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Exercise training upregulates CD55 to suppress complement-mediated synaptic phagocytosis in Parkinson's disease

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Abstract

The primary pathological change in Parkinson's disease (PD) is the progressive degeneration of dopaminergic neurons in the substantia nigra. Additionally, excessive microglial activation and synaptic loss are also typical features observed in PD samples. Exercise trainings have been proven to improve PD symptoms, delay the disease progression as well as affect excessive microglial synaptic phagocytosis. In this study, we established a mouse model of PD by injecting mouse-derived α-synuclein preformed fibrils (M-α-syn PFFs) into the substantia nigra, and demonstrated that treadmill exercise inhibits microglial activation and synaptic phagocytosis in striatum. Using RNA-Seq and proteomics, we also found that PD involves excessive activation of the complement pathway which is closely related to over-activation of microglia and abnormal synaptic function. More importantly, exercise training can inhibit complement levels and complement-mediated microglial phagocytosis of synapses. It is probably triggered by CD55, as we observed that CD55 in the striatum significantly increased after exercise training and up-regulation of that molecule rescued motor deficits of PD mice, accompanied with reduced microglial synaptic phagocytosis in the striatum. This research elucidated the interplay among microglia, complement, and synapses, and analyzed the effects of exercise training on these factors. Our work also suggested CD55 as a complement-relevant candidate molecule for developing therapeutic strategies of PD.

Keywords Parkinson's disease, Microglia, Proteomics and RNA-seq analysis, Complement pathway, Synapses, CD55

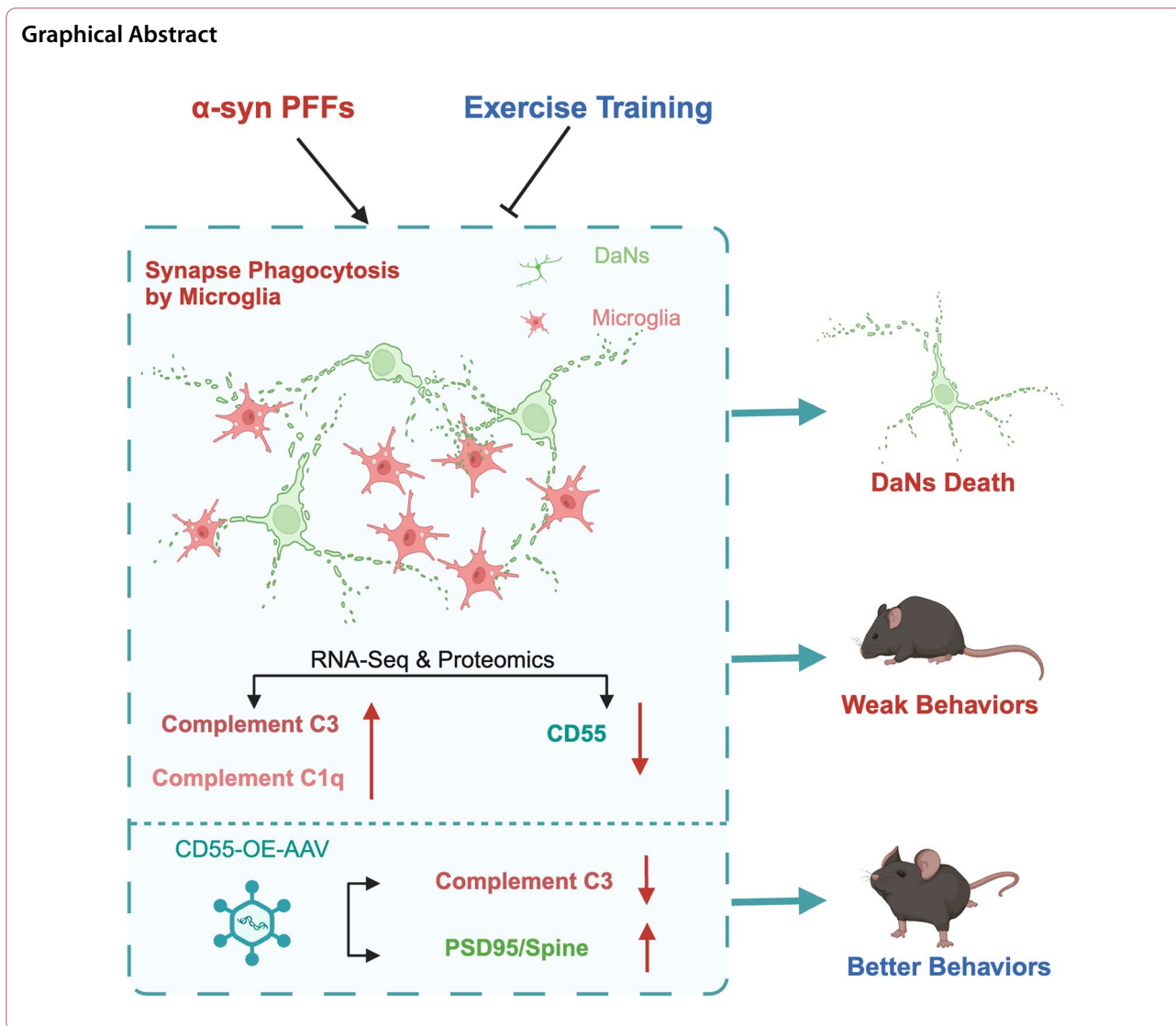*Correspondence:
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Graphical Abstract

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, with incidence and mortality rates increasing globally each year [1]. Clinically, PD manifests through hallmark symptoms such as resting tremors, muscular rigidity, reduced movement, and abnormal posture and gait [2–4]. The primary pathological change in PD is the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta of the midbrain [5–7]. Additionally, excessive microglial activation [8–13] and synaptic loss [14, 15] are also typical features observed in PD samples. In the early stages of PD, there is already a loss of synapse in the striatal region [16], accompanied by a reduction of dopamine transporter protein [17]. Loss of synapse accelerated the progress of PD, but no pharmacological treatments which

can reverse the decline of synapse has been developed currently [18, 19].

Exercise trainings have been proven to improve symptoms and delay the progression of PD [20]. Previous studies have shown that exercise trainings can enhance the balance, gait, and overall functional capacity [20–22], and reduce the incidence of falls in PD patients [23]. Our own group has also validated these findings, showcasing significant enhancements in motor abilities post-rehabilitation training [24]. Through animal experiments, researchers have not only observed related molecular changes, but also explored underlying mechanisms which may help to enhance the efficacy of exercise training and lay the groundwork for identifying new therapeutic targets for PD [25–28]. After exercise training, the number of microglia in the striatum tends to decrease, with more

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of those shifting into the resting state [29] compared to the MPTP-PD mice [30]. Concurrently, exercise trainings rescue the down-regulation of PSD95 and synaptophysin in the striatum of MPTP-treated mice [31]. That indicated synaptic function in the striatal region is enhanced. Experiment with electron microscopy also revealed significant neurogenesis of dendrites and axons in the striatal region following exercise [32]. Nevertheless, further research is needed to determine whether microglia associate with synaptic loss in PD and whether exercise training reduces synaptic loss in PD by regulating microglia.

Microglia are known to participate in synaptic phagocytosis in various diseases, and synaptic alterations are early events in many neurodegenerative diseases. In models of Alzheimer's disease, microglia act as potential and key cellular mediators of synaptic loss [33]. Increased microglia-mediated synaptic loss through phagocytosis exacerbates cognitive impairment [34]. Also, persistent microglial phagocytic activity results in decreased synaptic density and cognitive decline after stroke [35, 36] and in multiple sclerosis [37]. Similarly, excessive microglial synaptic phagocytosis has been observed in various PD animals [38–41]. Exercise training was reported to effectively restrict microglia-synapse engulfment. For example, long-term voluntary exercise can protect hippocampal synapses in APP/PS1 mice, reduce microglial activity and dendritic spine loss [42, 43]. In a TDP-43-induced model of ALS, treadmill exercise was found to prevent the worsening of motor dysfunction and further inhibit microglial synaptic phagocytosis [44]. However, how exercise training regulates microglial synaptic phagocytosis in PD is still not fully understood.

In this study, we employed treadmill training for PD mice to investigate how exercise regulates microglia-mediated synaptic phagocytosis in PD. We found that exercise rescues motor deficit, protects dopaminergic neurons, inhibits microglial activation and complement-mediated synaptic phagocytosis in PD utilizing immunocytochemistry, RNA-seq and proteomics. Additionally, CD55 probably accounts for the attenuation of complement-mediated synaptic phagocytosis in the striatum after treadmill training. Our work elucidated the interplay among microglia, complement and synapses. Moreover, a novel complement-related mechanism via which physical exercise exert neuroprotection in a mouse model of Parkinson's disease was explored.

Materials and methods
Animals
C57BL/6 J mice (6 or 8 weeks old, males) were purchased from Shanghai SLAC Experimental Animal Co., Ltd. 5 mice per cage were housed in a SPF environment equipped with a ventilation system. The ambient temperature was maintained at 22 °C ± 2 °C, and relative humidity ranged from 40 to 70%. The mice were kept in a 12-h light/12-h dark cycle (7:00–19:00) with access to ample food and water. All animal experiments were conducted in accordance with the guidelines for the care and use of laboratory animals from the National Institutes of Health and were approved by the Ethics Committee of Tongji University.

Mouse model of Parkinson's disease
The mouse model of PD was established by injecting mouse-derived alpha-synuclein preformed fibrils (M-α-syn PFFs) (AnaSpec, AS-56082-100) into the substantia nigra. Eight-week-old male mice were divided into Sham and PFFs groups. Animals were assigned a number, then use a random number table to assign the animals into groups. PFFs group received bilateral injections of PFFs into the substantia nigra (P3.0 mm, L/R1.3 mm, V4.5 mm), with a dosage of 2 µg per side at a continuous flow rate of 0.2 µl/min, while Sham group received an equivalent volume of saline. Behavior tests were performed weekly.

Behavior test
Behavior tests included pole test and rotarod test. The rotarod test included three training sessions, with speeds of 10 rpm/min constant, 2–20 rpm/min constant acceleration, and 4–40 rpm/min constant acceleration. Mice were then tested on the rotarod at 4–40 rpm/min constant acceleration for 5 min. Each experiment was conducted in triplicate, and the intervals between experiments exceeded thirty minutes. In the pole test, mice were trained to climb a vertical pole (50 cm height, 0.5 cm diameter) with a textured surface. The time taken for mice to climb from the top to the bottom was recorded in three repetitions, with intervals exceeding fifteen minutes between trials.

Tissue preparation
After behavior tests, brain tissues of mice were immediately collected. Following sodium pentobarbital anesthesia, blood was removed from the body using 1 × PBS, and the tissues were fixed by perfusion with PFA. After sucrose gradient dehydration, the tissues were embedded in OCT and stored at − 80 °C. For another subset of mice, brain tissues were dissected under a stereomicroscope after cervical dislocation, and the substantia nigra and striatum were isolated. The obtained tissues were rapidly frozen in liquid nitrogen and transferred to − 80 °C for storage.

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Western blot analysis

Brain tissues were lysed using RIPA lysis buffer (Beyotime,P0013B) supplemented with protease inhibitor mixture (Roche, 4693116001) and phosphatase inhibitor mixture (Roche, 4906845001). BCA assay kit (Beyotime, P0010) was used to measure the total protein concentration. Proteins were separated by SDS-PAGE, and then transferred to a PVDF membrane. Membranes were blocked in 5% skimmed milk for 1 h, incubated overnight with specific primary antibodies at 4 °C, washed three times in $1 \times$ TBST, incubated with secondary antibodies at room temperature for 1 h, and finally detected using the ECL protein blotting substrate kit. ImageJ software was used for quantitative analysis of band density.

Immunofluorescence

Frozen brain section (30 µm thickness) were collected for immunofluorescence. The substantia nigra and striatum brain slices were washed three times for 10 min each in $1 \times$ PBS and then blocked with immunofluorescence blocking solution (Beyotime, P0260) for 1 h. After blockage, the slices were incubated overnight with primary antibodies at 4 °C. After three washes with $1 \times$ PBS, the slices were incubated with secondary antibodies at room temperature for 1 h. Following three washes with $1 \times$ PBS, the slices were coverslipped using mounting medium and imaged using a Leica confocal laser scanning microscope. $63 \times$ oil objective was utilized to collect microglia confocal images. The microglia were scanned from top to bottom with 0.3-µm steps in the z direction with 2048×2048-pixel resolution and all collected images were merged to one image. ImageJ software was employed for image processing.

Treadmill exercise protocol

After three weeks of PFFs administration, PFFs group was divided into PFFs and Exer groups. Exer group participated in treadmill exercise training, consisting of 5 days per week, 40 min per day, with a speed ranging up to 15 m/min (6 m/min for 5 min, 9 m/min for 5 min, 12 m/min for 20 min, 15 m/min for 5 min, and 12 m/min for 5 min) for 4 weeks. Behavior tests were conducted after each exercise session, and after completion of the exercise regimen, mice were sacrificed to collect samples for subsequent analysis.

RNA-sequence

RNA-Seq was conducted by GENE DENOVO. Total RNA was extracted using Trizol reagent kit. After reverse transcription and RNA amplification, RNA libraries were constructed. Prior to information analysis, raw data underwent data filtering to reduce analysis interference

caused by invalid data. Preliminary filtering was performed on the obtained valid data, removing genes with zero expression in over half of the samples, and data normalization was conducted to ensure greater consistency within samples. PCA analysis was performed on three groups to ensure good clustering within each group. Further, differential expression gene (DEGs) analysis was conducted pairwise among the three groups, and then KEGG and GO functional enrichment analysis using DEGs were conducted. Foldchange ≥ 2 or ≤ -2, and p-value ≤ 0.05 were criteria for DEGs.

Proteomics

4D-DIA relative quantitative proteomics by GENE DENOVO were applied in our study. Proteins were extracted from the samples, and the total concentration was determined using BCA assay. Subsequently, peptide samples were prepared and subjected to mass spectrometry detection. This process involved comparing the obtained mass spectrometry data to the reference library to identify and quantify the proteins present in each sample. The obtained protein quantification results were statistically analyzed and subjected to bioinformatics analysis. After inter-sample correction using the Limma package [45], differential multiples were calculated using the Limma package, and Gene Set Enrichment Analysis (GSEA) was performed using the ClusterProfiler package [46]. Foldchange ≥ 1.2 or ≤ 0.8, and p-value ≤ 0.05 were criteria for Differential Expression Protein.

Enzyme-linked immunosorbent assay (ELISA)

After blood collection, samples were put on ice for 30 min. Subsequently, centrifugation was performed at 12,000 rpm for 15 min to obtain the serum. Striatal tissues were homogenized using lysis buffer, and the supernatant was collected after centrifugation at 12,000 rpm for 15 min. ELISA kits were used to quantify dopamine (DA) level in the striatum and complement levels (C3 and C1q) in the serum. The experimental procedures were performed according to the manufacturer's instructions.

Golgi staining

FD Rapid Golgi Stain Kit (FDNeurotech, PK401) was used for Golgi staining according to the manufacturer's instructions. After cervical dislocation, mouse brain tissues were separated and washed in PBS to remove blood. The brain tissues were immersed in a mixture of Solution A and Solution B (1:1) for 14 days and then transferred to Solution C for dehydration, with the entire process conducted in the dark. After dehydration, brain tissues were sliced into 200 µm sections using a vibratome, and the sections were collected on chrome-alum-gelatin-coated slides. Stained slices

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were imaged using a Zeiss confocal microscope under bright-field illumination, and images of the striatum were collected for subsequent analysis.

Virus injection

An adeno-associated virus (AAV) expressing CD55 (pAAV-EF1A-mCD55-mCherry:WPRE) (the AAV was constructed and packaged by VectorBuilder) was constructed and injected into the striatum (A0.8 mm, L/R1.2 mm, V3.6 mm). Virus injection included treatment and pre-treatment experiments. In the treatment experiment, eight-week-old male C57BL/6 J mice were divided into Sham, PFFs, and CD55-OE (overexpression) groups. PFFs and CD55-OE groups underwent PD modeling by receiving PFFs in the substantia nigra. CD55-OE group received AAV injection in the striatum, while Sham group and PFFs group received equivalent volumes of saline. Behavior tests, including pole test and rotarod test, were performed weekly, and mice were sacrificed for brain retrieval after three weeks. In the pre-treatment experiment, six-week-old mice were divided into Sham, PFFs, and CD55-OE groups. CD55-OE group received AAV injection in the striatum, while the PFFs and Sham groups received equivalent volumes of negative virus in the striatum. After two weeks, PFFs and CD55-OE groups underwent PD modeling in the substantia nigra, while Sham group received saline. Behavior tests were conducted weekly, and mice were sacrificed for brain retrieval three weeks after PD modeling.

Antibody list

Antibody	Vendor	Catalog	Dilution	RRID
TH	Merck	T1299	WB1:5000 IF1:5000	AB_477560
TH	Abcam	ab76442	IF1:200	AB_1524535
iba1	Abcam	ab5076	IF1:200	AB_2224402
iba1	Wako	019-19741	IF1:200	AB_839504
GAPDH	Proteintech	81640-5-RR	WB1:5000	AB_3086559
β-actin	Proteintech	20536-1-AP	WB1:5000	AB_10700003
Tubulin	Proteintech	11224-1-AP	WB1:5000	AB_2210206
CD68	CST	#97778	IF1:200	AB_2928056
C3	Proteintech	21337-1-AP	WB1:1000	AB_2878843
C3	Invitrogen	PA1-29715	WB1:1000	AB_2066730
PSD95	CST	#36233	WB1:1000 IF1:200	AB_2721262
C1q	Abcam	ab11861	IF1:200	AB_298643
C1q	Abcam	ab182451	IF1:200	AB_2732849
CD55	Proteintech	26580-1-AP	WB1:1000	AB_2880559

Statistical analysis

GraphPad Prism V.9 was used for all statistical analyses. All data are presented as mean ± SD. Normality of data was assessed, and statistical analysis was performed using unpaired two-tailed t-tests or one-way ANOVA. Differences between two groups were determined by unpaired two-tailed Student's t-test. When comparing more than two groups, one-way ANOVA analysis was used, followed by Tukey's post hoc test. Significance between groups was evaluated as follows: ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

Results

Injection of α-syn PFFs into the substantia nigra effectively induces behavioral and pathological changes associated with Parkinson's disease in wild-type mice

Injection of pre-formed fibrils of α-synuclein (PFFs) has been an alternative method proposed for modeling PD by increasing the level of α-synuclein in the substantia nigra. This model can more closely replicate the physiological processes that may occur in the brain of PD patients [47]. Given that the substantia nigra is the primary brain region affected in PD, injection into the substantia nigra allows for a more direct observation of the impact of α-syn on dopaminergic neurons. Therefore, we chose the substantia nigra as the main injection site. Prior to PFFs injection, we conducted baseline Behavior tests on mice to ensure no significant intergroup differences. The modeling lasted for three weeks, during which we performed behavior tests including pole test and rotarod test every week. The results showed that after PFFs injection three weeks, mice in PFFs group exhibited a decline in behavioral abilities, with reduced latency time on the rotarod and prolonged climbing time compared to Sham group, which showed statistical differences (Fig. 1a, b). To further evaluate the pathological changes, we validated TH protein levels in the two core brain areas of PD, substantia nigra (SN) and striatum. The results showed a significant decrease in TH protein levels in both the substantia nigra and striatum of PFFs group compared to Sham group (Fig. 1 c). The number of TH-positive neurons in the substantia nigra also declined in PFFs group, according to the results of immunofluorescence assay. That reflected the loss and death of nigral dopaminergic neurons in PFFs-treated mice (Fig. 1d). Additionally, we assessed microglia, the central immune cells involved in the development of Parkinson's disease in various groups. We chose CD68 as an indicator of microglial activation, and detected an upregulation of nigral/striatal microglia in PFFs group, along with increased number of microglia and an elevated proportion of CD68 expression per microglia, suggesting that microglial aggregation and

over-activation occur in the substantia nigra and striatum in the PFFs-induced mouse model (Fig. 1e–h). Moreover, PFFs administration significantly suppressed the expression of postsynaptic density protein 95 (PSD95) in the striatum, indicating the synaptic loss under that condition (Fig. 1i). These results showed that PFFs administration into the substantia nigra of wild-type mice for three weeks can significantly induce motor skill decline, loss of dopaminergic neurons in the substantia nigra, activation of microglia, and loss of synapses in the striatum.

Exercise training improves behavioral and pathological outcomes in PFFs mice

In previous studies and earlier researches of our team, we found that exercise training could improve motor skill levels and slow down the disease progression in both PD patients and mouse models. To further explore the specific mechanism of exercise training, we conducted treadmill training for PFFs mice. After three weeks of α-syn PFFs administration, mice were forced to participate in a four-week treadmill training, five times per week, 40 min each time (Fig. 2a, b). Behavior tests were conducted weekly. After exercise training, we assessed the mice's body weight, and the results showed no significant differences among the groups (Fig. 2c). The results of pole test and rotarod test showed a significant decline in motor skills in PFFs group compared to Sham group, while exercise training partially rescued the motor ability of PFFs mice (Fig. 2d, e). Similarly, results of immunofluorescence staining showed that, compared to Sham group, PFFs mice exhibited a significant reduction in TH-positive neurons in the substantia nigra, while that change was recovered in PFFs group, indicating that exercise training could reduce damage to dopaminergic neurons and delay their loss (Fig. 2f). For microglia in the substantia nigra, they were prone to accumulate and shift into an activated state in PFFs group, compared to Sham group. While after exercise training, there was a downregulation of microglia in the substantia nigra region, accompanied by a decreased proportion of cells in the activated state (Fig. 2g, h). Also, we assessed the synaptic protein PSD95 in the striatum among various groups.

Western blot data indicated a significant reduction in PSD95 in PFFs group compared to Sham group, while exercise training attenuated the loss of PSD95. Similarly, results of Golgi staining also revealed dendritic spine loss in PFFs group, which could be alleviated by exercise training (Fig. 2i). In order to further explore the relationship between microglia and synapses as well as the effect of exercise training, we conducted immunofluorescence staining and observed the co-localization of CD68 and PSD95 in the striatum. The results showed that activated microglia were more abundant in the striatum of PFFs group compared to Sham group. The co-localization level of CD68 and PSD95 was increased after PFFs injection, which suggested an increased phagocytosis of microglia. After exercise training, the co-localization of CD68 and PSD95 in microglia decreased, indicating that exercise training could suppress microglial synaptic phagocytosis (Fig. 2j). Overall, these results demonstrated that exercise training can effectively inhibit the activation of microglia and reduce the loss of synapse in the striatum.

Proteomics and RNA-seq reveal that exercise improves synaptic function and inhibits upregulation of the complement pathway in PD mice

To further investigate how exercise training regulates microglial synaptic phagocytosis in PD, we analyzed the nigral RNA-Seq data and found that Sham group and Exer group clustered closer together in PCA dimensional reduction analysis, suggesting that exercise corrected transcriptional changes induced by PFFs (Fig. 3a). 201 differential genes were identified between Sham and PFFs group, and 179 were achieved between PFFs and Exer group (Fig. 3b). Functional analysis of upregulated genes in PFFs group compared to Sham group revealed significant enrichment in pathways such as Phagosome and Complement and coagulation (Fig. 3c), while downregulated genes were enriched in the neuroactive ligand-receptor interaction pathway (Figure S1a). Compared to PFFs group, Exer group showed downregulation of chemokine-related pathways (Fig. 3d) and upregulation of immune response pathways (Figure S1b). We confirmed these results in the transcriptomic sequencing

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Fig. 1 Behavioral and pathological changes in the PFF-induced PD mouse model. **a** Results of rotarod test in Sham and PFFs groups over time and at day21 (Sham group n = 8, PFFs group n = 21). **b** Results of poles test in Sham and PFFs groups over time and at day21. (Sham group n = 10, PFFs group n = 22) **c** Western blot showing the expression of TH protein in the substantia nigra (SN) and striatum. (n = 6 per group) **d** Representative images of immunostaining and relative quantitative analysis of dopamine neurons in the substantia nigra. Scale bar = 1000 μm. (n = 4 per group) **e**, **f** Representative images of immunostaining and relative quantitative analysis of active microglia in the substantia nigra. Scale bar = 20 μm. (n = 4 per group) (n = 20 per group from 4 mice each group) **g**, **h** Representative images of immunostaining and relative quantitative analysis of active microglia in the striatum. Scale bar = 20 μm. (n = 4 per group) (n = 20 per group from 4 mice each group) **i** Results of western blot analysis for the striatal expression of PSD95 protein in Sham and PFFs group. (n = 6 per group) All data are presented as mean ± SD. Statistical analysis was performed using unpaired two-tailed t-tests
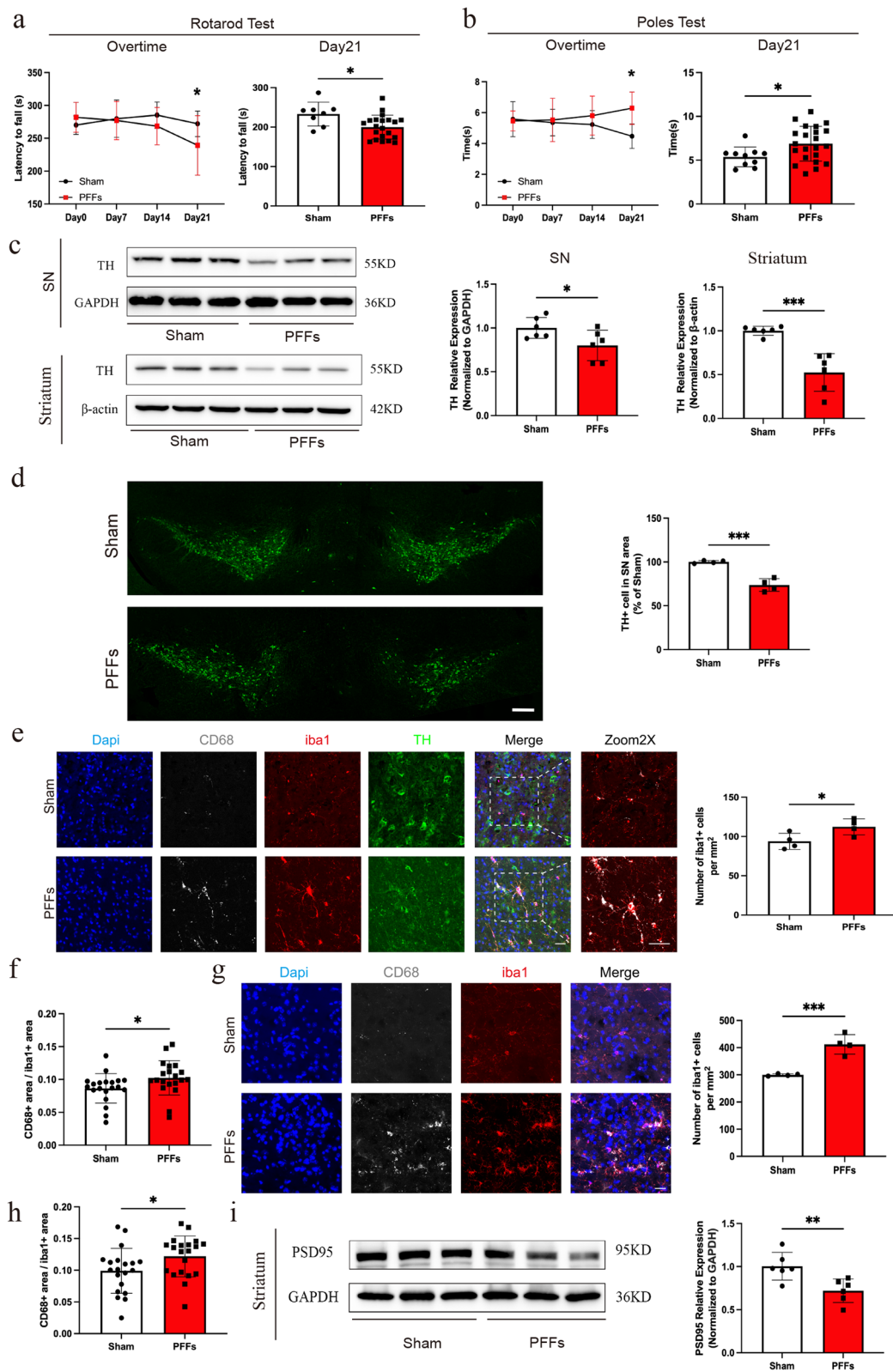
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data. We found that lysosome-related protein CD68 was upregulated in PFFs group but downregulated after exercise (Figure S1c). Additionally, inflammatory indicators of microglia (Cst3 and Cst7) exhibited a similar trend for expression patterns (Figure S1d). Various components of the complement cascade, including C1q, C3, and C4b, were significantly upregulated in PFFs group, while exercise training suppressed this abnormal upregulation (Figure S1e). Further, we conducted proteomics in the substantia nigra and striatum regions using 4D-DIA technology. From results of the nigral proteomics, we detected 96 differentially expressed proteins between Sham and PFFs group, as well as 235 between PFFs and Exer group (Fig. 3e). Results of GESA also pointed to pathways "complement and coagulation cascades", which was upregulated in PFFs group compared to Sham group, and downregulated after exercise training (Fig. 3f). In agreement with transcriptomic sequencing data, various components of the "complement and coagulation cascades" (such as key protein C3) were upregulated in PFFs group but downregulated after exercise training (Fig. 3g) in Proteomics. The expression trend of proteins involved in the "complement and coagulation cascades" pathway in the striatum was similar to that in the substantia nigra (Fig. 3h, i). Taken together, we believe that changes related to the complement system play a crucial role in the onset and progression of PD, as well as in the improvement of the motor deficit after physical exercise. Moreover, complement may be an important node to connect microglia and synapse loss.

Exercise training suppresses upregulation of complement C3 and reduces synaptic phagocytosis by microglia

Based on RNA-Seq data and proteomic results described above, we first evaluate the alterations of the complement system in the peripheral blood of mice under various conditions. As we expected, serum concentrations of complement C3 and C1q were higher in PFFs group compared to Sham group, while treadmill exercise training markedly reduced those complement levels in the peripheral blood of PFFs-treated mice (Fig. 4a, b). Furthermore,

we conducted a correlation analysis and found both serum C3 and C1q levels are negatively related to latency to fall in the rotarod test (Fig. 4c, d). In the substantia nigra or the striatum, two critical brain regions with PD, the loss of TH protein in PFFs group was accompanied by an increase in complement C3, while exercise training reduced the level of complement C3 (Fig. 4e, f). The microglial expression of complement C1q in the striatum exhibited a similar pattern. More C1q expression in microglia were observed after PFFs treatment, and exercise training significantly reversed the change (Fig. 4g). Furthermore, we performed Elisa to detect dopamine level in the striatum, and found that decreased in PFFs group compared to Sham group, and covered after treadmill training (Fig. 4h). Therefore, we figured out that complement pathway was definitely over-activated in PFFs mouse, resulting in the increased microglial phagocytosis of synapse. And exercise training on PD mice may downregulate complement pathway to improve the synapse survival and motor function. In agreement with that hypothesis, the striatal level of CD55, known as a negative regulator of complement system, was markedly downregulated in response to PFFs treatment and rebounded after exercise (Fig. 4i). All of those indicated CD55 might be the key molecule participating in PD progress as well as exercise-induced protective effects, probably via the regulation of complement and microglial synaptic phagocytosis.

Overexpression of CD55 effectively improves PD motor skills and reduces synaptic phagocytosis by microglia

Based on the results above, we constructed AAV virus overexpressing CD55 to further explore its role in affecting the progress of PD. The genetic modulation of CD55 were introduced on mice before or at the same time of PFFs treatment, respectively.

Under the simultaneous treatment condition, we conducted behavioral experiments, including pole and rotarod tests, on three groups of mice. The results showed significant behavioral differences between Sham group and PFFs or CD55-OE group. However, there was

(See figure on next page.)
Fig. 2 Exercise training pattern and behavioral, pathological evaluation. **a** Experimental paradigm of exercise training. **b** Exercise training pattern. **c** Analysis of mouse body weight. (n = 7 per group) (**d**) Results of rotarod test among three groups over time and the last of exercise training (Day49). (Sham group n = 11, PFFs group n = 10, Exer group n = 11) **e** Results of poles test among three groups over time and at the last day of exercise training (Day49). (Sham group n = 10, PFFs group n = 11, Exer group n = 11) **f** Representative images of immunostaining and relative quantitative analysis of dopamine neurons in the substantia nigra. Scale bar = 1000 μm. (n = 4 per group) **g** Representative images of immunostaining and relative quantitative analysis of dopamine neurons and microglia in the substantia nigra. Scale bar = 20 μm. (n = 4 per group) (n = 20 per group from 4 mice each group) **i** Western blot and Golgi staining results of striatal PSD95 expression and spine density in the striatum. (n = 6 per group) (n = 18 per group from 3 mice each group) **j** Representative images of immunostaining and relative quantitative analysis of phagocytic function of microglia in the striatum. Scale bar = 20 μm. (n = 4 per group) (n = 20 per group from 4 mice each group) (n = 4 per group) All data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA
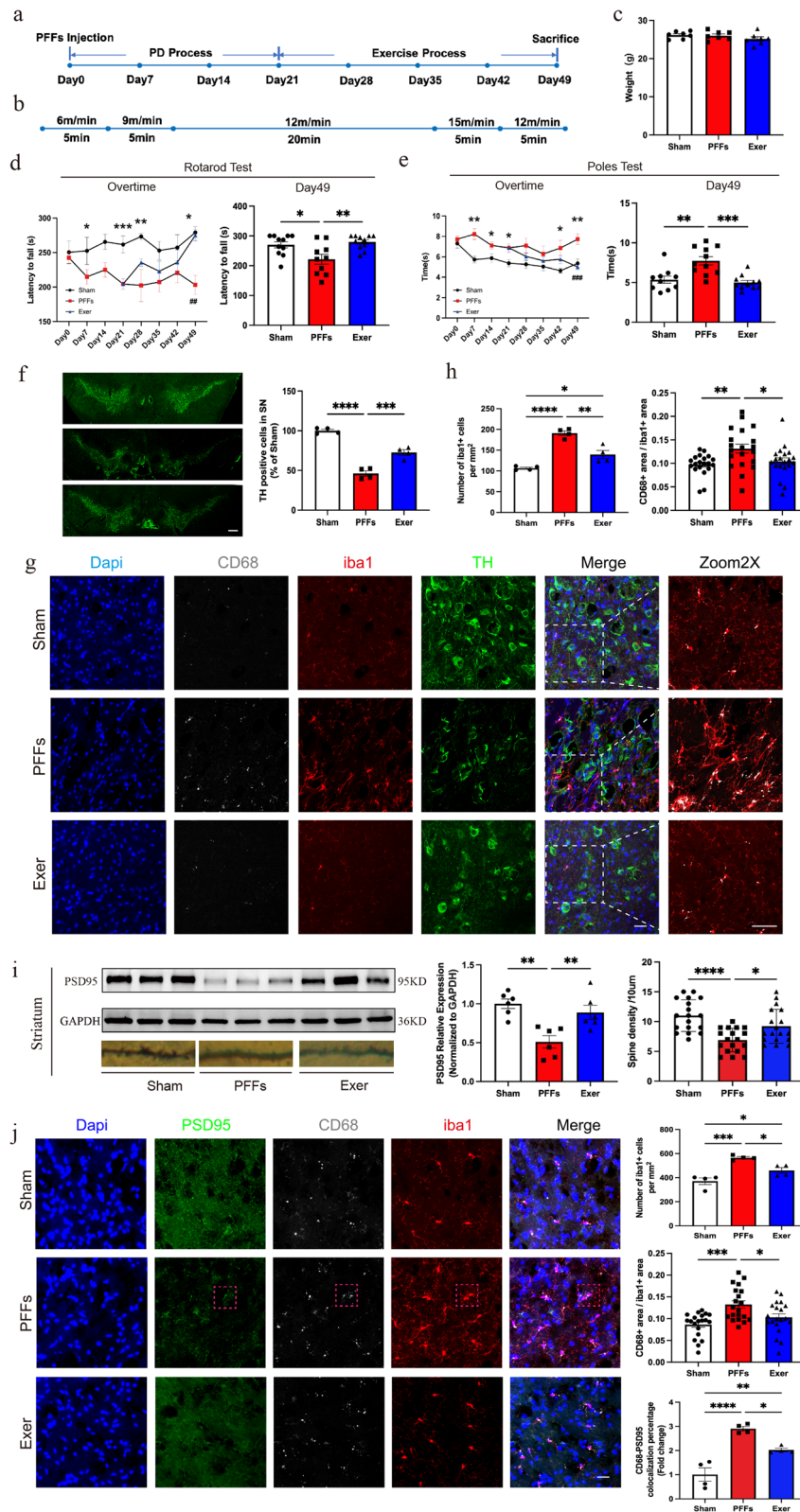
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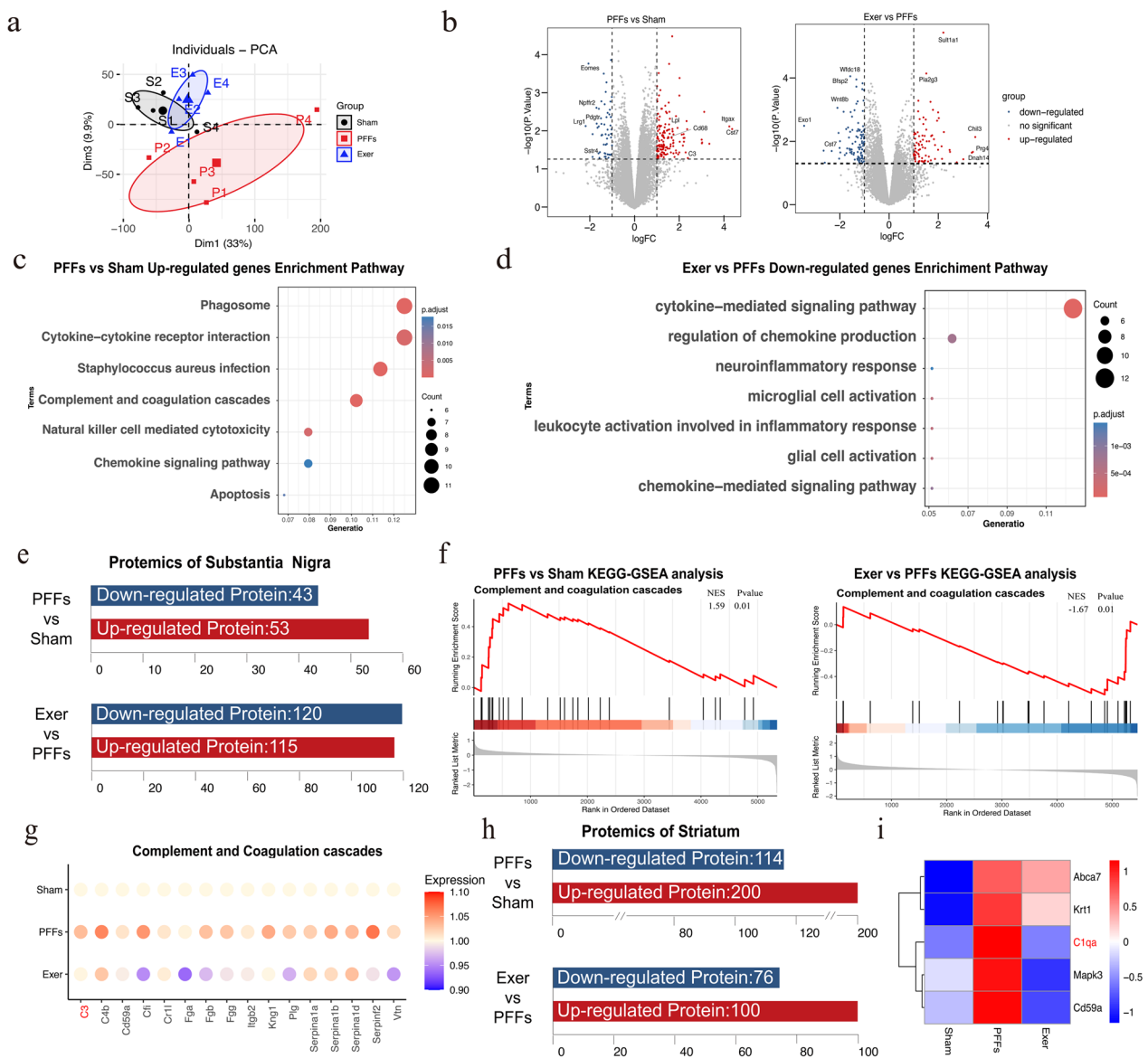
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Fig. 3 Proteomic and RNA-Seq data analysis revealed the protective effect of exercise and de-regulated complement pathway after exercise. **a** PCA plot of RNA-Seq data for three groups. **b** Volcano plot of DEGs. **c** PFFs group vs Sham group up-regulated genes enrichment pathway. **d** Exer group vs PFFs group down-regulated genes enrichment pathway. **e** Differential expression proteins in the substantia nigra of three groups. **f** GSEA showing the significant changed pathway in the substantia nigra of Sham group vs PFFs group and PFFs group vs Exer group. **g** Relative expression of proteins associated with "Complement and Coagulation cascades" pathway in the substantia nigra of three groups. **h** Differential expression proteins in the striatum of three groups. **i** Heatmap of relative expression of proteins associated with "Complement and Coagulation cascades" pathway in the striatum of three groups

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Fig. 4 Exercise training suppressed complement C3 and C1q levels and inhibited complement-induced phagocytic function of microglia. Level of serum C3 (**a**) (Sham group n=6, PFFs group n=5, Exer group n=7) and C1q (**b**) (Sham group n=5, PFFs group n=7, Exer group n=7) concentration measured by Elisa. The correlation of latency of rotarod test and serum C3 (**c**) and C1q (**d**) concentration. **e** Western blot showing the expression of TH protein and complement C3α in the substantia nigra. (n=6 per group) **f** Western blot showing the expression of TH protein and complement C3α in the striatum. (n=6 per group) **g** Immunofluorescence results and relative quantitative analysis of C1q and microglia co-localization in the striatum. Scale bar=20 μm. (n=20 per group from 4 mice each group) **h** Level of DA concentration measured by Elisa in the striatum. **i** Western blot showing the expression of CD55 protein in the striatum. (n=6 per group) All data are presented as mean±SD. Statistical analysis was performed using one-way ANOVA
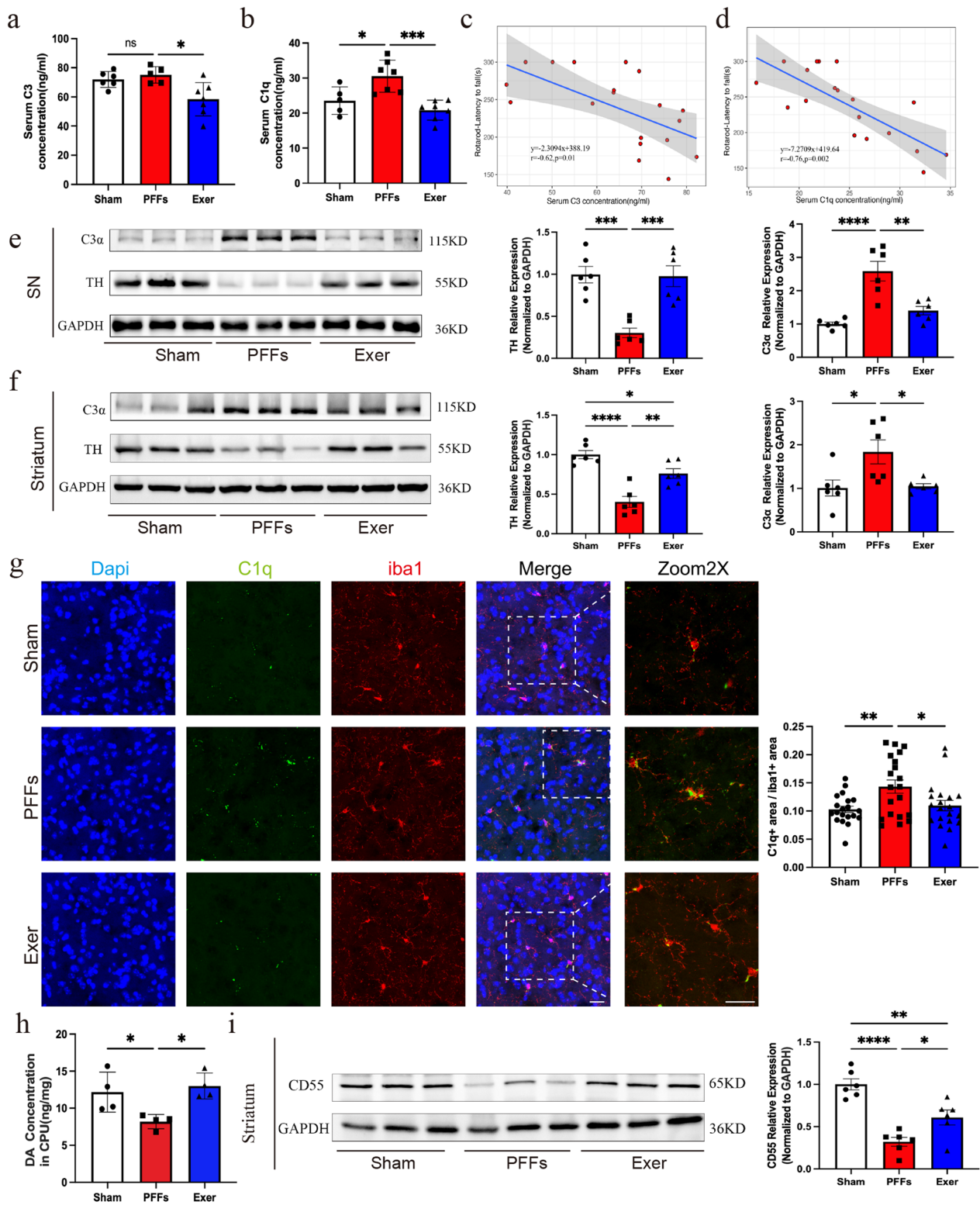
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no statistical difference in behavior between the CD55-OE and PFFs group (Fig. 5a, b), indicating that simultaneous administration of PFFs to the substantia nigra and CD55-OE-AAV into the striatum did not improve the motor deficits in the mouse model of PD. Results of quantitative western blot analysis revealed that striatal CD55 level was downregulated in PFFs group compared to Sham group, while the CD55-OE-AAV injection elevated that expression (Fig. 5c). This validated the efficacy of our virus infection as well as the potential impact of CD55 on PD. The complement C3α expression level in the striatum exhibited a decreasing trend in response to CD55-OE-AAV treatment. When analyzing the core pathological areas of PD, the substantia nigra and striatum, we found that CD55-OE-AAV could reduce the loss of TH protein (Fig. 5d). Also, we investigated the synapses after CD55-OE-AAV treatment. Western blot results indicated a significant reduction in PSD95 in PFFs group compared to Sham group. CD55-OE-AAV introduction partly attenuated the loss of PSD95, but it didn't show a statistical significance. While, results of Golgi staining revealed dendritic spine loss in PFFs group, which was rescued by CD55 overexpression (Fig. 5e). This was further confirmed by immunofluorescence staining, where staining for iba1, CD68 and PSD95 in the striatum revealed a significant inhibition in microglial activation and a decrease in microglial phagocytosis of synapses in the CD55-OE group compared to PFFs group (Fig. 5f). Based on the result above, we found that increased CD55 expression can to some extent attenuate PD-related pathological changes.

Further, we conducted the pre-treatment experiment in which the virus was injected into mice two weeks before PFFs modeling to observe the impact of high CD55 expression on the progression of PD. Behavioral results from the pre-treatment experiment showed a significant difference in pole and rotarod test, with the CD55-OE group exhibiting prolonged retention time and shortened climbing time compared to PFFs group (Fig. 6a, b). Next, we assessed CD55 and complement C3α levels in the striatum. A significant upregulation of CD55 in the CD55-OE group compared to both the Sham and PFFs groups was detected. The CD55-OE group showed a significant downregulation of complement C3α level compared to PFFs group, confirming that the overexpression of CD55 indeed inhibited complement C3α (Fig. 6c). Quantitative analysis of TH protein in the substantia nigra and striatum indicated that PFFs-treated mice exhibited TH protein loss as expected, while the CD55-OE-AAV pretreatment delayed this process (Fig. 6d). Western blot analysis disclosed that striatal PSD95 was markedly suppressed after PPFs injection, while CD55-OE-AAV pretreatment attenuated the loss of PSD95.Similarly, results of Golgi staining also revealed dendritic spine loss in the stratum in PFFs group, which was reversed by CD55-OE-AAV pre-treatment (Fig. 6e). Meanwhile, immunofluorescence staining for CD68 and iba1 revealed a reduction in microglial activation in the CD55-OE-AAV group compared to PFFs group (Fig. 6f), suggesting that CD55-OE-AAV could restrict microglial phagocytic activity towards synapses in the striatum. These results indicate that overexpressing CD55 in the striatum can improve behavioral and pathological aspects of PFFs-induced PD to some extent, probably via tuning down excessive microglial phagocytosis of synapses.

Proteomics comprehensively reveals the mechanism of overexpression of CD55 and explores the similarity between exercise and CD55

To further explore the role of CD55 in PD treatment and investigate the commonalities between CD55-OE-AAV and exercise, we performed proteomics of the striatum from the CD55-OE-AAV pre-treatment experiment. PCA revealed that samples from the same group clustered together, indicating similar protein expression patterns within groups. Additionally, the distinct separation between groups suggests that CD55 altered the protein differences caused by PFFs (Fig. 7a). Based on the PCA results, we identified differentially expressed proteins between pairs of groups. PFFs pretreatment upregulated 401 proteins and downregulated 324 proteins, compared to the Sham group. While CD55-OE group upregulated 190 proteins and downregulated 99 proteins compared to PFFs group (Fig. 7b). Furthermore, we found the "complement activation pathway" was upregulated after PFFs pretreatment. While in CD55-OE

(See figure on next page.)

Fig. 5 Behavioral and pathological evaluation of AAV treatment experiment. **a** The latency time of the rotarod test and pole test in Sham, PFFs, and CD55-OE groups over time and at day21. (Sham group n = 8, PFFs group n = 7, CD55-OE group n = 11) **b** Time of the pole test in the three groups over time and at day21. (Sham group n = 9, PFFs group n = 8, CD55-OE group n = 11) **c** Results of western blot for the expression of CD55 and complement C3α in the striatum. (n = 6 per group) **d** Results of western blot for the expression of TH protein in the substantia nigra and striatum. (n = 6 per group) **e** Results of western blot for the expression of PSD95 protein in the striatum and spine density count in the striatum. (n = 6 per group) (n = 18 per group from 3 mice each group) **f** Changes in microglial states and synaptic engulfment function in the striatum among Sham, PFFs, and CD55-OE groups. Scale bar = 10 μm. (n = 15 per group from 3 mice each group) (n = 3per group) All data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA
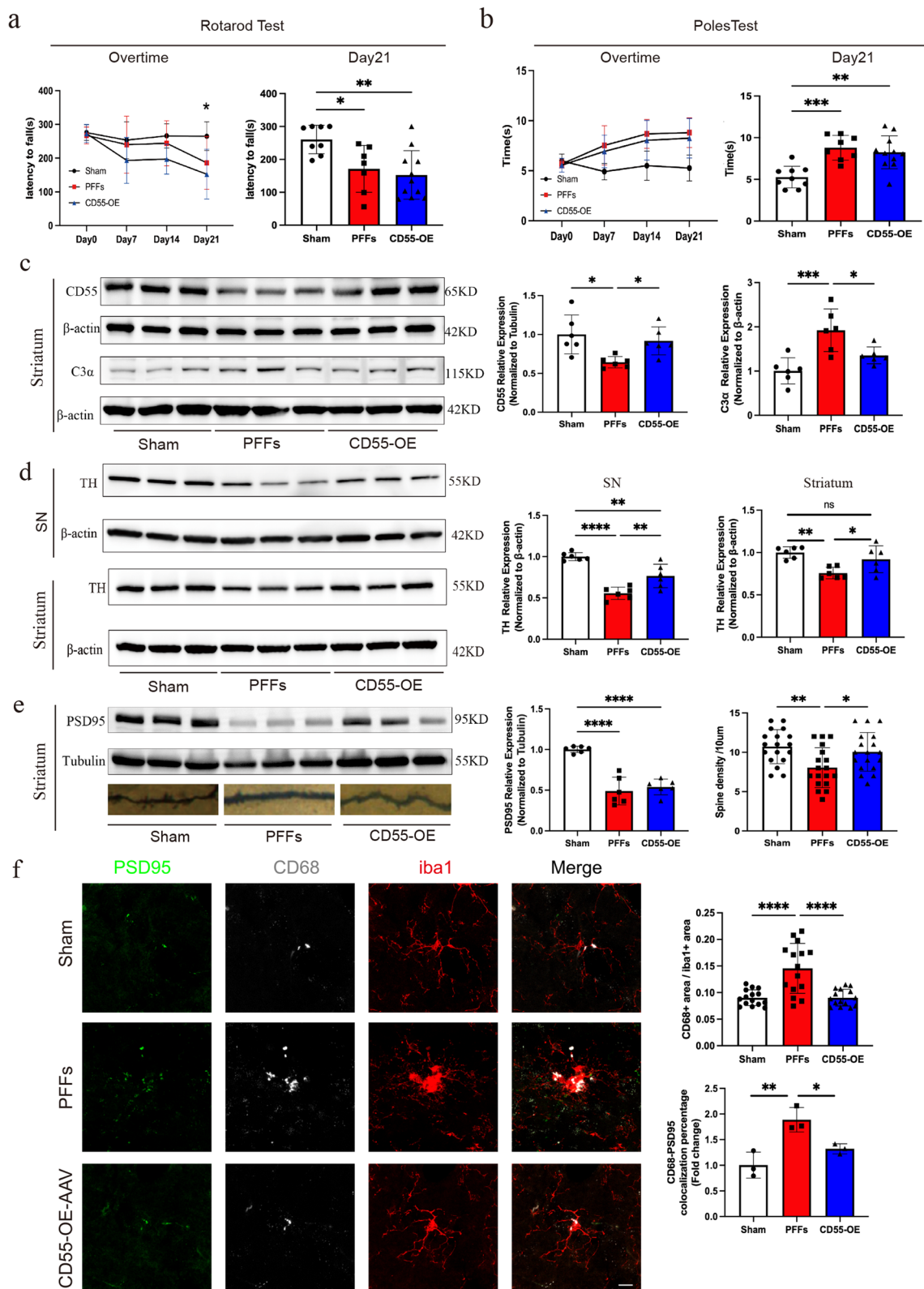
Fig. 5 (See legend on previous page.)

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group that pathway was downregulated compared to the Sham group (Fig. 7c). To comprehensively investigate how CD55-OE-AAV improves PD, we conducted an intersection analysis of differentially expressed proteins across the groups. First, we analyzed the subset of proteins upregulated in PFFs group but downregulated in CD55-OE group, identifying 32 differentially expressed proteins. GO functional enrichment analysis showed that these 32 proteins were mostly enriched in RNA splicing and metabolism pathways, such as "mRNA processing", "RNA splicing", and "nuclear-transcribed mRNA catabolic process, nonsense-mediated decay". Similarly, we analyzed the proteins downregulated in PFFs group but upregulated in CD55-OE group, identifying 63 differentially expressed proteins. These proteins were primarily associated with dopaminergic neurons and dopamine-related pathways, including "dopamine transport" and "dopamine biosynthetic process" (Fig. 7d). Additionally, we aimed to identify similarities in proteomics between exercise intervention and CD55-OE-AAV pre-treatment. We found that compared to Sham group, the "neuropeptide signaling pathway" was significantly upregulated in both Exer group and CD55-OE group (Fig. 7e), while "synaptic transmission, glutamatergic" was significantly downregulated (Fig. 7f). These results suggest that the shared mechanisms by which exercise and AAV improve PD may involve enhancing neuropeptide signaling in the striatum and inhibiting the excessively upregulated glutamatergic system. Also, the relationship between neuropeptide, glutamate and complement still remains to be explored.

Discussion

Exercise training attenuates motor deficits and pathological features of PD. The potential causes might be related to neuronal survival, synaptic plasticity, neuroinflammation, oxidative stress, neurotrophic factors, etc. [48, 49]. However, the understanding of the specific process mediating the exercise-induced protective effect on PD is still rare. In this study, we found PFFs mice actually exhibited over-activated microglia and synapse loss in the striatum, and exercise training definitely reversed that phenomenon. Furthermore, using RNA-Seq and proteomics, we observed excessive activation of the complement pathway, which is closely related to excessive microglial synaptic phagocytosis in PD mice. Treadmill running on PFFs-treated mice markedly inhibited complement levels, and exercise-induced impacts on motor functions, microglia and synapse were mimicked by overexpressing CD55. Therefore, we proposed that complement may be the key element through which exercise suppresses microglial synapse elimination and slows the PD progress.

As mentioned earlier, PD exhibits abnormalities in synaptic structure and function. Here, we verified these changes through various methods. We detected the decreased expression level of PSD95 in the striatum in PFFs group compared to Sham group, and concurrently observed a significant loss of spines in the striatum through Golgi staining. The reduction of spine density in the striatum is regarded as the consequence of dopamine denervation under the condition of PD, which might further disturb the connectivity of the corticostriatal and thalamostriatal systems [50]. The striatal spine pathology in PFFs mice was ameliorated by treadmill exercise or the overexpression of CD55 in our study. That at least partially pointed out the repair of basal ganglia neurocircuitry.

Induction of α-synuclein into the brain are suggested to trigger neuroinflammation. Striatal CD11b + cells presenting a pro-inflammatory state were detected when AAV9 for α-synuclein was injected into the substantia nigra of mice [51]. A recent study unraveled that chronic expose to PD patient-derived α-synuclein fibrils (FPD) promoted the microglial release of IL-1b, IL-6, TNF-a and glutamate. Moreover, treatment of FPD together with Tumor necrosis factor-α (TNFα) and prostaglandin E2 (PGE2) resulted in a microglial metabolic reprogramming closely linked to M1-type polarized cells [52]. Consistent with these results, we also found microglia in the striatum are more active in PFFs mouse, while exercise can reverse the change. Excessive synaptic pruning by microglia has been proposed as one of the potential causes for many neurodegenerative diseases [33, 53]. In order to discern the correlation between microglia and synapse in our study, we conducted immunofluorescence

(See figure on next page.)

Fig. 6 Behavioral and pathological evaluation of AAV pre-treatment experiment. **a** The latency time of the rotarod test and pole test in Sham, PFFs, and CD55-OE groups over time and at day21. (Sham group n = 7, PFFs group n = 6, CD55-OE group n = 8) **b** Time of the pole test in the three groups over time and at day21. (Sham group n = 8, PFFs group n = 6, CD55-OE group n = 7) **c** Results of western blot for the expression of CD55 and complement C3α in the striatum. (n = 5 per group) **d** Results of western blot for the expression of TH protein in the substantia nigra and striatum. (n = 5 per group) **e** Results of western blot for the expression of PSD95 protein in the striatum and spine density count in the striatum. (n = 5 per group) (n = 18 per group from 3 mice each group) **f** Changes in microglial states and synaptic engulfment function in the striatum among Sham, PFFs, and CD55-OE groups. Scale bar = 10 μm. (n = 15 per group from 3 mice each group) (n = 3 per group) All data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA
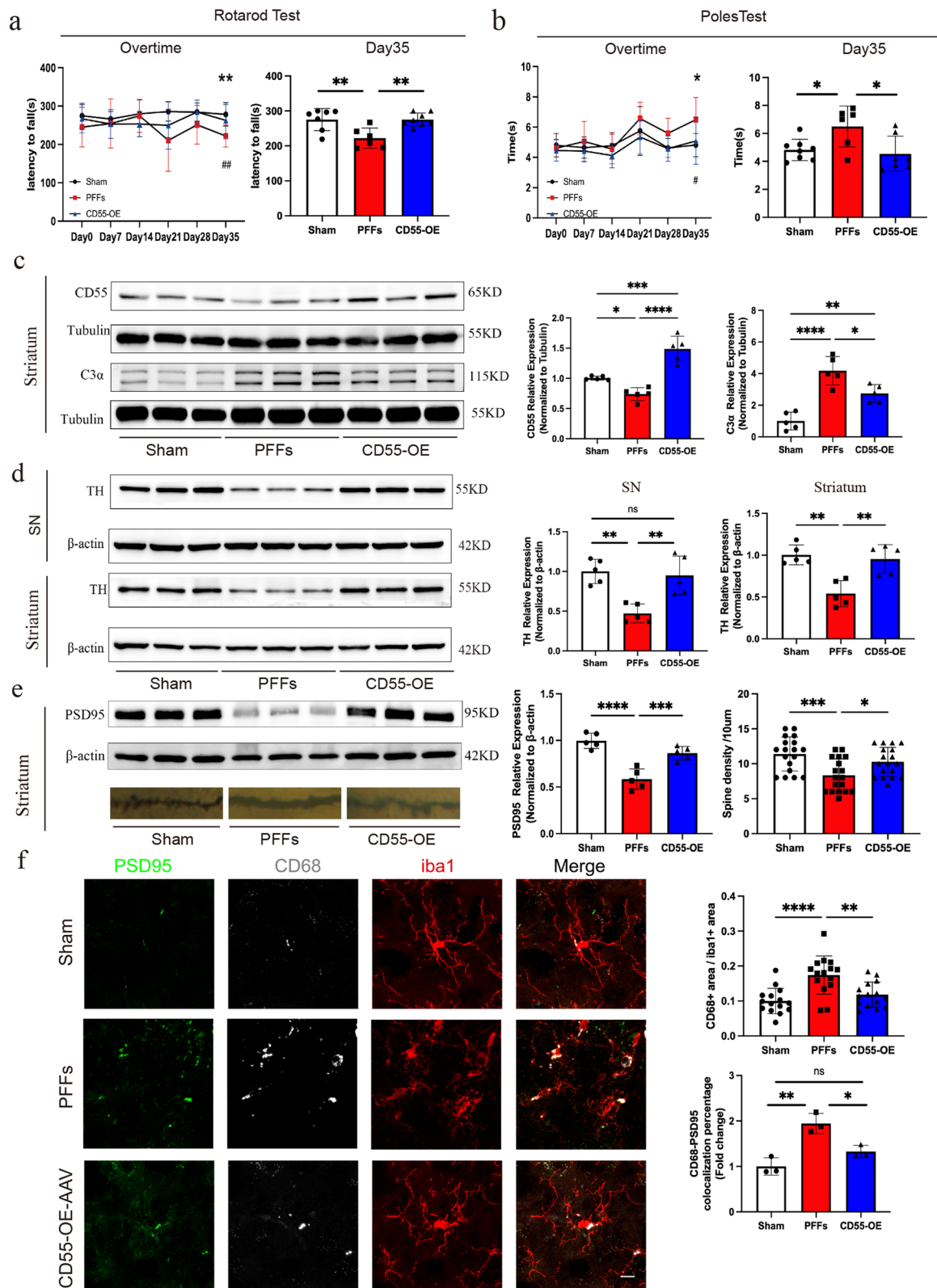
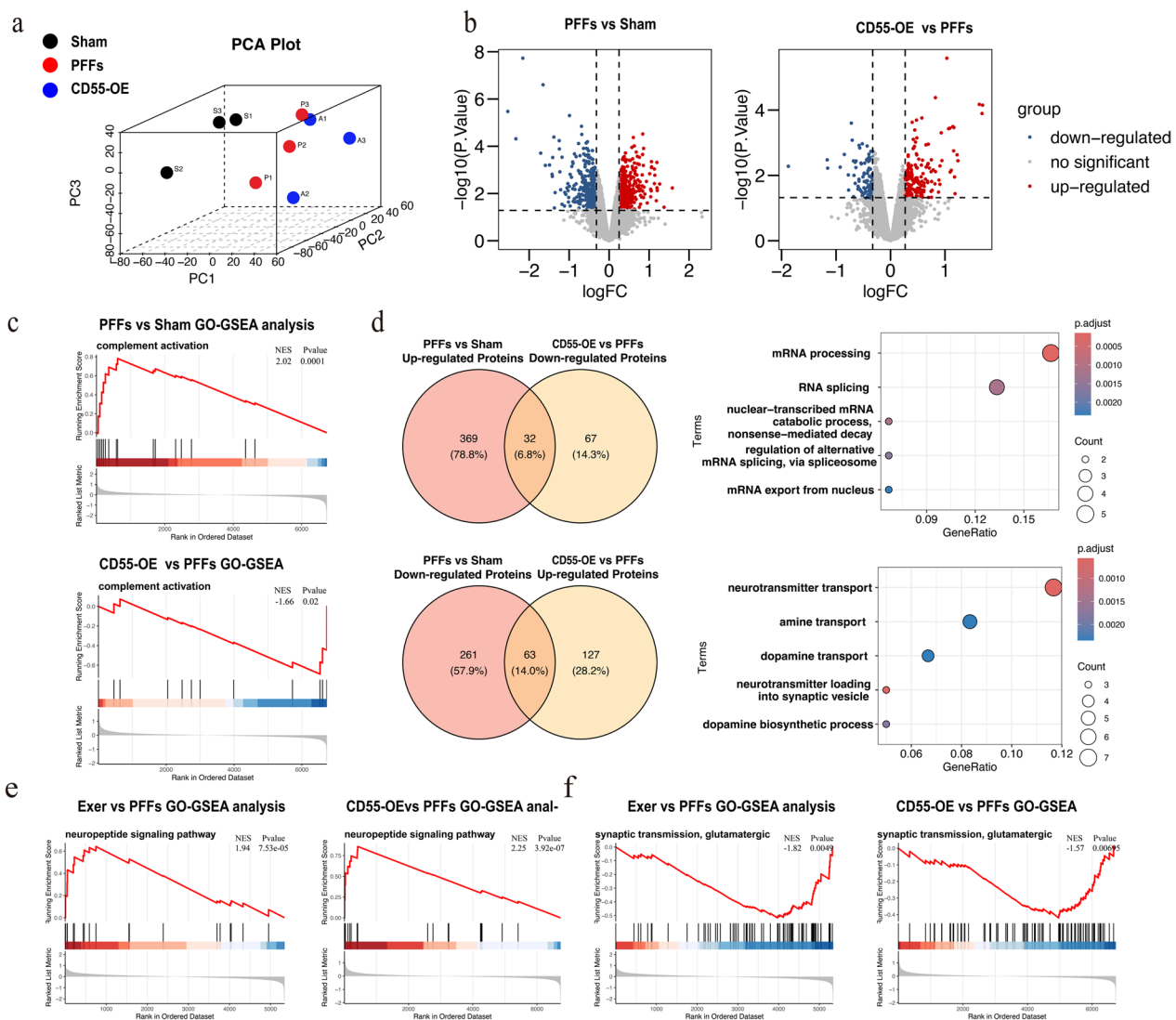**Fig. 6** (See legend on previous page.)

Fig. 7 Proteomics analysis of CD55-OE-AAV pre-treatment group revealed the protective effects of CD55 and potential shared pathways between CD55-AAV overexpression and exercise intervention. **a** PCA analysis of Sham, PFFs and CD55-OE groups. **b** Volcano plot of DE Proteins between pairs of groups. **c** Results of "complement activation pathway" from GSEA of three groups. **d** Different expression module of three groups and relative pathway enrichment. **e** Exercise and CD55-OE-AAV common up-regulated GSEA pathway. **f** Exercise and CD55-OE-AAV common down-regulated GSEA pathway

co-staining. As a result, phagocytosis of synapse by microglia increased after PFFs induction and decreased after exercise training.

Our main discovery is that the complement pathway, negatively regulated after exercise, plays a crucial role in mediating microglial phagocytosis of striatal synapse related to the PD status. From the data of RNA-Seq and proteomics, we observed an upregulation of the complement cascade reaction in PD and a downregulation of it after exercise training. Similarly, we detected elevated levels of complement in both the substantia nigra-striatum region and peripheral blood serum in PD samples

through WB and ELISA assays. After exercise, decrease in complement expressions were observed in PD mice. Additionally, the performance in behavior tests was negatively associated with serum concentration of C1q and C3, which also suggests the potential of complement in affecting motor ability. In previous studies, C1q or C3 knockout as well as application of endogenous neuronal complement inhibitor SRPX2 were found to suppress synaptic elimination, indicating that the complement cascade is involved in synapse loss [54, 55]. Specially, C1q and C3 may bind to adjacent weaker synapses and eliminate them through the phagocytosis of activated

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microglia [56]. Upregulation of complement levels has been observed in various neurodegenerative diseases. In brain samples of Alzheimer's disease (AD) subjects, C3 transcription level was significantly higher than controls, and was also significantly correlated with cognitive decline [57]. Blocking C1 activation using genetic-editing techniques or by antibodies can alleviate Aβ synaptic toxicity [33]. Multiple sclerosis shows synaptic phagocytosis by microglia and severe synaptic loss, associated with increased complement component C3. In a mouse model of depressive disorder, behavioral impairments were rescued by using C1q neutralizing antibody suppress the C1q/C3-CR3 pathway and reduce abnormal microglial phagocytosis of synapse [58]. Survival and loss of dopaminergic neurons in the substantia nigra are also linked to C3, as reported by Bodea and colleagues, complement C3-deficient mice were resistant to LPS-induced dopaminergic denervation [59]. Complement system are supposed to be regulated by physical training, which might contribute to the exercise-related effects on various diseases. For example, exercise can alleviate neuropathic pain, primarily by reducing the level of complement C3 [60]. In AD, voluntary exercise was suggested to effectively reduce the number of hippocampal microglia, synaptic colocalization with microglia, as well as the C1q/C3 complement system [42, 43]. In a TDP-43-induced ALS model, treadmill exercise prevented the worsening of motor dysfunction, enhanced the release of clusterin to block the complement pathway, and further inhibited complement-mediated microglial synaptic phagocytosis [44]. In our study, we found that complement levels increased in PD mice, within peripheral blood and multiple brain regions, while exercise training inhibited the activation of complement and related excessive microglial phagocytosis of striatal synapses.

Furthermore, we validated the alteration of CD55, an important complement deactivator on PD. We found that in the striatum region of PFFs group, CD55 level were significantly decreased compared to Sham group, but they increased after exercise training. Changes of CD55 in the central nervous system have been rarely researched. An ultra-deep sequencing on brain predicted a variant transcription factor binding sites upstream of the CD55 gene, which contributes to AD pathogenesis by affecting the complement system [61]. Also, multifocal motor neuropathy (MMN) immunopathology is associated with CD55 promotor mutation and lower CD55 expression. CD55 can decrease complement activity, protect the integrity of the blood–brain barrier, and mitigate blast-induced neurotrauma [62]. In primary cultured hypoxic neurons, exogenously added CD55 can reduce cell death and apoptosis [63]. Overexpression of CD55 on cultured IMR32 neuronal

cells was also found to protect neurons from complement attack and cell death [64]. To our knowledge, the changes in CD55 in PD have not yet been reported. We herein report that in the striatum of PFFs-treated mice, CD55 level was significantly decreased compared to sham-operated mice, but that expression increased after exercise training. In order to figure out the significance of CD55 in PD, we utilized an AAV-mediated overexpression technique on PFFs-treated mice. The results showed that elevation in CD55 expression can to some extent improve behavioral and pathological aspects of PD, probably via reducing excessive microglial phagocytosis of striatal synapses. CD55 might also couple exercise and microglial function during the intervention on symptoms of Parkinson's disease.

Previous research has described various mechanisms through which exercise training slow PD progress [25, 65–69]. Here, we proposed that exercise may alleviate motor and pathological impairments of PD by reducing complement levels, thereby mitigating microglia and subsequent phagocytosis. Complement-mediated microglial phagocytosis in the striatum is one of the central alterations in PFF-treated mouse model of PD, giving rise to dopaminergic denervation. Elevation in CD55 level by treadmill training or by genetic modification would antagonize complement activation and bring beneficial effects to PD subjects. This study investigated the interactions among microglia, complement system and synapses during the development and exercise intervention of Parkinson's disease. CD55 targeting on complement can be considered as the critical molecule of exercise therapy in PD, as well as the potential pharmaceutical target.

Abbreviations
PD   Parkinson's disease
PSD95   Post-synaptic density-95
AD   Alzheimer's disease

Supplementary Information

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Additional file 1.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study about animals was approved by the ethics committee of Tongji University (TJBC00321101).

Competing interests
The authors declare no competing interests.

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