



Nanoplastics cause an increased risk of Parkinson's disease compared to microplastics at environmental exposure levels

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ABSTRACT

Recent evidence suggests a potential link between airborne microplastics (MP) and nanoplastics (NP) and neurodegenerative diseases, yet direct in vivo demonstration of their neurotoxicity remains limited. This study aimed to assess the potential risk of Parkinson's disease (PD) associated with chronic inhalation exposure to polystyrene nanoplastics (PS-NP), by examining PD-relevant behavioral and neuropathological alterations compared to polystyrene microplastics (PS-MP). Adult mice were exposed daily to 100 ppm/200 μ L of 20 nm PS-NP or 0.25 μ m PS-MP via inhalation for 16 weeks. Brain uptake and exposed dose were quantified using ⁶⁴Cu-labeled PS and gamma counting. Behavioral assessments, histology, immunohistochemistry, western blotting, and RNA sequencing were performed to evaluate PD-relevant outcomes. Only 0.094 % of the estimated human-equivalent environmental dose was exposed to the substantia nigra and striatum. PS-NP exposure induced greater behavioral deficits compared to PS-MP. RNA-seq revealed dysregulation of PD-related genes, overlapping with those in MPTP-induced PD models. Western blot and immunohistochemistry confirmed reduced tyrosine hydroxylase, increased phospho- α -synuclein (Ser129) aggregation, and elevated markers of glial activation (GFAP, IBA1) and pro-inflammatory cytokines in the PS-NP group. Inhaled PS-NP induced PD-relevant neuropathological and behavioral changes, suggesting that chronic exposure may contribute to increased PD risk under environmentally realistic conditions. These findings emphasize the need to evaluate airborne NP exposure as a novel environmental risk factor for PD.

1. Introduction

Microplastics (MP) in the air are particularly released from sources such as synthetic fibers, car tires, and building wear (Hong and Kim, 2024). According to the study by Prata et al. (2020), MP are present at concentrations of 0.3 to 1.5 particles/mm³ outdoors and 0.4 to 56.5 particles/mm³ indoors. Airborne MP may enter the body through inhalation and have been linked to Parkinson's diseases (PD) (Prata et al., 2020; Aderinto et al., 2025).

Once considered rare, PD is now the fastest-growing neurological disorder in the world, leading to immense social and economic costs. In 1990, the estimated number of PD patients were 2.5 million, but by 2015, it had more than doubled to 6.2 million. Due to aging, this number

is expected to double again to 12.9 million by 2040. Various factors (such as pesticides, chemicals, air pollution, and reduced smoking) have led to an increase in PD incidence that outpaces the current rate of aging, and this number could be even higher due to disproportionate increases in emerging industrial regions worldwide (De Miranda et al., 2022). The risk of PD is associated with various exposures over decades, including pesticides, organic solvents, and metals, combined with air pollution. The relationship between exposure and disease is variable, indicating that gene-environment and environment-environment interactions, along with other exposure dynamics, contribute to disease phenotypes (De Miranda et al., 2022). Neuroinflammation is a central contributor to the pathogenesis of PD, where chronic inflammation accelerates dopaminergic neuron degeneration (Tansey and Goldberg, 2010).

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polystyrene(PS)-MP may act as a novel environmental risk factor for neurodegenerative diseases by entering neuronal and glial cells via endocytosis and inducing pro-inflammatory cytokine release, oxidative stress, and mitochondrial dysfunction (Jin et al., 2022; Han et al., 2024). These disturbances activate chronic neuroinflammation, thereby increasing neuronal vulnerability and promoting degeneration in PD (Tansey and Goldberg, 2010; Jin et al., 2022; Han et al., 2024).

Recently, significant research has been conducted on the toxicity of MP and nanoplastic (NP) based on their size. These studies include investigations examining the cytotoxicity and pro-inflammatory effects of PS-MP and PS-NP in immune cells such as RAW264.7 cells (Wang et al., 2023), as well as research analyzing the effects of PS-MP and PS-NP on human intestinal organoids (Wang et al., 2024). However, there is currently no literature comparing the impact of size differences in MP on the occurrence of specific brain diseases. In our previous study, we found that when radiolabeled PS-MP (0.25 μm) and PS-NP (20 nm) were exposed via inhalation, PS-NP exhibited nearly twice the distribution in the brain compared to PS-MP (Fig. 4 in the ref (Shanmugiah et al., 2024)), showing a significantly higher accumulation (Shanmugiah et al., 2024). Therefore, in the present study, we selected PS as a representative polymer and employed the same two particle sizes to ensure continuity with our previous work, aiming to further evaluate whether PS-NP, which accumulates more readily in the brain, poses a higher risk of PD-like neurotoxicity than PS-MP.

2. Methods

PS, a synthetic polymer made from monomers of hydrocarbon styrene with IUPAC name as poly(1-phenylethane-1,2-diyl) was used throughout the study. For longitudinal exposure, PS-MP (0.25 μm , Fluorescent Yellow conjugated, excitation 470 nm / emission 500 nm; SpheroTech, USA) and PS-NP (20 nm, FITC conjugated, excitation 495 nm / emission 519 nm; NANOCs, USA) were used.

2.1. Characterization of micro- and nano plastics

2.1.1. Scanning electron microscopy imaging

To characterize the PS-MP and PS-NP, scanning electron microscopy (SEM) imaging was performed. The samples were prepared by dropping PS-MP and PS-NP dissolved in deionized water onto aluminum foil and allowing them to air dry completely at room temperature. Subsequently, SEM images were acquired using an SU8010 ultra-high-resolution field emission scanning electron microscope (FE-SEM; Hitachi, Japan) after the samples were coated with platinum in a low-vacuum atmosphere. The coating process was conducted using a Cressington 208 HR (Cressington Scientific Instruments, UK) at a current of 20 mA for 120 s to enhance conductivity and imaging quality.

2.1.2. Zeta potential measurement

The zeta potential of PS-MP and PS-NP (200 ppm in deionized water) was measured in quintuplicate using a Zetasizer Nano ZS (Malvern Instruments, UK) with disposable folded capillary cells (DTS1070, UK) at 25 °C, and analyzed with Zetasizer software (v8.02).

2.2. In vitro study

2.2.1. SH-SY5Y cell culture

SH-SY5Y cells, which are widely used as a model for PD and can differentiate into dopaminergic neuron-like cells upon treatment with retinoic acid (RA) (Korecka et al., 2013), were employed as a mechanistically informative and reproducible in vitro platform to validate the in vivo findings and to directly assess the size-dependent neurotoxic effects of PS-MP and PS-NP under controlled conditions. Cells were cultured in a medium consisting of 45 % Minimum Essential Medium Eagle, 45 % Ham's F-12 (Welgene, Republic of Korea), 10 % fetal bovine serum (FBS; Corning, USA), and 1 $\times$ Antibiotic-Antimycotic (Gibco,

USA) at 37 °C in a 5 % CO₂ incubator. The culture medium was replaced twice per week, and cells were subcultured at 80 % confluence.

2.2.2. Cytotoxicity assay

Laminin-coated 96-well plates were seeded with SH-SY5Y cells (0.3×10^4 /well). Cells were differentiated with 10 μM RA and treated with PS-MP or PS-NP for 7 days (medium changed on day 4). Viability was assessed using the EZ-Cytox WST assay (10 μL /well, 3 h at 37 °C), and absorbance was measured at 450 nm.

2.2.3. Imaging of dendritic branching of neurons

The 4-well chamber slides (Lab-Tek, USA) were coated with 2 $\mu\text{g}/\text{cm}^2$ laminin and incubated overnight at 4 °C. SH-SY5Y cells (1.2×10^4 cells/well) were cultured for 24 h, with the PS-MP group receiving 10 μM RA and PS-MP, and the PS-NP group receiving 10 μM RA with varying PS-NP concentrations. After 7 days of culture with a medium change on day 4, cells were fixed in 2.5 % glutaraldehyde, permeabilized with 0.3 % Triton X-100, and blocked with 2.5 % goat serum and BSA. Cells were incubated overnight with a 1:500 β -tubulin primary antibody (Table S1), followed by Alexa Fluor® 555 secondary antibody (1:500; Abcam, UK) for 1 h. Nuclei were stained with NucBlue™ (Hoechst 33342; Invitrogen, USA), and samples were observed under a fluorescence microscope AxioImagerM2 (Zeiss, Germany). The quantification of immunocytochemistry (ICC) images is described in detail in the Supplementary Information.

2.3. Animal model

Five-week-old C57BL/6 J male mice (Shizuoka Laboratory Center, Japan) were used in the experiments. Mice were maintained under controlled conditions (12 h light/dark cycle) and acclimatized for 1 week before experiments. All procedures followed KIRAMS institutional guidelines and were approved by the IACUC (KIRAMS 2023–0126, 2024–0146). At the initiation of the inhalation study, a total of 54 mice were included, with 18 mice assigned to each group (control (Ctrl), PS-MP, PS-NP). For subsequent analyses, animals were randomly selected, and no specific selection criteria were applied. A total of 100 ppm / 200 μL (20 μg) of PS-MP and PS-NP were administered via inhalation per mouse daily for 16 weeks using a complex compressor nebulizer (Omron Healthcare, Republic of Korea).

2.4. Biodistribution of PS-MP and PS-NP

To identify PS-MP and PS-NP in the striatum and substantia nigra (SN) after inhalation exposure, the brain was extracted and microscopic imaging was performed. PBS Ctrl ($n = 3$), PS-MP ($n = 3$), and PS-NP ($n = 3$) groups were used. Tissue was cryo-sectioned, dried, and stained with NucBlue™ for 20 min, followed by PBS washes. After mounting, samples were observed using a laser scanning confocal microscope (LSM-710, Zeiss, Germany) with a 63 $\times$ objective lens.

To quantify the accumulation of PS-MP and PS-NP, we labeled ^{64}Cu to PS-MP and PS-NP (Shanmugiah et al., 2024). The method was briefly summarized as follows. ^{64}Cu was produced in a cyclotron at KIRAMS. For radiolabeling, amino-functionalized PS-MP (0.2–0.3 μm ; SpheroTech, USA) and PS-NP (20 nm; NANOCs, USA) were used. The radiochemical purity was 95.33 % for PS-MP and 83.25 % for PS-NP. Mice ($n = 5$ per group) were intratracheally administered ^{64}Cu DOTA PS-MP (^{64}Cu -PS-MP) or ^{64}Cu DOTA PS-NP (^{64}Cu -PS-NP) using an aerosol sprayer needle (Natsume, Japan). After 72 h, the mice were euthanized, and the striatum and SN were collected. Radioactivity was measured using a WIZARDS2 γ -counter (PerkinElmer, USA) and expressed as % injected dose per gram (%ID/g).

2.5. Behaviors study

Mice were exposed to PS-MP or PS-NP by daily inhalation for 16

weeks as described in Section 2.3 (Ctrl, $n = 10$; PS-MP, $n = 10$; PS-NP, $n = 10$).

2.5.1. Rotarod test

For training, mice were placed on the rotating rod of a rotarod device (Jeungdo Bio & Plant Co., Ltd., Seoul, Republic of Korea) at a speed of 20 m/s for 10 min. Following training, the test was conducted with the rotation speed accelerating from 10 to 46 m/s over one-min intervals. The cumulative number of falls per mouse was recorded, and the average of three readings was calculated.

2.5.2. Balance beam test

Mice in each experimental group were trained to perform the beam walk task. The apparatus consisted of a 10 mm round beam (Jeungdo Bio & Plant Co., Ltd., Seoul, Republic of Korea) positioned approximately 50 cm above the ground and connected to two poles. One pole featured a starting platform, while the other was equipped with a dark box containing food and nesting material to encourage mice to reach the endpoint. Trial data included the time each mouse took to traverse the beam and the number of paw slips. Performance was assessed based on the average of three consecutive trials for each mouse.

2.5.3. Splay reflex

The splay reflex is a neurophysiological response involving the extension of limbs in reaction to external stimuli (Hatzipetros et al., 2015). The splay reflex helps mice maintain balance and stability. Modified splay reflex scores were used to assess disease onset. Mice were suspended by their tails, and the hindlimb splay was scored from 0 to 3. A score of 0 (normal, 80–90° splay) indicated full hindlimb extension for at least 2 s. Scores of 1 (55–45°), 2 (25–15°), and 3 (10–0°) reflected increasing severity of hindlimb collapse, trembling, or retraction. Each mouse was measured three times, and the average score was recorded.

2.5.4. Grip strength test

For measurement of muscle decline, the mice underwent evaluation for forelimb and overall paw strength by assessing their muscular force and ability to grip a grid while suspended by their tails (Montilla-García et al., 2017; Alonso et al., 2018; Olczak et al., 2022). Each group of mice was positioned on a 10 cm x 10 cm grid, held by the tail, and allowed to grip the grid with their forelimbs. Subsequently, they were gently pushed backward until losing their grip. The grip meter (Jeungdo Bio & Plant Co., Ltd, Seoul, Republic of Korea) recorded the peak kilogram-force (kgf). Similarly, the total force was evaluated by enabling all four paws to grasp the grid. The mice were pulled backward via the tail, and the peak kgf was recorded. The average of three consecutive measurements for each mouse was documented.

2.5.5. Treadmill test

The mice were tested after 3 days of training in the treadmill (Jeungdo Bio & Plant co, Ltd, Seoul, Republic of Korea). At the back of the treadmill, a grid was installed so that there was an electric shock of 0.2 mA when it touched. The shock intensity and frequency were fixed at 200 ms/pulse and 3 Hz. The mice were trained for 10 min at a rate of 8, 18, and 28 m/min for 1–3 days, respectively, and were tested on day 4. During the test, the treadmill started at a rate of 8 m/min and accelerated every 10 m, and was operated at a maximum rate of 28 m/min. The time, distance, and maximum speed of the mouse when it stayed in the "fatigue zone" for >5 s were recorded. The fatigue zone, defined as the belt region within approximately one body length of the shock grid as well as the grid, itself.

2.5.6. Open field test

This experiment was conducted using open field test equipment (Jeungdo Bio & Plant, Seoul, Korea). The apparatus consisted of a rectangular white box with dimensions of 40 cm × 40 cm × 40 cm. Each mouse was individually placed in one corner of the box, and its activity

was recorded for 10 min using a USB sniper camera mounted above the box. The recorded video was analyzed by dividing the arena into central and peripheral zones. Locomotor activity was automatically analyzed using SMART software (Panlab, Spain), and the time spent in each zone and the distance traveled were measured.

2.5.7. Forced swim test

The experiment was conducted in transparent cylindrical tanks with a height of 20 cm and a diameter of 15 cm, with the water temperature set at 28 °C. The water level was adjusted to ensure that the animal's torso was submerged. Dividers were installed between the tanks to prevent the rats from seeing each other and potentially altering their behavior. On the day of the experiment, the animals were placed in the tanks, and the experiment was conducted for 6 min. A video camera was mounted on a tripod to record the session. Behavioral analysis was performed by measuring the time spent by the mice without movement (floating time in the water).

2.5.8. Adhesive removal test

In this experiment, a piece of sticker was attached to the sole of each mouse's paw, and the time taken to remove it was measured. The mice were placed in a bedding-free polycarbonate cage (17 cm × 28 cm × 12 cm). The mice detected and removed the sticker attached to their paws. The time taken by mice in each group to remove the sticker was measured to assess their ability.

2.5.9. Marble burying test

The experiment was conducted using polycarbonate cages (17 cm × 28 cm × 12 cm) with fitted lids. The cages were filled with fresh bedding to a depth of 5 cm. Standard glass toy marbles (assorted styles and colors, 15 mm in diameter, 5.2 g in weight) were arranged in five horizontal rows of four marbles on the surface of the bedding. The cages were placed in a clean, quiet room, and the behavior of the mice was recorded at 10, 20, and 30 min. During the experiment, food and water were withheld from the mice. The cages were covered with a filter top and left undisturbed. During the marble burying test, mouse behavior was observed, and a marble was considered buried if at least two-thirds of its surface was covered by bedding. The average score for each group was then calculated.

2.5.10. Nestlet shredding test

In this experiment, commercially available cotton fiber nestlets (Central Lab Animal Inc., Seoul, Republic of Korea) were used. Each nestlet measured approximately 5 cm × 5 cm, with a thickness of 5 cm and a weight of 2.5 g. The nestlets were placed in the center of the bedding. Mice were allowed to move freely within the cage, and water and food were withheld during the experiment. The activities of the mice were observed and recorded at 30 and 60 min. At the end of the experiment, the shredded nestlets were removed. The percentage of nestlet shredding was calculated as follows:

$$\% \text{ nestlet shredding} = (\text{initial intact nestlets} - \text{unshredded nestlets}) / \text{initial intact nestlets} \times 100$$

2.6. Transcriptome analysis

RNA-sequencing (RNA-Seq) via next-generation sequencing (NGS) was used to compare transcriptomes of the Ctrl, PS-MP, and PS-NP groups ($n = 3$ each). Striatum and SN tissues were collected, stored in RNAlater™, and processed by MacroGen (Republic of Korea) using an Illumina NovaSeq 6000 system. Differentially expressed genes (DEGs) were defined as those exhibiting a log2 fold change > 1 and an FDR adjusted p -value < 0.05. To visualize the expression patterns of DEGs, volcano plot and heatmap analyses were conducted. Gene expression data were normalized based on read counts, followed by log2 transformation. Volcano plots were generated using GraphPad Prism software (v8.0.2; GraphPad Software, USA), where the x-axis represents the

log₂(fold change) and the y-axis represents $-\log_{10}(\text{p-value})$. Heatmaps were generated using the heatmap package in R software (v4.4.0, Austria), based on log₂-transformed, normalized expression values to illustrate the clustering of DEGs across samples. Functional Annotation Clustering (FAC) was conducted using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases ($p < 0.05$). GENEMANIA (v3.5.2, <https://genemania.org/>) was used to visualize gene interaction networks (Warde-Farley et al., 2010).

2.7. Western blot

The striatum and SN tissue samples, stored at -70°C , were homogenized in $1\times$ protein extraction buffer (Abcam, UK) and centrifuged at 4°C for 15 min at 13,000 rpm. The supernatant was collected, and protein concentration was quantified using the Bio-Rad Protein Assay Dye Reagent. Protein samples were loaded into Pierce Lane Marker Reducing Sample Buffer (ThermoFisher, USA) and separated by SDS-PAGE (8 %–12.5 % gels), then transferred to a nitrocellulose membrane. The membrane was blocked with 5 % skim milk in TBST for 1 h, incubated overnight at 4°C with a 1:1000 diluted primary antibody (the antibodies used are listed in Table S1), washed, and treated with an HRP-conjugated secondary antibody (1:10,000; Abcam, UK). After additional washing, results were detected using Pierce Pico Western blotting substrate (ThermoFisher, USA) and visualized with the e-BLOT Touch Imager (e-BLOT, China).

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

The striatum and SN tissue samples, stored at -70°C , were homogenized with 1 ml of TRI Reagent (Molecular Research Center, USA). After incubation and addition of chloroform, the mixture was vortexed and centrifuged at 4°C for 15 min at 14,000 g. The supernatant was mixed with isopropanol, incubated, and centrifuged again. The RNA pellet was washed with cold 75 % ethanol, dried, and resuspended in Ultra-Pure Water for quantification using NanoDrop. 2.5 μg of RNA was used to synthesize cDNA (Superscript IV kit, Invitrogen, Lithuania) and then analyzed via qRT-PCR using SYBR Green Master-Mix (Applied Biosystems, Lithuania) with a real-time PCR system (Applied Biosystems 7500). Relative gene expression levels were calculated using the $\Delta\Delta\text{Ct}$ method. The primers used are shown in Table S2.

2.9. Immunohistochemistry (IHC)

Section samples were washed twice with PBS, permeabilized in 0.3 % Triton X-100 in TBS for 15 min, and treated with 0.3 % hydrogen peroxide for 10 min. After washing with PBS, a blocking solution (2.5 % normal goat serum and 2.5 % BSA in TBST) was applied for 1 h. The primary antibody (1:300), as listed in Table S1, was added and incubated overnight at 4°C . After washing with TBST, samples were incubated with an HRP secondary antibody (1:500) for 1 h. The reaction was developed using a DAB Substrate Kit (Abcam, UK) for 1 min, followed by PBS washes. Finally, the samples were mounted on slides and left to dry for over a day.

2.9.1. Nissl staining

After IHC, the slides were air-dried for 24 h, then immersed in a 1:1 alcohol/chloroform solution overnight. They were rehydrated through a series of alcohol solutions (100 %, 95 %, 80 %, 50 %) and rinsed in distilled water. The sections were stained with pre-warmed Cresyl violet solution (VibroVivoBiotech, Maryland) for 5 min, rinsed in water, and differentiated in 95 % alcohol. The sections were dehydrated in 100 % alcohol, cleared in xylene (5 min), and mounted with a coverslip using mounting solution (Abcam, UK).

2.10. Mouse model of polystyrene stereotaxic injection

Mice were anesthetized using alfaxalone (100 mg/kg) and xylazine (10 mg/kg) and secured on a stereotaxic frame (Laiyu Biotech, China) for the surgical procedure. For the striatum model, 10 μg / 1 μl of PS-MP or PS-NP solution was administered into the each striatum of each mouse using a Hamilton syringe (Hamilton Company, USA) at the following coordinates relative to the bregma: anteroposterior (AP) $+0.62\text{ mm}$, mediolateral (ML) $\pm 1.72\text{ mm}$, dorsoventral (DV) -3.5 mm . For the SN injection model, 3 μg / 0.3 μl of PS-MP or PS-NP solution was injected into the each SN of mice in each group at the following coordinates relative to the bregma: AP -3.2 mm , ML $\pm 1.2\text{ mm}$, DV -4.3 mm .

2.11. Statistical analysis

Statistical analyses were conducted using GraphPad Prism software (version 5.0, GraphPad Software; USA). Data are presented as mean $\pm$ standard error of the mean (SEM). Data normality was assessed prior to analysis, and parametric or nonparametric tests were applied as appropriate. For comparisons between two groups, unpaired two-tailed t -tests were performed. For comparisons involving more than two groups, one-way analysis of variance (ANOVA) was applied, followed by Tukey's multiple comparisons test, depending on the experimental design. The * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.005$.

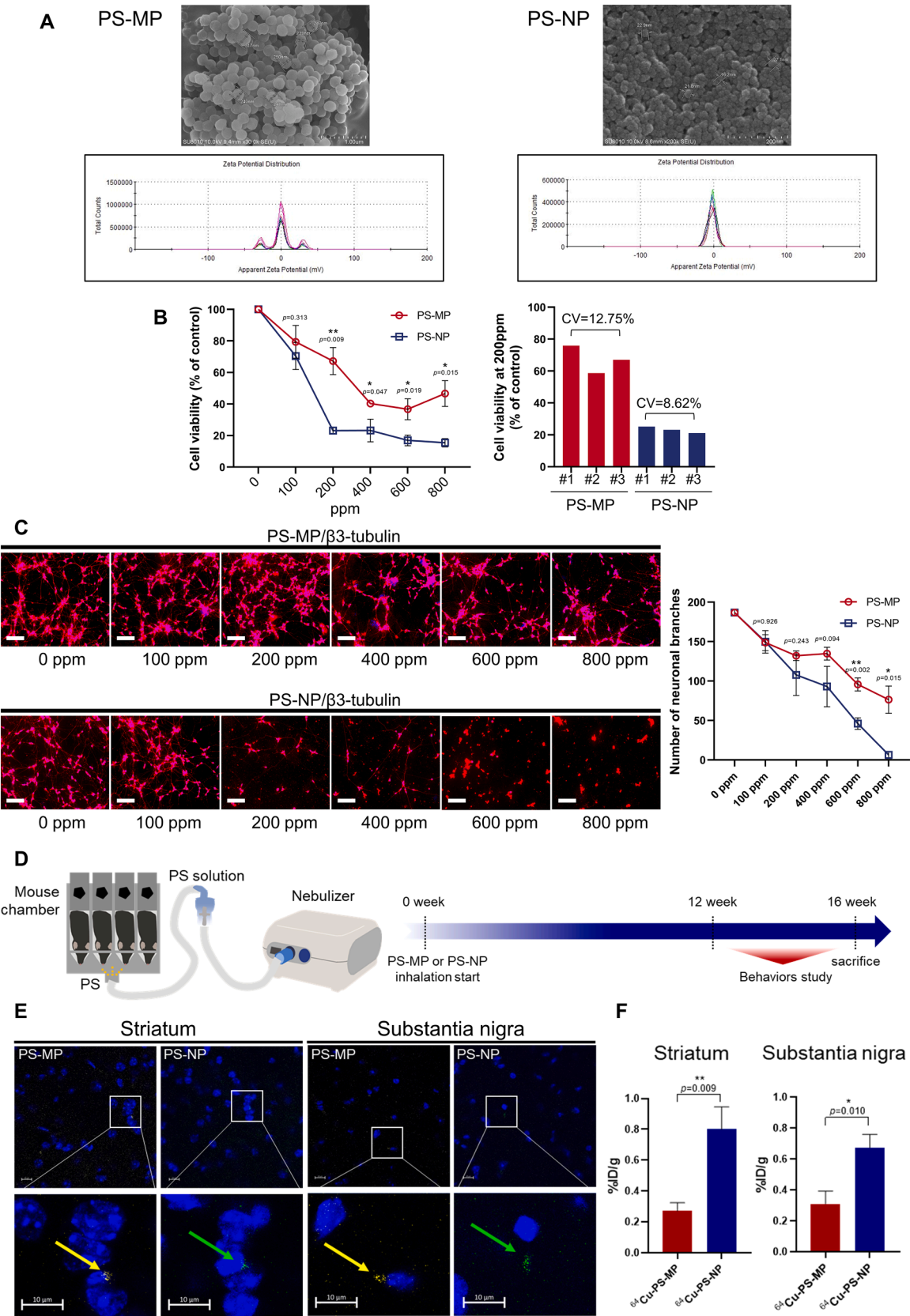
3. Results

3.1. PS-NP induces greater cytotoxicity than PS-MP and leads to neuronal morphological damage

The sizes of the purchased PS-MP and PS-NP were confirmed using FE-SEM (Fig. 1A). Zeta potential was measured to evaluate the surface charge characteristics of PS-MP and PS-NP. PS-MP exhibited a zeta potential of $-0.31 \pm 0.10\text{ mV}$, while PS-NP showed $-1.7 \pm 0.16\text{ mV}$. These results suggest that both PS-MP and PS-NP possess surface charge properties that are nearly electrically neutral (Fig. 1A). PS-NP demonstrated greater cytotoxicity than PS-MP at all concentrations used in the experiment, starting from 200 ppm (Fig. 1B, * $p < 0.05$, ** $p < 0.01$). PS-NP induced significantly higher cytotoxicity than PS-MP at 200 ppm, reducing cell viability to $23.12 \pm 4.95\%$ compared to $67.21 \pm 1.15\%$ for PS-MP. ICC staining of $\beta 3$ -tubulin showed that PS-NP caused greater neuronal morphological damage than PS-MP in a dose-dependent manner (Fig. 1C). Exposure to PS-NP significantly reduced the number of neuronal branches in a dose-dependent manner compared with PS-MP, with notable decreases observed at 400 and 600 ppm (* $p < 0.05$, ** $p < 0.01$) (Fig. 1C and Figure S1). qRT-PCR analysis revealed that exposure to 400 ppm PS-NP, but not PS-MP, significantly upregulated pro-inflammatory cytokines *TNF- α* , *IL1B*, and *IL6* in dopaminergic neurons (Figure S2).

3.2. Inhalation of PS-NP resulted in greater accumulation compared to PS-MP

The experimental timeline was illustrated in Fig. 1D. As shown in Fig. 1E, confocal microscopy demonstrated the intracellular localization of PS-NP and PS-MP in the striatum and SN regions. In the striatum, the uptake of ^{64}Cu -PS-MP was $0.27 \pm 0.05\%$ ID/g, while ^{64}Cu -PS-NP was $0.80 \pm 0.15\%$ ID/g. In the SN region, ^{64}Cu -PS-MP was $0.31 \pm 0.08\%$ ID/g, while ^{64}Cu -PS-NP was $0.67 \pm 0.09\%$ ID/g. ^{64}Cu -PS-NP exhibited 2.9-fold and 2.16-fold higher distribution in the striatum and SN regions, respectively, compared to ^{64}Cu -PS-MP (Fig. 1F, * $p < 0.05$, ** $p < 0.01$).



(caption on next page)

Fig. 1. Characterization of polystyrene (PS), in vitro experimental analysis, and detection and quantitative analysis of PS after in vivo experiments. (A) Field emission scanning electron microscope (FE-SEM) images and zeta potential graphs of polystyrene microplastics (PS-MP) and polystyrene nanoplastics (PS-NP). Both PS-MP and PS-NP exhibited nearly neutral surface charge properties. (B) Cell viability (% of control) of retinoic acid (RA)-differentiated SH-SY5Y cells after exposure to PS-MP and PS-NP (0–800 ppm). The right graph shows cell viability at 200 ppm with coefficient of variation (CV) values. Data are expressed as a percentage of control (Ctrl) cells ($n = 3$). PS-NP induced markedly greater cytotoxicity than PS-MP ($*p < 0.05$, $**p < 0.01$). (C) Immunocytochemistry (ICC) analysis of dendritic branching and morphological changes in dopaminergic neurons following exposure to PS-MP and PS-NP. Representative images of cultured neurons treated with varying concentrations of PS-MP or PS-NP for 7 days. Neurons were stained with β 3-tubulin, a neuronal marker (red), and counterstained with Hoechst (blue) to visualize nuclei. Scale bar = 100 μ m. PS-NP exposure resulted in dose-dependent neuronal morphological damage and greater reduction in dendritic branching compared to PS-MP. The number of dopaminergic neuron branches was quantified and is presented as a graph. (D) Schematic of the inhalation exposure system and experimental schedule for animals. Mice were divided into three groups: Ctrl, PS-MP, and PS-NP. They were exposed to inhalation once daily for 16 weeks. Behavioral tests were conducted between weeks 12 and 16. At the end of week 16, the mice were sacrificed for tissue collection. (E) Detection of PS-MP (yellow arrow indicated) and PS-NP (green arrow indicated) in tissue samples using confocal microscopy. Representative images showing the intracellular localization of PS-MP and PS-NP in the brain sections ($n = 3$). The nuclei were stained with Hoechst (blue). Scale bar = 10 μ m. PS-NP showed greater accumulation in brain regions compared with PS-MP. (F) In vivo distribution of 64 Cu-labeled PS-MP and PS-NP in the striatum and SN ($n = 5$). The graph represents the percentage of injected dose per gram (%ID/g) measured in the striatum and SN tissue of mice exposed to 64 Cu-labeled PS-MP or PS-NP. PS-NP exhibited 2.9-fold higher accumulation in the striatum and 2.16-fold higher accumulation in the SN than PS-MP. Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed using an independent t -test. $*p < 0.05$, $**p < 0.01$.

3.3. PS-NP were more likely to induce Parkinson's disease compared to PS-MP (Behavioral study)

The rotarod test was performed to assess motor coordination (Deacon, 2013). In the rotarod test, the Ctrl group's motor coordination was 134.19 ± 13.11 s, while the PS-MP and PS-NP groups showed 123.13 ± 11.01 s and 75.34 ± 8.29 s, respectively. PS-NP group showed approximately 1.63-fold lower motor coordination time compared to the PS-MP group (Fig. 2A, $**p < 0.01$).

Fine motor coordination and balance were evaluated using the balance beam test, which assesses a mouse's ability to traverse a high and narrow beam to reach a safe platform (Luong et al., 2011). In the balance beam test, the PS-NP group (0.95 ± 0.08 count) exhibited a significantly higher number of foot slips, with a 1.42-fold increase compared to the PS-MP (0.67 ± 0.11 count) group. (Fig. 2B, $**p < 0.01$, $***p < 0.001$). However, no significant differences were observed among the groups in the time taken to cross the beam.

The splay reflex test, a method for assessing neuromuscular function, allows evaluation of the onset, progression, and severity of paralysis (Hatzipetros et al., 2015). The splay reflex score in the PS-NP group (0.46 ± 0.08) was significantly elevated, showing a 2.19-fold increase compared to the Ctrl group (0.21 ± 0.05) (Fig. 2C).

Grip strength and treadmill tests were conducted to assess neuromuscular function (Castro and Kuang, 2017). The PS-NP group (67.15 ± 5.84 kgf) exhibited significantly reduced forelimb grip strength, measuring 1.28 times lower than the Ctrl group (88.99 ± 4.68 kgf) and 1.26 times lower than the PS-MP group (87.61 ± 4.10 kgf) ($*p < 0.05$). Additionally, overall grip strength was significantly decreased, showing a 1.33-fold reduction compared to the Ctrl group and a 1.30-fold reduction compared to the PS-MP group (Fig. 2D, $*p < 0.05$, $***p < 0.001$). In the treadmill test, only the PS-NP group (321.27 ± 40.82 s) demonstrated a significant decrease in the time to reach a fatigued state, which was reduced by 1.41-fold compared to the Ctrl (452.27 ± 15.44 s) (Fig. 2E, $*p < 0.05$).

The open field test and forced swim test were conducted to evaluate NMS of PD, such as anxiety, exploratory behavior, and depression. In the open field test, mice exhibiting higher anxiety levels tend to spend more time near the walls of the field's perimeter and display reduced exploratory behavior (Horka et al., 2024). The results of the open field test showed that mice exposed to PS-MP and PS-NP exhibited reduced activity in the central area of the field. The distance traveled in the center was reduced by 2.03-fold in the PS-NP group (7861.37 ± 1151 cm) compared to the Ctrl (16013.30 ± 1681 cm), which was a greater reduction than the 1.67-fold decrease observed in the PS-MP group (9587.6 ± 1369 cm). Additionally, the total exploration distance within the field was significantly reduced in the PS-MP- and PS-NP-exposed groups, indicating increased anxiety in these mice. (Fig. 2F, $*p < 0.05$, $**p < 0.01$).

The forced swim test evaluates depression-related behaviors and the effects of neurobiological manipulations by observing the animal's tendency to lose hope in escaping a stressful environment (Can et al., 2012). The test results showed a significant increase in immobility time in the PS-NP group (132.00 ± 10.44 s), which was 1.47 fold higher than the Ctrl group (90.00 ± 13.87 s), indicating a reduced motivation to escape and suggesting heightened depressive-like behavior (Fig. 2G, $*p < 0.05$).

The adhesive removal test, which evaluates sensorimotor function in mice, revealed that the PS-NP group (286.57 ± 55.23 s) took significantly longer to remove the adhesive from the paw compared to the Ctrl group (101.57 ± 24.68 s), with a 2.82-fold increase in removal time. This delay was also greater than that observed in the PS-MP group (151.00 ± 17.42 s), which showed a 1.90-fold increase relative to the Ctrl. This result indicates impaired sensorimotor function in mice exposed to PS-NP (Fig. 2H, $**p < 0.01$) (Bouet et al., 2009).

The marble burying test, which assesses anxiety-driven compulsive behavior by measuring the number of marbles buried by mice, revealed a significant increase in marble burying behavior in both PS-MP- and PS-NP-exposed groups. The PS-MP group (6.25 ± 0.86 counts) buried 2.08 times more marbles than the Ctrl group (3.00 ± 0.94 counts), while the PS-NP group (6.38 ± 0.94 counts) buried 2.13 times more, indicating heightened anxiety-related behaviors in the PS-MP- and PS-NP-exposed mice. This result suggests increased compulsive behavior associated with anxiety in these groups (Fig. 2H, $*p < 0.05$) (Jimenez Chavez and Szumlanski, 2024).

In the nestlet shredding test, the PS-NP group (6.75 ± 0.82 %) exhibited a significant reduction in nestlet shredding behavior, with the shredding percentage being 2.63 times lower than the Ctrl group (17.79 ± 3.25 %) and 1.90 times lower than the PS-MP group (12.81 ± 1.60 %). This suggests a decreased interest in novel materials in the PS-NP-exposed mice (Dorninger et al., 2020). This behavior may be associated with depression, a NMS commonly observed in PD (Fig. 2H, $**p < 0.01$) (Barnes et al., 2017).

3.4. PS-NP inhalation alters PD-related genes more than PS-MP

Heat map analysis revealed a total of 109 significant DEGs in the PS-MP group and 128 significant DEGs in the PS-NP group compared to the Ctrl (Fig. 3A), and identified PD-related genes, with two genes (*Kcnn4*, *Neurod1*) in the striatum and two genes (Toll-like receptor 7 (*Tlr7*), *Cartpt*) in the SN (Fig. 3B). PD-related genes were also shown in volcano plot (Fig. 3C). qRT-PCR confirmed the upregulation of *Kcnn4* and downregulation of *Neurod1* in the striatum, as well as the upregulation of *Tlr7* and downregulation of *Cartpt* in the SN. Western blot analysis confirmed increased KCNN4 and decreased NEUROD1 levels in the striatum, along with increased TLR7 and decreased CART levels in the SN (*Cartpt* encodes the CART protein). (Fig. 3D, E, Figure S3, $*p < 0.05$,

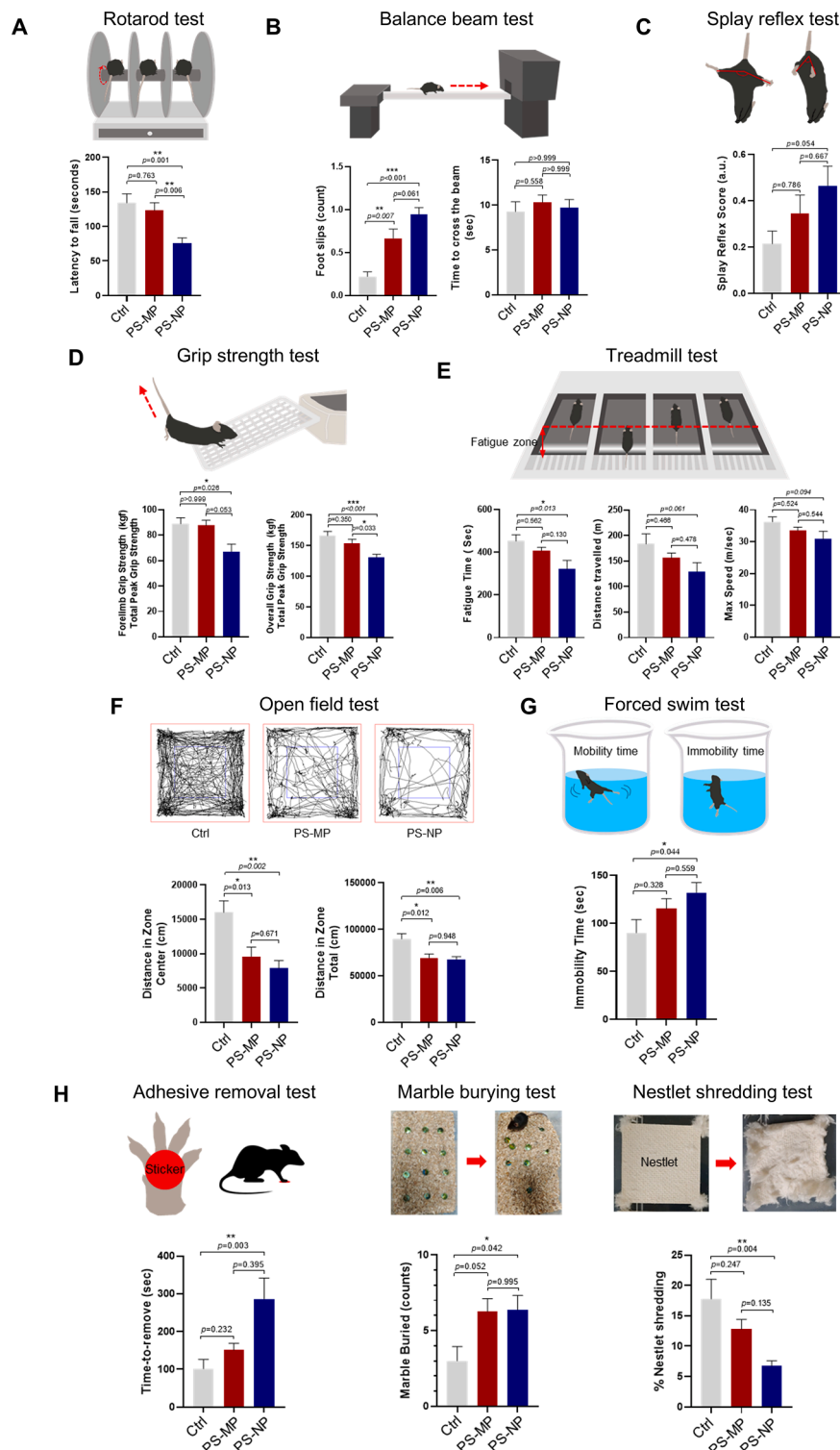
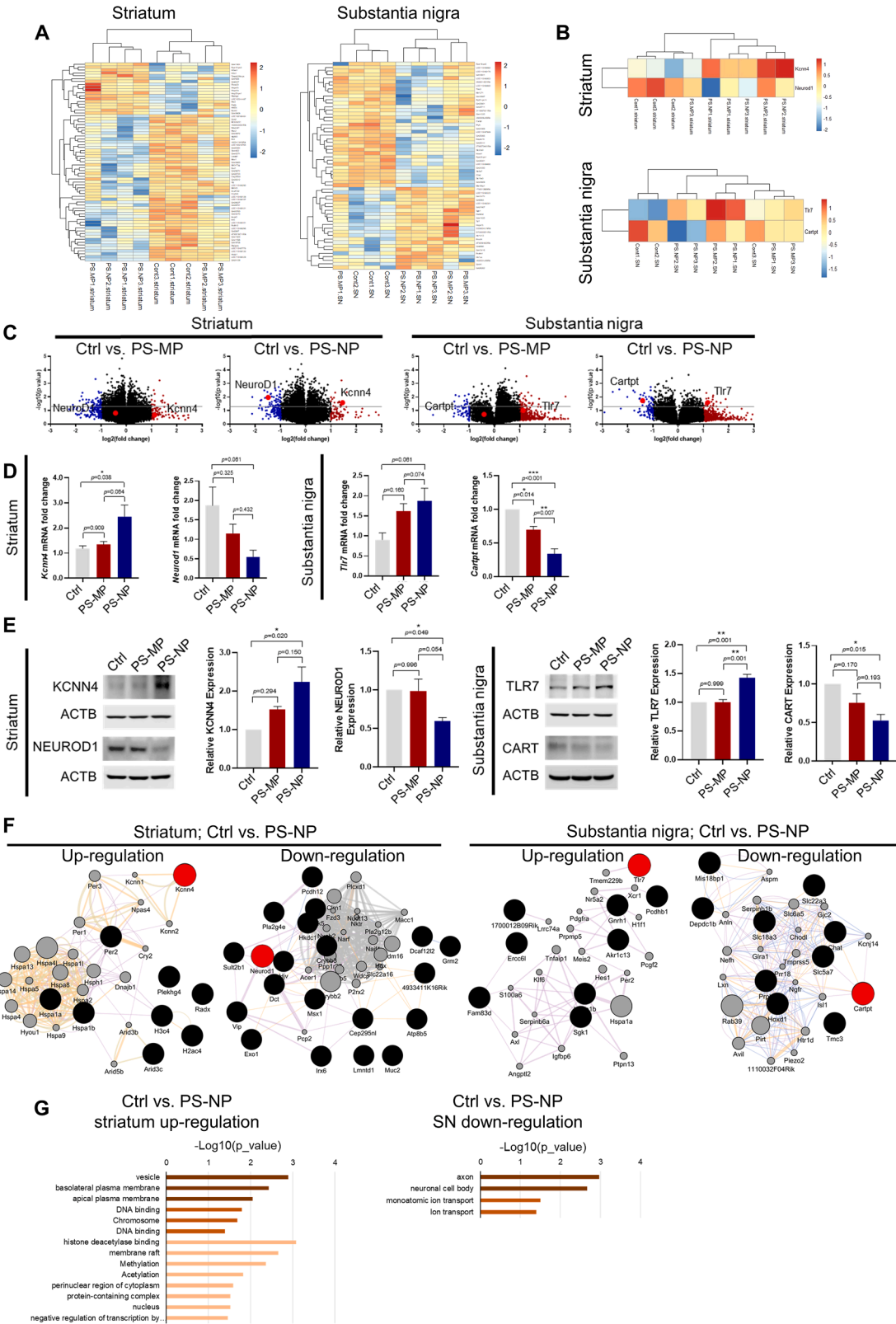


Fig. 2. Behavioral analysis results of mice exposed to polystyrene microplastics (PS-MP) and polystyrene nanoplastics (PS-NP) ($n = 10$). (A) Rotarod test assessing motor coordination. PS-NP-exposed mice showed significantly shorter latency to fall compared with PS-MP and control (Ctrl) groups. (B) Balance beam test evaluating fine motor coordination and balance. PS-NP exposure resulted in a higher number of foot slips compared with PS-MP and Ctrl groups. (C) Splay reflex test. PS-NP-exposed mice exhibited a significantly increased splay reflex score compared with Ctrl. (D) Grip strength test. PS-NP exposure significantly reduced forelimb grip strength compared with both PS-MP and Ctrl groups. (E) Treadmill test. PS-NP-exposed mice showed a significantly reduced time to fatigue compared with Ctrl. (F) Open field test assessing anxiety and exploratory behavior. PS-NP exposure led to a reduction in central zone activity and total exploration distance compared with PS-MP and Ctrl groups. (G) Forced swim test for depression-like behavior. PS-NP-exposed mice exhibited significantly increased immobility time compared with Ctrl. (H) Adhesive removal test, Marble burying test and Nestlet shredding test. PS-NP-exposed mice showed delayed adhesive removal, increased marble burying behavior, and significantly reduced nestlet shredding compared with PS-MP and Ctrl groups ($*p < 0.05$, $**p < 0.01$), indicating impaired sensorimotor function, heightened anxiety-related behavior, and depressive-like phenotypes. Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed using an one-way ANOVA. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

*****p* < 0.01, ****p* < 0.001).**

GeneMANIA and FAC analysis identified major upregulated genes in the striatum of the PS-NP group was related with metabolic exchange and stress responses (enrichment score of 1.59), chromatin remodeling,

DNA damage repair, maintenance of chromosome structure, and transcriptional regulation (enrichment score of 1.37), and epigenetic regulation, cellular signaling, and transcriptional repression (enrichment score of 1.36). However, no significant functional clusters were



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Fig. 3. Transcriptomic and validation analysis of Parkinson's disease (PD)-related genes in striatum and substantia nigra (SN) of mice. (A) Heatmap of RNA sequencing analysis showing 109 significant differentially expressed genes (DEGs) in the polystyrene microplastics (PS-MP) group and 128 DEGs in the polystyrene nanoplastics (PS-NP) group compared with control (Ctrl) ($n = 3$). (B) Heatmap highlighting PD-related DEGs, including *Kcnn4* and *Neurod1* in the striatum, and toll-like receptor 7 (*Tlr7*) and *Cartpt* in the SN. (C) Volcano plot of RNA sequencing results showing PD-related DEGs validated in subsequent assays. Bright red closed circle indicated PD-related genes that were validated by qRT-PCR and Western blot analysis. (D) qRT-PCR validation confirming upregulation of *Kcnn4* and *Tlr7* and downregulation of *Neurod1* and *Cartpt* in PS-NP-exposed mice ($n = 3$). Data are presented as fold change relative to the Ctrl group. (E) Western blot validation of protein expression, showing increased *KCNN4* and *TLR7* and decreased *NEUROD1* and *CART* in PS-NP-exposed mice ($n = 3$). Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed using a one-way ANOVA. (F) GeneMANIA network analysis of DEGs in the striatum and SN between the Ctrl and PS-NP groups. Node colors represented different functional categories, and edge thickness indicated interaction strength. PS-NP exposure alters genes related to stress response, metabolism, neural connectivity, inflammation, and neurotransmission. (G) Functional characteristics of DEGs in Ctrl and PS-NP groups. The figure shows the functional annotation and enrichment analysis of DEGs identified in the striatum and SN tissues across the experimental groups. Genes are clustered based on their functional similarities, with each color representing a distinct cluster. PS-NP exposure altered pathways linked to stress responses, DNA repair, epigenetic regulation, and axonal and neuronal body structure. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

observed among the downregulated genes in the striatum of the PS-NP group (Fig. 3F, G).

In the SN of the PS-NP group, no significant clusters were observed among the upregulated genes. However, two major functional clusters were identified among the downregulated genes: cholinergic neurotransmission, axonal and neuronal body structure, and metabolism (enrichment score of 1.07), and ion transport and neurotransmission pathways (enrichment score of 0.57).

3.5. Decrease of tyrosine hydroxylase and increase of phospho- α -synuclein (Ser129)

Reduced tyrosine hydroxylase (TH) expression indicates the loss of dopamine-producing neurons, a hallmark of the progressive neurodegenerative disorder PD (Jin et al., 2022). α Syn is known to undergo phosphorylation at Ser129, which is a hallmark of synucleinopathies, such as PD (Fujiwara et al., 2002). To assess the effect of PS inhalation exposure on TH and phospho- α -synuclein (phospho- α Syn (Ser129)), western blot and IHC experiments were conducted. In the SN, the PS-NP group exhibited a significant reduction in TH expression, as shown by both Western blot analysis (1.35 ± 0.06 -fold decrease compared to the Ctrl, Fig. 4A, Figure S4, * $p < 0.05$) and IHC staining (Fig. 4B). Western blot analysis showed no significant change in TH expression in the striatum (Fig. 4A, Figure S4). In the striatum, phospho- α Syn (Ser129) was 1.12-fold increase for PS-NP compared to PS-MP. 1.28-fold increase for PS-MP and 1.43-fold increase for PS-NP compared to the Ctrl (Fig. 4C, Figure S4, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). In the SN, phospho- α Syn (Ser129) was 1.53-fold increase for PS-MP and 1.91-fold increase for PS-NP compared to the Ctrl (Fig. 4C, Figure S2, * $p < 0.05$, ** $p < 0.01$). The IHC results of phospho- α Syn (Ser129) in the striatum and SN were shown in Fig. 4D. To investigate the potential for more severe neurological effects induced by PS-MP and PS-NP, a stereotaxic injection model was employed to directly deliver PS into the mouse brain. This approach allowed simulation of conditions in which PS-MP and PS-NP have crossed or bypassed the blood-brain barrier (BBB), either due to long-term systemic exposure or pathological BBB disruption. By enabling localized and high-concentration exposure, the model facilitates the evaluation of potential neurotoxic outcomes under scenarios of chronic accumulation and intensified brain burden. IHC analysis was performed to examine the expression of TH and the aggregation of phospho- α Syn (Ser129). The results revealed that, compared to the PS-MP and Ctrl groups, the PS-NP group exhibited reduced TH expression and increased phospho- α Syn (Ser129) aggregation. (Fig. 5A, B).

3.6. PS-NP inhalation increased inflammation

In the striatum region, western blot analysis revealed that glial fibrillary acidic protein (GFAP) levels increased by 2.88 ± 0.41 -fold, and ionized calcium-binding adapter molecule 1 (IBA1) levels increased by 7.28 ± 2.18 -fold in the PS-NP group compared to the Ctrl group (Fig. 6A, Figure S5, * $p < 0.05$). IHC staining for GFAP and IBA1 showed an

increase in astrocyte staining and a higher proportion of activated microglia in the PS-NP group (Fig. 6B). Western blot analysis was performed. In the PS-NP group, TNF- α increased by 2.98 ± 0.32 -fold, IL-1 β by 2.57 ± 0.35 -fold, and IL-6 by 2.36 ± 0.42 -fold in the striatum (Fig. 6C, Figure S5, * $p < 0.05$, ** $p < 0.01$).

In the SN, western blot analysis revealed that GFAP levels increased by 1.75 ± 0.15 -fold, and IBA1 levels increased by 1.59 ± 0.16 -fold in the PS-NP group (Fig. 6D, Figure S5, * $p < 0.05$). IHC staining for GFAP and IBA1 in the SN also showed increased astrocyte staining and a higher prevalence of activated microglia in the PS-NP group (Fig. 6E). Western blot analysis revealed that TNF- α increased by 2.12 ± 0.62 -fold, IL-1 β by 1.95 ± 0.26 -fold, and IL-6 by 1.79 ± 0.15 -fold in the PS-NP group compared to the Ctrl group (Fig. 6F, Figure S5, * $p < 0.05$). There was a trend of increased inflammatory responses in the PS-NP exposed groups compared to the PS-MP group.

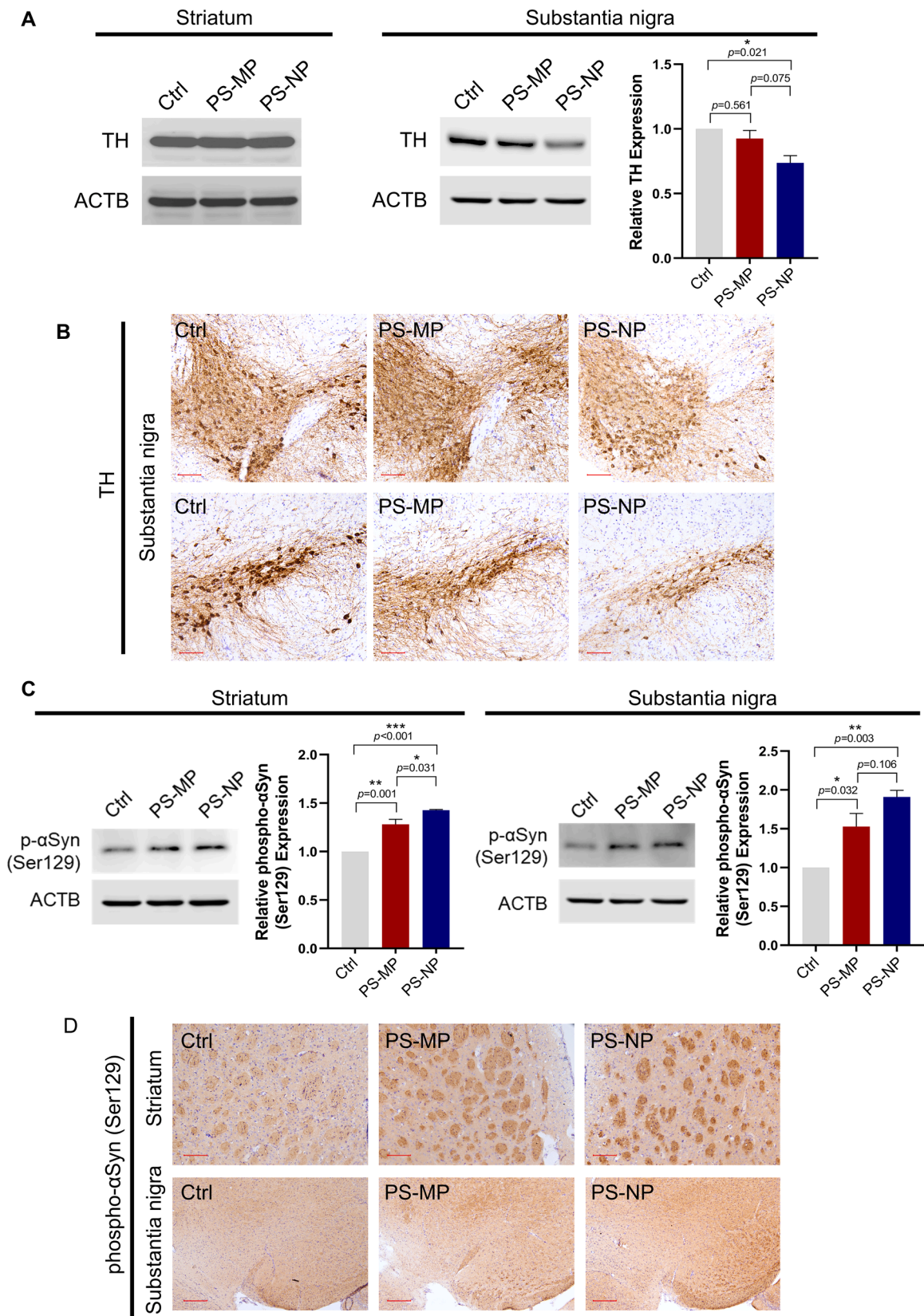
4. Discussion

Recent attention has been drawn to MP and NP as emerging airborne pollutants, yet their impact on the central nervous system via inhalation remains poorly understood. In this study, we provide the first in vivo evidence that chronic inhalation of PS-NP, even at doses below estimated human environmental exposure levels, may increase the risk of PD in mice. To the best of our knowledge, this is the first study to demonstrate that PS-NP pose a higher risk of inducing PD than PS-MP. As inhalation is a primary route of MP exposure and recent evidence indicates a potential link between airborne MP and PD (Hong and Kim, 2024; Prata et al., 2020; Aderinto et al., 2025), we investigated the size-dependent neurotoxic effects of PS-MP and PS-NP using dopaminergic neuronal models and environmentally relevant inhalation paradigms.

The rationale for comparing PS-MP and PS-NP lies in their size-dependent physicochemical properties and biological behaviors (Hong and Kim, 2024). Compared to MP, NP exhibit enhanced mobility and bioavailability owing to their smaller size and higher surface reactivity. These properties enable them to penetrate biological barriers, accumulate in internal organs such as the brain and placenta, and induce systemic toxicity, immune dysregulation (Hong and Kim, 2024). Therefore, we hypothesized that chronic PS-NP inhalation promotes oxidative stress, neuroinflammation, and neuronal dysfunction, thereby contributing to increased vulnerability to PD.

Our study demonstrated that PS-NP are more toxic to neurons than PS-MP (Fig. 1C, D), and that PD symptoms are further exacerbated by PS-NP in in-vivo experiments (Fig. 2-6). The toxicity of PS-NP observed in this study, which affects the number of neurites, is likely to have a significantly detrimental impact on the function of dopamine neurons (Volpicelli et al., 2020). In light of our findings with PS-NP, it is therefore critical to consider inhalation not only as an exposure route but as a potential mechanistic driver of PD pathogenesis.

While previous studies have reported that MP ingestion induces locomotor deficits in *C. elegans*, supporting their neurotoxic potential (Jeong et al., 2024). Our study substantially advances this field by using



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Fig. 4. Western blot and immunohistochemistry (IHC) analysis of tyrosine hydroxylase (TH) and phospho- α Syn (Ser129) expression in striatum and substantia nigra (SN) of mice. (A) Western blot analysis of TH expression in striatum and SN tissues ($n = 3$). The representative Western blot images show TH expression in the striatum and SN from control (Ctrl), polystyrene microplastics (PS-MP), and polystyrene nanoplastics (PS-NP) groups. PS-NP exposure reduced TH expression in the SN, while no significant change was observed in the striatum. The graph represents the quantification of TH protein expression levels normalized to ACTB. Data are presented as fold change relative to the Ctrl group. (B) IHC staining of TH in the SN region ($n = 3$). TH-positive neurons are visualized as brown-stained cells. The PS-NP group showed reduced TH immunoreactivity compared with PS-MP and Ctrl groups. Scale bar = 100 μ m. (C) Western blot analysis of phospho- α Syn (Ser129) expression in striatum and SN tissues ($n = 3$). PS-NP exposure increased phospho- α Syn levels in both striatum and SN compared with PS-MP and Ctrl groups. (D) Representative IHC images of phospho- α Syn (Ser129) in striatum and SN ($n = 3$). PS-NP-exposed mice showed increased phospho- α Syn immunoreactivity. Scale bar = 100 μ m. Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed using a one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

a mammalian model with a realistic inhalation exposure route and environmentally relevant doses. Comprehensive details regarding the dosing regimen are provided in the Methods section (2.3. Animal model). This study provides the first *in vivo* evidence that PS-NP exposure is associated with increased PD risk in a size-dependent manner. This association is supported by reduced TH, α -synuclein accumulation, PD-related behavioral deficits, and transcriptomic alterations observed in PD-relevant brain regions in mice. These findings highlight the elevated neurotoxic potential of nanoscale particles when delivered via respiratory exposure.

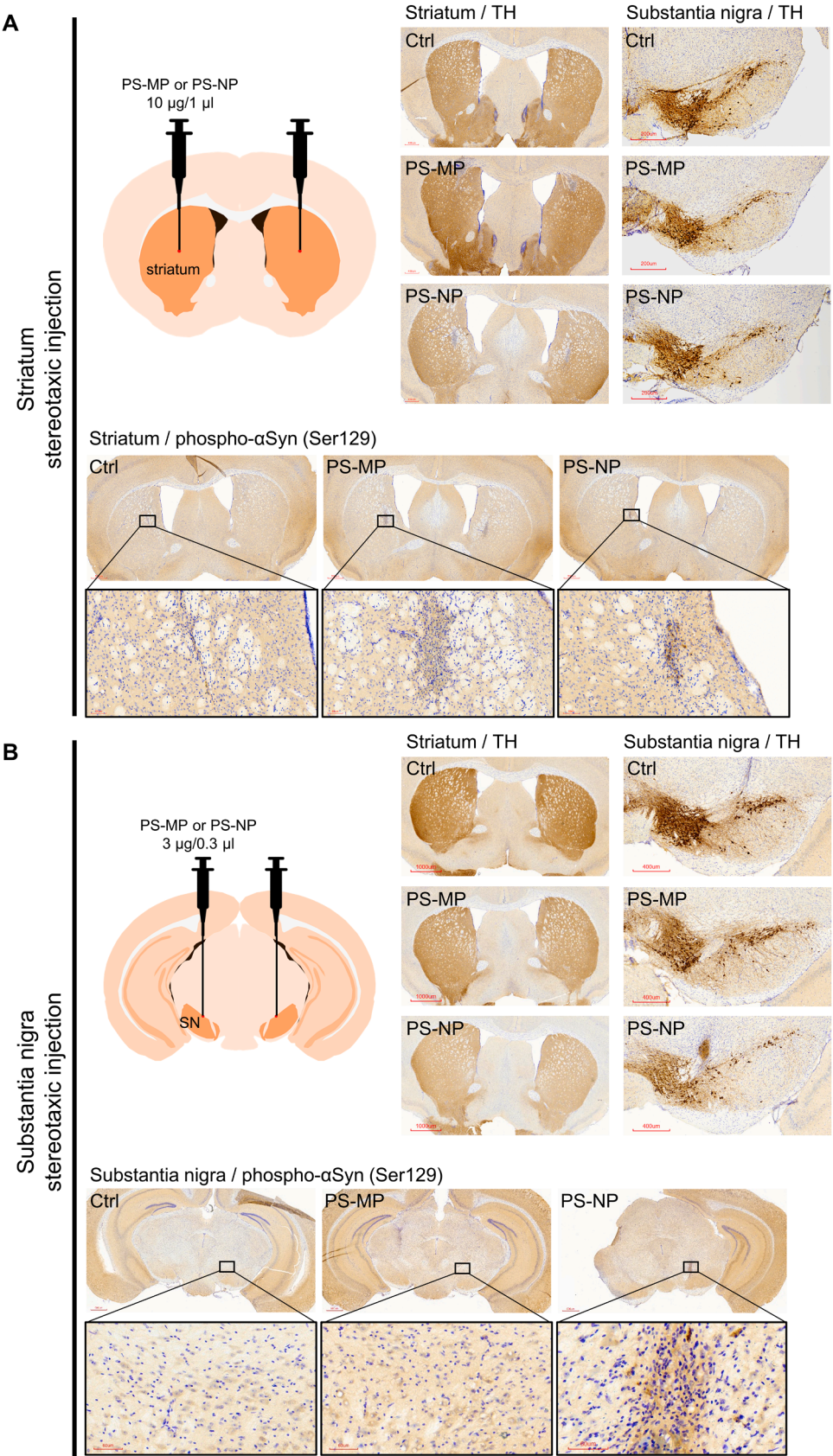
In terms of exposure, despite the administration of 20 μ g of PS-NP per mouse per day, radiotracing analysis revealed that only 3.87 ng of the administered dose accumulated in the striatum and SN combined. When compared to the human-equivalent environmental inhalation dose—calculated as 4.11 μ g/day for a 20 g mouse using interspecies conversion from reported indoor air MP exposure levels (16.71 μ g/kg/day) (Nawab et al., 2024; Nair and Jacob, 2016)—the amount that reached the striatum and SN corresponds to approximately 27-fold lower than the total environmental exposure level reported by Song et al. (460.20 μ g/kg/day, in the ref (Song et al., 2024)), yet it is essentially identical to the estimated daily human respiratory intake of MP from indoor dust (17 μ g/kg/day, in the ref (Nawab et al., 2024)), thereby underscoring the environmental relevance and conservative nature of the dose applied in this study.

Given that this low-level accumulation coincided with PD-relevant molecular, histopathological, and behavioral alterations, our findings indicate a potential increase in PD risk under environmentally realistic exposure conditions. Recent reports have confirmed the presence of MP in human lungs and postmortem brain tissues, suggesting their ability to translocate to and accumulate within the central nervous system (Nihart et al., 2025). However, the biodistribution of MP following inhalation remains largely uncharacterized. In our previous study, we employed 64 Cu-labeled PS-MP and PS-NP to track their *in vivo* distribution in a murine inhalation model using PET imaging and γ -counting (Shanmugiah et al., 2024). Positron Emission Tomography (PET) imaging up to 72 h after intratracheal instillation revealed that PS-MP showed sustained retention in the lungs, whereas PS-NP cleared more rapidly and accumulated in extrapulmonary organs including the brain (Shanmugiah et al., 2024). The mean %ID/g of 64 Cu-PS-NP in the brain was 2.26-fold higher than that of PS-MP (Shanmugiah et al., 2024), suggesting that PS-NP may have a greater propensity to penetrate the brain and exert neurotoxic effects. In this study, we employed 64 Cu-labeled PS-MP and PS-NP to quantitatively evaluate in PD-relevant brain regions, including the striatum and SN following inhalation exposure. Radiotracing measurements confirmed that both PS-MP and PS-NP reached the brain, with significantly higher accumulation of PS-NP in the striatum and SN than PS-MP. To our knowledge, this is the first study to quantitatively demonstrate size-dependent accumulation of inhaled PS in PD-relevant brain regions, such as the striatum and SN, using radiotracer-based biodistribution analysis.

In the present study, we have demonstrated that inhalation exposure to nanoscale PS particles induces PD-like neuropathology and behavioral deficits in a size-dependent manner. These findings fit within a broader context in which inhaled neurotoxins have long been implicated in dopaminergic neurodegeneration. In 1983, young

individuals who either intravenously injected or inhaled heroin contaminated with the protoxin MPTP developed an acute parkinsonian syndrome; following metabolic conversion by monoamine oxidase-B in astrocytes, MPP⁺ selectively enters and destroys nigrostriatal dopamine neurons, recapitulating key features of PD (Langston et al., 1983). Studies using animal models with intranasal MPTP administration have further substantiated the notion that the olfactory and respiratory mucosa can act as entry routes for airborne neurotoxins (Prediger et al., 2006). Likewise, agricultural chemicals such as inhaled paraquat (McCormack et al., 2005) and inhaled rotenone (Emmrich et al., 2013)—both capable of aerosolization in dusty or spray-drift environments—have been shown to produce dopaminergic cell loss when delivered via inhalation, through mechanisms involving oxidative stress, mitochondrial complex I inhibition, and microglial activation. Taken together, these classic examples underscore the plausibility that recurrent, low-level inhalation of environmental particulates (including—but not limited to—engineered NP) may cumulatively compromise nigrostriatal integrity and precipitate PD-relevant pathology over time. Beyond discrete chemical agents, ambient air pollution and airborne heavy metals have emerged as additional inhalation-mediated risk factors for PD. Epidemiological studies have linked chronic exposure to fine particulate matter (PM_{2.5}) and traffic-derived ultrafine particles with increased incidence of PD (Krzyzanowski et al., 2023; Rhew et al., 2021). Heavy metals commonly present in urban aerosols—such as manganese, lead, and copper—have been associated with an increased risk of PD (Pyatha et al., 2022). These findings align with the concept that "the risk is in the air," emphasizing the potential for inhalation exposure to bypass the BBB and directly induce dopaminergic neurodegeneration in the SN, thereby contributing to Parkinsonian pathology.

PS-NP, due to their small size, can enter cells via endocytosis (Han et al., 2024). In fact, research has shown that 80 nm PS-NP are more toxic to cells, with higher ROS production and increased inflammatory responses, compared to 3 μ m PS-MP, which are too large to enter cells (Han et al., 2024). In this way, airborne NP play a leading role in deteriorating human health. NP have also been reported in deep-sea environments and soil, and the toxicity of airborne NP increases as the size of the plastic particles decreases (Choudhury et al., 2023). Recent *in vivo* studies have shown that oral exposure to PS-NP (30–50 nm) induces memory impairment in mice through microglial uptake, activation, and inflammatory responses, implicating NP as potential drivers of cognitive dysfunction (Paing et al., 2024). Notably, in these studies, PS-NP were administered at doses of 10–20 mg/kg/day, which are substantially higher than the 20 μ g/day dose used in the present study, highlighting the relevance of evaluating neurotoxicity at environmentally realistic exposure levels. Consistently, 50 nm PS-NP disrupted the BBB by inducing ROS, NF- κ B activation, and necroptosis (Araújo et al., 2025). Complementary evidence from a human postmortem brain cohort further revealed greater accumulation of MP and NP in individuals with dementia, with notable deposition in cerebrovascular walls and immune cells (Nihart et al., 2025). These findings highlight the importance of particle size and exposure dose in shaping PS neurotoxicity, emphasizing that MP and NP, which differ markedly in their physicochemical properties, environmental behavior, and biological impacts (Allen et al., 2022; Hong and Kim, 2024), may exert distinct effects on the nervous



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Fig. 5. Immunohistochemistry (IHC) analysis of tyrosine hydroxylase (TH) and phospho- α Syn (Ser129) aggregation following stereotaxic injection of polystyrene microplastics (PS-MP) or polystyrene nanoplastics (PS-NP) into the striatum and substantia nigra (SN) of mice. (A) IHC analysis of TH and phospho- α Syn (Ser129) aggregation following stereotaxic injection of PS-MP or PS-NP into the striatum ($n = 3$). Eight weeks after injection, PS-NP exposure led to reduced TH expression and increased phospho- α Syn aggregation compared with PS-MP and control (Ctrl) groups. (B) IHC analysis of TH and phospho- α Syn (Ser129) aggregation following stereotaxic injection of PS-MP or PS-NP into the SN ($n = 3$). Analysis was performed 8 weeks after PS-MP or PS-NP injection into the SN of mice. Similar to the striatum, PS-NP exposure decreased TH levels and increased phospho- α Syn aggregation. Scale bar was colored in red on individual images.

system. A clearer understanding of these size-dependent differences is crucial, and epidemiological studies are needed to determine their contribution to the risk of neurodegenerative and systemic disorders.

While engineered particles differ chemically from classic neurotoxicants, they share key features—small size, high surface area, and potential for oxidative surface chemistry—that can elicit neurotoxic cascades once deposited in the respiratory tract.

Future work should aim to delineate the physicochemical properties that govern olfactory versus pulmonary uptake, track translocation to brain regions of interest, and define threshold exposure regimes beyond which dopaminergic resilience is overwhelmed. Ultimately, integrating insights from MPTP, pesticide, and air pollution research with emerging data on NP will sharpen our understanding of how inhaled particulates contribute to sporadic PD and guide development of targeted preventive strategies.

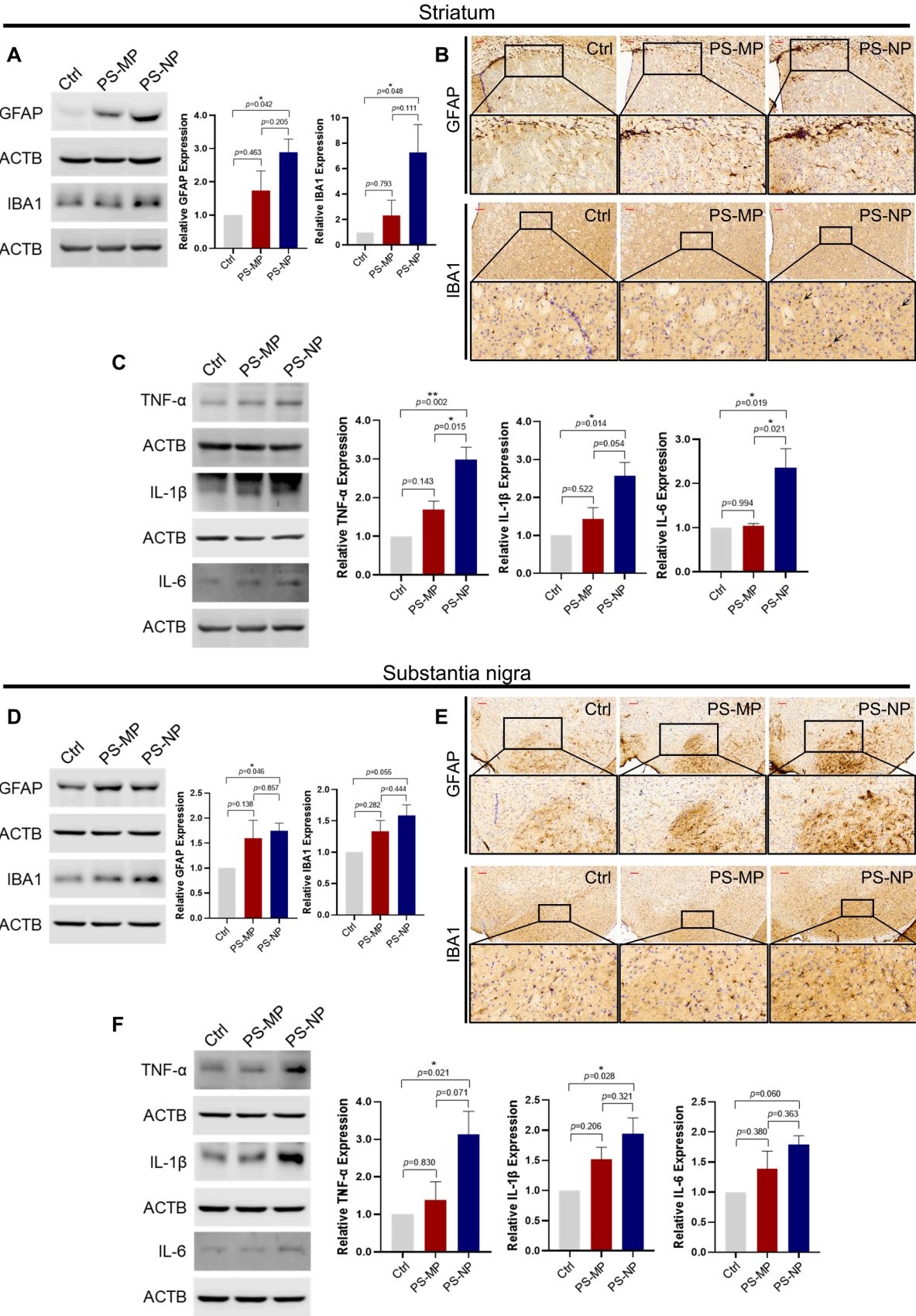
This study provides a comprehensive evaluation of motor and non-motor symptoms (NMS) of PD in PS-MP- and PS-NP-exposed mice. Motor impairments were assessed using five distinct tests—treadmill, balance beam, splay reflex, rotarod, and grip strength tests—demonstrating PS-induced deficits in motor coordination and strength (Fig. 2). Notably, we also performed a broad set of behavioral assays, including the forced swim, open field, adhesive removal, marble burying, and nestlet shredding tests, to systematically investigate NMS such as depression, anxiety, cognitive dysfunction, and apathy. These findings clearly show that PS exposure induced a spectrum of PD-relevant behavioral phenotypes. This multi-dimensional behavioral evaluation highlights the unique strength of our study in addressing PD as a complex, multi-system neurodegenerative disorder encompassing both motor and non-motor domains (Lee and Koh, 2015).

Akinesia (lack of movement, difficulty initiating movement), bradykinesia (slowness of movement), and hypokinesia are considered the primary motor symptoms of PD. These symptoms typically refer to a reduction in movement and overall slowness, occurring without any actual weakness (Moustafa et al., 2016; Mazzoni et al., 2012). Gait disturbances are a common issue for patients with PD. Typically, individuals with this condition experience a shuffling gait, struggling significantly to lift their feet off the ground, with impaired ability to activate proper leg swing and propulsion. Those with PD also experience various episodic deficits in walking, where the normal gait cycle is interrupted during an active exercise program, causing their feet to freeze, exacerbating balance issues, and leading to falls (Moustafa et al., 2016). Additionally, "postural instability" refers to the impaired response when balance is disrupted. For example, when a patient falls on uneven pavement, a lack of response in recovering balance can result in falling. "Rigidity" refers to increased muscle tone, which is typically experienced as stiffness and discomfort (Mazzoni et al., 2012). Individuals with PD exhibit lower rates of force development (RFD) and rates of force relaxation (RFR) compared to age-matched adults. Grip strength models are widely used in rehabilitation research, providing information about muscle strength loss that may accompany aging or disease. Additionally, grip strength is associated with a person's overall health status and physical function (Daniels et al., 2024).

The NMS of PD encompasses a range of cognitive, neuropsychiatric, sleep, autonomic, and sensory dysfunctions. Neuroanatomically, NMS can be subdivided into cortical symptoms (psychosis and cognitive impairments), basal ganglia symptoms (impulse Ctrl disorders, apathy, restlessness, or akathisia), brainstem symptoms (depression, anxiety, and sleep disturbances), and peripheral nervous system disorders

(orthostatic hypotension, constipation, pain, and sensory abnormalities) (Lee and Koh, 2015).

RNA-Seq analysis identified four PD-associated genes—*Kcnn4* and *Neurod1* in the striatum, and *Tlr7* and *Cartpt* in the SN (Fig. 3B). Notably, these genes, altered in our PS-MP- and PS-NP-exposed mice, overlap with those identified in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD models—the gold standard for experimental PD research (Figure S6) (Lu et al., 2019; Campolo et al., 2020; Mao et al., 2013) as well as in transcriptomic datasets from PD patients (Termine et al., 2022). In our study underscores the pathophysiological relevance of our plastic exposure model to human PD. *Kcnn4* encodes the KCa3.1 protein, which is involved in inducing Ca^{2+} overload and endoplasmic reticulum stress during microgliosis via the protein kinase B (AKT) signaling pathway. In MPTP-induced PD mouse models, pharmacological blockade of KCa3.1 using either gene deletion or senicapoc has been shown to improve motor function, increase the number of TH-positive neurons, and attenuate microgliosis and neuroinflammation in the SNpc (Lu et al., 2019). *Neurod1* plays a role in mediating the conversion of astrocytes into functional neurons, and the loss of *Neurod1* signaling contributes to the loss of dopaminergic neuron phenotypes (Wang et al., 2023; Haynes et al., 2021). The loss of *Neurod1* signaling has been reported to contribute to the loss of dopaminergic neuron phenotypes (Wang et al., 2023; Haynes et al., 2021; Chen et al., 2023). In a study analyzing RNA-seq data from postmortem human PD brain samples (GSE136666, GSE135036, GSE134390, GSE110716, and GSE68719), *Neurod1* was identified as a key regulator within the gene network and was reported as a differentially expressed gene across PD subtypes (Termine et al., 2022). *Tlr7* is a receptor widely expressed in various cells and has been reported to be upregulated in MPTP-induced PD models. It is also being discussed as a potential target for PD therapeutics (Campolo et al., 2020). When *Tlr7* was knocked out in mice and then subjected to MPTP treatment to model PD, a notable reduction in microglial and astrocyte activation was observed compared to the PD group. *Tlr7* has also been suggested as a potential target for PD therapy (Campolo et al., 2020). *Cartpt* is a gene encoding the CART protein, which was been reported that CART treatment in mouse PD models can reduce dopamine neuronal loss (Mao et al., 2013). CART was reported as a potential biomarker for PD (Mao et al., 2013). Mao et al. showed that pre-treating mice with CART before daily MPTP administration for seven days resulted in reduced loss of dopaminergic neurons in the SN (Mao et al., 2013). Analysis of the RNA expression profiling by high-throughput sequencing dataset from the midbrain of MPTP-induced PD mouse models (GSE214542) revealed a similar pattern to our PS inhalation model, showing a decrease in *Cartpt* and an increase in *Tlr7* expression in the SN (Figure S6). These findings suggest that inhalation exposure to PS-NP exacerbates both motor and NMS of PD in mice, along with histopathological changes. Additionally, PS-NP exposure influenced gene expression in the brain, with increased *Kcnn4* and *Tlr7* and decreased *Neurod1* and *Cartpt*, supporting its role as a contributing factor to PD pathogenesis. According to GeneMANIA and FAC analysis, in the striatum of the PS-NP group, upregulated genes were mainly associated with metabolic stress, DNA repair, and epigenetic regulation, while no significant downregulated clusters were identified (Fig. 3F, G). Mitochondrial metabolic dysfunction and disruption of metabolic pathways in PD are directly linked to the increased energy demands of dopaminergic neurons. This metabolic stress contributes to elevated reactive oxygen species (ROS) production and neuronal cell death (Dias et al., 2013). Stress response pathways are



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Fig. 6. Western blot and immunohistochemistry (IHC) analysis of glial fibrillary acidic protein (GFAP), ionized calcium-binding adapter molecule 1 (IBA1), and cytokine expression in the striatum and substantia nigra (SN) of mice. (A) The western blot analysis of GFAP and IBA1 expression in the striatum of control (Ctrl), polystyrene microplastics (PS-MP), and polystyrene nanoplastics (PS-NP) groups ($n = 3$) and the quantification of GFAP and IBA1 protein expression levels normalized to ACTB. PS-NP exposure increased GFAP and IBA1 levels compared with PS-MP and Ctrl groups. (B) The IHC staining results of GFAP and IBA1 in the striatum of Ctrl, PS-MP, and PS-NP groups ($n = 3$). PS-NP exposure led to stronger astrocyte staining and a higher proportion of activated microglia. Scale bar = 200 μm . (C) Western blot image of the expression of TNF- α , IL-1 β , and IL-6 in the striatum region for each group ($n = 3$). PS-NP exposure significantly elevated pro-inflammatory cytokine levels. The quantification of the cytokine protein expression levels normalized to the Ctrl group. (D) The western blot analysis of GFAP and IBA1 expression in the SN of Ctrl, PS-MP, and PS-NP groups ($n = 3$). PS-NP exposure increased GFAP and IBA1 expression compared with PS-MP and Ctrl groups. The quantification of GFAP and IBA1 protein expression levels normalized to ACTB. (E) The IHC staining results of GFAP and IBA1 in the SN of Ctrl, PS-MP, and PS-NP groups ($n = 3$). PS-NP exposure resulted in enhanced astrocyte staining and greater microglial activation. Scale bar = 200 μm . (F) Western blot image of TNF- α , IL-1 β , and IL-6 in the SN region for each group ($n = 3$). PS-NP exposure increased cytokine expression, indicating enhanced neuroinflammation. The quantification of the cytokine protein expression levels normalized to the Ctrl group. Data are presented as mean $\pm$ standard error of the mean (SEM), and statistical analysis was performed using a one-way ANOVA. * $p < 0.05$, ** $p < 0.01$.

also activated, leading to disturbances in cellular homeostasis and promoting neurodegenerative changes (Dias et al., 2013). Expression products of PD-related genes interact with molecular components of lysosomes and mitochondria, thereby inducing neuronal apoptosis and degeneration in PD, while epigenetic modifications contribute significantly to neurodegeneration and neuronal loss by regulating gene expression (Song et al., 2023). Aberrant histone modifications further exacerbate the onset and progression of neurodegenerative and neuropsychiatric disorders (Park et al., 2022). Increased ROS and mitochondrial dysfunction result in DNA damage and chromosomal instability, which in turn activate DNA repair pathways such as non-homologous end joining (NHEJ) and homologous recombination (HR). Upregulation of transcription factors associated with DNA repair genes has also been reported (Pan et al., 2014). Notably, PD-related proteins, including α -synuclein, have been suggested to influence DNA repair processes (Arnold et al., 2025). In the SN, no significant upregulated clusters were found, but downregulated genes were enriched in pathways related to cholinergic neurotransmission, neuronal structure, metabolism, and ion transport (Fig. 3F, G). In patients with PD, a widespread loss of the cholinergic system and impaired cholinergic signaling have been reported (Perez-Lloret and Barrantes, 2016). One of the major pathological hallmarks of PD is axonal degeneration and somatic loss of dopaminergic neurons (Burke and O'Malley, 2013), which leads to disruption of neuronal connectivity and reduced neurotransmission, ultimately contributing to motor impairments and functional decline (Gcwensa et al., 2021). In addition, dysfunction of ion channel activity has been considered a contributing factor in PD pathogenesis (Zhang et al., 2020). Taken together, these findings support that the pathway enrichment analysis results provide a potential mechanistic link between NP exposure and PD-related neurotoxicity.

In this study, we observed a decrease in TH levels and an increase in phospho- α Syn (Ser129), indicating neurodegenerative effects associated with exposure (Fig. 4-5). In PD, the target neurons that are susceptible to infection are those containing catecholamines in the SN or sympathetic ganglia, where TH is specifically expressed. TH is the rate-limiting enzyme in the biosynthesis of catecholamines, catalyzing the hydroxylation of the side chain of tyrosine to form dihydroxyphenylalanine (DOPA). Through this pathway, substances such as dopamine, epinephrine, and norepinephrine are produced (Jin et al., 2022; Daubner et al., 2011). Additionally, the phospho- α Syn (Ser129), which is considered specific to PD, is widely used to assess the degree and extent of degeneration in animal models and humans (Kontaxi and Edwards, 2023). In particular, the post-translational modification of α Syn through phosphorylation at serine 129 may be significant in PD. In Lewy bodies of patients with both sporadic and genetic forms of PD, α Syn is predominantly phosphorylated at S129. Furthermore, pS129 alters the properties of α Syn, including its aggregation propensity, toxicity, and interactions with other proteins (Stewart et al., 2015). Aggregated α Syn was characterized by increased production of inflammatory mediators (cytokines, such as IL-1 β , IL-6, and TNF- α) (Pajares et al., 2020). Extensive evidence from human samples and animal models supports the involvement of inflammation in the pathogenesis of PD (Pajares

et al., 2020). GFAP (Norden et al., 2016) and IBA1 (Hopperton et al., 2018) were upregulated in PD patients (Liu et al., 2023; Tang et al., 2023; Lai et al., 2021). In this study, we revealed a greater upregulation of GFAP, IBA1, TNF- α , IL-1 β , and IL-6 in the PS-NP-exposed group compared to the PS-MP group or Ctrl group (Fig. 6).

PS exposure has been reported to induce neuroinflammation and oxidative stress (Han et al., 2024), the present study provides molecular evidence supporting these mechanisms. Transcriptomic and enrichment analyses revealed that PS-NP exposure upregulated genes associated with metabolic stress, while downregulating genes related to cholinergic neurotransmission, neuronal structural maintenance, and ion transport and neurotransmission. These transcriptional alterations were concordant with histopathological findings of increased phosphorylated α -synuclein, decreased TH, and elevated inflammatory markers in the PS-NP group, suggesting that PS-NP induces neurotoxicity through interconnected pathways of and neuroinflammation, oxidative stress, and acetylcholine metabolism activity. In line with this, our transcriptome analysis revealed significant dysregulation of oxidative stress-related and cholinergic signaling-related genes. Specifically, *Hspa1a* (Samani et al., 2023), *Hspa1b* (Samani et al., 2023), and *Sgk1* (Kwon et al., 2021) were upregulated, while *Vip* (Hu et al., 2021) was downregulated, supporting the involvement of oxidative stress and impaired proteostasis in PS-NP-induced neurotoxicity (Table S3). Moreover, *Kcnn4* (Lu et al., 2019) was upregulated in the striatum, whereas *Grm2* (Samadi et al., 2009), *Slc5a7* (CHT1) (Arnold et al., 2018), and *Slc18a3* (VACHT) (Bohnen et al., 2025) were downregulated in the SN, indicating disruption of cholinergic neurotransmission and reduced acetylcholinesterase (AChE) activity (Table S4). Collectively, these findings suggest that PS-NP exposure elicits neurotoxic effects through interconnected pathways involving oxidative stress, proteostasis imbalance, and cholinergic dysfunction.

Furthermore, in vitro experiments showed that PS-NP, but not PS-MP, enhanced inflammatory responses and upregulated pro-inflammatory cytokines (TNF- α , IL1 β , IL6) (Figure S2). In agreement with these findings, a previous study demonstrated that exposure to MP induced inflammatory responses, including increased expression of inflammatory genes and cytokines, in astrocytes (Marcellus et al., 2024), radial neural stem cells (rNSCs) (Marcellus et al., 2024), and the human microglial cell line human microglial cell line-3 (HMC-3) (Mahmud et al., 2024). Another investigation using the human neural stem cell line hNS1 reported that exposure to 30 nm PS-NP for 4 days triggered oxidative stress, cellular stress, DNA damage, alterations in inflammatory responses, and apoptosis, ultimately increasing the risk of tissue damage and neurodevelopmental disorders (Martin-Folgar et al., 2024). Collectively, these findings indicate that PS-NP disrupts neuronal homeostasis through the interplay of oxidative stress, neuroinflammation, and impaired neurotransmission, which may contribute to the initiation and progression of PD pathology.

Nevertheless, several limitations of this study should be acknowledged. First, the experiments were conducted using a single polymer type and particle size, which may not fully capture the heterogeneity of plastics encountered under real-world conditions. In addition, although

behavioral and neuropathological outcomes were evaluated, causal relationships in humans cannot be established. Further longitudinal and epidemiological studies will therefore be necessary to clarify the translational relevance of these findings.

The present findings highlight the dual impact of PS-NP exposure on both neurons and glial cells, underscoring the importance of neuron–glia interactions in the pathogenesis of neurodegenerative disease. In addition to direct neuronal injury, characterized by reduced neurite branching and loss of dopaminergic markers such as TH, PS-NP exposure led to pronounced activation of astrocytes and microglia, accompanied by upregulation of pro-inflammatory cytokines. This glial activation provides a mechanistic link between environmental NP exposure and sustained neuroinflammation, which is increasingly recognized as a driving force in the progression of PD pathology. In humans, activated microglia and astrocytes secrete pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 (Kam et al., 2020), and chronic microglial activation further exacerbates disease progression by impairing phagocytosis, sustaining the release of inflammatory cytokines, and reducing the clearance of α -synuclein (Hong et al., 2024). By integrating neuronal dysfunction with glial-mediated inflammatory responses, our results align with current knowledge that chronic inflammation serves as a critical environmental stressor contributing to dopaminergic neurodegeneration, thereby reinforcing the relevance of neuroinflammation as a convergent pathway in PD development.

From a regulatory perspective, our findings underscore the need to establish reference values for airborne MP and NP, as no such standards currently exist despite the well-defined categories of the U.S. EPA Air Quality Index for PM_{2.5} (AQI, 2024).

In sum, we evaluated the presence of PS-MP and PS-NP in the striatum and SN of exposed mice. Long-term exposure to PS-NP resulted in PD-related behavioral impairments and expression changes in PD-associated genes, including *Kcnn4*, *Neurod1*, *Tlr7*, and *Carpt*, even at environmental levels. In the striatum and SN. Additionally, we demonstrated a significant decrease in TH, an increase in phospho- α Syn (Ser129), and a notable elevation in neuroinflammatory responses. These changes were more pronounced in the PS-NP group than in the PS-MP group, suggesting that PS-NP may pose a greater risk of PD-related neurodegenerative outcomes compared to PS-MP.

5. Conclusion

This study provides the first in vivo evidence that chronic inhalation of PS-NP can induce PD-related neuropathological and behavioral alterations, even at environmentally relevant exposure levels. Direct comparison with PS-MP revealed a clear size-dependent increase in neurotoxicity, with PS-NP causing greater neuroinflammation, dopaminergic neuronal loss, α -synuclein aggregation, and microglial activation. These findings indicate that airborne nanoplastics pose a substantially higher risk of PD than microplastics and should be recognized as an emerging environmental factor contributing to neurodegenerative disease pathogenesis.

Glossary

ACHe: acetylcholinesterase; AKT: protein kinase B; ANOVA: analysis of variance; BBB: blood–brain barrier; Ctrl: control; DAB: 3,3'-diaminobenzidine; DEG/DEGs: differentially expressed gene(s); DOPA: dihydroxyphenylalanine; DV: dorsoventral; FAC: functional annotation clustering; FE-SEM: field emission scanning electron microscope; GFAP: glial fibrillary acidic protein; GO: Gene Ontology; HMC-3: human microglial cell line-3; HR: homologous recombination; IACUC: Institutional Animal Care and Use Committee; IBA1: ionized calcium-binding adapter molecule 1; ICC: immunocytochemistry; IHC: immunohistochemistry; ID/g: injected dose per gram; KEGG: Kyoto Encyclopedia of Genes and Genomes; ML: mediolateral; MP: microplastics; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NGS: next-generation

sequencing; NHEJ: non-homologous end joining; NMS: non-motor symptoms; NP: nanoplastics; PD: Parkinson's disease; PET: Positron Emission Tomography; PM_{2.5}: particulate matter ≤ 2.5 μ m in diameter; PS: polystyrene; PS-MP: polystyrene microplastics; PS-NP: polystyrene nanoplastics; phospho- α Syn (Ser129): phosphorylated α -synuclein at serine 129; qRT-PCR: quantitative real-time polymerase chain reaction; RA: retinoic acid; RFD: rates of force development; RFR: rates of force relaxation; RNA-Seq: RNA sequencing; ROS: reactive oxygen species; SEM: scanning electron microscopy; SN: substantia nigra; SNpc: substantia nigra pars compacta; TH: tyrosine hydroxylase; Tlr7: toll-like receptor 7

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CRediT authorship contribution statement

Hyeju Jeong: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Joycie Shanmugiah:** Methodology. **Jaehye Jang:** Methodology. **Do Hee Lee:** Validation. **Jonghoon Choi:** Validation. **Jin Su Kim:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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Data availability

Data will be made available on request.

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