Reduced Interaction of VAMP2 and Aggregated α-synuclein by Environmental Enrichment Alleviates Hyperactivity and Anxiety in a Model of Parkinson's Disease.

Kyungri Kim
Yonsei University College of Medicine
Soohyun Wi
Yonsei University College of Medicine
Jung Hwa Seo
Yonsei University College of Medicine
Soonil Pyo
Yonsei University College of Medicine
Sung-Rae Cho (srcho918@yuhs.ac)
Yonsei University College of Medicine https://orcid.org/0000-0003-1429-2684

Research

Keywords: Parkinson's disease, Environmental enrichment, Alpha-synuclein, VAMP2, Nucleus accumbens

Posted Date: August 27th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-66186/v1

Version of Record: A version of this preprint was published at Genes on March 10th, 2021. See the published version at https://doi.org/10.3390/genes12030392.

Abstract

Background

Although Parkinson's disease (PD) is one of the prevalent motor diseases caused by the accumulation of mutated α-Synuclein (α-Syn), it is characterized by non-motor symptoms such as olfactory loss, cognitive decline, depression and anxiety in the early stage of the disease. Environmental enrichment (EE) provides a complex environment comprising physical, cognitive, and social stimuli that improve synaptic plasticity and behavioral functions. The therapeutic effects of EE on behavioral recovery of motor function in a mouse model of PD pathology have been reported. However, the effects of EE on non-motor symptoms in human A53T α-Syn overexpressing transgenic (hA53T α-Syn) mice have yet to be determined. In this study, we revealed the beneficial effect of EE on changes in synaptic plasticity in the striatum and nucleus accumbens (NAc) during the initial phase of PD.

Method

To investigate therapeutic effects of EE in abnormalities during the early phase of PD, we randomly assigned eight-month-old hA53T α-Syn mice to either EE (PD-EE) or standard conditions (PD-SC) for two months. Next, we performed behavioral tests, biochemical and histological analysis at 10 months of age.

Results

EE significantly alleviated locomotor hyperactivity and anxiety in the early stage of PD. EE normalized the level of tyrosine hydroxylase (TH), phosphorylated and oligomeric forms of α-Syn (pSer129 α-Syn), and SNARE complex-forming proteins including SNAP-25, Syntaxin1 and VAMP2. Moreover, the close link between VAMP2 and pSer129 α-Syn was significantly reduced after exposure to EE.

Conclusions

Our results showed that EE attenuates aversive mood state on the early stage of PD mouse model. These results were parallel with the reduced expression of pathological α-Syn and the increased expression of neurotransmitter transporter proteins in EE. Interestingly, the interaction between pSer129 α-Syn and VAMP2 also decreased in EE. The restoration of synaptic vesicle transportation status may be responsible for the neuroprotective effects of EE in hA53T α-Syn mice.

Background

α-Synuclein (α-Syn) is the main constituent of the neuropathological lesions found in patients with Parkinson's disease (PD), Lewy body dementia, multiple system atrophy and other disorders collectively known as α-synucleinopathies (1–3). The point mutation A53T in α-Syn has been identified in the familiar form of PD, and A53T mutant α-Syn is associated with early stages of PD and Lewy bodies with increased phosphorylated α-Syn (4, 5). PD is typically defined by gradual destruction of the dopaminergic nigrostriatal pathway and by the occurrence of rigidity, tremor, and bradykinesia (6). However, PD also

presents non-motor symptoms, involving loss of olfactory senses, cognitive decline, sleep disorders, gastrointestinal disorders, sensory disorders, depression, and anxiety, which often appear in the early stages including the pre-motor phase (7).

The pathogenesis of PD is associated with the dysfunction of various brain regions such as substantia nigra, striatum, and cortex with the increased degree of alpha-synuclein pathology (8). Among those brain regions, the nucleus accumbens (NAc), a part of ventral striatum, is the brain region that is mainly responsible for reward and emotional processes and has been implicated in psychiatric and neurodegenerative diseases such as PD (9–11). Previous studies have shown that the manifestation of non-motor symptoms is related to neurochemical changes and the brain reward circuit including the NAc in PD (9, 12). Given the increasing interest in the NAc, various treatments and interventions to this brain region have been conducted to alleviate the non-motor symptoms of PD (13–15).

Environmental enrichment (EE) is a way of breeding animals in an enormous cage containing running wheels, novel objects, and providing social interactions as a form of a complex stimuli mixture of physical, cognitive, and social experiences (16). In clinical studies, EE is used as a rehabilitation therapy for human patients (17, 18). Epidemiological studies supported a link between hard exercise and lessened risk for PD (19, 20). Additionally, many studies about the effect of exercise on normal aging or PD supported the advantages of exercise, physical activity, and EE (21–23). The therapeutic effects of EE in behavioral recuperation of motor function on a mouse model of PD pathology induced by the administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydrophyridine (MPTP) have been reported (24, 25). However, there are no studies on the effects of EE on non-motor symptoms during the pre-motor phase in mice overexpressing human A53T α-Syn (hA53T α-Syn). Through this transgenic mouse model of PD, our study showed the influence of EE on the induction of synaptic plasticity in the striatum and NAc in the initial phase of PD.

Materials And Methods

Mice

The hA53T α-Syn transgenic line G2-3 (B6.Cg-Tg (Prnp-SNCA*A53T) 23Mkle/J; Jackson Laboratories, stock no. 006823, Bar Harbor, ME, USA) was used to generate both wild-type (WT) and transgenic mice. The transgenic mice bred heterozygous progeny that overexpressed one copy of hA53T α -Syn. All animals were raised in a facility qualified by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and provided food and water ad libitum with 12-hour light/dark cycles, according to animal protection regulations. The experimental procedures were permitted by the Yonsei University Health System Institutional Animal Care and Use Committee (YUHS-IACUC approval number; 2017-0039).

Genotyping

Genotyping of mice was done based on a manufacturer's protocol from Jackson Laboratories. Genomic DNA was obtained from a 2-mm piece of each mouse tail using the prepGEM Tissue Kit (ZyGEM, New Zealand). The mouse tail tissue was incubated with 1 µL of prepGEM, 10 µL of Buffer Gold, and 89 µL of autoclaved 3' distilled water at 75 °C for 15 minutes and 95 °C for 5 minutes. The following primers were used for polymerase chain reaction (PCR): transgene forward, 5'-TCA TGA AAG GAC TTT CAA AGG C-3'; transgene reverse, 5'-CCT CCC CCA GCC TAG ACC-3' (transgene = ~ 500 bp); internal positive control forward, 5'-CTA GGC CAC AGA ATT GAA AGA TCT-3'; and internal positive control reverse, 5'-GTA GGT GGA AAT TCT AGC ATC C-3' (internal positive control = 324 bp). Electrophoresis was performed by loading 10 µL of each PCR product on a 1.5% agarose gel.

Housing conditions

At 10 months of age, mice were randomly separated into standard cage (SC) or EE. The standard housing environments with common housing cage ($27 \times 22.5 \times 14$ cm^3). The control mice were housed for two months in standard cages (5 mice/cage). EE mice were housed in a large cage ($86 \times 76 \times 31$ cm^3) including tunnels, shelters, toys and running wheels for voluntary exercise, and conditions allowing for social interactions (10 mice/cage) for the same duration.

Behavioral assessment

Grip strength test

A grip strength test was exerted using the SDI Grip Strength System (San Diego Instruments Inc., San Diego, CA), which consist of a push-pull strain gauge. Each animal grasped a triangular metal wire 2 mm in diameter with its forepaws, then pulled its tail until the animal lose the bar. The machine automatically recorded peak force in gram-force.

Hanging wire test

Mice held by their forepaws from a horizontal rod (5×5 mm^2 area, 35 cm long, between two poles 50 cm high) tend to assist themselves with their hind limbs to prevent themselves from falling and to aid their progression along the rod. For this test, suspension latencies were recorded for five minutes (26).

Cylinder test

When a mouse is placed in the cylinder, it will spontaneously rear and use its forepaws for support. For this test, the number of each forelimb touched the cylinder wall (Jeung Do B&P, Seoul, Korea) while the animal was rearing was counted over a period of five minutes (27).

Open field test

An open field test was performed for 25 minutes when mice were 10 months of age to determine whether EE exposure influenced locomotor activity. Activity was recorded in a square area measuring $30 \times 30 \times 30$ cm^3. The total distance traveled by mouse was recorded during 25 minutes as an index of hyperactivity (28). The area's floor was composed of 16 sectors. The four inner sectors represented the

center, the 12 outer sectors were described as the periphery. Total time spent in four inner sections was recorded as an sign of anxiety (29, 30). Mice were individually put into the periphery of the area and let to explore spontaneously for 25 minutes while being recorded with a video camera. The resulting data were examined using the Smart Vision 2.5.21 (Panlab, Barcelona, Spain) video tracking system.

RNA extraction

Mice were sacrificed perfused with cold 1x PBS at 10 months after birth. Total RNA was extracted from the striatum and NAc of mice brains using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Extracted RNA purity was assessed using the Nanodrop-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Purified total RNA (1 μg) was used as a template to produce the complementary DNA (cDNA) using The ReverTra Ace qPCR RT master mix with gDNA remover (TOYOBO, Osaka, Japan).

Quantitative real time polymerase chain reaction (qRT-PCR)

The following reaction used 1 μL of cDNA in a total volume of 20 μL. qRT-PCR was done in triplicate on a LightCycler 480 (Roche Applied Science, Mannheim, Germany) using the LightCycler 480 SYBR Green master mix (Roche Applied Science, Mannheim, Germany), and the thermocycler conditions were as follows: amplifications were started with a 5-minute template preincubation step at 95 °C, followed by 40 cycles at 95 °C for 20 seconds, 62 °C for 20 seconds, and 72 °C for 15 seconds. Melting curve analysis initiated at 95 °C for five seconds, followed by one minute at 60 °C. The specificity of the product was confirmed by melting curve analysis, which showed a distinctive single sharp peak with the expected Tm for all samples. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as the internal control. The expression level of each gene of interest was acquired using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Striatum and NAc were lysed in 500 μL of cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate) with a protease inhibitor cocktail (Sigma Aldrich, St Louis, MO, USA). Tissue lysates were centrifuged at 13,000 rpm at 4 °C for 20 minutes, the supernatant was extracted, and it was measured by the Bradford method. Extracted protein (50 μg) was dissolved in sample buffer (Invitrogen Life Technologies, Carlsbad, CA, USA), incubated for 10 minutes at 80 °C, and separated on a 4–12% SDS reducing polyacrylamide gel (Invitrogen Life Technologies, Carlsbad, CA, USA). Separated proteins were equally loaded and moved to polyvinylidene difluoride membranes (Invitrogen Life Technologies, Carlsbad, CA, USA) using a trans-blot system (NovexR Mini-Cell; Invitrogen Life Technologies, Carlsbad, CA, USA). Blots were blocked for one hour in Tris-buffered saline (TBS) (10 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 5% nonfat dry milk (Bio-Rad, Berkeley, CA, USA) at room temperature and washed three times with TBS. Blots were then incubated at 4 °C overnight with the following antibodies: synaptosomal-associated protein, 25 kDa (SNAP-25, 1:1,000, Abcam, Cambridge, UK); syntaxin1 (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA); vesicle-associated membrane protein 2 (VAMP2, 1:1,000, Synaptic systems, Goettingen, Germany); dopamine transporter (DAT, 1:1,000, Chemicon, Pemecula, CA, USA); dopamine receptor D1 (DRD1,

1:1,000, Proteintech, Manchester, UK); dopamine receptor D2 (DRD2, 1:1,000, Abcam, Cambridge, UK); human α-Syn (1:1,000, Abcam, Cambridge, UK); human pSer129 α-Syn (1:1,000, Abcam, Cambridge, UK); and tyrosine hydroxylase (TH, 1:1,000, Sigma Aldrich, St Louis, MO, USA) in TBST (10 mM Tris pH 7.5, 150 mM NaCl, and 0.02% Tween 20) with 3% nonfat dry milk. After incubation, the blots were rinsed three times with TBST and incubated for one hour with horseradish peroxidase-conjugated secondary antibodies (1:3,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature. A housekeeping gene was evaluated with an Actin antibody (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with TBST, blots were visualized with an ECL detection system (Amersham Pharmacia Biotech, Little Chalfont, UK). The western blot results were analyzed using Multi Gauge (Fuji photo firm, version 3.0, Tokyo, Japan).

Immunohistochemistry (IHC)

Animals were euthanized and perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. Brains were removed and post-fixed for 1 hour, followed by cryoprotection in 30% sucrose in TBS containing 0.02% sodium azide. Harvested brain tissue was cryo-sectioned with a slice thickness of 16 µm along the sagittal or coronal plane and IHC was performed on four sections. For immunofluorescence double labeling, sections were stained with the following antibodies: human α-Syn (1:400, Abcam, Cambridge, UK); human pSer129 α-Syn (1:100, Abcam, Cambridge, UK); DAT (1:400, Chemicon, Pemecula, CA, USA), and secondary antibodies such as Alexa Fluor® 488 goat anti-rabbit (1:400, Invitrogen, Carlsbad, CA, USA), Alexa Fluor® 594 anti-mouse (1:400, Invitrogen), and Alexa Fluor® 594 anti-rat (1:400, Invitrogen). Sections were mounted on glass slides with fluorescent mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI; Vectorshield, Vector, Burlingame, CA, USA). To examine the expression of the TH, sections were permeabilized with 1% bovine serum albumin (BSA), 0.5% Triton-X in PBS for 30 minutes. After overnight incubation with TH antibody (1:2000, Sigma Aldrich, St Louis, MO, USA), the sections were washed with 0.1M phosphate buffer solution and biotinylated with alkaline phosphatase-conjugated secondary antibody (1:400, Vectorshield, Vector, Burlingame, CA, USA) for 1 hour. Followed by the peroxidase with avidin-biotin complex with phosphate buffer solution (1:200, Vectorshield, Vector, Burlingame, CA, USA), the immunostaining visualized with 0.01% of 3,3-diaminobenzidine (DAB; Sigma Aldrich, St Louis, MO, USA) and 0.012% of H_2O_2 in phosphate buffer solution. Stained sections were examined using a fluorescent microscope (Axio Imager M2, Zeiss, Gottingen, Germany) and confocal microscopy (LSM700, Zeiss, Gottingen, Germany).

In situ proximity ligation assay (*In situ* PLA)

Animals were euthanized and perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. Brains were removed and post-fixed for 1 hour, followed by cryoprotection in 30% sucrose in TBS containing 0.02% sodium azide. Harvested brain tissue was cryo-sectioned with a slice thickness of 16 µm along the sagittal or coronal plane. The sections were stained with VAMP2 (1:400, Abcam, Cambridge, UK) 1:1,000, Synaptic systems) and human pSer129 α-Syn (1:1,000, FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) primary antibodies overnight at 4 °C to detect interacting VAMP2 and human

pSer129 α-Syn proteins. After rinsing, the sections were simmered with the secondary oligonucleotide-linked antibodies (The Duolink® kit, Olink Bioscience, Uppsala, Sweden) provided in the kit. The oligonucleotides attached to the antibodies were detected using a fluorescent probe (Detection Kit 563). The specks were detected by confocal imaging (LSM700, Zeiss, Oberkochen, Germany).

Statistical analysis

Statistical analysis was conducted using Statistical Package for Social Sciences (SPSS) software IBM Corp., released 2017. IBM SPSS Statistics for Windows (Version 25.0. Armonk, NY: IBM Corp.). Data are expressed as the mean ± standard error of the mean (SEM). The results of behavioral tests, qRT-PCR, western blot, and immunohistochemistry were analyzed by the one-way ANOVA followed by a post-hoc Bonferroni to adjust the variance for multiple testing effects (WT, PD-SC, and PD-EE). A P-value < 0.05 was considered statistically significant.

Results

EE ameliorates hyperactivity and anxiety, but not motor function, in hA53T α-Syn transgenic mice

Eight-month-old WT or hA53T α-Syn mice were randomly allocated to either the EE group or SC group (n = 15–25 per each group) for two months (Fig. 1a and b). Tests of motor function were performed to determine whether there were motor symptoms in hA53T α-Syn mice at 10 months of age. Grip strength test results showed did not differ significantly between WT (n = 5, 120.20 ± 2.13 g × force), PD-SC (n = 10, 117.50 ± 3.45 g × force), and PD-EE (n = 10, 119.50 ± 1.55 g × force) mice (Fig. 1c). The hanging wire test endurance time did not differ significantly between WT (n = 5, 14.00 ± 4.46 seconds), PD-SC (n = 10, 12.17 ± 2.50 seconds), and PD-EE (n = 10, 13.77 ± 1.78 seconds) mice (Fig. 1d). The cylinder test count did not significantly differ between WT (n = 10, 15.60 ± 2.37), PD-SC (n = 10, 21.60 ± 2.32), and PD-EE (n = 16.80 ± 2.21) mice (Fig. 1e).

The open field test is commonly used to estimate locomotor activity and spontaneous exploration in a new environment (31, 32). During the 25 minutes, total distance traveled significantly increased in PD-SC (n = 15, 38,313 ± 1,614) compared to WT (n = 12, 14,377 ± 512 cm) mice (Fig. 1f). However, the total distance significantly decreased in PD-EE (n = 12, 21,910 ± 2,274 cm) compared to PD-SC mice, and increased in PD-EE (n = 12, 21,910 ± 2,274 cm) compared to WT mice.

To determine whether EE exposure affected anxiety, the percentage of spending time in the inner zone compared to the outer zone (Fig. 1g). The percentage was significantly decreased in PD-SC (n = 12, 7.3 ± 0.8%) compared to WT (n = 10, 20.2 ± 2.7%) mice. However, the percentage significantly increased in PD-EE (n = 11, 13.1 ± 2.4%) compared to PD-SC mice, and decreased in PD-EE (n = 11, 13.1 ± 2.4%) compared to WT mice.

EE reduces degeneration of dopaminergic nerve terminals in NAc of hA53T α-Syn mice

To investigate whether dopaminergic neurons in the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) were degenerated at 10 months of age, immunostaining was performed for analysis of TH-positive cells (Fig. 2a). The immunostaining results showed that TH-positive cell bodies (% of WT) in SNpc did not change significantly among the three groups: WT (n = 8, 100.00% ± 8.05), PD-SC (n = 7, 95.19% ± 6.41), and PD-EE (n = 3, 95.05% ± 3.73). Also, TH-positive cell bodies (% of WT) in the VTA did not differ significantly between the three groups: WT (n = 5, 100.00% ± 13.26), PD-SC (n = 3, 104.74% ± 5.07), and PD-EE mice (n = 3, 96.54% ± 5.07) (Fig. 2b).

Immunostaining was performed for analysis of TH density to investigate dopaminergic nerve terminals in the striatum and NAc were degenerated at 10 months of age and the effects of EE exposure on dopaminergic nerve terminals. Immunostaining results showed that dopaminergic nerve terminals in the striatum decreased significantly in PD-SC (n = 5, 57.11% ± 4.40) and PD-EE (n = 5, 95.38% ± 6.82) compared to WT (n = 5, 100.00% ± 5.31) mice (Fig. 2c).

EE increases SNARE expression and alters the expression of dopamine transporter and dopamine receptor in NAc of hA53T α-Syn mice

qRT-PCR and western blot showed the effects of EE on the expression of SNARE genes and associated proteins SNAP-25, Syntaxin1, and VAMP2 in the striatum and NAc of 10-month-old hA53T α-Syn mice. The qRT-PCR results showed that the decreased level of SNAP-25, Syntaxin1, and VAMP2 mRNA expression levels in PD-SC (SNAP-25; n = 4, 0.74 ± 0.04, Syntaxin1; n = 4, 0.39 ± 0.03, VAMP-2; n = 3, 0.64 ± 0.07) tended to restored in PD-EE (SNAP-25; n = 4, 0.93 ± 0.04, Syntaxin1; n = 3, 0.56 ± 0.02, VAMP2; n = 3, 0.89 ± 0.04) compared to WT (SNAP-25; n = 4, 1.00 ± 0.06, Syntaxin1; n = 4, 1.00 ± 0.05, VAMP2; n = 4 1.00 ± 0.06) mice (Fig. 3a). Western blot results indicated that relative protein levels of SNARE proteins significantly increased in PD-EE (n = 5, 0.84 ± 0.01, 0.74 ± 0.01, 1.01 ± 0.01) compared to PD-SC (n = 5, 0.77 ± 0.01, 0.61 ± 0.01, 0.67 ± 0.03) (Fig. 3b).

Next, we investigated the effects of EE on expression of dopamine receptors and dopamine transporters such as DRD1, DRD2, and DAT in the striatum and NAc of 10-month-old hA53T α-Syn mice. The RT-qPCR results showed that the relative mRNA expression of DRD1 was significantly decreased in PD-EE (n = 4, 1.17 ± 0.08) compared to PD-SC (n = 4, 1.53 ± 0.12) mice, but the expression of DAT significantly increased in PD-EE (n = 4, 0.69 ± 0.09) compared to PD-SC (n = 4, 0.28 ± 0.03) mice (Fig. 3c). The expression of DRD2 in PD-SC (n = 3, 1.24 ± 0.10) and PD-EE (n = 4, 1.08 ± 0.07) mice did not differ significantly. Western blot results showed that the relative protein expression of DRD1 was significantly decreased in PD-EE (n = 5, 0.72 ± 0.03) compared to PD-SC (n = 5, 0.72 ± 0.03) mice (Fig. 3d). The relative protein expression of DAT was significantly increased in PD-EE (n = 5, 0.77 ± 0.05) compared to PD-SC (n = 5, 0.6 ± 0.05) mice. Expression of DRD2 in PD-SC (n = 5, 0.87 ± 0.2) and A53T EE (n = 5, 0.77 ± 0.05) mice did not differ significantly.

EE reduces aggregated α-Syn and the interaction between α-Syn to VAMP-2 in NAc of hA53T α-Syn mice

Next, we examined if EE can block α-Syn aggregation since the A53T mutation of α-Syn is known to increase its tendency to aggregate (33). The intensity of α-Syn monomer band (14-kDa) was increased in PD-SC and PD-EE then WT (Fig. 4a). Monomeric and α-Syn aggregates slightly decreased in PD-EE(n = 5, 59.59 ± 21.91, 2.24 ± 0.91) then PD-SC (n = 5, 222.70 ± 50.2, 22.46 ± 8.36). Interestingly, these tendencies that α-Syn monomer in PD-EE was higher than PD-SC was consistent with the result exercise reduced α-Syn oligomer but not monomer in human α-Syn expressing transgenic mice (34).

IHC was performed to investigate the effects of EE on aggregation of α-Syn in striatum and NAc in 10-month-old mice. Both of IHC results showed that pSer129 α-Syn counts tended to decrease in PD-EE (striatum; n = 6, 42.85 ± 7.48, NAc; n = 5, 48.80 ± 22.82, pSer129 α-Syn count / mm^3) contrast to PD-SC (striatum; n = 8, 78.50 ± 15.98, NAc; n = 6, 162.67 ± 34.02, pSer129 α-Syn count / mm^3) mice (Fig. 4b).

Previous studies showed that pSer129 α-Syn directly bound to the SNARE-protein VAMP2 (35), and α-Syn overexpressed mice showed inhibited intersynaptic vesicle mobility and trafficking (36). To confirm that EE can affect to the interaction between pSer129 α-Syn and VAMP2, *in situ* PLA assay was conducted in striatum and NAc of PD-SC or PD-EE groups. Red puncta showed the complexes formed between pSer129 α-Syn and VAMP2. Abundant signal was observed in PD-SC of NAc (n = 4, 0.27 ± 0.06), but not in PD-EE group (n = 3, 0.11 ± 0.03) (Fig. 4c). There were no differences between PD-SC (n = 4, 0.19 ± 0.01) and PD-EE (n = 4, 0.13 ± 0.03) in striatum of the hA53T α-Syn mice. We confirm that EE can reduce the interaction of VAMP2 and pSer129 α-Syn in NAc by *in situ* PLA assay.

Discussion

Motor impairments in PD patients are primarily due to a 50 to 70% loss of dopamine neurons in the SNpc (37). The mouse model used in this study, which expresses hA53T α-Syn under the mouse Prnp promoter, does not show degeneration in the SNpc even at the age of 13- to 14-months (38). Dopaminergic neurons in the striatum of 10-month-old hA53T α-Syn mice showed only mild degeneration (~ 20%) compared to WT mice.

The pathogenesis of PD is associated with the increased degree of alpha-synuclein pathology (8). Previous studies have shown that the manifestation of non-motor symptoms like depression and anxiety is related to neurochemical changes and the brain reward circuit including the NAc in PD (9, 12). Anxiety is frequent and can be the starting signals of the disease before the onset of motor defects in PD (39, 40). The dysregulation of dopamine levels in NAc related with hyperactivity disorder (41).

The abnormal accumulation of Lewy bodies is the characteristic of Parkinson's disease and is associated with many neurodegenerative diseases. The recent study has revealed that raised the level of impulsive compulsive behaviors in PD is associated with the increased expression of α-Syn proteins and excessive stimulation of dopamine receptors in the NAc of PD patients (42). However, the psychological roles of α-Syn have yet to be fully identified. Consistent with the previous study, the present study showed that the mice did not show motor deficits in grip strength test, hanging wire test or cylinder test, but non-motor

symptoms, like anxiety and hyperactivity were revealed in the mice as shown by the open field test results (Fig. 1).

There are numerous lines of evidence have been linked with α-Syn in the control center of neurotransmitter delivery through regulating the formation of the SNARE complex and the size of the synaptic vesicle pool (35, 36, 43, 44). The recent studies showed pSer129 α-Syn proteins can attach to SNARE proteins and interrupt their functions (35, 45). In this study, exposure to EE improved the expression of SNAP-25, Syntaxin1, and VAMP2, decreased pathological α-Syn simultaneously (Fig. 3), contrary to hA53T α-Syn mice at 10 months of age (Fig. 4a and b). Then, we confirmed that EE can ameliorate the close link of VAMP2 and pSer129 α-Syn by *in situ* PLA assay (Fig. 4c).

The upregulation of dopamine transporter is compensatory responses associated with deficient dopamine signaling (46, 47). Kurz et al. reported that the increased striatal dopamine levels in young (8 months) and old (18 months) hA53T α-Syn mice and revealed elevated striatal DRD1 and DRD2 levels in the absence of neurodegeneration (46). Furthermore, as a compensatory effect, the expression of DRD1 could be increased to receive more synaptic signals.

In this study, we explained progressively increasing striatal dopamine levels as an early effect of hA53T α-Syn overexpression prior to neurodegeneration. Our work suggests that EE exerts therapeutic effects on the early symptoms of PD, which includes hyperactivity and anxiety, mainly responsible for the expression of synaptic proteins, dopamine transporters, and dopamine receptors (Fig. 5).

Conclusion

The original functions of v- and t-SNARE proteins help synaptic vesicle to dock and fuse for exocytosis. This event can be interrupted by the aggregated α-Syn proteins in striatum and NAc of PD-SC group. On the other hand, EE can reduce the aggregated α-Syn and enhance the expression level of SNARE proteins, recovering the behavioral functions.

Abbreviations

PD: Parkinson’s disease; EE: environmental enrichment; hA53T a-Syn: human A53T α-synuclein overexpressing transgenic mouse; NAc: nucleus accumbens; TH: tyrosine hydroxylase; a-Syn: α-synuclein; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydrophyridine; WT: wild-type mice; PCR: polymerase chain reaction; SC: standard cage; qRT-PCR: quantitative reverse transcription polymerase chain reaction; TBS: tris-buffered saline; IHC: immunohistochemistry; PFA: paraformaldehyde; PLA: proximity ligation assay; SNpc: substantia nigra pars compacta; VTA: ventral tegmental area; cDNA: complementary DNA; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SNAP-25: synaptosomal-associated protein, 25kDa; VAMP2: vesicle-associated membrane protein 2; DAPI: 4’,6-diamidino-2-phenylindole; SNARE: soluble N-

ethylmaleimide-sensitive factor attachment protein receptor; DRD1: dopamine receptor D1; DRD2: dopamine receptor D2; DAT: dopamine transporter

Declarations

Acknowledgments

We thank Medical Illustration & Design, part of the Medical Research Support Services of Yonsei University College of Medicine for all artistic supports related to this work.

Authors' contributions

KK and SW equally contributed to this study. SW performed most of the experiments, analyzed data, and wrote the manuscript; KK performed most of molecular study, analyzed data, and wrote the manuscript; JHS performed animal experiments and wrote manuscript; SP wrote the manuscript and contributed to English editing; S-RC developed the study concept and design, wrote the manuscript, and supervised the project. All authors read and confirmed the manuscript.

Funding

This work was supported by grants from the National Research Foundation (NRF-2019R1I1A1A01062098), the Ministry of Science and Technology, Republic of Korea, the Korean Health Technology R&D Project (HI16C1012), the Ministry of Health & Welfare, Republic of Korea, and a faculty research grant of Yonsei University College of Medicine (6-2020-0104).

Availability of data and materials

All relevant data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval

All animals were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC 2017-0039).

Consent for publication

Not applicable.

Competing interests

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

References

1. Spillantini MG, Crowther RA, Jakes R, Cairns NJ, Lantos PL, Goedert MJNl. Filamentous α-synuclein inclusions link multiple system atrophy with Parkinson's disease and dementia with Lewy bodies. 1998;251(3):205-8.
2. Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert MJPotNAoS. α-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. 1998;95(11):6469-73.
3. Spillantini MG, Schmidt ML, Lee VM-Y, Trojanowski JQ, Jakes R, Goedert MJN. α-Synuclein in Lewy bodies. 1997;388(6645):839-40.
4. Anderson JP, Walker DE, Goldstein JM, De Laat R, Banducci K, Caccavello RJ, et al. Phosphorylation of Ser-129 is the dominant pathological modification of α-synuclein in familial and sporadic Lewy body disease. 2006;281(40):29739-52.
5. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, et al. Mutation in the α-synuclein gene identified in families with Parkinson's disease. 1997;276(5321):2045-7.
6. Jankovic JJJon, neurosurgery, psychiatry. Parkinson's disease: clinical features and diagnosis. 2008;79(4):368-76.
7. Chaudhuri KR, Schapira AHJTLN. Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. 2009;8(5):464-74.
8. Riley BE, Gardai SJ, Emig-Agius D, Bessarabova M, Ivliev AE, Schüle B, et al. Systems-based analyses of brain regions functionally impacted in Parkinson's disease reveals underlying causal mechanisms. PLoS One. 2014;9(8):e102909.
9. Carriere N, Besson P, Dujardin K, Duhamel A, Defebvre L, Delmaire C, et al. Apathy in Parkinson's disease is associated with nucleus accumbens atrophy: a magnetic resonance imaging shape analysis. Movement disorders. 2014;29(7):897-903.
10. Juárez Olguín H, Calderon Guzman D, Hernandez Garcia E, Barragán Mejía G. The role of dopamine and its dysfunction as a consequence of oxidative stress. Oxidative Medicine and Cellular Longevity. 2016;2016.
11. Russo SJ, Nestler EJ. The brain reward circuitry in mood disorders. Nat Rev Neurosci. 2013;14(9):609-25.
12. Kano O, Ikeda K, Cridebring D, Takazawa T, Yoshii Y, Iwasaki Y. Neurobiology of depression and anxiety in Parkinson's disease. Parkinson's disease. 2011;2011.
13. Iarkov A, Barreto GE, Grizzell JA, Echeverria V. Strategies for the treatment of Parkinson's disease: Beyond dopamine. Frontiers in Aging Neuroscience. 2020.
14. Salgado S, Kaplitt MG. The nucleus accumbens: a comprehensive review. Stereotactic and functional neurosurgery. 2015;93(2):75-93.
15. Williams N, Short B, Williams E, Jeffery A, Kerns S, Sahlem G, et al. Deep Brain Stimulation of the Nucleus Accumbens Has Positive Effects on Parkinson's Disease-Related Apathy (P7. 050). AAN

Enterprises; 2014.
16. Rosenzweig MR, Bennett EL, Hebert M, Morimoto HJBr. Social grouping cannot account for cerebral effects of enriched environments. 1978;153(3):563-76.
17. Janssen H, Ada L, Bernhardt J, McElduff P, Pollack M, Nilsson M, et al. An enriched environment increases activity in stroke patients undergoing rehabilitation in a mixed rehabilitation unit: a pilot non-randomized controlled trial. 2014;36(3):255-62.
18. White JH, Alborough K, Janssen H, Spratt N, Jordan L, Pollack MJD, et al. Exploring staff experience of an “enriched environment” within stroke rehabilitation: a qualitative sub-study. 2014;36(21):1783-9.
19. Chen H, Zhang S, Schwarzschild M, Hernan M, Ascherio AJN. Physical activity and the risk of Parkinson disease. 2005;64(4):664-9.
20. Xu Q, Park Y, Huang X, Hollenbeck A, Blair A, Schatzkin A, et al. Physical activities and future risk of Parkinson disease. 2010;75(4):341-8.
21. Berchtold NC, Castello N, Cotman CWJN. Exercise and time-dependent benefits to learning and memory. 2010;167(3):588-97.
22. Cotman CW, Berchtold NCJAs, Dementia. Physical activity and the maintenance of cognition: learning from animal models. 2007;3(2):S30-S7.
23. Petzinger GM, Fisher BE, Van Leeuwen JE, Vukovic M, Akopian G, Meshul CK, et al. Enhancing neuroplasticity in the basal ganglia: the role of exercise in Parkinson's disease. 2010;25(S1):S141-S5.
24. Faherty CJ, Shepherd KR, Herasimtschuk A, Smeyne RJJMbr. Environmental enrichment in adulthood eliminates neuronal death in experimental Parkinsonism. 2005;134(1):170-9.
25. Goldberg N, Haack A, Meshul CJN. Enriched environment promotes similar neuronal and behavioral recovery in a young and aged mouse model of Parkinson's disease. 2011;172:443-52.
26. Fan L-W, Lin S, Pang Y, Lei M, Zhang F, Rhodes PG, et al. Hypoxia-ischemia induced neurological dysfunction and brain injury in the neonatal rat. Behavioural brain research. 2005;165(1):80-90.
27. Im S, Yu J, Park E, Lee J, Kim H, Park KI, et al. Induction of striatal neurogenesis enhances functional recovery in an adult animal model of neonatal hypoxic-ischemic brain injury. Neuroscience. 2010;169(1):259-68.
28. Valvassori SS, Varela RB, Quevedo J. Animal models of mood disorders: focus on bipolar disorder and depression. Animal Models for the Study of Human Disease: Elsevier; 2017. p. 991-1001.
29. Carola V, D'Olimpio F, Brunamonti E, Mangia F, Renzi P. Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. Behavioural brain research. 2002;134(1-2):49-57.
30. Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. European journal of pharmacology. 2003;463(1-3):3-33.
31. Denenberg VH. Open-field behavior in the rat: What does it mean? Annals of the New York Academy of Sciences. 1969;159(3):852-9.

32. Walsh RN, Cummins RA. The open-field test: a critical review. Psychological bulletin. 1976;83(3):482.
33. Ostrerova-Golts N, Petrucelli L, Hardy J, Lee JM, Farer M, Wolozin B. The A53T α-synuclein mutation increases iron-dependent aggregation and toxicity. Journal of Neuroscience. 2000;20(16):6048-54.
34. Zhou W, Barkow JC, Freed CR. Running wheel exercise reduces α-synuclein aggregation and improves motor and cognitive function in a transgenic mouse model of Parkinson's disease. PloS one. 2017;12(12).
35. Burré J, Sharma M, Tsetsenis T, Buchman V, Etherton MR, Südhof TC. α-Synuclein promotes SNARE-complex assembly in vivo and in vitro. Science. 2010;329(5999):1663-7.
36. Scott D, Roy S. α-Synuclein inhibits intersynaptic vesicle mobility and maintains recycling-pool homeostasis. Journal of Neuroscience. 2012;32(30):10129-35.
37. Fearnley JM, Lees AJ. Ageing and Parkinson's disease: substantia nigra regional selectivity. Brain. 1991;114(5):2283-301.
38. Daher JPL, Pletnikova O, Biskup S, Musso A, Gellhaar S, Galter D, et al. Neurodegenerative phenotypes in an A53T α-synuclein transgenic mouse model are independent of LRRK2. Human molecular genetics. 2012;21(11):2420-31.
39. Pellicano C, Benincasa D, Pisani V, Buttarelli FR, Giovannelli M, Pontieri FE. Prodromal non-motor symptoms of Parkinson's disease. Neuropsychiatric disease and treatment. 2007;3(1):145.
40. Shiba M, Bower JH, Maraganore DM, McDonnell SK, Peterson BJ, Ahlskog JE, et al. Anxiety disorders and depressive disorders preceding Parkinson's disease: a case-control study. Movement disorders: official journal of the Movement Disorder Society. 2000;15(4):669-77.
41. Dagher A, Robbins TW. Personality, addiction, dopamine: insights from Parkinson's disease. Neuron. 2009;61(4):502-10.
42. Barbosa P, Hapuarachchi B, Djamshidian A, Strand K, Lees AJ, de Silva R, et al. Lower nucleus accumbens α-synuclein load and D3 receptor levels in Parkinson's disease with impulsive compulsive behaviours. Brain. 2019;142(11):3580-91.
43. Darios F, Ruiperez V, Lopez I, Villanueva J, Gutierrez LM, Davletov B. α-Synuclein sequesters arachidonic acid to modulate SNARE-mediated exocytosis. EMBO reports. 2010;11(7):528-33.
44. Fernandez-Chacon CSGG, TC RSOS. Alpha-synuclein cooperates with CSPalpha in preventing neurodegeneration. Cell. 2005;123(3):38396.
45. Garcia-Reitböck P, Anichtchik O, Bellucci A, Iovino M, Ballini C, Fineberg E, et al. SNARE protein redistribution and synaptic failure in a transgenic mouse model of Parkinson's disease. Brain. 2010;133(7):2032-44.
46. Kurz A, Double KL, Lastres-Becker I, Tozzi A, Tantucci M, Bockhart V, et al. A53T-alpha-synuclein overexpression impairs dopamine signaling and striatal synaptic plasticity in old mice. PloS one. 2010;5(7).
47. Seeman P, Niznik HB. Dopamine receptors and transporters in Parkinson's disease and schizophrenia. The FASEB Journal. 1990;4(10):2737-44.

Figures

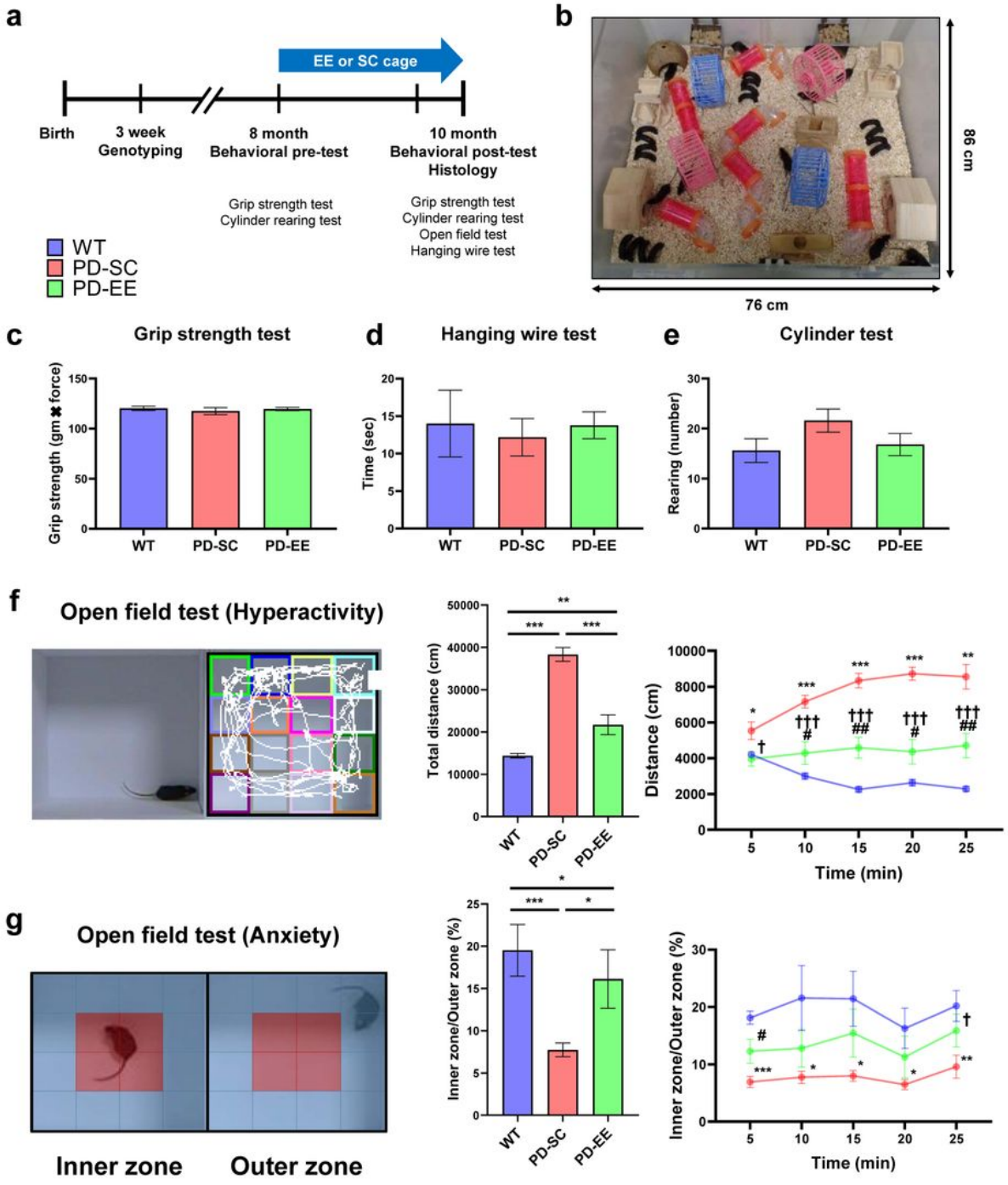

Figure 1

Environmental enrichment ameliorates hyperactivity and anxiety, but not motor function, in hA53T α-Syn mice. (a) Experimental scheme. (b) Environmental enrichment (86 × 76 × 31 cm3). (c) The grip strength test. (d) The hanging wire test. (e) The cylinder test (f, g) Open field test that conducted in a square area

(30 × 30 × 30 cm3). The floor was divided into 16 sectors. The four red sectors were the inner zone, and the 12 blue sectors were the outer zone. (f) Open field test (Hyperactivity). (g) Open field test (Anxiety). *P < 0.05, **P < 0.01, and *P < 0.001 WT versus PD-SC. †P < 0.05, ††P < 0.01, and †††P < 0.001 WT versus PD-EE. #P < 0.05, ##P < 0.01, and ###P < 0.001 PD-SC versus PD-EE. Data in all panels represent mean ± SEM. SC: standard cage, EE: environmental enrichment, WT: wild-type

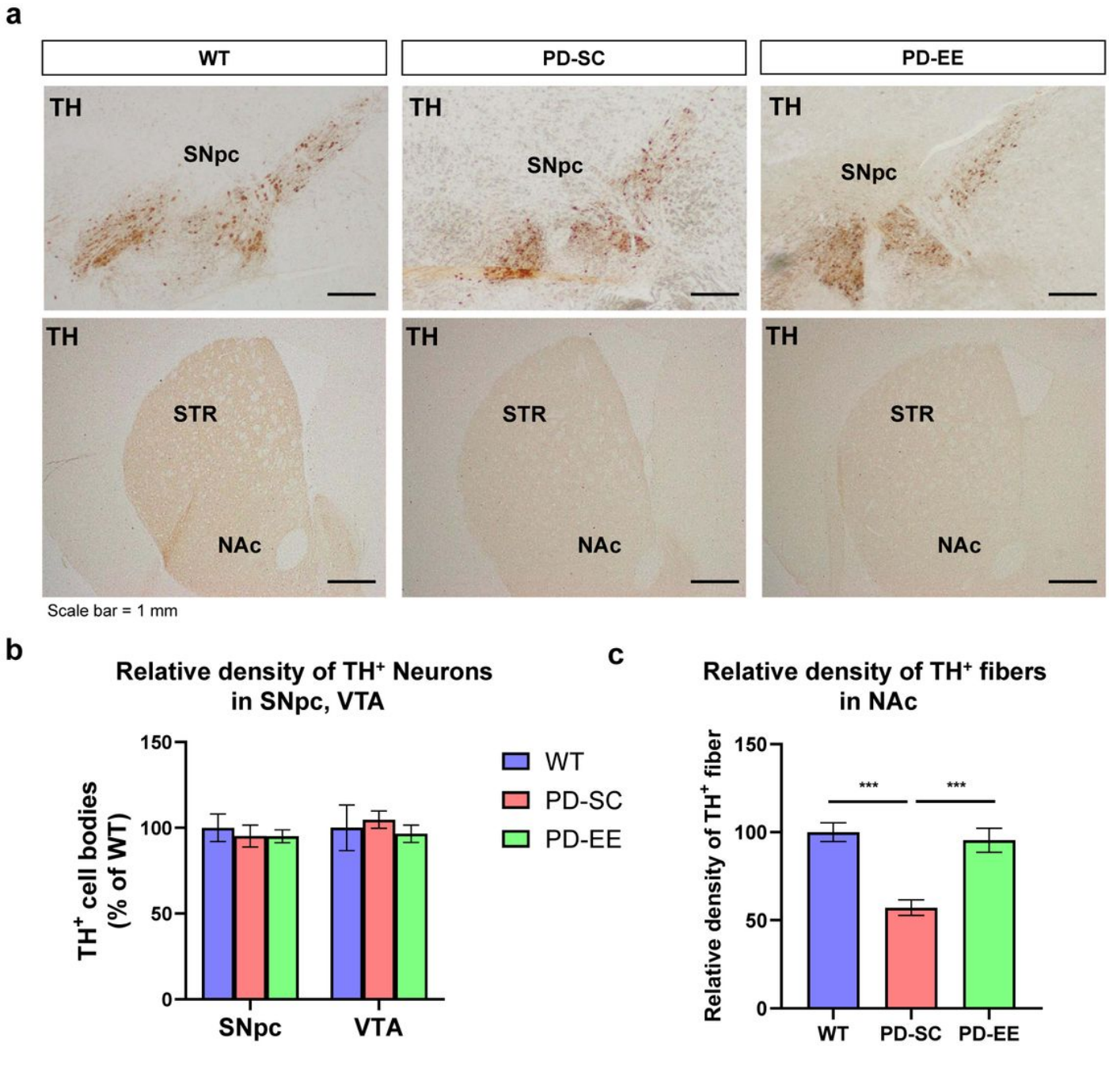

Figure 2

EE reduces the degeneration of dopaminergic nerve terminals in NAc of hA53T α-Syn mice. (a) Representative images of TH-positive cells in SNpc and VTA in the three groups (scale bar = 1 mm). (b) Relative density of TH-positive cells in SNpc, VTA. (c) Relative density of TH-positive dopaminergic nerve terminals in NAc. *P < 0.05, **P < 0.01, and *P < 0.001 versus WT. Data in all panels represent mean ± SEM. TH: tyrosine hydroxylase, SC: standard cage, EE: environmental enrichment, WT: wild-type, SNpc: substantia nigra pars compacta, VTA: ventral tegmental area, STR: striatum, NAc: nucleus accumbens

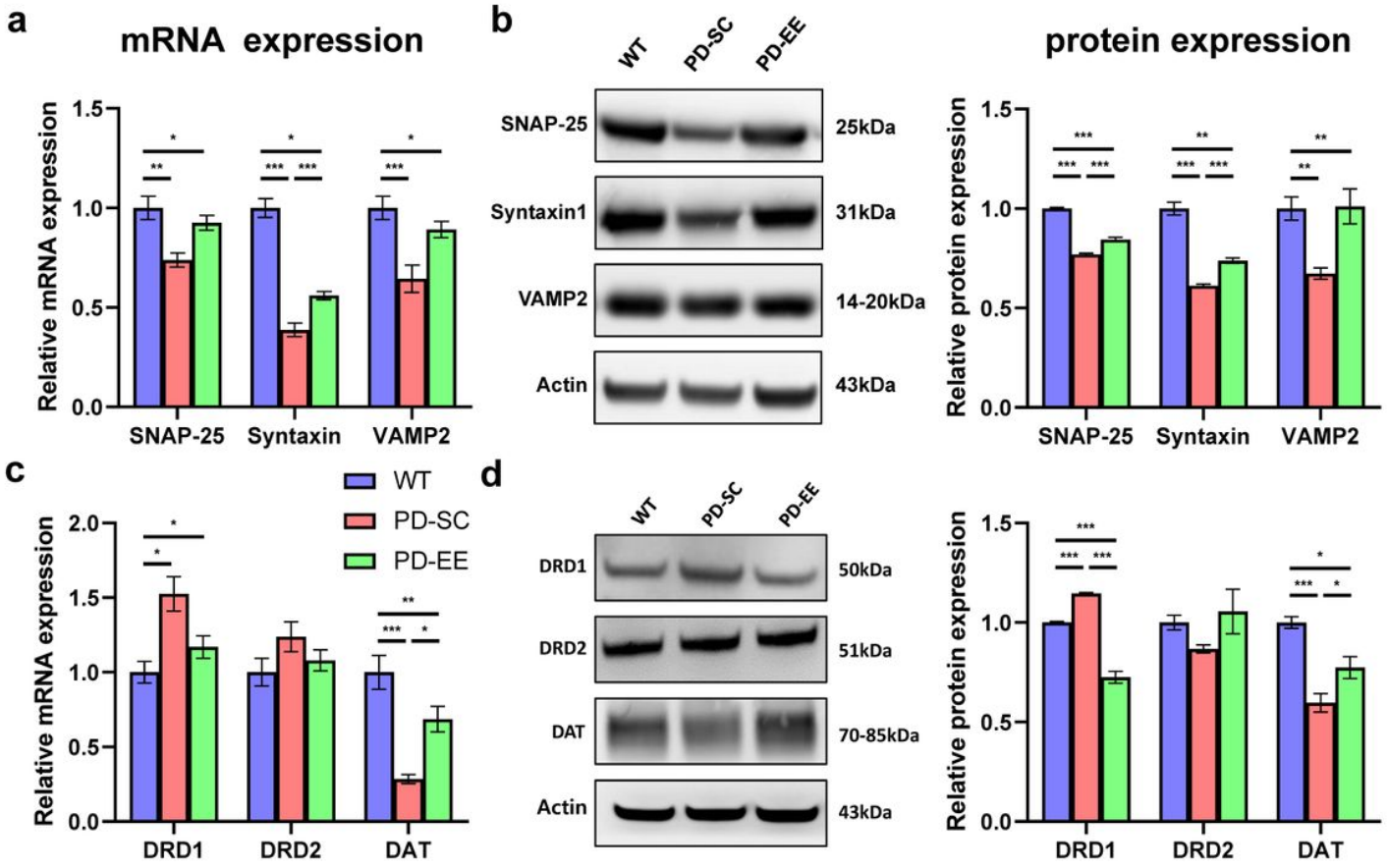

Figure 3

EE increases SNARE expression and alters the expression of DAT and DRDs in NAc. (a) qRT-PCR results showed the relative mRNA expression of SNAP-25, Syntaxin1 and VAMP2 expression that were significantly increased in PD-EE compared to PD-SC mice. (b) Western blot results showed the relative protein expressions of both Syntaxin1 and VAMP2 that were significantly increased in PD-EE compared to PD-SC mice. The expression of SNAP-25 tended to increase in PD-EE compared to PD-SC mice. (c) qRT-PCR results showed the relative mRNA expression of DRD1, DRD2 and DAT expression that were significantly increased in PD-EE compared to PD-SC mice. (b) Western blot results showed the relative protein expressions of DRD1, DRD2 and DAT. *P < 0.05, **P < 0.01, and *P < 0.001 versus WT. Data in all panels represent mean ± SEM. SC: standard cage, EE: environmental enrichment, WT: wild-type, SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor, SNAP-25: synaptosomal-

associated protein 25, VAMP2: vesicle-associated membrane protein 2, DRD1: dopamine receptor D1, DRD2: dopamine receptor D2, DAT: dopamine transporter

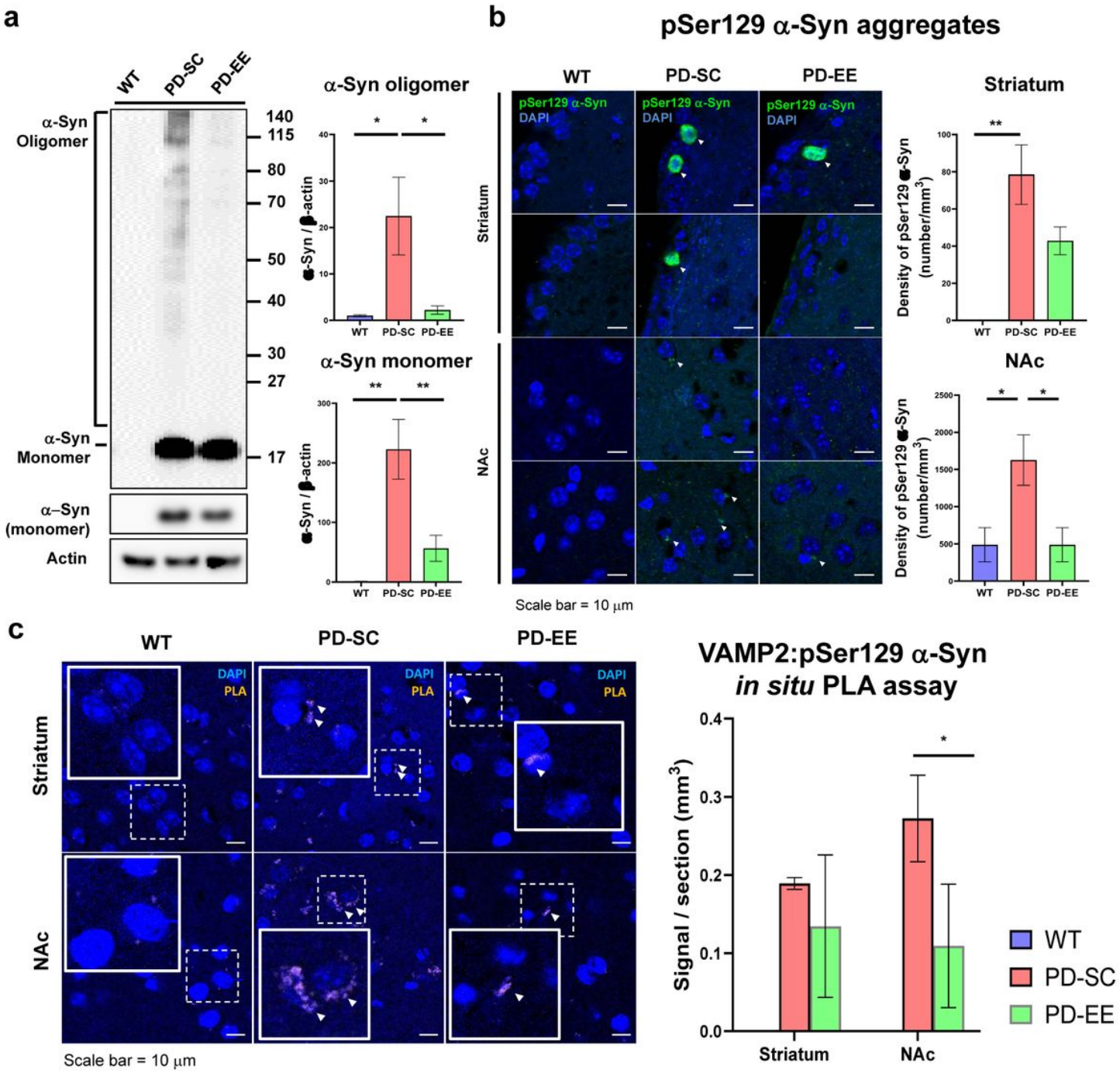

Figure 4

EE reduces aggregated α-Syn and the interaction between α-Syn to VAMP-2 in NAc. (a) Western blot results indicated the relative expressions of α-Syn monomer and oligomer. The expression of α-Syn monomer tends to increase but aggregated α-Syn is decreased in PD-EE than PD-SC mice. (b) Representative images of pSer129 α-Syn immunohistochemistry in striatum and NAc of the three groups.

The density of pSer129 α-Syn tended to decrease in PD-EE compared to PD-SC mice in both region (scale bar = 10 μm). (c)The proximity of VAMP2 and pSer129 α-Syn in the striatum and NAc assessed by proximity ligation assay shows the decreased PLA signals in PD-EE relative to PD-SC mice (scale bar = 10 μm). *P < 0.05, **P < 0.01, and *P < 0.001 versus WT. Data in all panels represent mean ± SEM. α-Syn: α-synuclein, pSer129 α-Syn: phosphorylated serine 129 α-synuclein, PLA: proximity ligation assay, NAc: nucleus accumbens

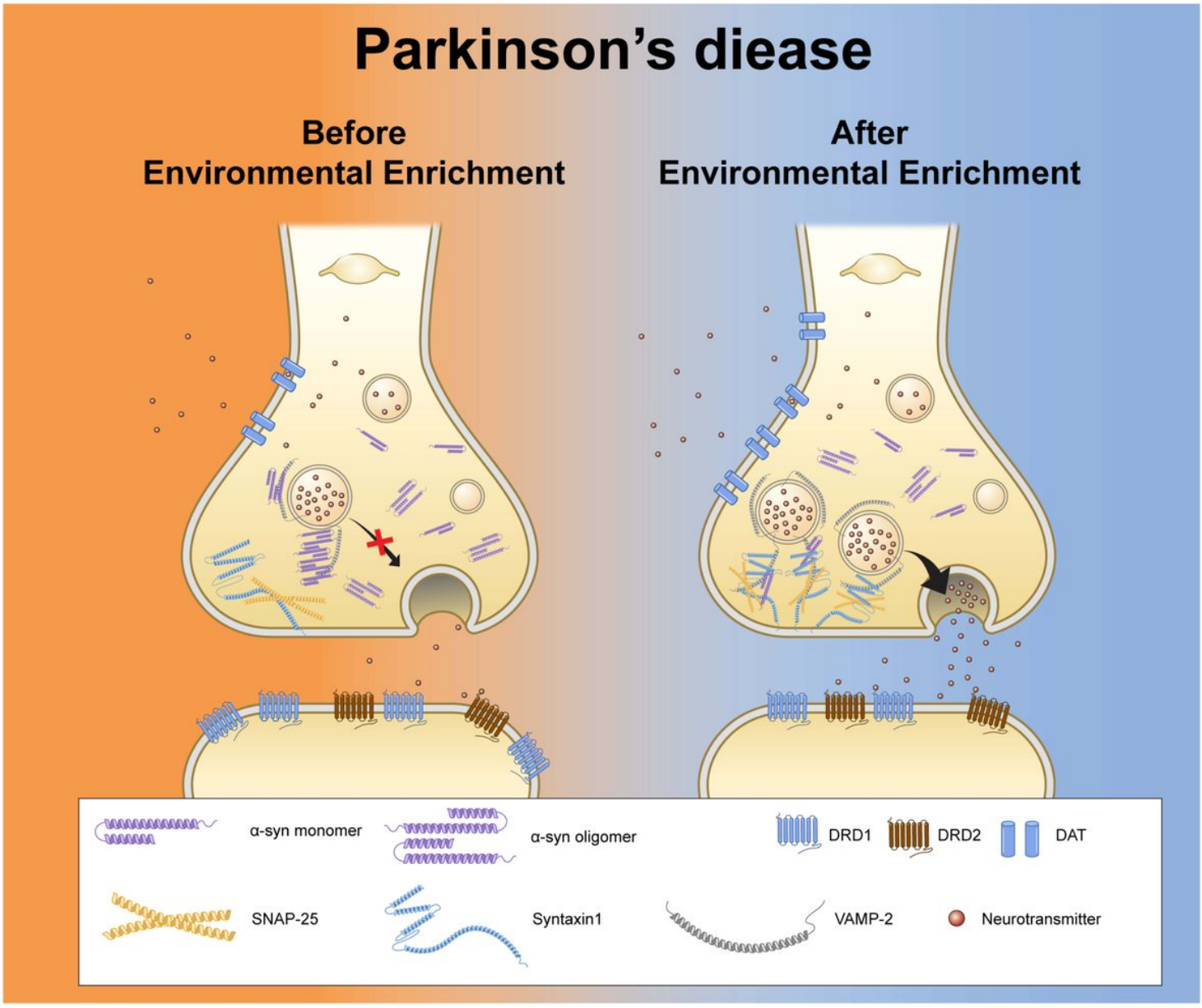

Figure 5

Synopsis of aberrant synaptic signaling in hA53T α-Syn mice recovered by EE. This schematic illustration summarizes the progressive pathology of dopaminergic nerve terminals in the striatum and NAc in PD. Originally, the functions of SNAP-25, Syntaxin1 and VAMP2 help synaptic vesicles to dock and fusion to permit exocytosis. These functions were interrupted by aggregated □-Syn proteins; therefore, dopamine

release was decreased in the PD-SC group. As a compensatory effect, DRD1s were increased to receive more synaptic signals. EE simultaneously reduced aggregated α-Syn and induced the expression of SNARE proteins like syntaxin1 and VAMP2. SNARE proteins may recover their functions. After all, synaptic vesicles were re-circulated and normalized, so cytosolic dopamine levels may be normalized in NAc. Since dopamine transporters and/or dopamine receptors were normalized, pre-motor symptoms were ameliorated, and the progress of the disease can be delayed.