterisks indicate the injection site; n = 6 per group). **b-e** Percentage of brain areas (ipsilateral to the injection side) covered by phospho-α-synuclein (pS129) immunoreactivity 10 weeks after injection with WT-syn fibrils and V40G multimers in the Striatum (WT-syn, n=6; V40G, n=6) (**b**), the motor cortex (WT-syn, n=6; V40G, n=6) (**c**), the rhinal cortex (WT-syn, n=6; V40G, n=6) (**d**), and the amygdala (WT-syn, n=6; V40G, n=6) (**e**). Data are expressed as the mean ± SEM, two-tailed paired t-test, **P*<0.05, ***P*<0.01, ****P*<0.0001.

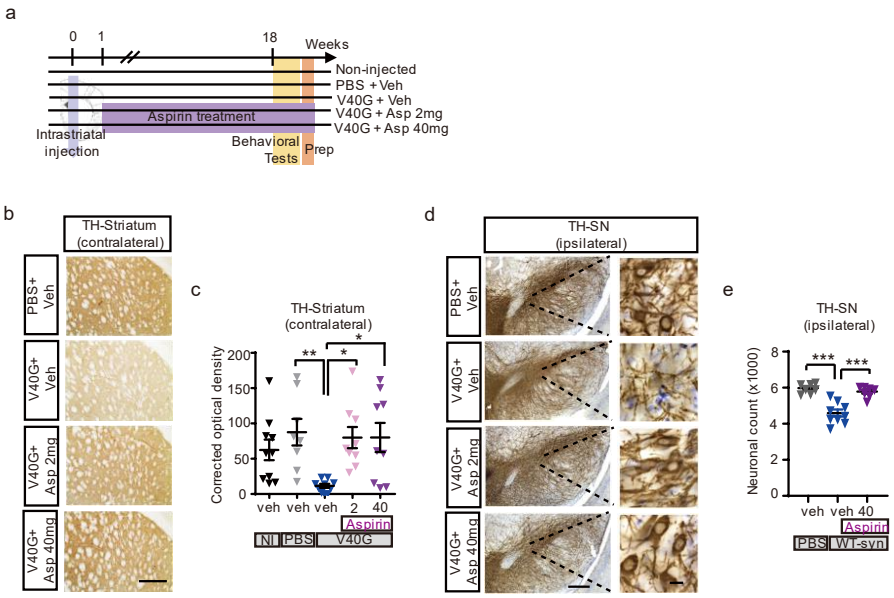


Supplementary Fig. 5 Overview of DEGs after injection of WT-syn fibrils or V40G multimers. a Heat map representing the expression levels (log2 read count) of all mapped genes in the rhinal cortex from PBS-, WT-syn, and V40G-injected mice. **b** Heat map representing the Euclidean distances between the samples. Distances were analyzed by logarithmic transformation in DESeq2. **c** Mean Average (MA) plots of WT-syn *vs.* PBS and V40G *vs.* PBS. The log 2 foldchange for each comparison is shown on the y-axis and the average counts normalized by size factor is plotted on the x-axis. **d** Volcano plots of WT-syn *vs.* PBS and V40G *vs.* PBS. DEGs were selected by their fold changes (>1.5) and p values

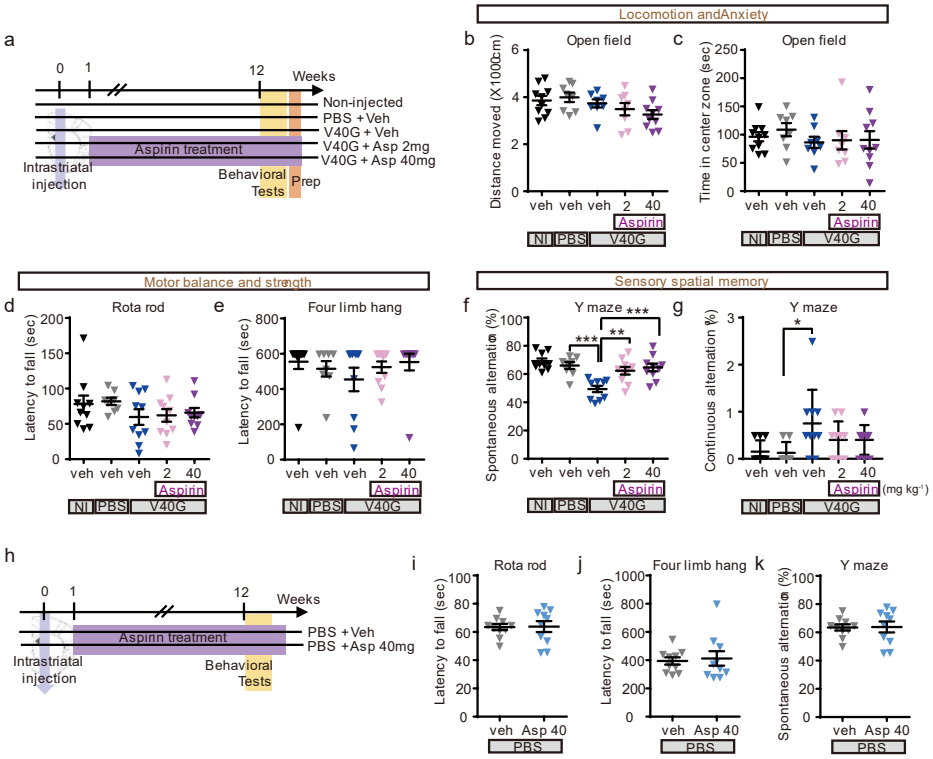
(<0.05). Green dots represent downregulated DEGs, and red dots indicate upregulated DEGs. **e, f** Enriched GO (**e**) and KEGG (**f**) analyses for 418 DEGs in WT-syn-injected mice compared with PBS-injected mice. **g, h** Enriched GO (**g**) and KEGG (**h**) analyses for 485 DEGs in V40G-injected mice compared with PBS-injected mice.



Supplementary Fig. 6 Representative images of astroglial activation. a Representative images of α-synuclein propagation regions 2, 4, and 10 weeks after injection of WT fibrils and V40G multimers stained with GFAP (marker of astrogliosis). Scale bar, 50 μm. **b-e** Optical density of areas covered by GFAP immunoreactivity at 2, 4, and 10 weeks after injection in the striatum (at 2, 4, and 10 weeks, respectively: PBS, n=6, 6, 6; WT-syn, n=5, 6, 6; V40G, n=6, 7, 7) (**b**), the motor cortex (PBS, n=6, 6, 6; WT-syn, n=5, 7, 7; V40G, n=6, 7, 7) (**c**), the rhinal cortex (PBS, n=5, 6, 5; WT-syn, n=4, 7, 6; V40G, n=6, 7, 7) (**d**), and the amygdala (PBS, n=5, 6, 6; WT-syn, n=5, 7, 7; V40G, n=6, 7, 7) (**e**). Data are expressed as the mean ± SEM, one-way ANOVA with Tukey's post hoc test, two-sided, **P*<0.05, ***P*<0.01, ****P*<0.0001.



Supplementary Fig. 7 Representative images of dopaminergic terminal and cell bodies by aspirin treatment. a Oral administration of aspirin at approximately 2 mg/kg and 40 mg/kg via drinking water starting 1 week after intrastriatal injection in C57BL/6 mice. **b** Representative images of the striatal region stained with TH. Scale bar, 200 μm. **c** Optical density of the contralateral striatal region covered by TH immunoreactivity. (Noninjected, n=10; PBS+vehicle, n=8; V40G+vehicle, n=10; V40G+aspirin 2 mg, n=9; V40G+aspirin 40 mg, n=9). Data are expressed as the mean ± SEM, one-way ANOVA with Tukey's post hoc test, two-sided. **d** Representative images of the substantia nigra pars compacta stained with TH. Scale bar, low magnification, 200 μm; high magnification, 10 μm. **e** Stereological cell counts of TH-immunoreactive dopaminergic neurons in the substantia nigra pars compacta of mice that were injected with either PBS or WT-syn fibrils. Following these injections, animals were treated with vehicle or aspirin (PBS+vehicle, n=7; WT-syn+vehicle, n=9; WT-syn+aspirin 40 mg, n=7). Data are expressed as the mean ± SEM, one-way ANOVA with Tukey's post hoc test, two-sided, **P*<0.05, ***P*<0.01, ****P*<0.0001.



Supplementary Fig. 8 Behavioral assessment of V40G-injected and PBS-injected mice after 12 weeks and 18 weeks of oral aspirin administration. a Oral administration of aspirin at approximately 2 mg/kg and 40 mg/kg via drinking water starting 1 week after intrastriatal injection in C57BL/6 mice. **b-g** Twelve weeks after the intrastriatal injection, we performed open field, rotarod, four-limb hanging and Y maze behavioral tests. **b, c** Distance moved (cm) and time in center (s) for analysis of locomotion and anxiety (noninjected (NI), n=10; PBS+vehicle, n=8; V40G+vehicle, n=8; V40G+aspirin 2 mg, n=8; V40G+aspirin 40 mg, n=10). **d, e** Latency to fall (s) in rotarod and four-limb hanging tests for analysis of motor balance and strength (NI, n=10; PBS+vehicle, n=8; V40G+vehicle, n=10; V40G+aspirin 2 mg, n=10; V40G+aspirin 40 mg, n=10). **f, g** Spontaneous alteration (%) and continuous alteration (%) in the Y maze for analysis of sensory spatial memory (noninjected, n=10; PBS+vehicle, n=8; V40G+vehicle, n=10; V40G+aspirin 2 mg, n=10; V40G+aspirin 40 mg, n=10). **h** Oral administration of aspirin at 40 mg/kg via the drinking water for 17 weeks, starting 1 week after

injection of PBS in C57BL/6 mice. **i-k** Eighteen weeks after the initial intervention, we subjected the mice to the rotarod, four-limb hanging, and Y maze behavioral tests. (PBS+vehicle, n=10; PBS+aspirin 40 mg, n=10). Data are expressed as the mean ± SEM, one-way ANOVA with Tukey's post hoc test, two-sided, **P*<0.05, ***P*<0.01, ****P*<0.0001.

level	% area	
-	~ 0.5	No pathology
+	0.5 ~1.5	Mild pathology
++	1.5 ~ 2.5	Moderate pathology
+++	2.5 ~3.5	Dense pathology
++++	3.5 ~	Severe pathology

		2 weeks				4 weeks				10 weeks			
Bregma		WT-syn		V40G		WT-syn		V40G		WT-syn		V40G	
(mm)		cont	ipsi	cont	ipsi	cont	ipsi	cont	ipsi	cont	ipsi	cont	ipsi
0.86	cingulate cortex	-	-	-	-	-	-	+	+	-	+	++++	++++
	motor cortex	-	-	-	-	-	-	-	+	-	+	++	++++
	somatosensory cortex	-	-	-	+	-	+	-	+	-	+	+	++
	insular cortex	-	-	-	-	-	+	-	+	-	+	+	++
	piriform cortex	-	-	-	-	-	-	-	+	-	-	-	+
	claustrum	-	-	-	-	-	-	-	+	-	-	+	++
	striatum	-	-	-	+	-	+	-	++++	-	+	+	++++
	nucleus accumbens	-	-	-	-	-	-	+	-	-	-	-	-
-1.82	retrosplenial cortex	-	-	+	+	+	+	+	++	+	+	++++	++++
	parietal cortex	-	-	+	+	+	+	+	+	+	+	+	++++
	somatosensory cortex	-	-	-	+	-	+	+	+	-	+	+	++
	auditory cortex	-	-	-	-	-	-	-	-	-	-	+	++
	rhinal cortex	-	-	-	-	-	-	-	-	-	+	++++	++++
	piriform cortex	-	-	-	-	-	-	-	-	-	+	-	+
	endopiriform nucleus	-	-	-	-	-	-	-	-	-	-	-	-
	hippocampus	-	-	-	-	-	-	-	-	-	-	-	-
	habenular nucleus	-	-	-	+	-	+	-	+	-	+	+	+
	hypothalamus	-	-	-	-	-	-	-	-	-	-	-	-
	basolateral amygdala	-	-	-	-	-	-	+	++++	-	++	++++	++++
	central amygdala	-	-	-	-	-	-	-	-	-	-	-	++++
-2.8	retrosplenial cortex	-	-	-	-	-	-	-	-	-	+	+	+
	visual cortex	-	-	-	-	-	-	-	-	+	+	+	+
	auditory cortex	-	-	-	-	-	-	-	-	-	-	+	+
	rhinal cortex	-	-	-	-	-	-	-	+	-	+	++	++++
	piriform cortex	-	-	-	-	-	-	-	-	-	+	+	+
	hippocampus	-	-	-	-	-	-	-	-	-	-	-	-
	antpretectal nucleus	-	-	-	-	-	-	-	+	-	+	+	+
	substantia nigra	-	-	-	+	-	+	+	++	+	++	+	++
	retomammillary nucleus	-	-	-	-	-	-	-	-	-	-	-	-

Supplementary Table 1 Representative profiles of α-synuclein pathology in mouse brain regions. Semiquantitative grading of α-synuclein pathology in mice injected with WT-syn fibrils and V40G multimers. Phospho-α-synuclein (pS129) expression levels in each region were evaluated in brain sections from six animals on a scale of 1 to 5, where 1 is the lowest in the area with less than 0.5% and 5 is the highest in the area with at least 3.5%, and the average values for each region were rounded up or down.

level	% area	
-	~ 1.5	No pathology
+	1.5~3.0	Mild pathology
++	3.0~4.5	Moderate pathology
+++	4.5~6.0	Dense pathology
++++	6.0 ~	Severe pathology

		2 weeks						4 weeks						10 weeks					
Bregma		PBS		WT-syn		V40G		PBS		WT-syn		V40G		PBS		WT-syn		V40G	
(mm)		cont	ipsi	cont	ipsi	cont	ipsi	cont	ipsi	cont	ipsi	cont	ipsi	cont	ipsi	cont	ipsi	cont	ipsi
0.86	cingulate cortex	+	++	+	+++	+	+++	-	+	+	++	+	++++	-	-	-	-	-	-
	motor cortex	-	+	-	++	-	++	-	-	-	+++	-	+++	-	-	-	+	-	-
	somatosensory cortex	-	++	-	+	-	+	-	-	-	+++	-	+	-	-	-	-	-	+
	insular cortex	-	++	-	++	-	++	-	-	-	++++	-	+++	-	-	-	-	-	+++
	piriform cortex	+	++	-	++	-	+++	-	-	-	+++	-	++++	-	-	-	-	+	++++
	claustrum	-	++	-	++	+	+++	-	-	-	++++	-	++++	-	-	-	-	+	++++
	striatum	+	++	+	+++	-	+++	-	-	-	+++	-	++++	-	-	-	-	-	++
	nucleus accumbens	++	+++	-	++++	+	++++	-	-	-	++	-	+++	-	-	-	+	-	+++
-1.82	retrosplenial cortex	++	+	+	++	++++	++++	-	+	-	-	-	-	-	-	-	-	-	-
	parietal cortex	-	+	+	++	++++	++++	-	-	-	-	-	-	-	-	-	-	-	-
	somatosensory cortex	+	++	-	+	+++	++++	-	-	-	-	-	-	-	-	-	+	-	-
	rhinal cortex	-	++++	++	++++	+++	++++	-	-	-	+++	+	++	-	++	-	+	+	++
	piriform cortex	++	++++	+	++++	++	++++	-	-	++	++++	+	++	-	+	-	+	+	+++
	endopiriform nucleus	+	++++	+	++++	+++	++++	-	-	+	++++	+	+++	-	+	-	+	+++	++++
	hippocampus	+	++	+++	++++	+++	++++	-	-	-	-	-	+	-	-	-	-	-	+
	habenular nucleus	-	+	+	+	+	++++	-	-	+	+	+	++	-	-	-	-	+	+
	hypothalamus	+	+	++	++	+	++	-	-	-	+	-	+	-	-	-	-	++	+
	basolateral amygdala	+	++++	++	++++	++	++++	-	-	+	++++	+	+++	-	+	-	-	++	++++
	central amygdala	-	++++	+	++++	+	++++	-	+	+	++++	+	++++	-	+	-	-	++	++++
-2.8	retrosplenial cortex	-	-	++	++++	+	+	-	+	+	+	+	+	-	-	-	-	-	+
	visual cortex	-	+	-	++	-	+	-	-	-	+	-	+	-	-	-	+	-	-
	auditory cortex	-	+	-	++	-	++	-	-	-	+	-	++++	-	-	-	++	-	++
	rhinal cortex	-	++	+	++++	++	++++	+	+	+	++++	+	++++	-	-	-	+	+	++++
	piriform cortex	-	++	-	+	++	++++	-	+	+	++	+	++++	-	-	-	-	++	++++
	hippocampus	-	-	+	++	+	++	-	-	+	+	-	++	-	-	-	-	-	+
	antpretectal nucleus	-	-	+	++	-	-	-	-	-	+	-	-	-	-	-	-	-	-
	substantia nigra	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	retomammillary nucleus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Supplementary Table 4 Representative profiles of IL-1β pathology in mouse brain regions.

Semiquantitative grading of IL-1β-positive cells in mice injected with sham, WT-syn fibrils and V40G multimers. IL-1β expression levels in each region were evaluated in brain sections from six animals on a scale of 1 to 5, where level 1 is the lowest in the area with less than 1.5% and level 5 is the highest in the area with at least 6%, and the average values for each region were rounded up or down.