GM3 Ganglioside's Efficacy in LPS-Induced Parkinsonism: Neuroprotection and Gliosis Mitigation

Tsung-Ta Liu,PhD[a,b,1],Li-Yun Fann, PhD[c,d,1], I-HsunLi, PhD[e], Sheng-Huang Hsiao, MD, PhD[f],Yu-Chieh Chu, BS[a,d], Hao-Yuan Hung, PhD[d,g], Chih-Chien Cheng, MD, PhD[h], Ying-Che Huang, MD, PhD[i,j], Chuang-HsinChiu, MD, PhD[k], Ta-Kai Chou, MS[k]andJui-Hu Shih, PhD[a,d,*]

[a] School of Pharmacy, National Defense Medical Center, Taipei, Taiwan

[b]Department of Biology and Anatomy, National Defense Medical Center, Taipei, Taiwan

[c]Department of Nursing, Taipei City Hospital, Ren-Ai Branch, Taipei, Taiwan

[d]Department ofNurse-Midwifery and Women Health, National Taipei University of Nursing and Health Sciences. Taipei, Taiwan

[e] Department of Pharmacy Practice, Tri-Service General Hospital, Taipei, Taiwan

[f] Department of Neurosurgery, Taipei City Hospital, Taipei, Taiwan

[g] Department of Pharmacology, National Defense Medical Center, Taipei, Taiwan

[h]Department of Obstetrics/Gynecology, Taipei City Hospital, Taipei, Taiwan

[i]School of Medicine, College of Medicine, Fu Jen Catholic University, New Taipei City, Taiwan

[j]Department of Anesthesia and Critical Care Medicine, Taipei City Hospital, Ren-Ai Branch, Taipei, Taiwan

[k]Department of Nuclear Medicine, Tri-Service General Hospital, Taipei, Taiwan

[1]T.-T.L. and L.-Y.F. contributed equally to this article and both qualified as first authors.

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*Corresponding author:

Jui-Hu Shih, Department of Pharmacy Practice, Tri-Service General Hospital

No.325,Sec.2,Chenggong Rd.,Neihu District,Taipei City 11490,Taiwan

Tel: +886-2-8792-3311#17310 Fax: +886-2-87927154

E-mail:jtlovehl@gmail.com

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Abstract

Background: Parkinson's disease (PD) continues to be a neurological challenge with limited therapeutic options. This study aimed to investigate the potential therapeutic effects of GM3 ganglioside, focusing on its role in mitigating LPS-induced parkinsonism behaviors, gliosis, and neurotoxicity.

Methods: We employed a range of in vivo tests, including rotarod and beam-walking, to assess motor function improvements in LPS-induced parkinsonism following GM3 ganglioside pre-treatment. Dopaminergic neurotoxicity was examined using [^{18}F]FE-PE2I PET imaging and TH staining of the striatum. Further, we investigated the impact of GM3 ganglioside on LPS-induced gliosis by observing changes in microglial activation and astrocytic proliferation.

Results: Pre-treatment with GM3 ganglioside significantly improved motor functions, as evidenced by enhanced performance in rotarod and beam-walking tests. Our findings also showcased GM3 ganglioside's efficacy in countering LPS-induced dopaminergic neurotoxicity, with [^{18}F]FE-PE2I PET imaging and TH staining supporting its neuroprotective potential. Importantly, GM3 ganglioside pre-treatment notably reduced LPS-induced gliosis, demonstrating a significant decrease in both microglial activation and astrocytic proliferation.

Conclusions: GM3 ganglioside presents promising neuroprotective capabilities, effectively mitigating LPS-induced parkinsonism behaviors and gliosis. These findings underscore GM3 ganglioside's potential as a valuable therapeutic avenue for future Parkinson's disease interventions.

Keywords: GM3 ganglioside, Parkinson's Disease, PET, Microglia, Astrocyte

Introduction

Parkinson’s disease (PD) is projected to affect an estimated 12 million people by 2040, making it the second most prevalent neurodegenerative disease (Dorsey et al., 2018). This condition is characterized by the loss of dopaminergic neurons in the substantia nigra (SN), resulting in symptoms such as tremor, rigidity, postural instability, and bradykinesia (Anderson, 2004). In 2017, an estimated 1.04 million individuals were diagnosed with PD in the U.S., incurring a total economic cost of $51.9 billion, which includes direct medical costs of $25.4 billion and indirect/non-medical costs of $26.5 billion. However, it is projected that PD prevalence will exceed 1.6 million by 2037, with the total economic burden surpassing $79 billion (Yang et al., 2020).Given the staggering societal and economic costs associated with PD, preventive strategies have become a critical area of focus in order to alleviate the burden on both healthcare resources and manpower in the future.

The etiology of PD remains elusive, though numerous studies suggest that occupational, genetic, and environmental factors may play pivotal roles (Ben-Shlomo, 1997). The link between inflammation and PD was first reported in 1988, with the revelation of activated microglia in the midbrain of PD cases (McGeer et al., 1988). Subsequent research has further elucidated the involvement of a series of inflammatory responses in this disease (Kim and Joh, 2006; Miyazaki and Asanuma, 2020; Zhang et al., 2023).Administering LPS directly into the brain's substantia nigra induces microglial activation in rodents. Subsequently, activated astrocytes amplify the inflammatory response, leading to the degeneration of dopaminergic neurons through the heightened production of inflammatory cytokines (Kuter et al., 2018; Liu and Bing, 2011; Sharma and Nehru, 2011). Thus, in this study, we established a parkinsonism animal model by directly injecting LPS into the brain to investigate the

pathology of PD (Hunter et al., 2009; Esteves et al., 2023).

Gangliosides are glycosphingolipids composed of a glycan headgroup containing one or more acid residues attached to a hydrophobic ceramide tail. This tail serves as an anchor, attaching the gangliosides to the plasma membrane (van der Haar Àvila et al., 2023; Yu et al., 2011). Monosialotetrahexosylganglioside, which includes GM1, GM2, and GM3, belongs to a class of anionic glycosphingolipids. These molecules consist of one molecule of sialic acid (N-acetylneuraminic acid) linked to the sugar residues of a ceramide oligosaccharide (Galleguillos et al., 2022).Ganglioside GM3 has been reported to have effects on tumor cytotoxicity, HIV, and obesity (Akiyama et al., 2015; Ding et al., 1998; Kanoh et al., 2020). Recent studies have suggested that GM3 may also suppress LPS-induced inflammatory responses (TNF-α, IL-6, and IL-1β) in a rat macrophage cell line (Park et al., 2018).

In light of the profound societal and economic impact of PD, there is an urgent need for innovative approaches to prevent and mitigate its debilitating effects. This study seeks to address this imperative by investigating the potential protective effects of GM3 ganglioside in anLPD-induced parkinsonism animal model. By elucidating the role of GM3 in modulating inflammatory responses and neuronal degeneration, this research holds promise for the development of novel therapeutic interventions.

Materials and Methods

Animals

The animals utilized in this study were male C57BL/6 mice, aged eleven weeks, with an average weight of approximately 22-25 g. These mice were procured from BioLASCO Taiwan Co., Ltd., Taiwan. Prior to the formal experiment, three mice were accommodated in each cage and exposed to a 12-hour diurnal cycle for a duration of seven days to facilitate acclimatization. The ambient room temperature was meticulously maintained at 23±2°C. Throughout the study, the mice had unrestricted access to food and water.All experimental protocols strictly adhered to ethical guidelines stipulated by the Institutional Animal Care and Use Committee (approval protocol No. IACUC-22-190) of the National Defense Medical Center, Taipei, Taiwan.

Operation protocol

Prior to the injection of LPS (Salmonella enterica: L9764) (Figure 1), mice received intraperitoneal (i.p.) injections of GM3 at a dosage of 10 mg/kg once daily for a duration of 5 days. Following a single dose of GM3, the mice were anesthetized with sodium pentobarbital (45 mg/kg, i.p.) and securely positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The right and left striatum were subjected to four injections of 1μL LPS (5 μg dissolved in PBS, resulting in a total volume of 1 μL) or PBS, administered at a rate of 0.5 μL/min (Anteroposterior (AP) +1.18 mm, Lateral (LAT) ±1.5 mm, Dorsoventral (DV) −3.5 mm; AP −0.34 mm, Lateral ± 2.5 mm, DV−3.2 mm). Subsequently, the needle was left in place for 5 minutes to prevent reflux along the injection track. After the final PET imaging, mice (n = 6 in each group) were euthanized and underwent transcardial perfusion with

paraformaldehyde for subsequent immunohistochemical (IHC) staining.

Rotarodtests

Rotarod performance is assessed by placing mice on a rotating rod, requiring them to walk forward to prevent falling off the continuously spinning cylinder (Carter et al., 1999). The UGO BASILE (Model 7700) rotarod treadmill was employed to evaluate fine motor coordination and balance. Prior to LPS injections, mice underwent a pre-training phase encompassing three consecutive days of exposure to low rotational speeds (5, 20, 24, 28, 32, and 40 rpm), followed by a fourth day on a rod that accelerated from 0 to 50 rpm. The results obtained from the average performance on days four and five before LPS administration were established as the baseline for time spent on the Rotarod. Mice underwent testing once a week until euthanasia for tissue collection. During each trial, three measurements were taken, and the average time spent on the Rotarod for each mouse was utilized for data analysis. The time spent on the rod served as an indicator of fine motor coordination and balance.

Beam Walking tests

The mice underwent a two-day training period to traverse a narrow rectangular beam (100 cm in length, 1.2 cm in width) towards their home cage positioned at the far end of the beam. On the third day, the average time taken (tests were conducted three times at 10-minute intervals) to cross the beam was recorded as a measure of motor coordination. The maximum duration for the test was set at 60 seconds, and if a mouse was unable to complete the traversal within this time frame, the recorded testing time was noted as 60 seconds.The results are expressed as percentage differences (%).

$$\text{Percentage difference (\%)} = \frac{\text{Final test time - base line time}}{\text{Base line time}}$$

[^{18}F] FE-PE2I PET analysis

This study utilized [^{18}F]FE-PE2I as a specific radioligand for the dopamine transporter (DAT), which is responsible for reuptaking free dopamine molecules from the synaptic cleft. Employing this ligand for imaging enables the quantification of presynaptic dopaminergic neuron integrity, facilitating the assessment of clinical manifestations associated with dopamine depletion in patients.The small animal positron emission tomography (PET) scans were conducted at the animal center of National Defense Medical Center, Taipei, Taiwan. The radiopharmaceutical [^{18}F]FE-PE2I (with a purity > 95% and specific activity > 3 Ci/μmol) required for the experiments was prepared and supplied by the Department of Nuclear Medicine at Tri-Service General Hospital, Taipei, Taiwan. On the day of the experiment, mice were maintained under inhalation anesthesia with a mixture of isoflurane and oxygen (5% isoflurane for induction, 2% isoflurane for maintenance). Following intravenous injection via the tail vein of [^{18}F]FE-PE2I (14.8 – 18.5MBq; 0.4 – 0.5 mCi), the mice were returned to their cages for a 20-minute equilibration period. Subsequently, static 3D images were acquired using a PET scanner (BIOPET 105 imager, Bioscan, Inc., Washington, DC, USA) with an energy window of 250 – 700 keV for a duration of 20 minutes, as per the schedule (Park et al., 2020).

To ensure consistent volume of interest (VOI) placement across animals, MR images were obtained from a typical mouse brain and manually fused with six reconstructed [^{18}F]FE-PE2I PET images of normal mice to delineate VOIs based on a mouse brain atlas. These typical MR images, along with the VOIs, were saved as a template for further analysis. The [^{18}F]FE-PE2I images of each individual animal in

this study were manually co-registered to the corresponding MR template images using AMIDE software version 1.0.4 for measuring standardized uptake value (SUV) in various brain regions. The final data were expressed as specific uptake ratios (SURs), calculated as $SUR = (SUV_{striatum} - SUV_{cerebellum})/SUV_{cerebellum}$.

IHC staining

Brain coronal cryosections (20 μm thick) were affixed onto slides and subjected to a 5-minute wash with Dulbecco's phosphate-buffered saline (PBS) at pH 7.4. Subsequently, the slides were immersed in a blocking solution consisting of 5% BSA along with 0.1% Triton X-100 at room temperature for 30 minutes. This was followed by an overnight incubation with the primary antibodies at 4°C. The primary antibodies used included tyrosine hydroxylase (TH, 1:1000, Sigma-Aldrich), ionized calcium binding adaptor molecule 1 (Iba1, 1:400, Abcam), and glial fibrillary acidic protein (GFAP, 1:500, Genetex).Next, the brain sections underwent three 5-minute washes with PBS, followed by incubation with the respective secondary antibodies. To visualize the localization of the horseradish peroxidase -conjugated antibody, 3,3'-diaminobenzidine (Sangon Biotech, China) was applied. Finally, the slices were sealed with neutral resin and observed under a microscope for subsequent analysis.

Statistical analysis

The experimental results are presented as means ± standard error of the mean (SEM). The Shapiro-Wilk test was employed to assess the normality of all data. If the data followed a normal distribution, a one-way analysis of variance (ANOVA) was conducted. In cases of homogeneity of variances, post-hoc tests were performed using the Bonferroni method. In cases of heterogeneity of variances, post-hoc tests were conducted using the Games-Howell method. For data that did not exhibit a normal

distribution, the Kruskal-Wallistest was employed for analysis. All statistical tests were two-tailed, and the significance level (α) was set at 0.05.

Results

Pre-treatment with GM3 ganglioside improves motor functions in LPS-induced parkinsonism behaviors

To assess the effectiveness of GM3 ganglioside on behavior, we initially focused on evaluating LPS-induced motor dysfunction. For this purpose, we conducted rotarod and beam-walking tests following the protocol outlined by Nasuti et al. (2017) in our animal model. In the rotarod task, the latency (in seconds) to fall was consistently lower in the LPS group compared to the sham group, spanning from week 1 to week 5. Notably, the cotreatment group exhibited a significant improvement in the latency to fall compared to the LPS group, as depicted in Figure 2A.Subsequently, we transformed our data into area under the curve (AUC) and conducted statistical analysis as described by Taïwe et al. (2014). One-way analysis of variance (ANOVA) was employed to compare the differences in AUC among the groups, revealing a significant difference ($F_{(3,29)}$ = 52.531, p< 0.001). The Games-Howell post-hoc test for further comparison showed that the AUC of the LPS group was significantly lower than that of the sham group(p< 0.001), indicating notable motor impairment in mice from the LPS group. Conversely, the AUC of the cotreatment group was significantly higher than that of the LPS group(p< 0.01), suggesting that GM3 ganglioside may rescue this motor impairment, as illustrated in Figure 2B.

The beam-walking tests were conducted twice, once prior to LPS injection (pre-test) and then again six weeks after LPS injection (post-test). The difference time (post-test minus pre-test) was divided by the pre-test time, and expressed as a difference percentage. This percentage represents the ratio of behavioral performance change for each mouse between the pre-test and post-test. The Kruskal-Wallistest was

employed to compare the performance differences among the groups in the beam-walking test. The results revealed a significant difference among the groups($F_{(3,28)}$ = 11.046, p = 0.011). Further pairwise comparisons showed statistically significant differences between the sham group and the LPS group ($p < 0.05$), as well as between the LPS group and the cotreat group ($p < 0.05$). (Figure 2C).

GM3 ganglioside mitigates LPS-induced dopaminergic neurotoxicity*in vivo*: [^{18}F]FE-PE2I PET imaging analysis

To investigate whether GM3 ganglioside confers protective effects against LPS-induced dopaminergic neurotoxicity *in vivo*, we employed [^{18}F]FE-PE2I PET imaging to assess DAT availabilities in the striatum. [^{18}F]FE-PE2I is a widely recognized radioligand known for its proficiency in evaluating dopaminergic innervation, particularly in quantifying DAT expression (Sasaki et al., 2012). Different [^{18}F]FE-PE2I uptake patterns were observed in the sham, LPS, cotreat, and GM3 groups. The LPS group exhibited lower [^{18}F]FE-PE2I uptake compared to the sham group, indicating reduced DATavailability in the striatum (Figure 3A).

Quantitative results of [^{18}F]FE-PE2I radioactivity in the regions of interest are depicted in Figure 3B. A one-way ANOVA was conducted to compare differences in DAT levels among the groups within the striatum. The results revealed a significant difference in SURvalues ($F_{(3,28)}$ = 8.064, p = 0.001) among the groups. Subsequent Bonferroni *post-hoc* tests further demonstrated statistically significant differences, showing that the sham group differed significantly from the LPS group ($p< 0.01$) and the LPS group differed significantly from the cotreat group ($p< 0.01$).

Evaluation of dopaminergic neurons in the striatum using TH staining: implications of GM3 ganglioside in neuroprotection

To elucidate whether the elevated SURs of [^{18}F] FE-PE2I were indicative of the presence of dopaminergic neurons, TH staining of the striatum was carried out, as per the methodology detailed by Weng et al., 2020. Figure 4 (upper) showcases representative photomicrographs of the brain sections. Notably, the LPS group exhibited a reduced density of TH-positive fibers compared to the sham group. To provide a quantitative perspective, we analyzed the IHCresults using the OD ratio, aiming to evaluate the density of TH-positive cells. As depicted in Figure 4 (lower), the LPS group exhibited a significant reduction in staining intensity compared to the sham group ($p< 0.001$). However, the cotreat group showed some restoration of the staining intensity when compared to the LPS group($p< 0.05$). The GM3 group displayed similar staining intensity to that of the sham group, with no significant difference observed. This suggests that GM3 ganglioside may play a protective role in preserving dopaminergic neurons within the striatum.

GM3 ganglioside pre-treatment diminishes LPS-induced gliosis: attenuation of microglial and astrocytic activation

Microglia are the tissue-specific macrophages of the brain. They recognize pathogen-associated molecular patterns (PAMPs) via the TLR4 receptor, leading to cytokine production, antigen presentation, and the initiation of adaptive immunity (Boche et al., 2013). To determine if LPS induces an immune response, we utilized Iba1 as a marker for microglia (Singh et al., 2021). Representative photomicrographs of brain sections displayed an abundance of Iba1 positive cells, suggesting that the injection of LPS into the striatum led to significant microglial accumulation (Figure 5A, upper). Upon quantifying the Iba1 positive cells, we observed that the LPS group exhibited a markedly elevated cell count compared to the sham group ($p< 0.001$). Conversely, the cotreat group showed a significant reduction in cell count when

juxtaposed with the LPS group ($p< 0.05$). The GM3 group displayed a cell count that was comparable to the sham group, with no statistically significant differences detected. (Figure 5A, lower).

Astrocytes are the most prevalent glial cells in the brain, playing a pivotal role in supporting neuronal functions. They produce antioxidants such as glutathione, recycle neurotransmitters like glutamate and GABA, and help maintain the blood-brain barrier (BBB) to ensure a stable microenvironment (Miyazaki and Asanuma, 2020). However, under certain severe conditions, astrocytes can contribute to the formation of glial scars, which may impede axon regeneration (McGraw et al., 2001; Sofroniew, 2009). To investigate whether LPS induces an increase in astrocyte numbers, we employed GFAP as an astrocyte marker (Dong et al., 2021). Representative photomicrographs (Figure 5B, upper) show a surge in astrocyte numbers in the LPS group compared to the sham group. From the quantitative analysis depicted in Figure 5B (lower), it is evident that the LPS group demonstrated a significant increase in GFAP-positive astrocytes compared to the sham group ($p<0.001$). This increase indicates a heightened astrocytic reaction or astrogliosis in response to LPS. In comparison, the cotreat group showed a significant reduction in the number of GFAP-positive cells compared to the LPS group ($p<0.05$), although the cell count remained higher than the sham group. The GM3 group displayed a cell count akin to the sham group, indicating that standalone treatment with GM3 ganglioside does not noticeably influence astrocyte numbers. These results suggest that GM3 ganglioside may mitigate astrocyte proliferation, potentially reducing the likelihood of inhibiting axon regeneration.

Discussion

In our study, we initially established an animal model of parkinsonism by injecting LPS into the striatum. To ensure the successful establishment of parkinsonism, we conducted behavioral assessments, including the rotarod and beam-walking tests. Both tests revealed motor dysfunction in the LPS group, confirming the successful induction of parkinsonism. Remarkably, pre-treatment with GM3 ganglioside mitigated motor dysfunction in the co-treated group, suggesting potential benefits of GM3 for PD patients. Additionally, PET imaging showed impairment in the striatum of the LPS group, while the striatum in the co-treated group was comparable to the sham group. To elucidate the potential protective mechanism of GM3 against LPS-induced parkinsonism, we performed IHC staining. TH staining corroborated the PET findings, indicating dopaminergic neuron degeneration in the LPS group's striatum. Iba1 staining revealed a significant presence of activated microglia in the LPS group's striatum, but pre-treatment with GM3 ganglioside reduced microglial counts, suggesting that GM3 can counteract the LPS-induced inflammatory response. GFAP staining displayed an accumulation of astrocytes in the LPS group, while GM3 ganglioside pre-treatment appeared to prevent events that inhibit axon regeneration.

Three distinct methods have been employed to induce parkinsonism using LPS injection: intraperitoneal (i.p), intranasal, and intrastriatal (Bai et al., 2023; Deng et al., 2020; García-Revilla et al., 2022). All these techniques resulted in the degeneration of nigrostriatal dopaminergic neurons, motor dysfunction, and α-synuclein aggregation (Deng et al., 2020; Martins, 2015; Xu et al., 2021). However, from our observations, the intrastriatal injection of LPS exhibited a higher success rate and faster induction of parkinsonism. Therefore, despite the increased complexity of stereotaxic injections

compared to the other two methods, we opted for the intrastriatal injection of LPS in this study to induce parkinsonism.

PET imaging is a distinctive technology that allows for *in vivo* observation of organisms, though acquiring its radioligands can be challenging (Inubushi et al., 2020). [^{18}F]FE-PE2I is a notable radioligand that binds to the DAT of dopaminergic neurons (Kerstens et al., 2023). Previously, our group utilized [^{99}mTc]TRODAT-1, [^{18}F]FEPPA, and [^{18}F]FE-PE2I to capture in vivo images of dopaminergic neurons in both rodents and monkeys (Jhao et al., 2019; Ma et al., 2009; Shih et al., 2016). For this study, we employed [^{18}F]FE-PE2I PET imaging on mice, specifically examining LPS-induced parkinsonism *in vivo*. Additionally, our TH staining results from IHC were consistent with the PET imaging findings. This suggests that [^{18}F]FE-PE2I PET imaging can be used for ongoing monitoring of mouse brains over various weeks *in vivo*, reducing the need to sacrifice the animals.

A key pathophysiological aspect of PD is neuroinflammation in the striatum, resulting from the over-activation of microglia (Isik et al., 2023). Both animal and clinical studies have indicated an elevated presence of microglia during PD progression (Lazdon et al., 2020; Long-Smith et al., 2009; Stefanova, 2022). As a result, microglial inhibition has been targeted for PD prevention (Okuyama et al., 2016; Pisanu et al., 2014; Zhang et al., 2023). In our research, GM3 ganglioside was observed to decrease the heightened microglial levels, which were induced by a direct LPS injection into the striatum, potentially ameliorating the behavior of PD-afflicted mice. Beyond microglia, astrocytes also play a pivotal role in the neuroinflammation associated with PD. Kam and colleagues posited that activated microglia might influence astrocytes, leading them to release unidentified toxins that exacerbate dopaminergic neuron degeneration in PD (Kam et al., 2020). Moreover, astrocytes have been implicated in forming glial scars that could inhibit axon regeneration

(McGraw et al., 2001; Sofroniew, 2009). In our findings, GM3 ganglioside appeared to reduce the surge in astrocytes triggered by a direct LPS injection into the striatum, potentially decreasing events that prevent axon regeneration.

In summary, our study verified that GM3 ganglioside can counteract LPS-induced degeneration of dopaminergic neurons in the striatum and modify mouse behaviors. The effects of GM3 ganglioside likely stem from inhibiting microglia and astrocytes. While challenges related to the inhibition of inflammatory molecules (such as IL-1β, IL-6, and TNF-α) remain, our findings offer valuable insights into the potential neuroprotective benefits of GM3 ganglioside for clinical application in PD.

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Figure legends

Figure 1. Experimental timeline for assessing the effects of GM3 ganglioside in LPS-induced Parkinsonism in C57BL/6 mice.Note: Interventions and assessments are highlighted by their respective colored bars and icons on the timeline.

Figure 2. Assessment of motor functions across various treatment groups through rotarod and beam walking tests.(A) Time trend analysis of the rotarod performance (in seconds) across different weeks for four distinct groups: sham (n=7), LPS (n=8), cotreat (n=10), and GM3 (n=8).(B) Area under the curve (AUC) analysis for rotarod performance for each group, illustrating the total performance over 5 weeks. Statistical significance between groups is indicated by asterisks (**: $p<0.01$; ***: $p<0.001$).(C) Beam walking performance differences across treatment groups. The graph displays the difference percentages in the beam walking performance for the four groups: sham, LPS, cotreat, and GM3. Each dot represents an individual subject within the respective group, and the horizontal lines denote the mean of each group. Statistical significance between groups is indicated by asterisks (*: $p<0.05$).

Figure 3. [^{18}F] FE-PE2I PET imaging and associated uptake values across different treatment groups.(A) Representative PET images from each of the four groups: sham, LPS, cotreat, and GM3.(B) Scatter plot presenting the standardized uptake values (SUV) for each group. Each symbol signifies an individual subject, and horizontal lines indicate the group mean. Differences showing statistical significance between groups are marked by double asterisks (**: $p<0.01$).

Figure 4. Results of tyrosine hydroxylase (TH) staining across different treatment groups.Representative images of TH staining for each of the four groups: sham, LPS, cotreat, and GM3(upper).Bar chart showing the optical density (OD) ratio for each treatment group(lower). Statistical significance between groups is denoted by asterisks (*: $p<0.05$; ***: $p<0.001$).

Figure 5. Iba-1 and GFAP staining results across different treatment groups.

(A) Iba-1 staining:Representative micrographs of Iba-1 staining for each of the four groups: sham, LPS, cotreat, and GM3, depicting microglial activation (upper).Bar chart illustrating the number of Iba-1 positive cells per square millimeter (cells/mm^2) for each treatment group (lower). Statistical differences between groups are represented by asterisks (*: $p<0.05$; ***: $p<0.001$).(B) GFAP staining:Representative micrographs of GFAP staining for each of the four groups: sham, LPS, cotreat, and GM3, showcasing astrocyte activation(upper).Bar chart presenting the number of GFAP positive cells per square millimeter (cells/mm^2) for each treatment group(lower). Statistical variances between groups are highlighted by asterisks (*: $p<0.05$; ***: $p<0.001$).