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Supplemental information

Treadmill exercise reduces α-synuclein spreading via PPARα

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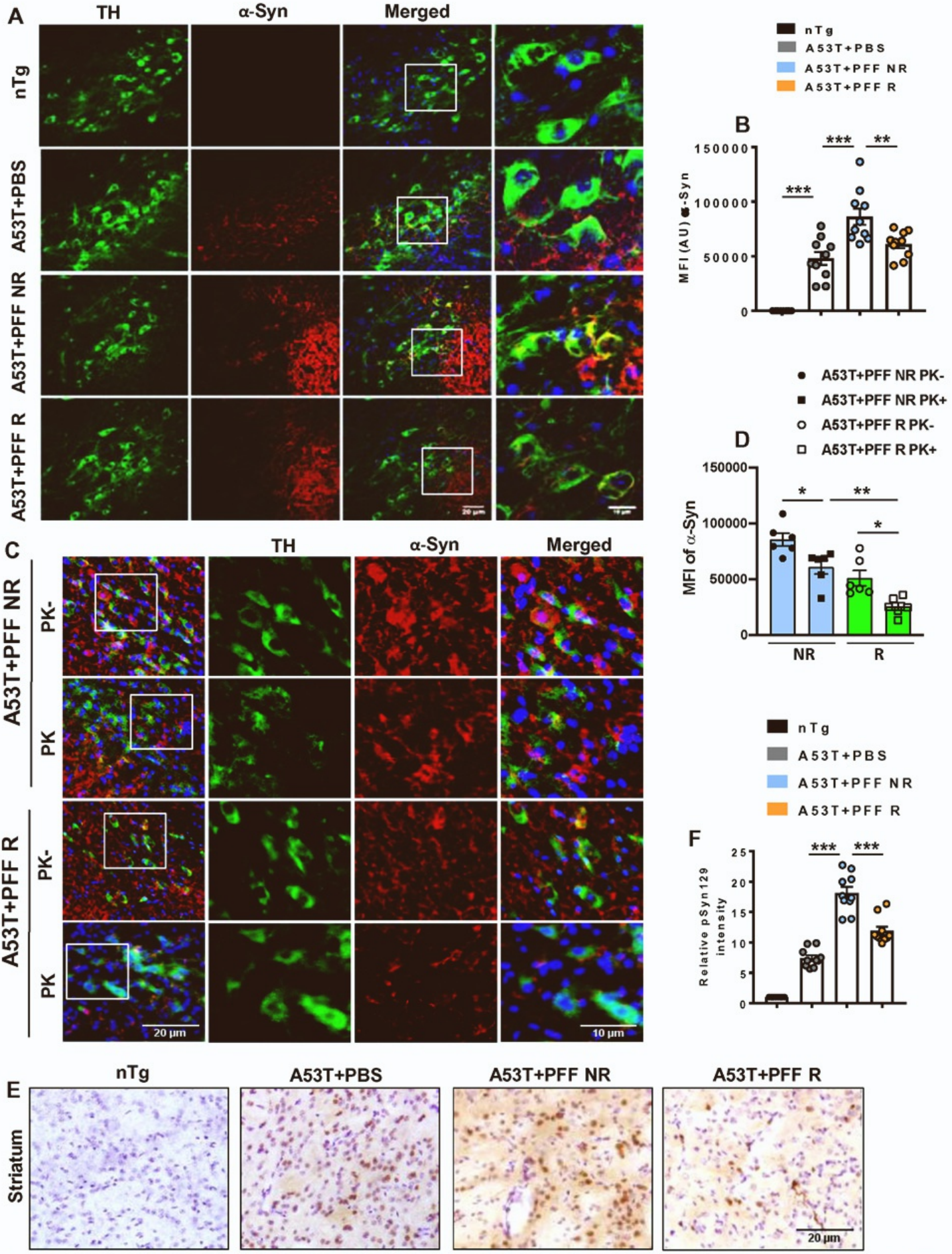

Figure S1. Related to Figure 1. Reduction of α-syn pathology in nigra and striatum of PFF seeded A53T mice by treadmill exercise. α-Syn PFF was seeded in internal capsule (IC)

region and accumulation of α-syn in nigral TH neurons of non-running (A53T+PFF NR) and running (A53T+PFF R) mice brain was monitored by immunofluorescence analysis of total α-syn (A) followed by analysis of mean fluorescence intensity (MFI) using ImageJ (B, n=5). Immunostaining of α-syn in nigral sections was also conducted with or without proteinase K (PK) treatment and the α-syn MFI was measured using ImageJ (C, D, n=3). At least two nigral sections from each brain were stained from each mouse brain and 10 α-syn positive cells were quantified from each section. Average value of MFI obtained from each section are shown in the diagrams. Images were captured at 20X and 60X magnifications and the scale bars were 20 μm and 10 μm respectively. One way ANOVA followed by Tukey's multiple comparison tests was performed for statistical analyses. *p< 0.05, **p< 0.01, ***p< 0.001 indicate significance compared to respective groups. Data are represented as mean ± SEM.

The accumulation of pSyn129 in dorsal striatum was assessed by immunohistochemistry (E). Two striatal sections from each brain were used for the experiment and 10 α-syn positive cells from each section were considered for density analysis. Optical density of pSyn129 was measured using Fiji and the relative fold change compared to the nTg was shown in the diagram (F). The value of fold change obtained from each section are shown in the diagram. Images were taken at 20X magnification, scale bar was 20 μm. One way ANOVA followed by Tukey's multiple comparison tests was performed for statistical analyses. ***p< 0.001 indicates significance compared to respective groups ($F_{3, 36}$=133.5). Data are represented as mean ± SEM (n=5 animals per group).

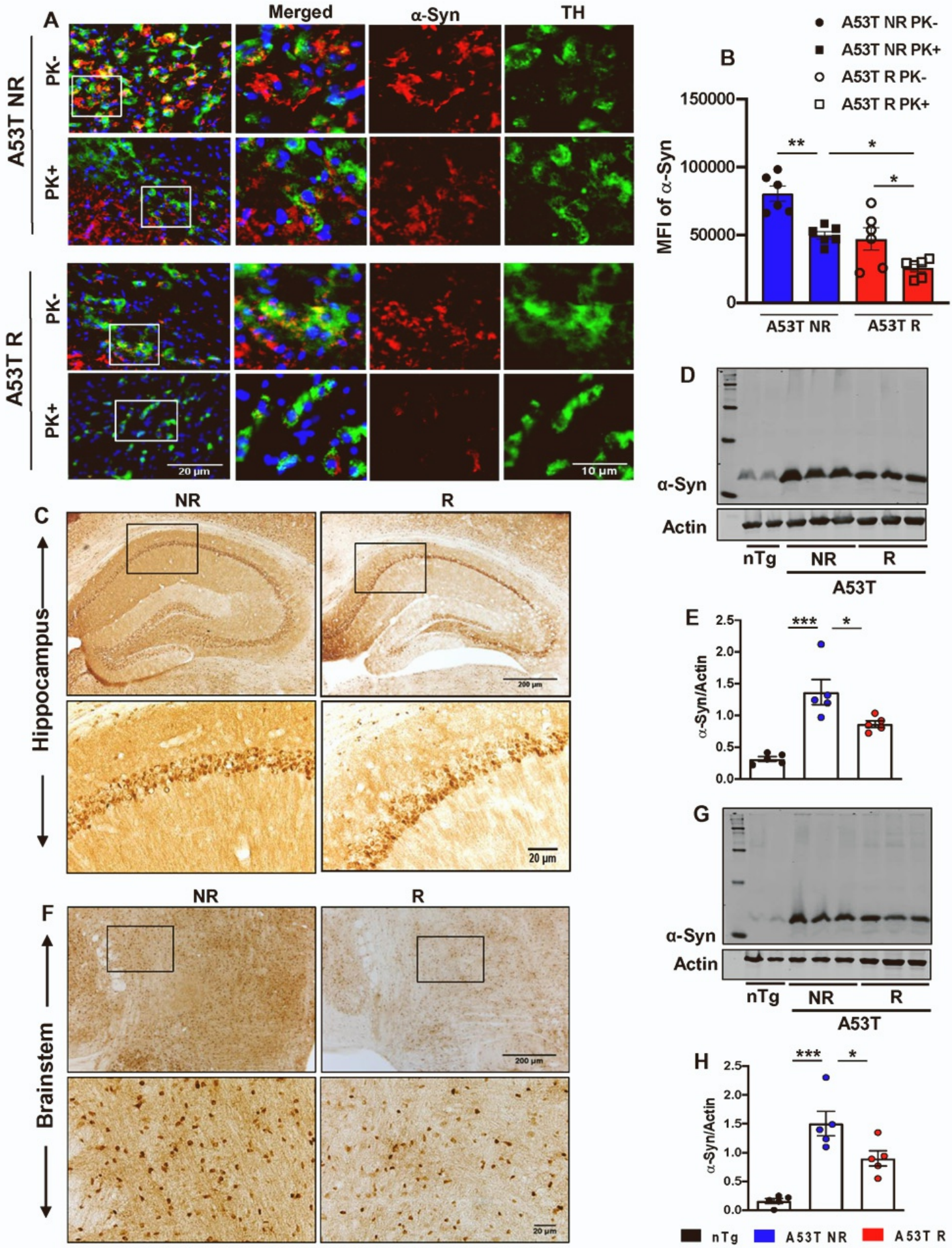

Figure S2. Related to Figure 1. Decrease in α-syn pathology in the brain of aged A53T mice

by treadmill exercise. A53T mice (8 months old) were subjected to run in the treadmill, every day for next 2 months, and α-syn pathology was compared in SN of non-running (A53T NR) and running (A53T R) mice by immunostaining of α-syn coupled with PK treatment (A). At least two nigral sections from each brain were stained from each mouse brain and 10 α-syn positive cells were quantified from each section. Average value of MFI obtained from each section are shown in the diagrams (B, n=3). One-way ANOVA followed by Tukey's multiple comparison tests was performed for statistical analyses. *$p< 0.05$, **$p< 0.01$ indicate significance compared to respective groups. Data are represented as mean ± SEM.

Immunohistochemistry of pSyn129 (C, F) and immunoblotting of total α-syn in RIPA fractions of tissues isolated from hippocampus ($F_{2, 12}=19.46$, $p< 0.05$, D, E) and brainstem ($F_{2, 12}=21.56$, $p< 0.05$, G, H). One-way ANOVA followed by Tukey's multiple comparison tests was performed for statistical analyses. *$p< 0.05$, ***$p< 0.001$ indicate significance compared to respective groups. Values are represented as mean ± SEM (n=5 animals per group).

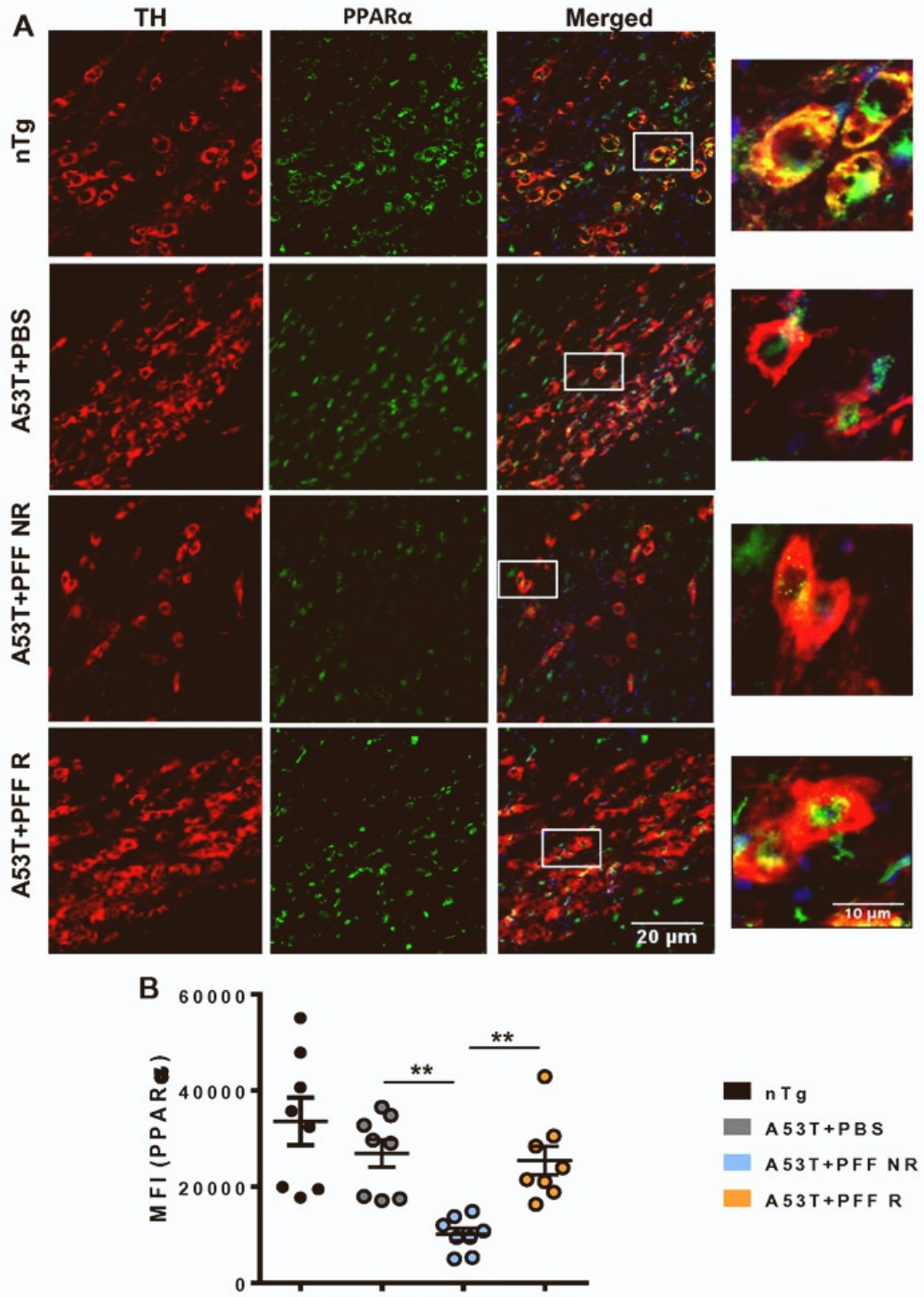

Figure S3. Related to Figure 2. Up-regulation of PPARα in SN of PFF-seeded A53T brains by treadmill. α-Syn PFF was seeded in IC region and PPARα level in nigral TH neurons of experimental mice brains was monitored by double immunofluorescence analysis of total TH and PPARα (A). MFI analysis of PPARα was conducted using ImageJ. At least two nigral sections from each brain were stained from each mouse brain and PPARα expression in 10 TH positive cells per section was quantified. Average value of MFI obtained from each section are shown in the diagrams (B, n=4). Images were captured at 20X and 60X magnifications and the scale bars were 20 µm and 10 µm respectively. One way ANOVA followed by Tukey's multiple comparison tests was performed for statistical analyses. **$p< 0.01$ indicates significance compared to respective groups. Data are represented as mean ± SEM.

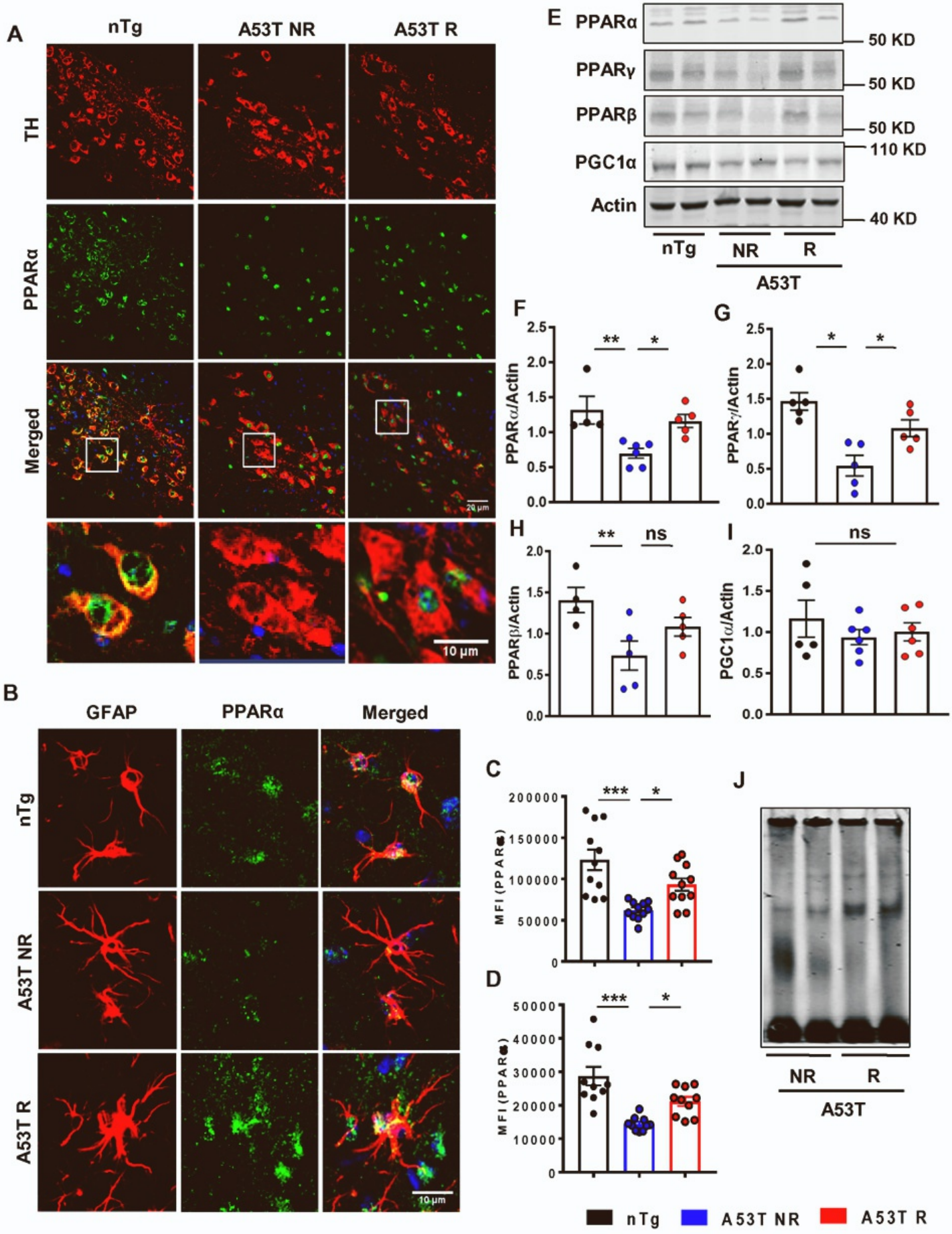

Figure S4. Related to Figure 2. Up-regulation of PPARα in A53T brains by treadmill. PPARα levels were assessed by double immunostaining of PPARα in TH positive

neurons of SN (A) and in GFAP positive astrocytes (B) from midbrain sections. Mean fluorescence intensity (MFI) of PPARα expression in TH neurons and astrocytes was measured by ImageJ ($F_{2,27}$=12.34, **p*<0.05 for TH neurons and $F_{2,27}$=15.47, **p*<0.05 for astrocytes A53T NR vs A53T R, C, D, n=5). Confocal images were captured at 20X and 60X magnifications and the scale bars were 20 µm and 10 µm respectively. At least two nigral sections from each brain were stained from each mouse brain and PPARα expression in 10 TH positive cells and 10 GFAP positive cells per section was quantified. Average value of MFI obtained from each section are shown in the diagrams. Total tissue level of PPARα ($F_{2, 12}$=8.113, **p*< 0.05), PPARγ ($F_{2, 12}$=12.32, **p*< 0.05), PPARβ and PGC1α was measured by immunoblotting, where actin was used as the loading control (E). Ratios of each protein to actin are represented in the bar diagrams (F-I, n=5). PPARα DNA binding to TFEB promoter region was evaluated by EMSA from midbrain tissue samples of A53T NR and A53T R mice (n=3). Statistical analyses were conducted by one way ANOVA followed by Tukey's multiple comparison tests. **p*< 0.05, ***p*< 0.01, ****p*< 0.001 indicate significance compared to respective groups, ns, nonsignificant. Values are given as mean ± SEM.

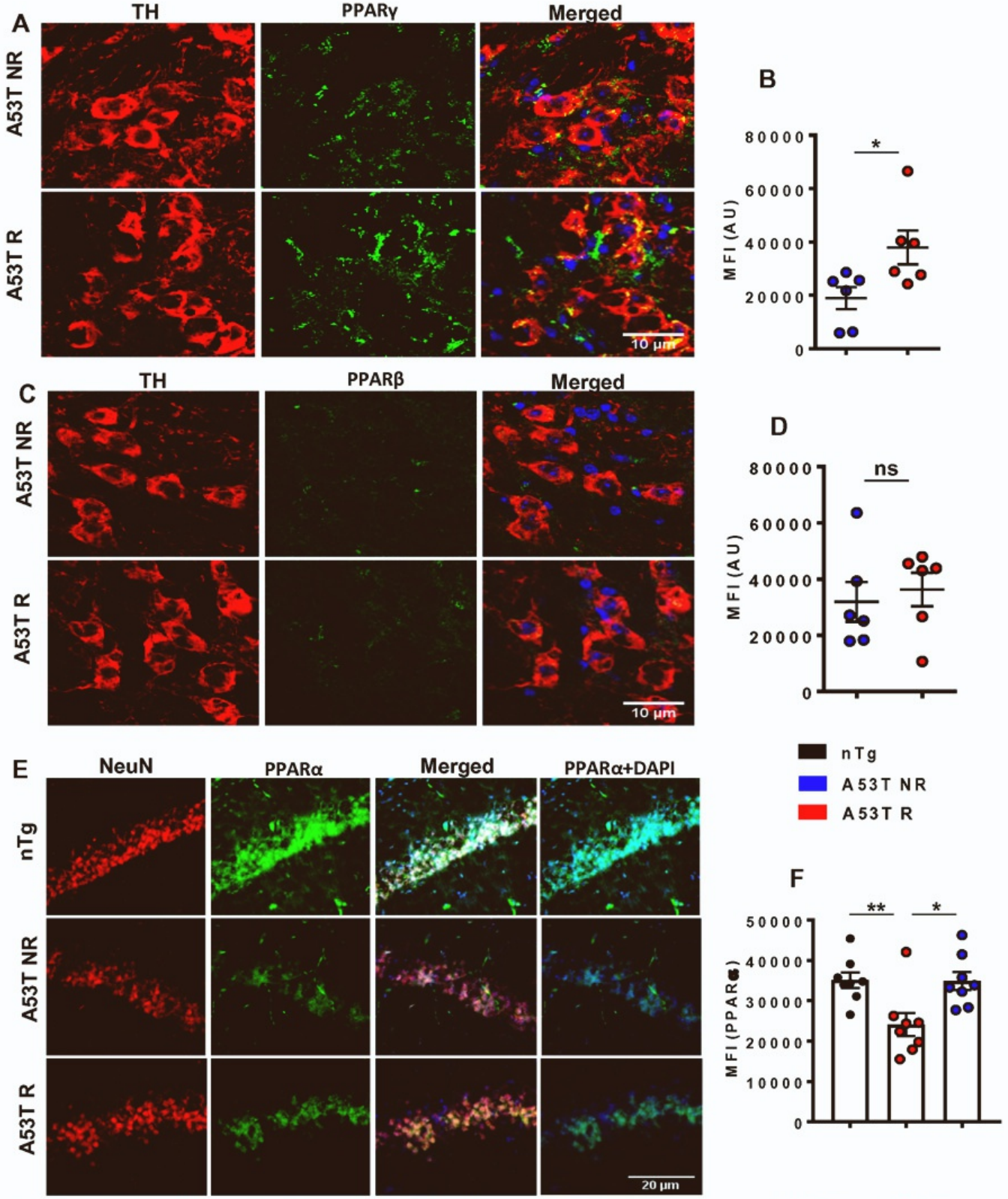

Figure S5. Related to Figure 3. Effect of treadmill exercise on expression of PPARα, PPARγ and PPARβ in A53T brains. Neuronal expression of PPARγ and PPARβ was monitored in TH positive neurons of SN by immunofluorescence analysis (A-D). Expression of PPARα in hippocampus of nTg, A53T NR and A53T R animals were shown by double immunostaining with NeuN followed by MFI analysis ($F_{2, 21}$= 6.97, A53T NR vs A53T R, *p< 0.05, E, F). Images were captured at 20X and 60X magnifications and the scale bars were set as 20 µm and 60 µm respectively. Statistics was conducted by unpaired t test and one way ANOVA followed by Tukey's multiple comparison tests. *p< 0.05, **p< 0.01 indicate significance compared to respective groups, ns, nonsignificant. Values are represented as mean ± SEM (n=6 for PPARγ and PPARβ analysis and n=4 for PPARα analysis).

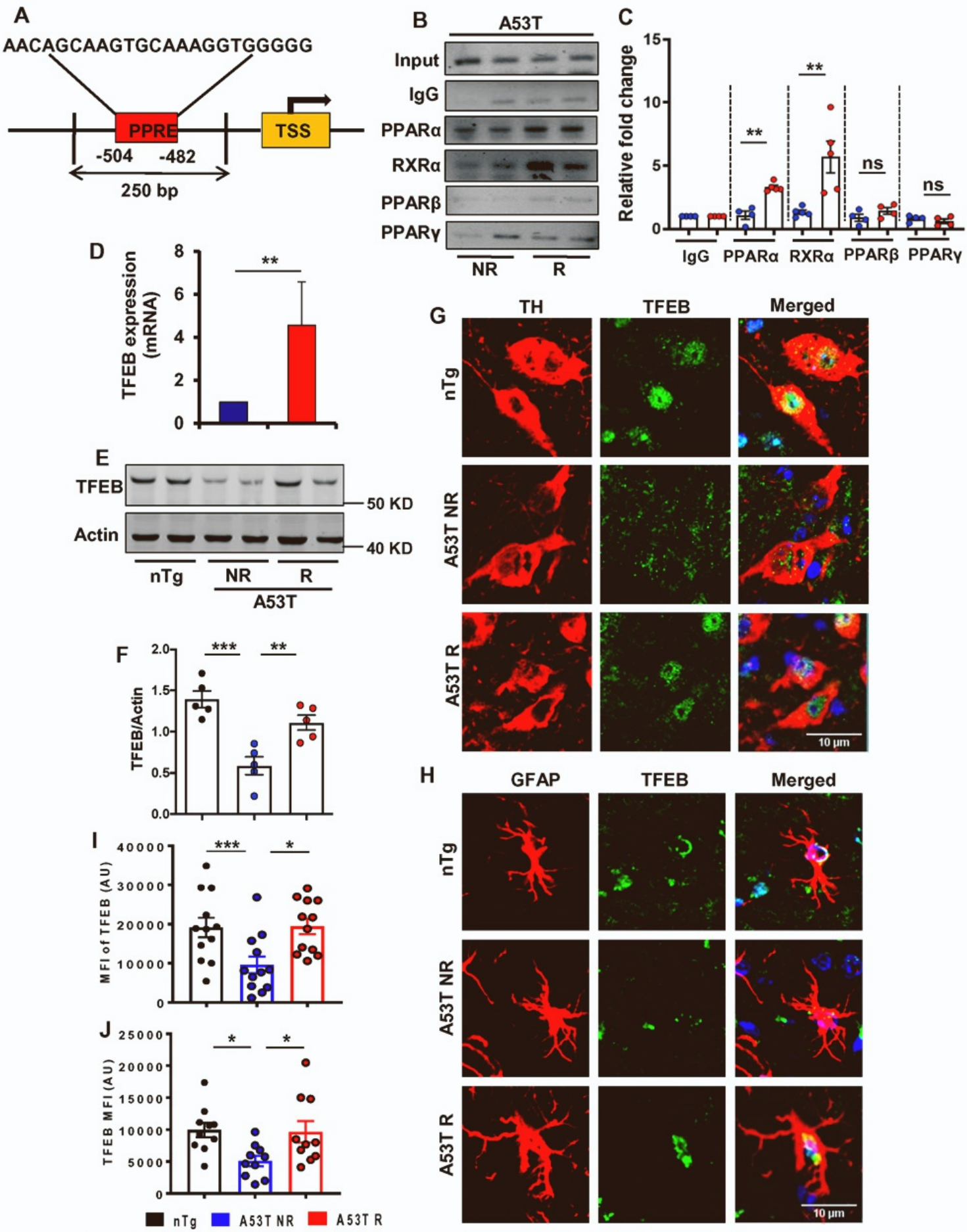

Figure S6. Related to Figure 4. PPARα activates TFEB expression in A53T brains. The schematic diagram shows presence of PPAR response element (PPRE) in the *Tfeb*

promoter region around 482 to 504 bp upstream of transcription start site (TSS) (A). Genomic DNA was isolated from midbrain tissues of A53T NR and R mice and binding of PPARα, RXRα, PPARβ and PPARγ in the PPRE site was analyzed by chromatin immunoprecipitation (ChIP) followed by semi-quantitative and real-time PCR (p< 0.01, B, C, n=4-5). Expression of TFEB at mRNA and protein level was monitored by real-time PCR (**p< 0.01, D, n=4) and immunoblotting ($F_{2, 12}$= 16.81, **p< 0.01, ***p< 0.001, E, F, n=5) from midbrain samples of nTg, A53T NR and A53T R mice. TFEB up-regulation was also demonstrated by double immunostaining in TH positive nigral neurons and GFAP positive astrocytes (G, H) followed by MFI analysis of TFEB expression by ImageJ ($F_{2, 27}$=4.653, *p< 0.05 and ***p< 0.001, I, J, n=5-6). Confocal images were captured at 60X magnification and the scale bar was 10 µm. Two sections from each brain per group were used for staining purpose, where TFEB mean fluorescence intensity (MFI) was quantified in at least 10 TH positive cells from each section. Average values of TFEB MFI obtained from each section of all the groups of mice are shown. Statistical significance was obtained by unpaired t-test for ChIP and real-time PCR analyses, and by one way ANOVA followed by Tukey's multiple comparison tests for immunoblotting and immunofluorescence analyses. *p< 0.05, **p< 0.01, ***p< 0.001 indicate significance compared to respective groups, ns, not significant. Data are represented as mean ± SEM.**

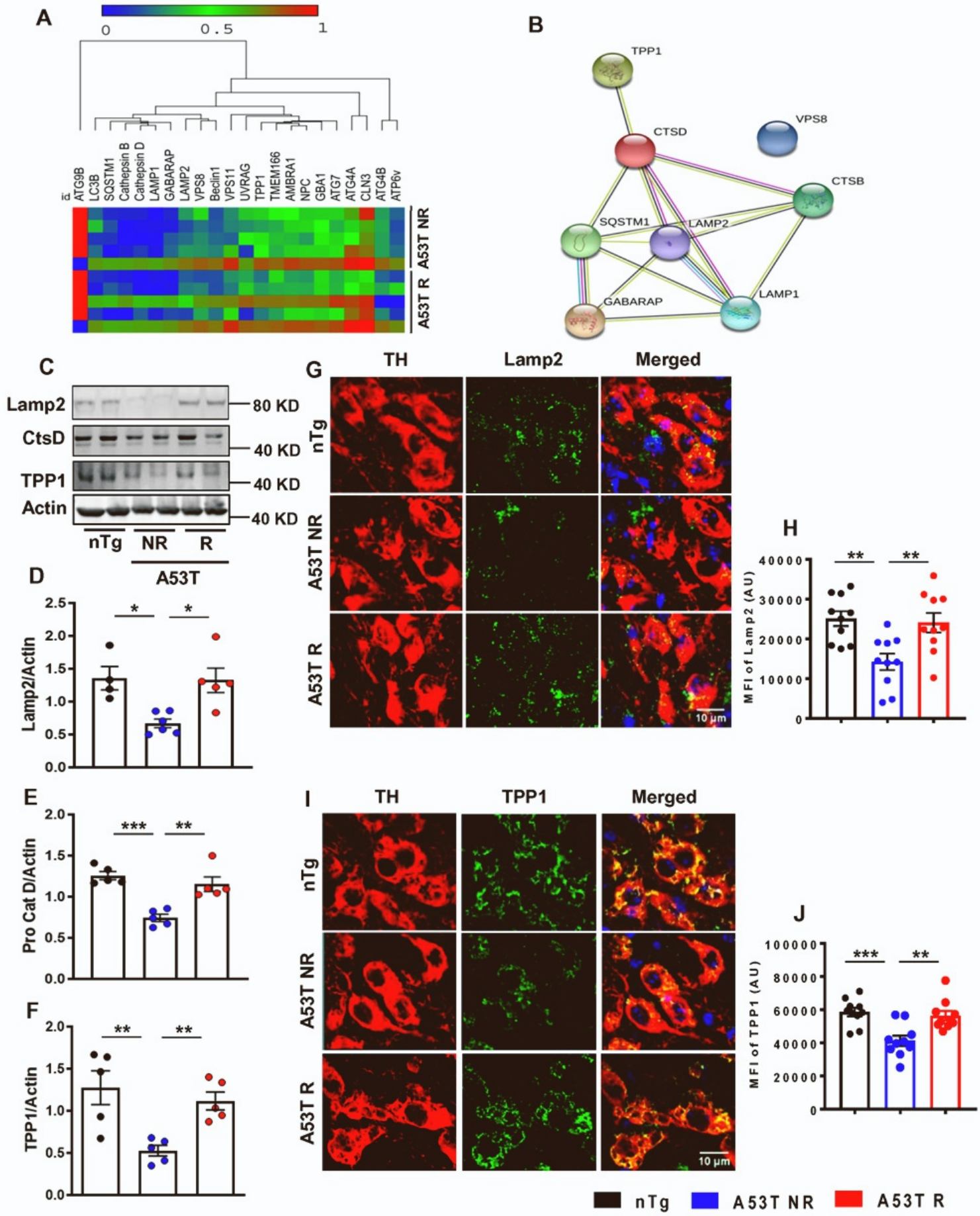

Figure S7. Related to Figure 4. Lysosomal biogenesis is increased by treadmill exercise. Real time PCR of 22 autophagy-lysosomal genes were carried out from midbrains of five individual A53T NR and R mice. Comparative gene expression of these genes is

represented through dendrogram to show hierarchical clustering of these selected genes based on the changes in expression in A53T NR and R brains (A). Protein interactome of up-regulated genes were made by String protein-protein interaction (B). Expression of Lamp2 ($F_{2, 12}$=8.042, A53T NR vs A53T R, *p< 0.05), cathepsin D (CtsD; $F_{2, 12}$=18.51, A53T NR vs A53T R, **p< 0.01) and TPP1 ($F_{2, 12}$=8.4, A53T NR vs A53T R, **p< 0.01) in midbrain tissues was evaluated by immunoblotting and relative quantification was performed with respect to actin (C-F). Neuronal up-regulation of Lamp2 ($F_{2,27}$=7.933, p<0.01) and TPP1 ($F_{2,27}$=11.27, **p<0.01) was validated by immunofluorescence analysis in TH positive neurons of SN followed by MFI analysis using ImageJ (G-J). At least two nigral sections from each brain were stained from each mouse brain and Lamp2 or TPP1 expression in 10 TH positive cells per section was quantified. Average value of MFI obtained from each section are shown in the diagrams. One way ANOVA followed by Tukey's multiple comparison tests was performed to determine statistical significance. *p< 0.05, **p< 0.01, ***p< 0.001 indicate significance compared to respective groups. Data are represented as mean ± SEM (n=5 animals per each group).

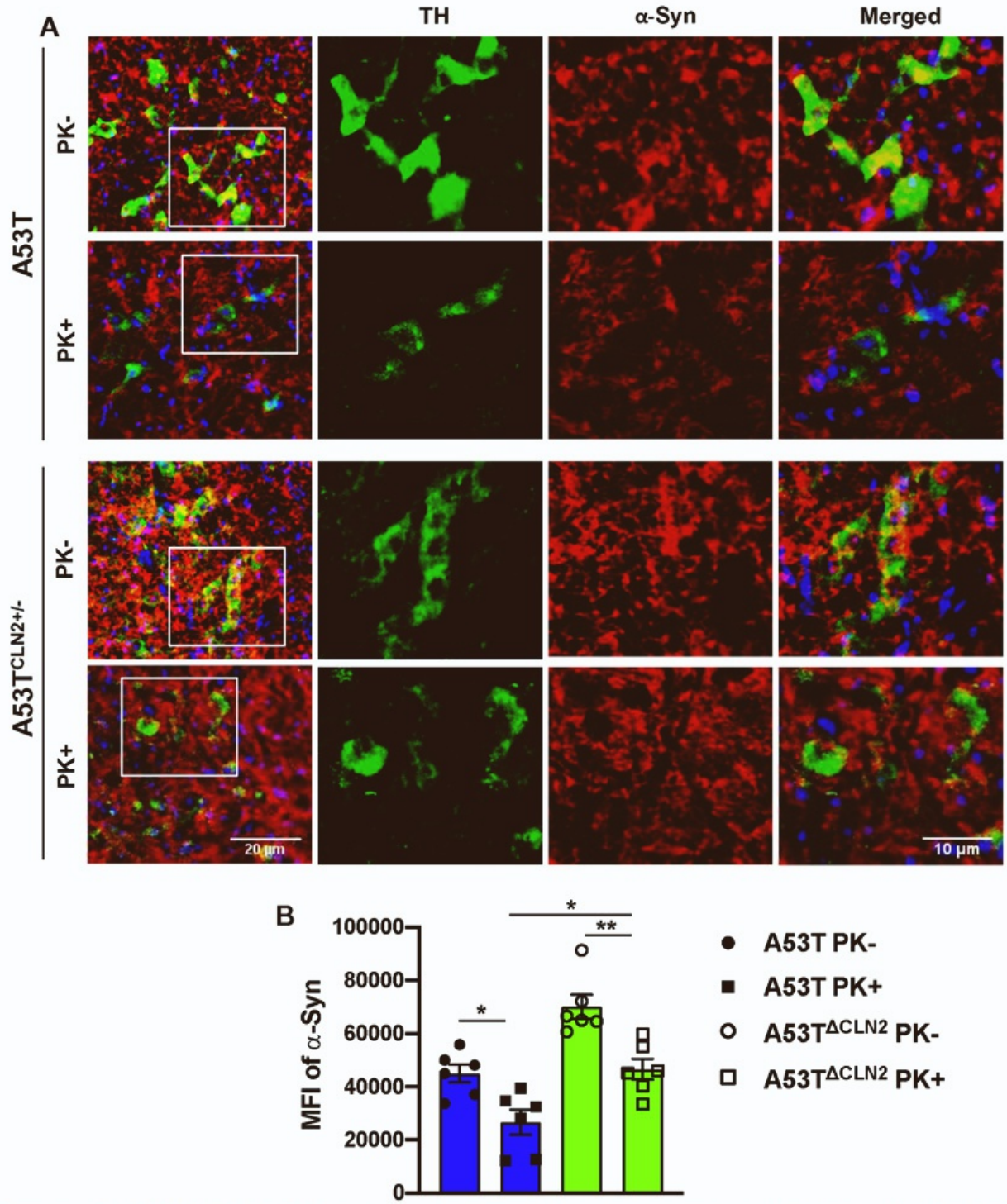

Figure S8. Related to Figure 4. Aggravation of α-syn pathology by heterozygous deletion of CLN2 in A53T mice. Accumulation of α-syn aggregates in SN of A53T and A53T$^{CLN2+/-}$ (A53T with heterozygous deletion of *CLN2*) mice was compared by immunostaining of α-syn coupled with PK treatment in nigral sections (A). At least two nigral sections from each brain were stained from each mouse brain and MFI of α-syn in 10 TH positive cells from each section was quantified and individual values are shown in the diagram (B). One way ANOVA followed by Tukey's multiple comparison tests was performed to determine statistical significance. *p< 0.05, **p< 0.01 indicate significance compared to respective groups. Data are represented as mean ± SEM (n=3 animals per group).

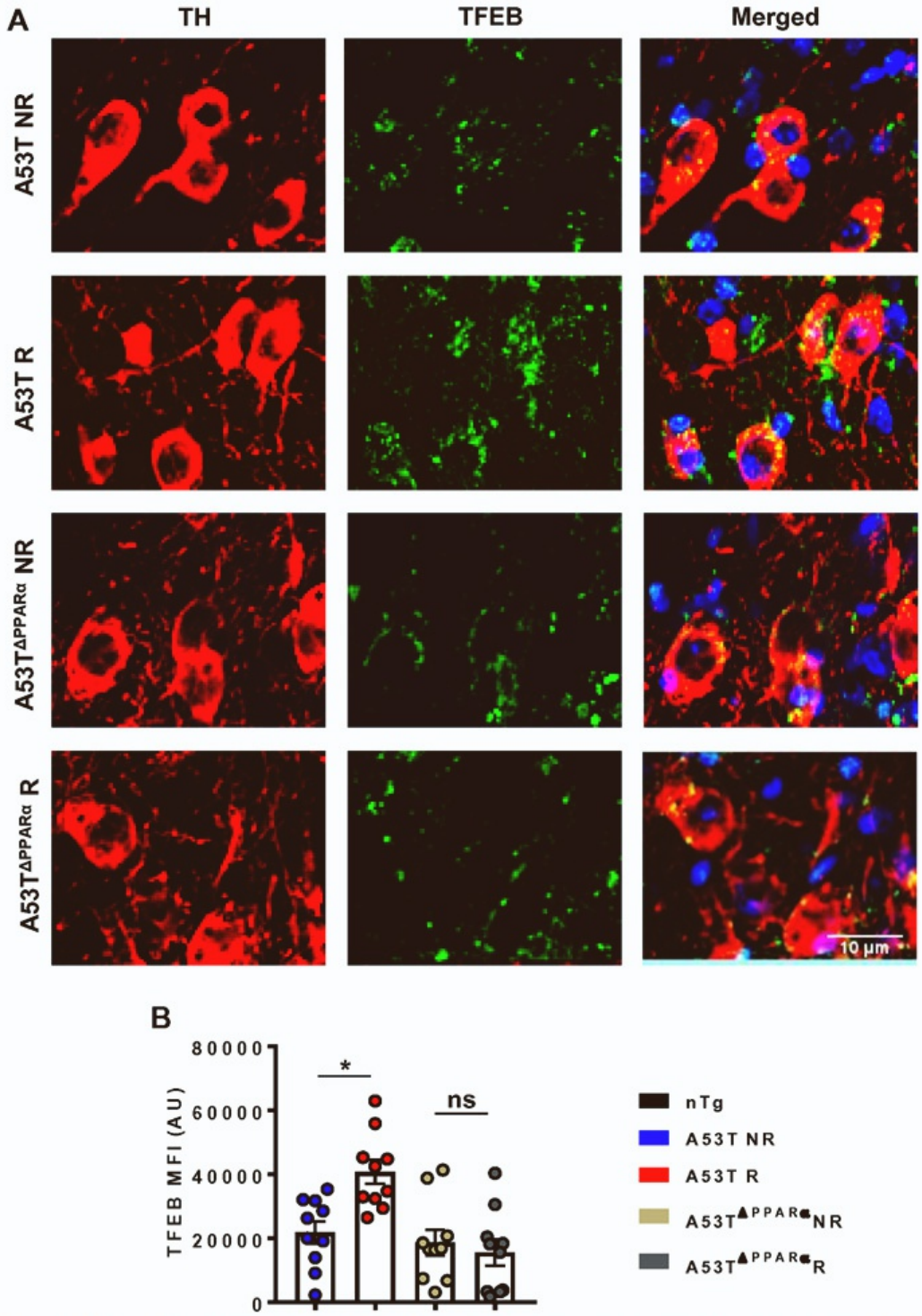

Figure S9. Related to Figure 5. TFEB level remained unchanged in A53T$^{\Delta PPAR\alpha}$ mice following treadmill exercise. TFEB expression in TH neurons of SN in aged A53T and A53T$^{\Delta PPAR\alpha}$ mice (NR and R) was demonstrated by immunofluorescence analysis followed by MFI quantification using ImageJ ($F_{1,36}$=4.25, A53T NR vs A53T R, **p*< 0.05, A, B). At least two nigral sections from each brain were stained from each mouse brain and TFEB MFI in 10 TH positive cells per section was quantified. Average value of MFI obtained from each section are shown in the diagrams. The significance of mean was compared using two way ANOVA considering NR/R and genotype as two independent factors. $F_{1, 20}$>Fc and *p*< 0.05 was considered as the statistical significance, where **p*< 0.05 indicates significance compared to A53T NR, ns, nonsignificant. Values are represented as mean ± SEM (n=5 animals per group).

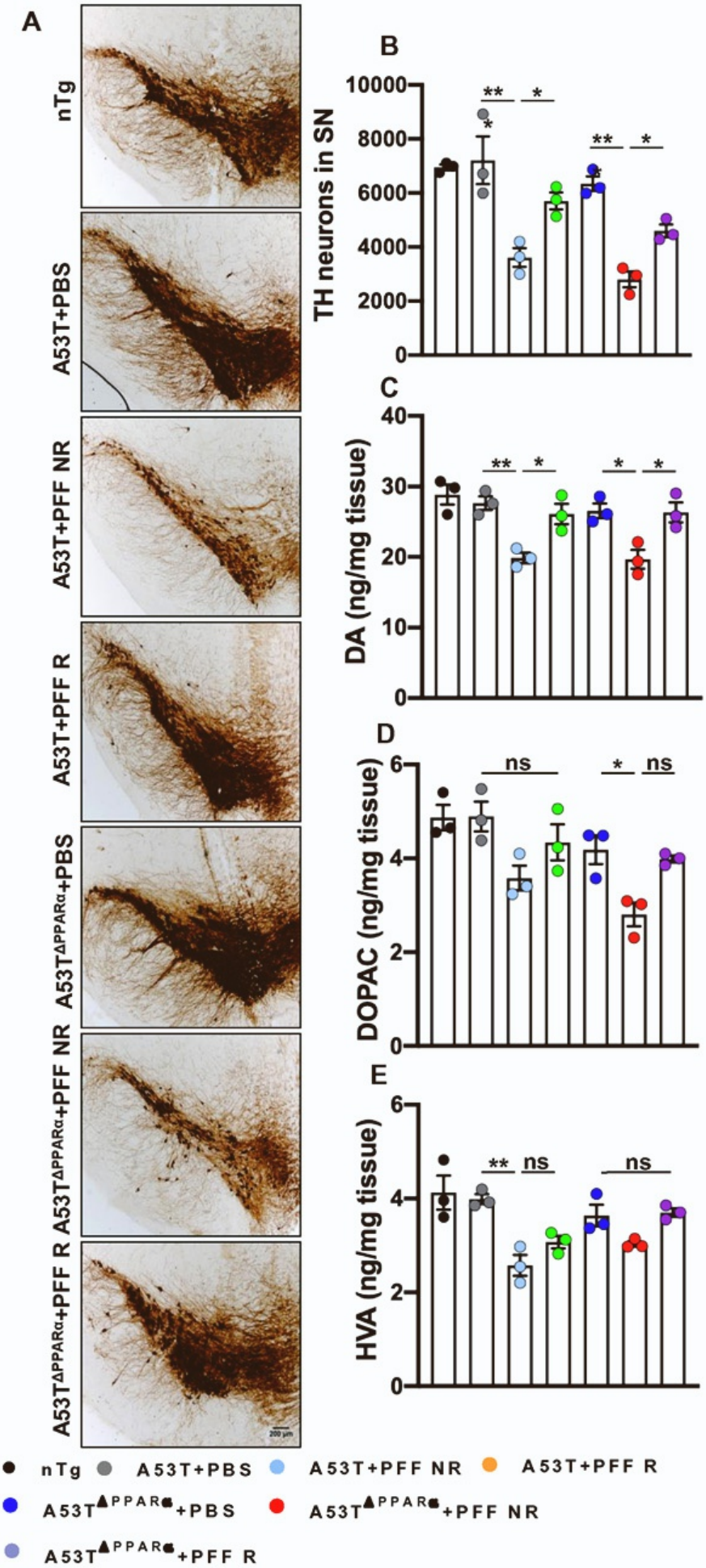

Figure S10. Related to Figure 6. Treadmill exercise attenuates loss of DAergic neurons in the absence of PPARα. Age-matched A53T and A53T mice lacking functional PPARα (A53T$^{\Delta PPAR\alpha}$) were injected with either PBS or PFF at the IC region of striatum and following 2 months of brain surgery, animals were run in the treadmill daily for next 2 months. Number of DAergic neurons in SN was monitored by immunohistochemistry for tyrosine hydroxylase (TH) followed by stereological counting in one hemisphere of the brain (A, B). Striatal dopamine (DA) and its metabolites, 3,4-dihyroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) was measured by HPLC-ECD (C-E). Statistical significance was measured using one-way ANOVA followed by Tukey's multiple comparison test. *p< 0.05, **p< 0.01 and ***p< 0.001 indicate significance compared to respective groups, ns, nonsignificant. Data are represented as mean ± SEM (n=3 animals per group).

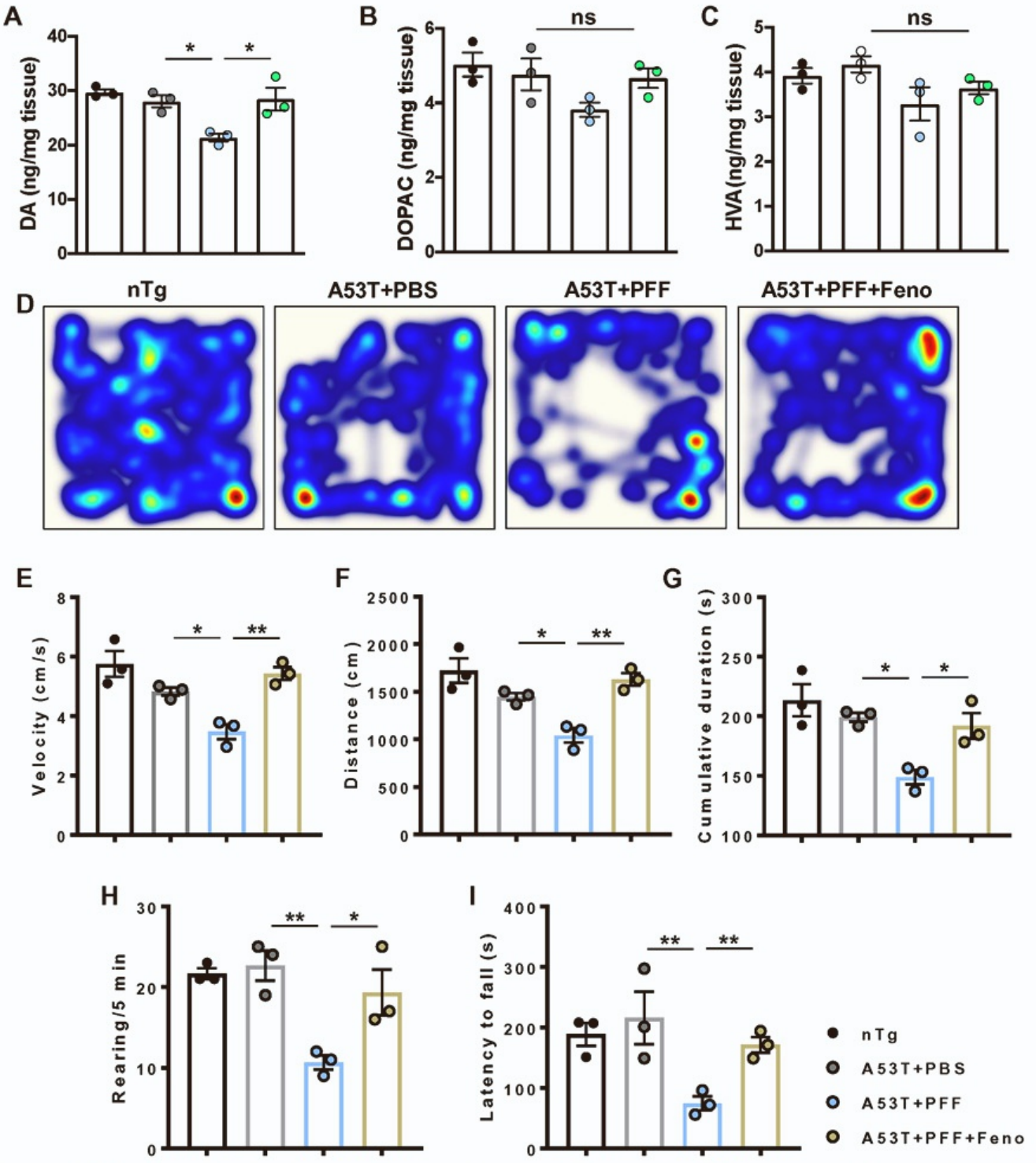

Figure S11. Related to Figure 7. Fenofibrate attenuates PFF-induced loss of striatal DA and behavioral deficits in A53T mice. The content of DA and its metabolites DOPAC and HVA in striatum of PFF-seeded control and fenofibrate-treated mice was measured by HPLC-ECD method and the values are given as ng/mg tissue ($F_{3, 11}$=8.779, **p*< 0.05 for DA, A). No significant change was found in the DOPAC and HVA levels (B, C). Behavioral performance of experimental animals was monitored by open field test ($F_{3, 8}$=12.98, for velocity, $F_{3, 8}$=12.98, for distance, $F_{3, 8}$=8.91, for cumulative duration, $F_{3, 8}$=9.33, for rearing and $F_{3, 8}$=6.939, for rotarod, D-I). Statistical significance was measured using one-way ANOVA followed by Tukey's multiple comparison test. **p*< 0.05, ***p*< 0.01 and ****p*< 0.001 indicate significance compared to respective groups, ns, nonsignificant. Data are represented as mean ± SEM (n=3 animals per group).

Table S1: Related to Figure 3. List of antibodies used for the study

Protein	Source	Catalogue No.	Application/ Dilution	Host Species
α-Synuclein	Abcam	ab138501 (MJFR1)	IFC/1:5000	Rabbit
α-Synuclein	BD Bioscience	610787	WB/1:1000	Mouse
Phospho Ser129 α-synuclein	Abcam	ab51253 (EP1536Y)	IHC/1:5000	Rabbit
Tyrosine hydroxylase (TH)	Pel-Freeze Biologicals	P40101	WB/1:2000 IFC/1:1000 IHC/1:1000	Rabbit
PPARα	Santacruz	sc-398394	WB/1:1000 IFC/1:250	Mouse
PPARβ	Santacruz	sc-74517	WB/1:1000 IFC/1:200	Mouse
PPARγ	Santacruz	sc-7273	WB/1:1000 IFC/1:200	Mouse
PGC1α	Millipore	AB3242	IFC/1:1000	Rabbit
TFEB	Abcam	ab2636	WB/1:1000 IFC/1:200	Goat
Lamp2	Millipore	MABC40	WB/1:1000 IFC/1:200	Rat
Cathepsin D	Santacruz	sc-6487	WB/1:1000	Goat
TPP1	Abcam	ab54685	WB/1:1000 IFC/1:300	Mouse
Actin	Abcam	ab8226	WB/1:10000	Mouse

WB, Western blot; IFC, Immunofluorescence, IHC, Immunohistochemistry

Table S2: Related to Figure 5. Sequence of primers used for the study

Gene name	Sequence of primers
SQSTM1	Sense: 5'-ATACCCACATCTCCCACCAGA-3' Antisense: 5'-ACAATGGTGGAGGGTGCTTC-3'
Cathepsin B	Sense: 5'-GGGTGCCTTCACTGTGTTTT-3' Antisense: 5'-ATGCCACAGTGGTTCTCTCC-3'
Cathepsin D	Sense: 5'-GTGCCTCTTATCCAGGGTGA-3' Antisense: 5'-ATTCCCATGAAGCCACTCAG-3'
LC3B	Sense: 5'-CCGAGAAGACCTTCAAGCAG-3' Antisense: 5'-CCATTCACCAGGAGGAAGAA-3'
VPS11	Sense: 5'-TCTGGCTGAGAATCATGCAC-3' Antisense: 5'-GGTTGTCTGCTCCGGTATGT-3'
VPS8	Sense: 5'-GTTCTGGACCCACAGCAAAT-3' Antisense: 5'-TCCTTCTGGAAGACGCTGTT-3'
Lamp1	Sense: 5'-GATGAATGCCAGCTCTAGCC-3' Antisense: 5'-CTGGACCTGCACACTGAAGA-3'
Lamp2	Sense: 5'-CACCCACTCCAACTCCAACT-3' Antisense: 5'-TTGTGGCAGGGTTGATGTTA-3'
ATG9B	Sense: 5'-ATGGCTTTGCCTGTATCCTG-3' Antisense: 5'-AGGAAGACCAACAGGGGACT-3'
UVRAG	Sense: 5'-GCAGAAGGACTCCCTGAGTG-3' Antisense: 5'-GGAAGTCCTCGGAATTAGGC-3'
AMBRA1	Sense: 5'-TAGTCCACGCTCGACCTTCT-3' Antisense: 5'-TGTGAACCAGCTTTCACTGC-3'
CLN2	Sense: 5'-TCGGATCCTAGCTCTCCTCA-3' Antisense: 5'-AAAGTCCTGGGTGGTCACTG-3'
TMEM166	Sense: 5'-CGAGCCGCTCTGTACTTTGT-3' Antisense: 5'-GTGTCCTCGCTACCATCCTC-3'
GBA1	Sense: 5'-ACCTACAGCAGGGCTCTTCA-3' Antisense: 5'-CAGCAAGCGTTGGTCATCTA-3'
GABARAP	Sense: 5'-TCCCGGTGATAGTGGAAAAA-3' Antisense: 5'-TGGGTGGAATGACATTGTTG-3'
ATG4A	Sense: 5'-CCGGATACAGATGAGCTGGT-3' Antisense: 5'-CATCAGATGAAGGGCCTGTT-3'
ATG4B	Sense: 5'-TGGGTGTTATTGGAGGGAAG-3' Antisense: 5'-CAGAAAAACCCCACAGCAAT-3'
ATG7	Sense: 5'-TCCGTTGAAGTCCTCTGCTT-3' Antisense: 5'-CCACTGAGGTTCACCATCCT-3'
Beclin1	Sense: 5'-GGCCAATAAGATGGGTCTGA-3' Antisense: 5'-GCTGCACACAGTCCAGAAAA-3'
ATP6v0d1	Sense: 5'-CAATGAAGCGTCACCTCTGA-3' Antisense: 5'-TCAGCTATTGAACGCTGGTG-3'
NPC	Sense: 5'-GGGATGCCCGTGCCTGCAAT-3' Antisense: 5'-CTGGCAGCTACATGGCCCCG-3'
CLN3	Sense: 5'-TGCTGCCCTGCCATCGAGTG-3' Antisense: 5'-GGCAGCGCTCAGCATCACCA-3'
TFEB	Sense: 5'-AAC AAA GGC ACC ATC CTC AA-3' Antisense: 5'-CAG CTC GGC CAT ATT CAC AC-3'