Methods

Animals

Adult male and female C57BL/6J mice (3–4 months old; RRID: IMSR_JAX:000664) were used (n = 5 per sex per group). Animals were housed under standard conditions (12 h light/dark cycle, food and water ad libitum). All procedures complied with ARRIVE guidelines and European Directive 2010/63/EU and were approved by the Italian Ministry of Health (Authorization n° 544/2023-PR).

6-OHDA Injection

Unilateral dopaminergic lesions were induced by intrastriatal injection of 6-hydroxydopamine (6-OHDA; 6 mg/mL in 0.02% ascorbic acid/saline) under ketamine–xylazine anesthesia (100/10 mg/kg). Using stereotaxic coordinates relative to bregma (AP +1.0 mm, ML −1.8 mm, DV −3.2 mm), 2 µL was infused at 0.2 µL/min. Control mice received vehicle injections.

Schallert Cylinder Test

Unilateral motor deficits were assessed using the Schallert cylinder test. Mice were recorded for 5 min in a transparent cylinder, and videos were analyzed by an experimenter blinded to the experimental group. Forelimb use asymmetry was expressed as the percentage of contralateral forelimb wall contacts relative to total contacts. Spontaneous turning behavior was quantified as the percentage of ipsilateral turns. Behavioral outcomes at 28 days post-lesion were normalized to each animal's pre-surgery baseline.

Histology

Mice were perfused transcardially with 4% paraformaldehyde. Brains were post-fixed, cryoprotected, and sectioned coronally (50 µm). Free-floating sections were immunostained using standard protocols. Dopaminergic degeneration was assessed in the striatum (CPu) and substantia nigra pars compacta (SNc) using anti-tyrosine hydroxylase (TH, Invitrogen PA5-85167). Cortical synaptic markers (VGLUT1,SYSY 135304; VGLUT2, SYSY 135403; VGAT, SYSY 131005) were analyzed in layer II/III of the primary motor cortex. Microglia and phagosomes were labeled in SNc with anti-Iba1 (Wako, 019–19741) and anti-CD68 (BioRad, 19#MCA1957) antibodies, respectively.

Image Analysis

Images were acquired using a Zeiss LSM 880 Airyscan confocal microscope (Carl Zeiss MicroImaging GmbH, Germany). Striatal dopaminergic fiber density was quantified as relative optical density from TH-immunoreactive signal using ImageJ (NIH, USA), whereas TH-positive neurons in the substantia nigra pars compacta were quantified using Neurolucida (MBF Bioscience). Cortical vesicular marker expression was analyzed from high-resolution confocal z-stacks (5 optical sections, 0.17 µm z-step) by measuring perisomatic synaptic puncta fluorescence intensity, with subtraction of somatic signal to correct for background. Microglial density was quantified via automated soma detection, and phagocytic activity was assessed by three-dimensional surface reconstruction of CD68-positive compartments using Imaris Bitplane software, with phagosome volume normalized to total Iba1-positive microglial volume (z-stacks ~10–15 µm, 0.3 µm z-step). For all histological analyses, values from the ipsilateral hemisphere were normalized to the contralateral side. For detailed information see Minetti et al., 2025.

Statistical Analysis

Data are presented as mean ± SEM. Two-way ANOVA followed by Sidak's post hoc test was used to assess the effects of sex and treatment ($\alpha = 0.05$). Principal component analysis (PCA) was performed in Python (scikit-learn) using all measured variables. Correlation analyses in 6-OHDA-treated mice were conducted on features contributing most strongly to the second principal component, which segregated animals by sex-specific parkinsonian phenotypes.