

Targeting Microglial Inflammation in PD Irisin Activates PAFAH1B1-RAGE Ubiquitination and TFEB Autophagy Alleviates Neurodegeneration

Corresponding Author: Dr Qin Yang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Dear Authors,

This manuscript explores the role of Irisin in ameliorating Parkinson's disease (PD) via modulation of autophagy, neuroinflammation, and ubiquitination. The study combines clinical, in vivo, and in vitro data to support a novel mechanism involving PAFAH1B1-mediated ubiquitination of RAGE and downstream regulation of the TFEB-NLRP3 axis. The study is well-conceived and methodologically sound, and it offers potentially impactful insights into therapeutic strategies for PD. However, there are several important concerns regarding mechanistic depth, in vivo validation, and translational relevance that should be addressed before the manuscript is suitable for publication.

Major Comments

1. Mechanistic Validation of PAFAH1B1-RAGE Axis In Vivo

While the in vitro data on RAGE ubiquitination are compelling, in vivo validation is lacking. The authors should consider performing co-immunoprecipitation assays from brain lysates to demonstrate RAGE ubiquitination and its regulation by Irisin in the mouse model.

2. Clinical Correlation with Irisin Levels

The authors report lower Irisin levels in PD patients, but no correlation with clinical features is provided. Inclusion of UPDRS scores or disease duration correlations would improve the translational significance of this finding.

3. Duration and Dosing in Animal Models

The in vivo Irisin treatment was relatively short (7 days). Given the chronic nature of PD, additional data on long-term outcomes or extended treatment paradigms would strengthen the relevance to therapeutic application.

4. Quantification of Autophagic Flux

TEM and mRFP-GFP-LC3 assays were appropriately used. However, quantitative data on the number of autophagosomes/autolysosomes per cell should be included, along with appropriate statistical analysis.

5. Clarification of Statistical Comparisons

In several figures, the specific group comparisons showing statistical significance are unclear. Please clarify comparisons in all figure legends (e.g., Control vs PD, PD vs PD+Irisin).

Minor Comments

- The Abstract could more explicitly highlight the central mechanism (PAFAH1B1-RAGE-TFEB-NLRP3).
- Please clarify if both sexes were used in animal studies and discuss the possible impact of Irisin's hormonal regulation in the Discussion.
- Consider confirming microglial polarization (CD86/CD206) with flow cytometry for higher quantitative accuracy.
- The Ubibrowser prediction method should be briefly described in the Methods or figure legend.
- Ensure consistent nomenclature throughout (e.g., PD+Irisin vs. Irisin-treated PD mice).

Reviewer #2

(Remarks to the Author)

Thank you for the opportunity to peer review. The authors elucidated the protective effects of Irisin against inflammatory in PD. This discovery could lead to the potential development of therapeutic drugs and functional supplements. Thus, this research is very important for the creation of a sustainable society in the future. Although the study is great, I have provided a few comments and suggestion that make the manuscript better. We appreciate the efforts of the authors in conducting this important study and look forward to seeing the final version of the manuscript.

Fig. 1: How did you detect the protein in human serum, ELIZA? Fig. 1C unit (pg/ml*1000) meant fg/mL?

Fig. 2I: It is interesting that the concentration of Irisin was different in each brain region. Is Irisin produced in the brain? If so, what are the producing cells?

Fig. 3A,3B The quantitative data should be submitted because only typical images did not express the decrease in HE and Nissl stained neuronal cells.

Fig. 5G: Since LPS damage is cell density dependent, areas of equal cell density should be taken. It would be helpful to indicate what GFP and RFP represent. Also, since the LC3 dot-like signal was not seen, the cell processing method should be reviewed and images taken at higher magnification should be added.

supplementary Fig. 3B: Why was only LC3 increased in Sup 3B bufA+LPS+Irisin?

Fig. 7F-H: β -actin misspelled.

Line 578-580: Is Irisin actin? Misspelling?

Reviewer #3

(Remarks to the Author)

This manuscript by Lijun Cai et al presents a comprehensive and well-structured investigation into the therapeutic potential of Irisin in Parkinson's disease (PD), integrating clinical data, in vivo mouse models, and in vitro mechanistic studies. The mechanistic focus on autophagy enhancement and RAGE ubiquitination via the PAFAH1B1 axis, along with TFEB-NLRP3 signaling, significantly contributes to the current understanding of immunomodulatory strategies in PD. The general purpose of this study is clear, and the experiments appear to be of interest. However, several critical aspects require further clarification. The statistical validity of the key finding—namely, the decreased serum Irisin levels in PD patients—should be substantiated with detailed statistical data. In addition, considering the experimental design, the use of one-way ANOVA for Figure 7E appears to be insufficient. In my opinion, therefore, this manuscript is not recommended for publication in its present form.

Major points

1. Fig1C

In the main text, it is stated that "We further measured serum Irisin levels in the peripheral blood of PD patients. The results indicated that serum Irisin levels in PD patients were significantly lower than those in controls ($p < 0.05$) (Figure 1C)." However, based on the variability and error bars shown in the graph, the difference does not appear to be statistically significant. As this is a critical point, I kindly request that the authors provide detailed statistical values (e.g., exact p-value, effect size, confidence intervals) to support the claim of significance.

2. Fig4

Interleukin-6 (IL-6) is a pleiotropic cytokine that can exhibit both pro-inflammatory and anti-inflammatory properties depending on the context. It may play roles in stimulating immune responses as well as regulating local and systemic acute-phase inflammatory reactions. In this manuscript, the authors appear to classify IL-6 as an anti-inflammatory cytokine. If this is the intended interpretation, I kindly request that the authors provide supporting evidence or citations to justify this classification within the context of their study.

3. Statistical analysis in Fig 7

The manuscript states that pain thresholds were analyzed using one-way ANOVA. However, for Figure 7E, a two-way ANOVA may be more appropriate given the presence of multiple factors. I recommend that the authors clearly specify the factors involved and reanalyze the data using two-way ANOVA to provide a more accurate statistical interpretation.

4. Discussion

The manuscript suggests that irisin suppresses the increase of pro-inflammatory cytokines and prevents the decrease of anti-inflammatory cytokines. Could the authors clarify which of these effects is considered the primary mechanism of irisin's anti-inflammatory action? Alternatively, is the dual regulation of both cytokine types essential to its function? Please discuss this non-selective activity in greater detail based on your data and supporting literature.

Additionally, I encourage the authors to elaborate on the potential effects of irisin on microglial polarization, if possible. Any insights into whether irisin promotes M2 (anti-inflammatory) phenotypes or suppresses M1 (pro-inflammatory) activation would strengthen the interpretation. Furthermore, please consider discussing the possible influence of irisin on other glial cell types, such as astrocytes, within the context of neuroinflammation or neuroprotection.

5. Abbreviations

Abbreviations used should be defined once the first time they appear in the text. Once a word is abbreviated, the abbreviation should be used consistently throughout the manuscript.

Ex Line57 Alzheimer's Disease $\Rightarrow$ Alzheimer's Disease (AD)

Line75 DA $\Rightarrow$ Dopamine ?

Line94 TNF— α 、IL-1 β 、IL-6 $\Rightarrow$ please spell out.

I hope these comments will be helpful.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed all of my previous concerns thoroughly and have made substantial improvements to the manuscript. The revised version is significantly clearer and robust. I have no further comments and recommend acceptance of the manuscript in its current form.

Reviewer #2

(Remarks to the Author)

There is nothing more to say. Thank you very much for your sincere response.

Reviewer #3

(Remarks to the Author)

The revised manuscript presents a comprehensive and well-structured study on the therapeutic potential of Irisin in Parkinson's disease, supported by consistent in vivo, in vitro, and clinical evidence. The authors have addressed the previous concerns with diligence and have significantly improved the clarity and scientific rigor of the manuscript.

However, the Discussion section remains somewhat lengthy and occasionally repetitive. The key conclusions regarding Irisin's neuroprotective and anti-inflammatory effects—particularly its ability to cross the blood-brain barrier, regulate microglial polarization, and modulate cytokine balance—are reiterated multiple times throughout the text. In addition, the mechanistic discussion of the PAFAH1B1–RAGE–TFEB–NLRP3 axis, while valuable, is at times over-explained across several paragraphs. Streamlining the narrative and removing redundancy would enhance the clarity and impact of the discussion.

Furthermore, the section on gender differences and hormonal regulation, although interesting, could be more concisely integrated as a future direction rather than occupying a substantial portion of the main discussion.

In conclusion, I believe the study is scientifically sound and presents valuable findings. However, I recommend minor revision prior to acceptance to improve the organization and conciseness of the Discussion section.

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Response Letter-Targeting Microglial Inflammation in Parkinson's Disease: Irisin Activates PAFAH1B1-RAGE Ubiquitination and TFEB-Dependent Autophagy to Alleviate Neurodegeneration

Editor:

Question 1. For all graphs depicting a single point value (e.g., mean) with error bars, you must add individual data points or convert the graph to a boxplot or dot-plot to show data distribution.

Reply: Thank you very much for your valuable comments on our manuscript. We have revised all the charts that depict single data points (e.g., means) with error bars according to your suggestions. These charts have now been replaced with dot plots to more clearly illustrate the distribution of the data. We believe that this modification will better present the integrity and accuracy of the data, helping readers to more fully understand our research findings.

Question 2. It's mandatory to provide access to the numerical source data for graphs and charts either through a repository or by providing the data in a Supplementary Data file (in Excel format).

Reply: Thank you very much for your valuable comments on our manuscript. Following your suggestions, we have organized the raw numerical data for all relevant figures and charts into Excel format and will upload these files when submitting the revised manuscript. These Excel files will include all the original data points for each experiment, as well as the statistical data used to generate the charts, ensuring that readers can fully reproduce our analysis process. We believe that providing this raw data will further enhance the transparency and reproducibility of our research.

Question 3. All blots/gels must be accompanied by size markers in every figure panel. Uncropped and unedited blot/gel images must be included as Supplementary Figure(s) in the Supplementary Information PDF.

Reply: Thank you very much for your valuable comments on our manuscript. We have clearly labeled the size markers in each figure panel and have compiled all the unedited and uncropped blot/gel images into a single PDF file, which we have uploaded as supplementary information. These original images will provide readers with a complete record of the experiments, ensuring the transparency and reproducibility of our research

results.

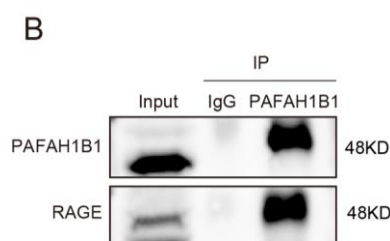
Reviewer 1:

This manuscript explores the role of Irisin in ameliorating Parkinson's disease (PD) via modulation of autophagy, neuroinflammation, and ubiquitination. The study combines clinical, in vivo, and in vitro data to support a novel mechanism involving PAFAH1B1-mediated ubiquitination of RAGE and downstream regulation of the TFEB-NLRP3 axis. The study is well-conceived and methodologically sound, and it offers potentially impactful insights into therapeutic strategies for PD. However, there are several important concerns regarding mechanistic depth, in vivo validation, and translational relevance that should be addressed before the manuscript is suitable for publication.

Question 1. Mechanistic Validation of PAFAH1B1-RAGE Axis In Vivo. While the in vitro data on RAGE ubiquitination are compelling, in vivo validation is lacking. The authors should consider performing co-immunoprecipitation assays from brain lysates to demonstrate RAGE ubiquitination and its regulation by Irisin in the mouse model.

Reply: Thank you for your important suggestions regarding the in vivo mechanism validation of this study. We fully agree with you that in vivo evidence of RAGE ubiquitination is necessary to complete the logical framework of our study, and we have conducted relevant experiments based on your suggestions. Given the current experimental conditions, we have completed the co-immunoprecipitation detection of PAFAH1B1 and RAGE in mouse brain lysates. The results provide indirect but crucial evidence for the in vivo regulation of RAGE ubiquitination, as detailed below:

1. After collecting brain tissue and extracting brain lysates, we performed co-immunoprecipitation using a PAFAH1B1-specific antibody and detected the expression level of RAGE in the precipitated complex by Western blot, with the Input group serving as a control. The co-immunoprecipitation results show an interaction between PAFAH1B1 and RAGE (Figure S6B).



Supplementary Figure 6. B: Co-IP results confirming the binding interaction between PAFAH1B1 and RAGE in mice.

2. We have detailed the specific steps and methods of these experiments in the Methods section of the manuscript and have updated the supplementary experimental section to ensure that readers can clearly understand our experimental design and procedures. We believe that these additional experimental results strongly support our conclusions regarding the in vivo PAFAH1B1-RAGE axis mechanism and provide a more comprehensive and in-depth evidence base for our study. The specific details are as follows:

“2.14 Co-immunoprecipitation: For the co-immunoprecipitation experiment, 500 µg of protein samples were mixed with agarose beads and antibodies, and the volume was adjusted with RIPA buffer...2.15 Cell and Tissue Protein Extraction: For cell protein extraction, cells were observed to calculate the required volume of lysis buffer...For tissue protein extraction (a mouse brain), fresh tissues were washed with PBS to remove impurities and placed in centrifuge tubes... and the samples were adjusted with RIPA and protein loading buffer for subsequent experiments.” Pages 15-16 in the manuscript.

“Co-IP and immunolocalization results confirmed their binding interaction (Figure 7C-D and Supplementary Figure 6B).” Page 27 in the manuscript.

We believe that by supplementing the in vivo interaction data of PAFAH1B1 and RAGE, combined with in vitro ubiquitination evidence, we can provide a more complete mechanistic chain for the neuroprotective effects of irisin through the regulation of RAGE ubiquitination.

Question 2. Clinical Correlation with Irisin Levels. The authors report lower Irisin levels in PD patients, but no correlation with clinical features is provided. Inclusion of UPDRS scores or disease duration correlations would improve the translational significance of this finding.

Reply: We are grateful for your meticulous review of our study and your constructive comments. Regarding the question you raised about the “correlation between clinical features and irisin levels,” we fully agree and have conducted additional analyses accordingly in our research. We have incorporated the Unified Parkinson’s Disease Rating Scale (UPDRS) score, an important clinical characteristic indicator, and systematically analyzed the correlation between irisin levels and this indicator. The specific analysis results are as follows:

Correlation between irisin levels and UPDRS score: Through correlation analysis, we found a significant negative correlation between irisin levels and UPDRS scores in Parkinson’s disease patients ($r = -0.3532$, $p < 0.001$) (Figure 1D). This indicates that lower irisin levels in patients are associated with higher UPDRS scores, suggesting

more severe disease. This result implies that irisin levels may be closely related to the degree of motor dysfunction in Parkinson's disease and may have potential clinical value for assessing disease progression and severity.

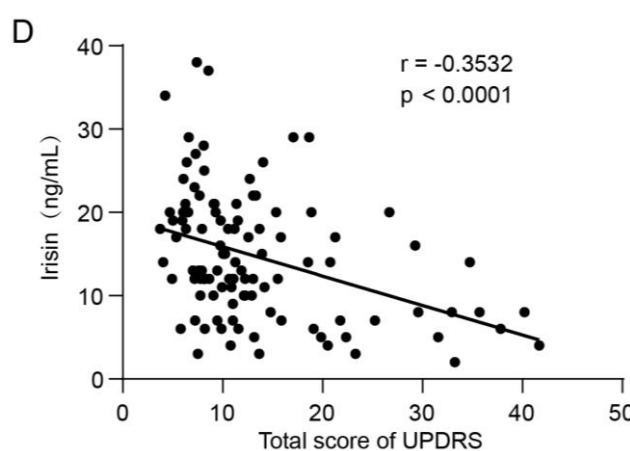


Figure 1. D. The correlation between irisin level and UPDRS score.

We have detailed the specific data and statistical results of these correlation analyses in the results section of the manuscript. The specific details are as follows:

“In addition, Pearson correlation analysis was used to explore the linear correlation between variable X and variable Y. The correlation coefficient (r) and p-value were calculated. A p-value < 0.05 was considered statistically significant.” Pages 17-18 in the manuscript.

“The correlation analysis indicated that the serum irisin level was negatively correlated with the UPDRS score ($r = -0.3532$, $p < 0.0001$) (Figure 1D).” Page 18 in the manuscript.

We believe that these additional correlation analyses further strengthen the significance of irisin as a potential biomarker for Parkinson's disease and contribute to the translation of this finding into clinical applications. We would like to thank the reviewer again for the valuable comments, which have significantly enhanced the depth of our study in terms of clinical relevance.

Question 3. Duration and Dosing in Animal Models. The in vivo Irisin treatment was relatively short (7 days). Given the chronic nature of PD, additional data on long-term outcomes or extended treatment paradigms would strengthen the relevance to therapeutic application.

Reply: We are grateful to the reviewer for raising this important point. Parkinson's disease (PD) is a chronic neurodegenerative disorder, and the assessment of long-term therapeutic effects is crucial for the clinical translation of potential therapeutic strategies. However, due to the following objective limitations, we have not yet

conducted long-term experiments, and we would like to explain as follows:

1. The primary objective of this study was to verify whether irisin regulates neuroprotective effects through the PAFAH1B1-RAGE axis. Therefore, we chose a short-term (7-day) treatment regimen to focus on mechanism validation rather than long-term efficacy assessment. In our preliminary experiments, we observed that a 7-day intervention with irisin significantly improved motor dysfunction (such as swimming and pole climbing analysis) (Figure 2) and molecular markers (such as the number of neurons, TH content, expression of α -synuclein, and changes in the expression of anti-inflammatory and pro-inflammatory factors) (Figure 3 and Figure S1) in the PD mouse model. This indicates that this time window is sufficient to detect key phenotypic changes.

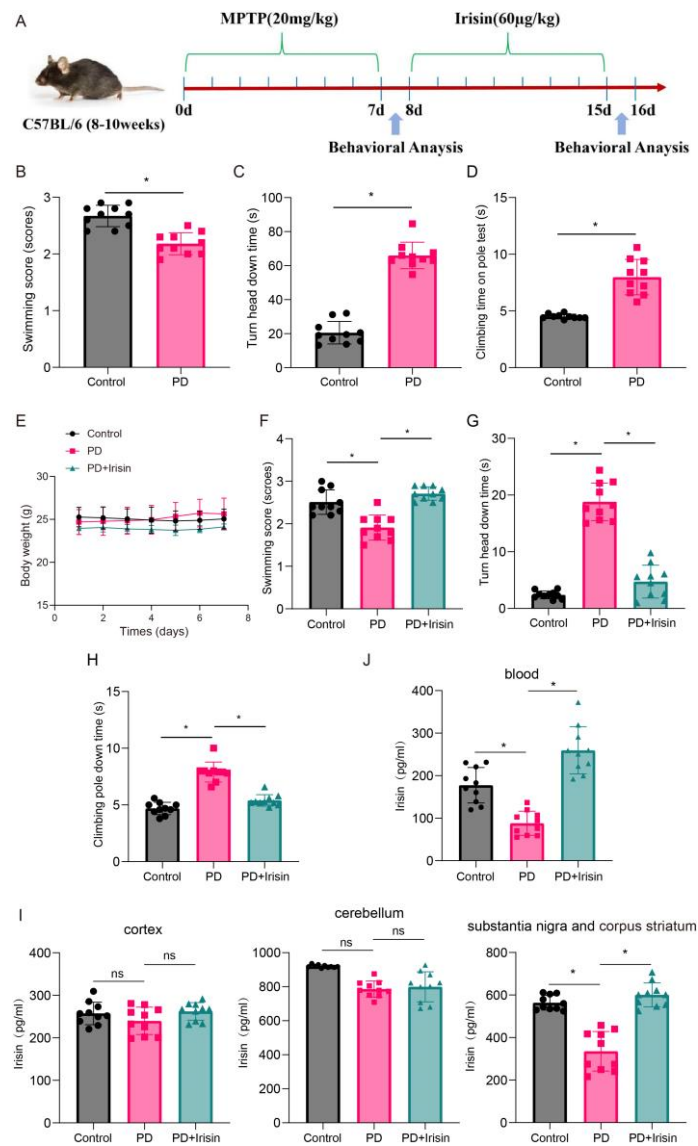


Figure 2. Improvement of PD-like symptoms and brain region distribution of irisin in mice model.

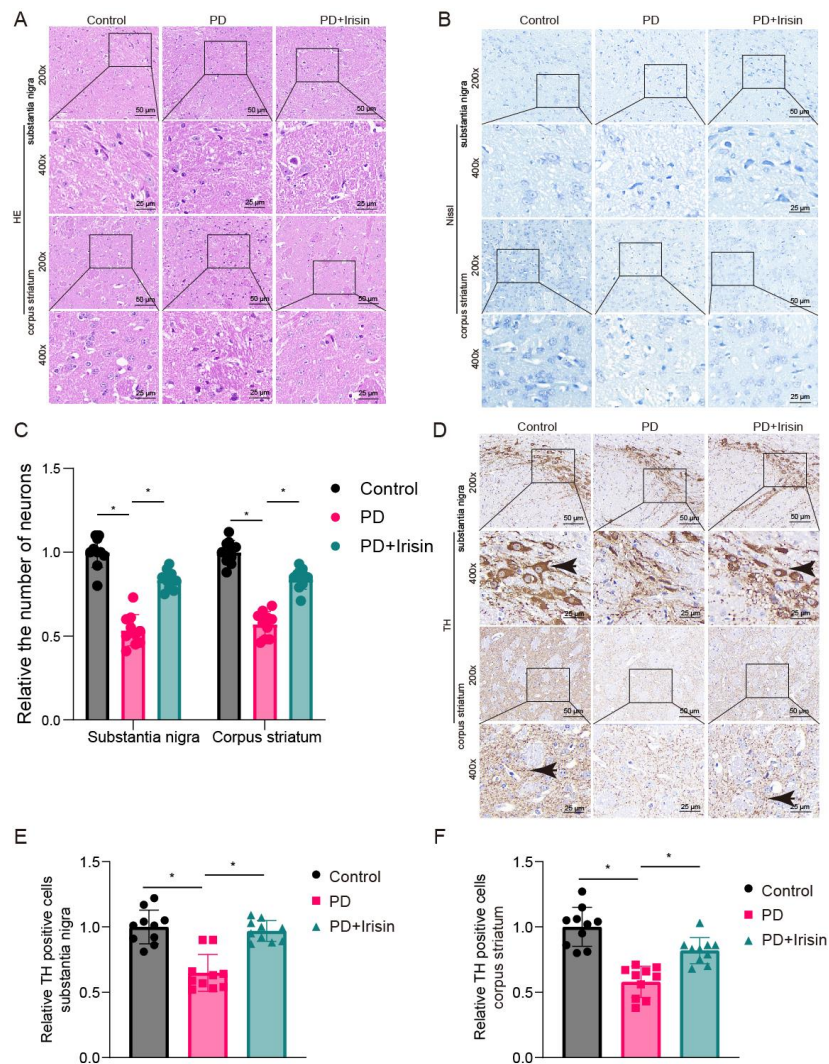
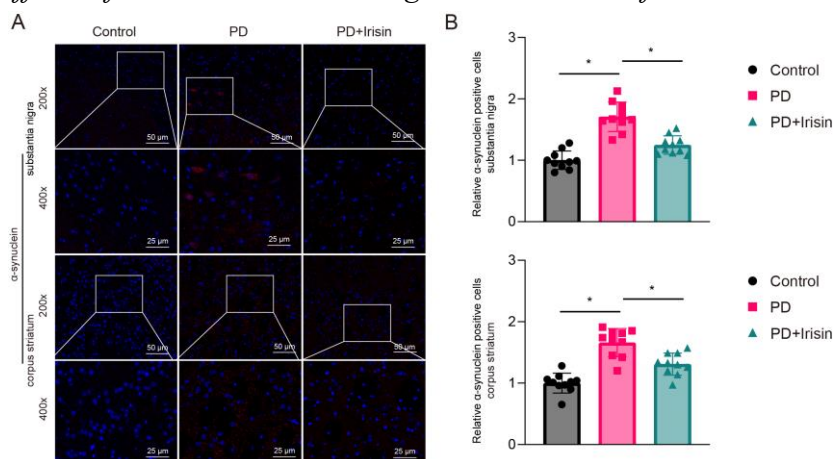


Figure 3. Effects of irisin on substantia nigra and striatum of midbrain in mice.



Supplementary Figure 1. Irisin suppresses α -synuclein expression in the substantia nigra and striatum of mice.

2. At present, we have already initiated long-term experimental studies in the clinical setting, aiming to evaluate the therapeutic effects and safety of irisin over a longer period. These experiments will provide more comprehensive evidence to support irisin

as a potential therapeutic means for Parkinson's disease. However, due to the complexity of long-term experiments and the rigorous requirements for data collection, complete data collection and analysis will take some time. We are actively advancing this work and plan to present the results of these long-term experiments in detail in subsequent research reports.

3. We fully agree with your view that more long-term results and data on extended treatment regimens will greatly enhance the relevance and reliability of irisin treatment for Parkinson's disease in clinical applications. We will continue to focus on research in this direction and will report the data and analysis results of the long-term experiments in detail in future papers.

Question 4. Quantification of Autophagic Flux. TEM and mRFP-GFP-LC3 assays were appropriately used. However, quantitative data on the number of autophagosomes/autolysosomes per cell should be included, along with appropriate statistical analysis.

Reply: Thank you for your meticulous comments on the detection method of autophagic flux. The quantitative analysis you proposed is crucial for improving the argumentation of the autophagy regulation mechanism. We have supplemented the quantitative data and statistical analysis of autophagosomes/autolysosomes as you requested. The specific details are as follows:

1. Quantitative analysis of mRFP-GFP-LC3 fluorescence spots: As shown in Figure 5G, green fluorescence punctate signals (GFP) can be detected at the early stage of autophagosome formation. After LPS treatment, autophagic flux is inhibited, leading to a decrease in autolysosomes and an increase in autophagosomes. However, autophagic flux is restored after Irisin treatment. (Autophagosomes (not fused with lysosomes) show GFP + RFP + double-positive yellow fluorescence; autolysosomes (fused) show RFP-single-positive red fluorescence.) By standardizing the fluorescence images with ImageJ software and counting the number of GFP+, RFP+, and GFP/mRFP double-positive spots in each cell (representing autophagosomes and autolysosomes, respectively), the quantitative results show that Irisin treatment based on LPS induction restores the number of autolysosomes ($p < 0.05$) and reduces the number of autophagosomes ($p < 0.05$), indicating that autophagy may play a key role in the therapeutic effect of Irisin (Figure 5G).

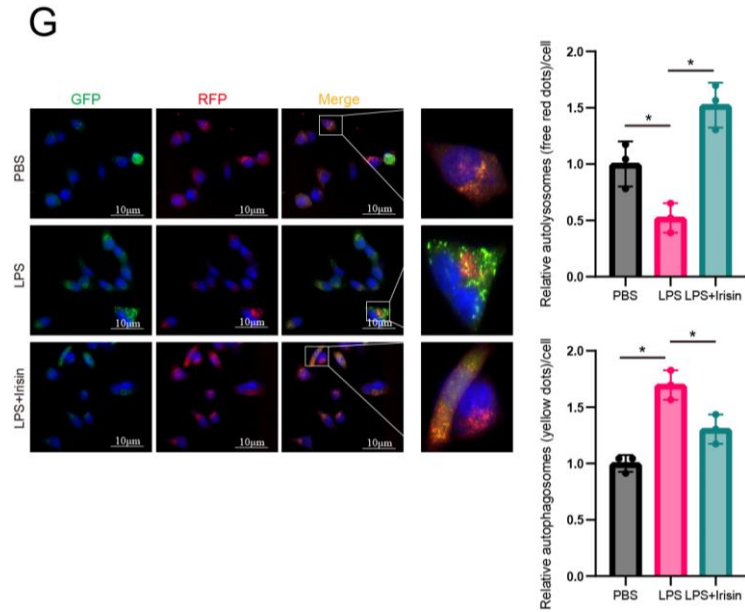


Figure 5. G: Restoration of autophagic flux in BV2 cells by irisin after labeling autophagosomes with RFP-GFP-LC3. Scale bars: 10 μ m. PBS vs LPS, $*p < 0.05$; LPS + Irisin vs LPS, $*p < 0.05$.

2. Quantitative analysis of transmission electron microscopy (TEM): A blind-method analysis was performed on the TEM images of cells in each group (PBS, LPS, and LPS + Irisin groups). Three cells were randomly selected from each sample, and the number of autophagosomes (double-membrane structure) and autolysosomes (single-membrane + electron-dense content) in individual cells was counted. The statistical results show that after LPS treatment, autolysosomes decrease while autophagosomes increase, but autophagic flux is restored after Irisin treatment (Figure 5H).

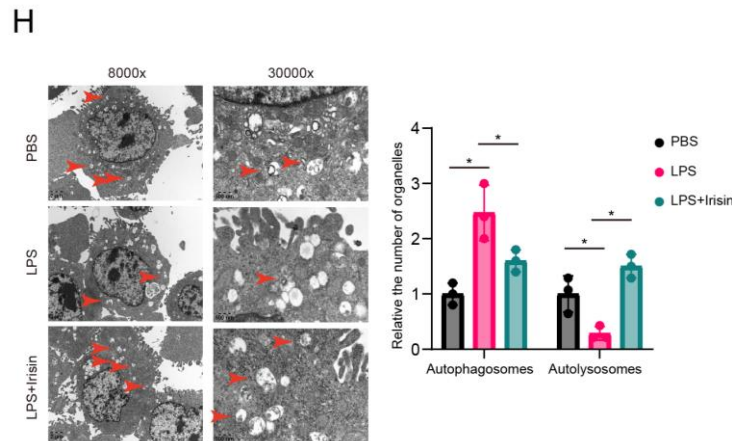


Figure 5. H: TEM results showing the number of autolysosomes in the LPS group and the restoration effect of irisin treatment. PBS vs LPS, $*p < 0.05$; LPS + Irisin vs LPS, $*p < 0.05$.

These quantitative data and statistical analysis results have been supplemented in the manuscript and detailed in the figure legends to ensure that readers can clearly

understand our experimental results and analysis methods. The specific details are as follows:

“After labeling autophagosomes with RFP-GFP-LC3, we found that irisin significantly restored autophagic flux in BV2 cells (Figure 5G). Further analysis of TEM results revealed a reduction in the number of autolysosomes in the LPS group, which was restored by irisin treatment (Figure 5H).” Page 24 in the manuscript.

Thank you again for your professional suggestions. These quantitative analyses have significantly enhanced the rigor of the autophagy mechanism argumentation.

Question 5. Clarification of Statistical Comparisons. In several figures, the specific group comparisons showing statistical significance are unclear. Please clarify comparisons in all figure legends (e.g., Control vs PD, PD vs PD + Irisin).

Reply: We are grateful for your meticulous review of our study and the valuable comments you have provided. In response to your concerns regarding the clarification of statistical comparisons, we have thoroughly revised and perfected all the figures and tables. We have ensured that the specific inter-group comparisons are clearly explained in the legends of each figure and table, so that readers can clearly understand the statistical significance of the experimental results. The specific modifications are as follows:

*“Figure 1. A-B: Comparison of IL-1 β (A) and TNF- α (B) levels in the peripheral blood serum between PD patients and the control group. Control vs PD, * $p < 0.05$.”* Page 46 in the manuscript.

*“Figure 2. F-H: Effects of irisin treatment on PD-like behaviors. Irisin treatment significantly improves PD-like behaviors, including increased swimming scores and decreased head-down time and pole-climbing time. Control vs PD, * $p < 0.05$; PD + Irisin vs PD, * $p < 0.05$.”* Page 46 in the manuscript.

*“Figure 5. A-D: Effects of irisin on the inflammatory response of LPS-activated BV2 cells. Treatment with 100 ng/mL irisin for 12 hours reduces the number of activated cells, alleviates cell swelling, and suppresses the inflammatory response of microglial cells. PBS vs LPS, * $p < 0.05$; LPS + Irisin vs LPS, * $p < 0.05$.”* Page 48 in the manuscript.

*“Figure 7. A: Effect of overexpression of PAFAH1B1 on RAGE protein expression and the reversal effect of MG132. NC vs oe-PAFAH1B1, * $p < 0.05$; oe-PAFAH1B1 + MG132 vs oe-PAFAH1B1, * $p < 0.05$.”* Page 49 in the manuscript.

Question 6. The Abstract could more explicitly highlight the central mechanism (PAFAH1B1-RAGE-TFEB-NLRP3).

Reply: We appreciate your meticulous review and valuable suggestions for this manuscript. Your suggestion that “the abstract needs to more explicitly highlight the core mechanism (PAFAH1B1-RAGE-TFEB-NLRP3)” is highly relevant and will significantly enhance the clarity and persuasiveness of the study’s mechanism. We have made targeted revisions to the abstract accordingly. The specific modifications are as follows:

“PD patients exhibited increased TNF- α and IL-1 β but decreased irisin levels. In PD mouse models, irisin improved motor deficits, increased nigrostriatal neuron numbers, restored tyrosine hydroxylase expression, and reduced α -synuclein aggregation. It also suppressed microglial inflammation and promoted anti-inflammatory polarization. Mechanistically, irisin enhanced autophagic flux, regulated RAGE ubiquitination mediated by PAFAH1B1, and inhibited neuroinflammation via the TFEB-NLRP3 axis. Specifically, PAFAH1B1 regulated RAGE expression through K61 and K169 sites on K48-linked polyubiquitin chains. Additionally, irisin restored lysosomal function by promoting TFEB nuclear translocation, enhancing NLRP3 inflammasome degradation, and reducing inflammatory factor secretion, thus alleviating neuroinflammation. This mechanism highlights irisin’s key role in regulating neuroinflammation and autophagy, offering a new potential target for PD treatment.” Pages 2-3 in the manuscript.

Question 7. Please clarify if both sexes were used in animal studies and discuss the possible impact of Irisin’s hormonal regulation in the Discussion.

Reply: We are grateful for the valuable comments and suggestions you have provided for our manuscript.

In our animal experiments, we indeed only used male C57BL/6 mice. This decision was primarily based on the following considerations: First, in the initial phase of our study, we aimed to minimize the complexity arising from sex differences to more clearly observe and analyze the mechanisms of action of the irisin hormone. Second, many previous studies in our research field have also mainly focused on male animal models, so we followed this traditional approach [1-3]. However, we are also well aware of the importance of sex as a factor in biomedical research, especially in studies related to hormonal regulation.

In the discussion section, we have thoroughly explored the potential influences of irisin hormone regulation and have specifically analyzed how sex differences might affect our research findings. We pointed out that, in previous studies, the metabolic processes and physiological functions of the irisin hormone may vary between female

and male animals. For example, in female animals, the secretion of the irisin hormone may be influenced by physiological states such as the menstrual cycle and pregnancy, while in male animals, its secretion pattern may be relatively more stable. These differences could lead to discrepancies between the results we observed in our study and the actual situation. The specific details are as follows:

“This study exclusively employed male C57BL/6 mice to circumvent the potential interference of the estrous cycle in female mice on inflammatory markers and the irisin signaling pathway, thereby ensuring model stability. Irisin, as a vital hormone, may have distinct regulatory mechanisms across different genders. Epidemiological data indicate that the incidence of Parkinson’s disease is significantly higher in males than in females²⁷, suggesting that the gonadal hormone milieu may be implicated in disease progression. Research has shown that in female animals, irisin may influence sexual maturation and reproductive functions by modulating luteinizing hormone (LH) and 17 β -estradiol levels²⁸. Moreover, in vitro experiments have confirmed that irisin can upregulate the expression of the CYP19A1 gene in human granulosa cells²⁹, further supporting its regulatory role in the female reproductive system. Future studies should incorporate female models and ovariectomized animals to elucidate the regulatory mechanisms of irisin on the PAFAH1B1-RAGE-TFEB-NLRP3 pathway against the backdrop of estrogen, which is of key significance for developing gender-differentiated PD treatment strategies.” Pages 32-33 in the manuscript.

We have proposed some directions for future research to further investigate the regulatory mechanisms of the irisin hormone in animals of different sexes. We believe that more experimental studies could be conducted to separately observe the secretion of the irisin hormone and changes in physiological functions in female and male animals under the same experimental conditions, thereby gaining a more comprehensive understanding of its regulatory effects. Additionally, we could also employ molecular biology techniques to delve into the differences in signaling pathways and gene expression involved in the irisin hormone in animals of different sexes, providing stronger evidence for elucidating its sex-specific regulatory mechanisms.

References:

- [1] Zhao M, Wang B, Zhang C, Su Z, Guo B, Zhao Y, Zheng R. The DJ1-Nrf2-STING axis mediates the neuroprotective effects of Withaferin A in Parkinson's disease. *Cell Death Differ.* 2021 Aug;28(8):2517-2535. doi: 10.1038/s41418-021-00767-2.
- [2] McFleder RL, Makhotkina A, Groh J, Keber U, Imdahl F, Peña Mosca J, Peteranderl A, Wu J, Tabuchi S, Hoffmann J, Karl AK, Pagenstecher A, Vogel J, Beilhack A, Koprich JB, Brotchie JM, Saliba AE, Volkmann J, Ip CW. Brain-to-gut trafficking of alpha-synuclein by CD11c+ cells in a

mouse model of Parkinson's disease. Nat Commun. 2023 Nov 20;14(1):7529. doi: 10.1038/s41467-023-43224-z.

[3] Hou YF, Shan C, Zhuang SY, Zhuang QQ, Ghosh A, Zhu KC, Kong XK, Wang SM, Gong YL, Yang YY, Tao B, Sun LH, Zhao HY, Guo XZ, Wang WQ, Ning G, Gu YY, Li ST, Liu JM. Gut microbiota-derived propionate mediates the neuroprotective effect of osteocalcin in a mouse model of Parkinson's disease. Microbiome. 2021 Jan 31;9(1):34. doi: 10.1186/s40168-020-00988-6. Erratum in: Microbiome. 2024 Jun 21;12(1):111. doi: 10.1186/s40168-024-01846-5.

Question 8. Consider confirming microglial polarization (CD86/CD206) with flow cytometry for higher quantitative accuracy.

Reply: Thank you for your valuable suggestions for this study. Your proposal to further verify the polarization phenotype of microglia (CD86/CD206) via flow cytometry is highly professional and of great significance for enhancing the quantitative accuracy of the data.

In response to your comments, we have conducted the following supplementary work:

1. We employed flow cytometry to examine the polarization status of microglia. After appropriate treatment, cell samples from both the experimental and control groups were labeled with specific antibodies against CD86 (a marker for M1-type polarization) and CD206 (a marker for M2-type polarization). The expression levels of CD86 and CD206 on the cell surface were quantitatively analyzed using a flow cytometer (Figure 4E).

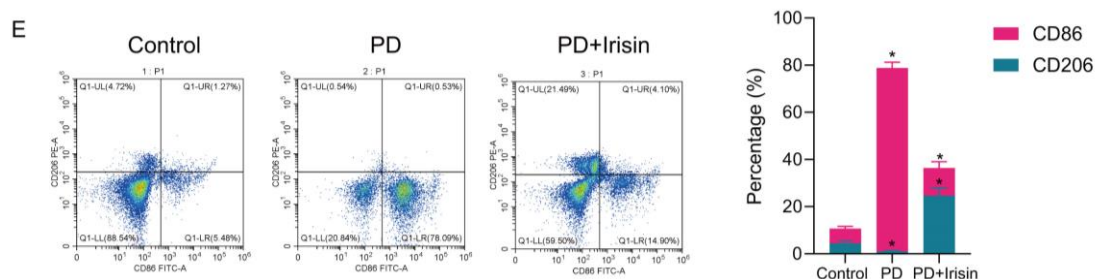


Figure 4. E: CD206+ and CD86+ were measured and analyzed by flow cytometry. Control vs PD, $*p < 0.05$; PD + Irisin vs PD, $*p < 0.05$.

2. The quantitative results from flow cytometry revealed significant changes in the expression levels of CD86 and CD206 in microglia under experimental conditions. These findings corroborate the observations from other methods, such as immunofluorescence, and further confirm the polarization status of microglia. The specific data are as follows: In the PD group, the proportion of CD86-positive cells was 78.09%, compared to 5.48% in the control group and 14.90% in the PD + Irisin group ($p < 0.05$); In the PD group, the proportion of CD206-positive cells was 0.54%, compared to 4.72% in the control group and 21.49% in the PD + Irisin group ($p < 0.05$) ($p < 0.05$ indicates a statistically significant difference). The quantitative analysis from

flow cytometry indicates that the polarization status of microglia is significantly altered under LPS treatment, which is consistent with our previous hypotheses and experimental observations. With the precise quantification provided by flow cytometry, we are able to more accurately assess the degree of microglial polarization, thereby providing stronger evidence for our study.

We have detailed the methods and results of the flow cytometry experiment in the revised manuscript. The specific details are as follows:

“2.12 Flow Cytometry: To explore the expression of cell surface markers after stimulation...Finally, cells were resuspended in 500 μ L PBS containing 3% fetal bovine serum and analyzed using a flow cytometer to obtain data on the expression levels of cell surface markers.” Pages 13-14 in the manuscript.

“Meanwhile, flow cytometry analysis showed that after irisin treatment, cells were able to reduce the elevated CD86+/CD206+ cell ratio in PD mice, indicating that irisin has an impact on the polarization transformation of microglia (Figure 4E).” Page 22 in the manuscript.

We believe that the quantitative analysis provided by flow cytometry has rendered our research findings more accurate and reliable, and our conclusions more persuasive. Thank you once again for your suggestions. Your comments have played an important guiding role in improving our study and enhancing the quality of our manuscript.

Question 9. The Ubibrowser prediction method should be briefly described in the Methods or figure legend.

Reply: We appreciate the valuable suggestions from the reviewer. Following your request, we have added relevant explanations for the prediction method of Ubibrowser in the Methods section of the manuscript. The specific details are as follows:

“We used UbiBrowser (<http://ubibrowser.ncpsb.org>) to predict the interactions between ubiquitin ligases and their substrates. This platform identifies potential E3-substrate pairs by integrating structural features, domain interactions, and co-expression data. We used the default parameters for prediction and selected high-confidence prediction results for subsequent experimental validation.” Page 17 in the manuscript.

These additional explanations enhance the transparency and reproducibility of our research methods. Thank you once again for your help in improving the quality of our manuscript.

Question 10. Ensure consistent nomenclature throughout (e.g., PD+Irisin vs. Irisin-treated PD mice).

Reply: We are grateful for your review of our manuscript and the valuable comments you have provided. Regarding the issue you have pointed out, we sincerely apologize. We take this matter very seriously and have conducted a comprehensive review and revision throughout the manuscript to ensure the consistency and accuracy of terminology.

During the revision process, we have standardized all references to “PD + Irisin” and “PD mice treated with Irisin” within the article, opting for the consistent use of the term “PD + Irisin”. We have meticulously examined the entire manuscript to ensure uniform terminology in the experimental design, methods, results description, and discussion sections. For example:

“After successful modeling, 20 PD mice were further divided into two groups (n = 10 each): the PD group and the PD + Irisin group.” Page 7 in the manuscript.

We have corrected all relevant expressions in the revised manuscript and ensured the consistency of terminology throughout the article. We believe that these modifications will render the manuscript clearer and more precise, facilitating readers' understanding and evaluation of our research work.

Reviewer 2:

Thank you for the opportunity to peer review. The authors elucidated the protective effects of Irisin against inflammatory in PD. This discovery could lead to the potential development of therapeutic drugs and functional supplements. Thus, this research is very important for the creation of a sustainable society in the future. Although the study is great, I have provided a few comments and suggestion that make the manuscript better. We appreciate the efforts of the authors in conducting this important study and look forward to seeing the final version of the manuscript.

Question 1. Fig. 1: How did you detect the protein in human serum, ELIZA? Fig. 1C unit (pg/ml*1000) meant fg/mL?

Reply: We are grateful for your review of our manuscript and the valuable comments you have provided. Regarding the issues you raised concerning the protein detection method in human serum and the unit representation in Figure 1C, we take them very seriously and would like to provide you with detailed explanations.

1. Protein Detection Method in Human Serum: In our study, we employed enzyme-linked immunosorbent assay (ELISA) to detect proteins in human serum. ELISA is a

widely used experimental technique for protein quantification, known for its high sensitivity, high specificity, and ease of operation. During the experiment, we strictly followed the instructions of the kit to ensure the reliability of the results.

2. Unit Explanation in Figure 1C: The actual unit of the data in the figure is pg/mL, and the notation “×1000” was used to adjust the range of the x-axis scale (i.e., the actual concentration is in pg/mL, but for the compactness of the chart, the values were divided by 1000 for display). We understand that this notation could lead to confusion. Since 1 ng/mL = 1000 pg/mL, we have changed the unit to ng/mL in the revised manuscript to avoid ambiguity. The specific details are as follows:

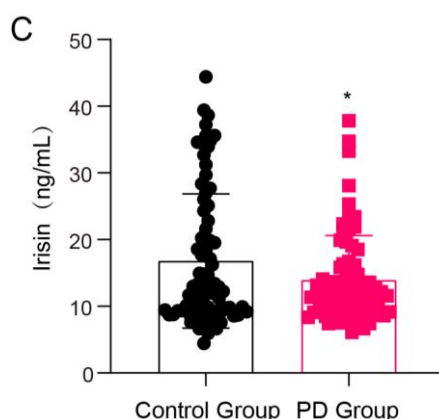


Figure 1. C: Comparison of serum Irisin levels in the peripheral blood between PD patients and the control group. Control vs PD, * $p < 0.05$.

Thank you for your meticulous review. We will refine the figures according to your suggestions.

Question 2. Fig. 2I: It is interesting that the concentration of Irisin was different in each brain region. Is Irisin produced in the brain? If so, what are the producing cells?

Reply: Thank you for your attention to and inquiry about the results in Figure 2I. First, it should be clarified that in the experiment depicted in Figure 2I, we assessed the ability of exogenous irisin to penetrate the blood-brain barrier via intravenous injection into the tail vein. The primary purpose of this experimental design was to verify whether exogenously administered irisin could effectively enter the central nervous system, thereby providing a basis for its potential therapeutic applications. The experimental results revealed differences in the distribution of irisin across various brain regions, which may reflect the regional specificity of blood-brain barrier permeability or varying capacities of different brain regions to take up irisin.

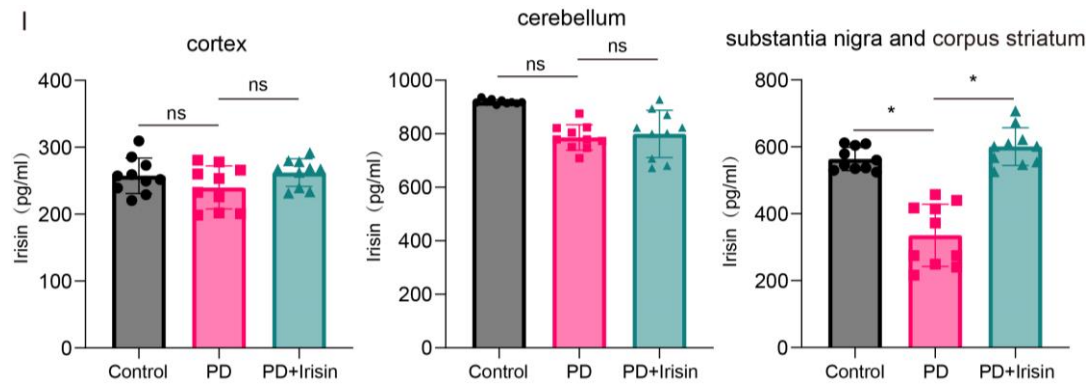


Figure 2. I: Levels of irisin in the tissue homogenates of the cortex, cerebellum, and substantia nigra striatum of each group of mice detected by ELISA. Cortex and cerebellum: Control vs PD, not significant; PD + Irisin vs PD, not significant. Substantia nigra striatum: Control vs PD, $*p < 0.05$; PD + Irisin vs PD, $*p < 0.05$.

Regarding the questions you raised about “whether irisin is produced in the brain” and “the cell types that produce it,” the following important findings have been reported in existing studies:

1. **Source of Brain-Derived Irisin:** According to the existing literature, irisin is primarily considered a hormone or myokine produced and released by muscle cells during exercise (especially endurance exercise). Exercise stimulates muscle cells to produce a membrane-bound protein called FNDC5, which, upon cleavage, releases irisin into the bloodstream. For instance, Bostrom et al. first discovered in their 2012 study that after prolonged endurance exercise in mice and humans, the expression of the FNDC5 gene in skeletal muscle is up-regulated, and serum irisin levels increase [1]. However, some studies also suggest the possibility of mechanisms for irisin production within the brain. For example, Professor Arancio’s team confirmed in their research published in *Nature Medicine* that irisin, a myokine induced by exercise, is released through the cleavage of the membrane-bound precursor protein fibronectin type III domain-containing protein 5 (FNDC5) and is also expressed in the hippocampus. In Alzheimer’s disease patients, the levels of FNDC5/irisin in the hippocampus are reduced, and exercise can increase the levels of FNDC5/irisin in the brain [2].

2. **Brain Cell Types That Produce Irisin:** Current research indicates the following regarding the specific brain cell types that produce irisin:

- (1) **Neurons:** Studies have found that FNDC5 is expressed in hippocampal neurons [2].
- (2) **Astrocytes and Microglia:** Some research suggests that these neuroglial cells may also be involved in the production and regulation of FNDC5/irisin [3].
- (3) **Choroid Plexus Cells:** Given that the choroid plexus is an essential component of the blood-brain barrier, it has been hypothesized that these cells may be involved in the trans-barrier transport of irisin [4].

References:

- 【1】 Boström P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, Rasbach KA, Boström EA, Choi JH, Long JZ, Kajimura S, Zingaretti MC, Vind BF, Tu H, Cinti S, Højlund K, Gygi SP, Spiegelman BM. A PGC1- α -dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature*. 2012 Jan 11;481(7382):463-8. doi: 10.1038/nature10777.
- 【2】 Lourenco MV, Frozza RL, de Freitas GB, Zhang H, Kincheski GC, Ribeiro FC, Gonçalves RA, Clarke JR, Beckman D, Staniszewski A, Berman H, Guerra LA, Forny-Germano L, Meier S, Wilcock DM, de Souza JM, Alves-Leon S, Prado VF, Prado MAM, Abisambra JF, Tovar-Moll F, Mattos P, Arancio O, Ferreira ST, De Felice FG. Exercise-linked FND5/irisin rescues synaptic plasticity and memory defects in Alzheimer's models. *Nat Med*. 2019 Jan;25(1):165-175. doi: 10.1038/s41591-018-0275-4.
- 【3】 Dun SL, Lyu RM, Chen YH, Chang JK, Luo JJ, Dun NJ. Irisin-immunoreactivity in neural and non-neural cells of the rodent. *Neuroscience*. 2013 Jun 14;240:155-62. doi: 10.1016/j.neuroscience.2013.02.050.
- 【4】 Ruan Q, Huang Y, Yang L, Ruan J, Gu W, Zhang X, Zhang Y, Zhang W, Yu Z. The effects of both age and sex on irisin levels in paired plasma and cerebrospinal fluid in healthy humans. *Peptides*. 2019 Mar;113:41-51. doi: 10.1016/j.peptides.2019.01.004.

Question 3. Fig. 3A,3B The quantitative data should be submitted because only typical images did not express the decrease in HE and Nissl stained neuronal cells.

Reply: We are grateful for your meticulous review of our study and the valuable comments you have provided. We fully agree with your concerns regarding the quantitative data for Figures 3A and 3B and would like to provide a detailed explanation here. Concerning the quantitative analysis of HE staining and Nissl staining:

1. Limitations of HE Staining: Hematoxylin and eosin (HE) staining is a commonly used histopathological technique primarily for observing the structure of tissues and the morphological characteristics of cells. In brain tissue, HE staining can reveal neuronal damage, such as cell swelling, pyknosis of the nucleus, and vacuolar changes [1-3]. However, HE staining is not suitable for accurately counting the number of neurons. This is because the staining effect of HE mainly reflects structural changes in cells rather than specifically staining neurons.

2. Advantages of Nissl Staining: Nissl staining is a specialized method for staining neural tissue that can specifically stain Nissl bodies within neurons, thereby clearly showing the cell bodies and nuclei of neurons [4]. The principle of Nissl staining involves using specific dyes (such as cresyl violet or toluidine blue) to stain RNA within neurons, highlighting their structure. Therefore, Nissl staining can be used for accurate neuron counting and quantitative analysis.

3. Supplementary Experimental Data: In our study, we conducted a quantitative analysis of neurons using Nissl staining. Through Nissl staining, we were able to accurately identify and count neurons and compare the number of neurons across different experimental groups. The specific data are as follows:

In HE staining, we observed neuronal damage but could not perform accurate cell counting. In Nissl staining, we conducted quantitative analysis of neurons and found significant differences between the experimental and control groups (Figure 3B-C).

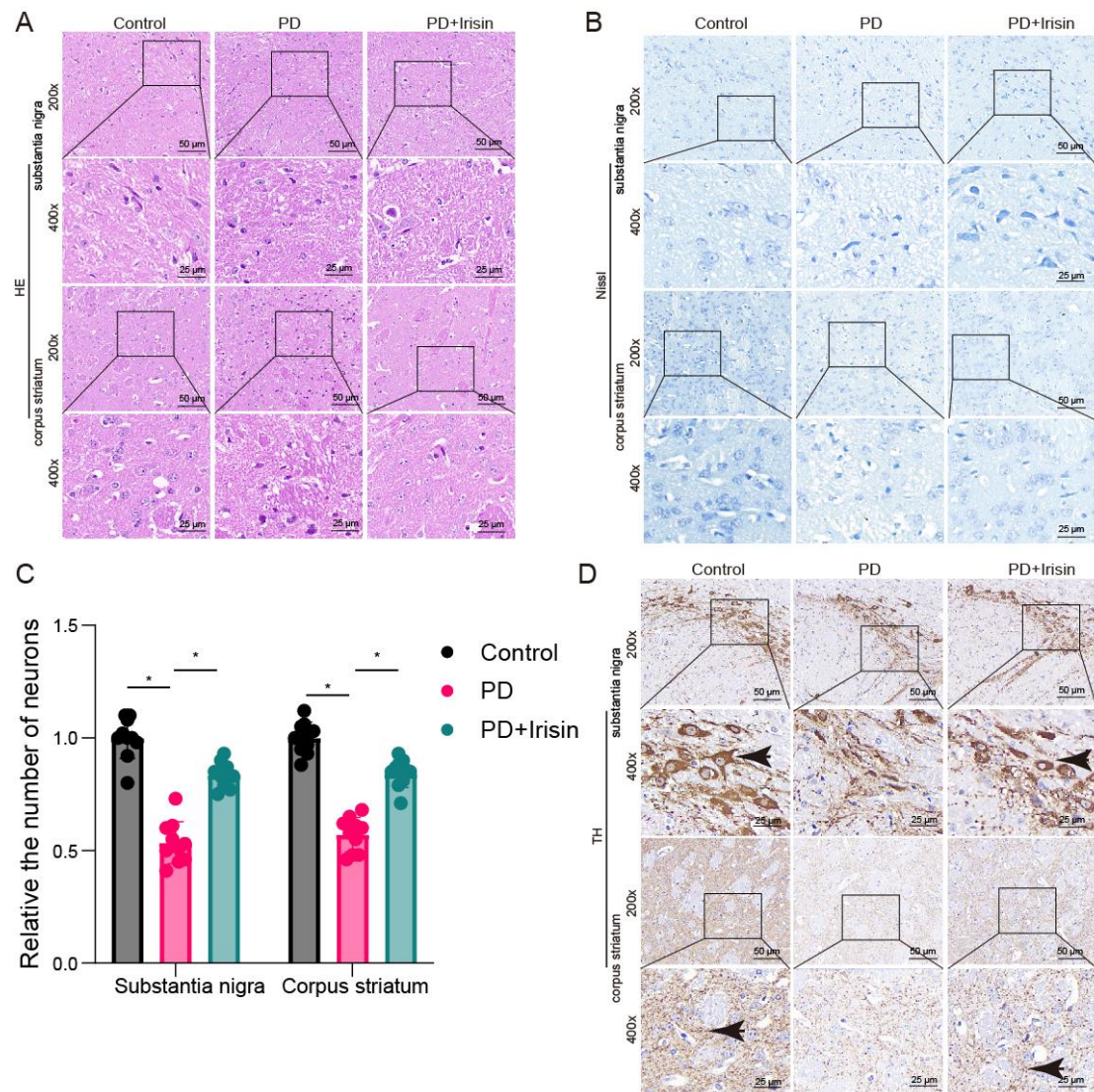


Figure 3. A: HE staining results of the substantia nigra and striatum of midbrain in each group. Scale bars: 50 μ m. B-C: Nissl staining results of neurons in the substantia nigra and striatum. Scale bars: 50 μ m. Control vs PD, $*p < 0.05$; PD + Irisin vs PD, $*p < 0.05$.

In summary, due to the limitations of HE staining, we could not accurately count the number of neurons using this method. Therefore, we adopted Nissl staining for

quantitative analysis to ensure the accuracy and reliability of our experimental results. We have supplemented the quantitative data from Nissl staining in the revised manuscript and provided detailed explanations in the figure legends to facilitate readers' clear understanding of our findings.

References:

- [1] Gao H, Geng Z. Calpain I activity and its relationship with hippocampal neuronal death in pilocarpine-induced status epilepticus rat model. *Cell Biochem Biophys*. 2013 Jun;66(2):371-7. doi: 10.1007/s12013-012-9476-5.
- [2] Wang ZK, Xue L, Wang T, Wang XJ, Su ZQ. Infiltration of invariant natural killer T cells occur and accelerate brain infarction in permanent ischemic stroke in mice. *Neurosci Lett*. 2016 Oct 28;633:62-68. doi: 10.1016/j.neulet.2016.09.010.
- [3] Zhang H, Xu R, Xie F, Xu W, Zeng MF, Wang X, Zhu J. Protective effects of perfluorooctyl-bromide nanoparticles on early brain injuries following subarachnoid hemorrhage in rats. *Am J Transl Res*. 2015 Aug 15;7(8):1404-16.
- [4] Fernandez-Garcia JM, Carrillo B, Tezanos P, Pinos H, Collado P. Genistein early in life modifies the arcuate nucleus of the hypothalamus morphology differentially in male and female rats. *Mol Cell Endocrinol*. 2023 Jun 15;570:111933. doi: 10.1016/j.mce.2023.111933.

Question 4. Fig. 5G: Since LPS damage is cell density dependent, areas of equal cell density should be taken.

Reply: We are grateful for your attention to our study and the valuable comments you have provided. We take very seriously the issue you raised regarding the correlation between LPS-induced damage and cell density in Figure 5G and have conducted a re-evaluation and processed accordingly.

To ensure the accuracy and reliability of the experimental results, we have re-selected areas with the same cell density for photography and analysis. The following steps were adopted to ensure consistent cell density:

1. Cell Counting and Density Measurement: Before the experiment, we conducted precise cell counting and adjusted the cell plating density based on the counting results to ensure consistent cell density across all experimental groups.
2. Microscopic Observation and Area Selection: When taking photographs, we used a microscope to observe the cells and selected areas with uniform and consistent cell density for imaging. We ensured that the selected areas had tightly packed and evenly distributed cells, avoiding density differences caused by cell clustering or sparsity.
3. Image Analysis and Validation: After the photography was completed, we re-analyzed all the original microscopic images.

We have supplemented the revised manuscript with the newly taken photographs and

related data. The revised Figure 5G is consistent with the original experimental results, further validating the stability of the conclusions. The specific details are as follows:

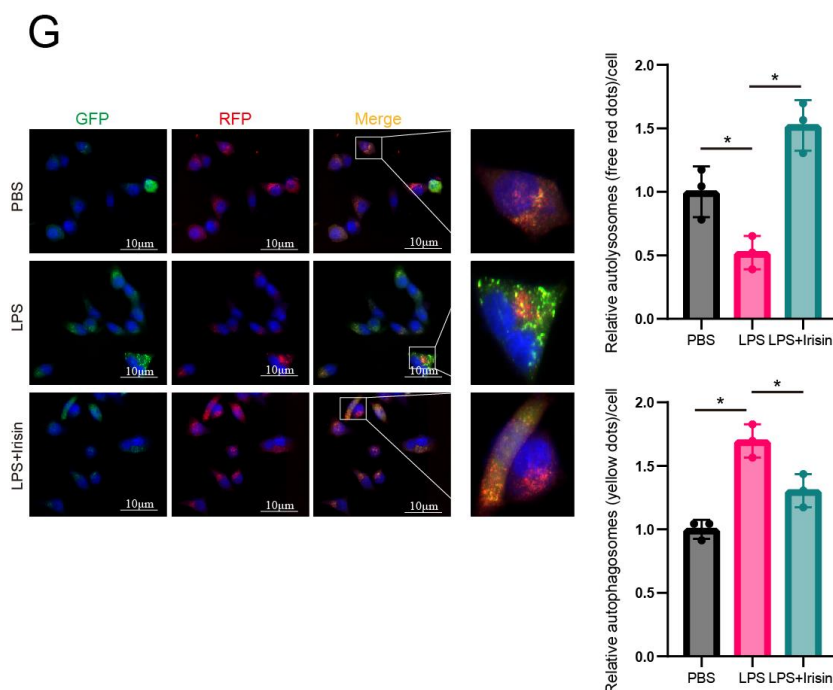


Figure 5. G: Restoration of autophagic flux in BV2 cells by irisin after labeling autophagosomes with RFP-GFP-LC3. Scale bars: 10 μ m. PBS vs LPS, $*p < 0.05$; LPS + Irisin vs LPS, $*p < 0.05$.

We believe that these improvements will make our experimental results more accurate and reliable.

Question 5. It would be helpful to indicate what GFP and RFP represent. Also, since the LC3 dot-like signal was not seen, the cell processing method should be reviewed and images taken at higher magnification should be added.

Reply: We appreciate the valuable comments you have provided for this manuscript. We have carefully considered your suggestions and made the following revisions and improvements to the manuscript:

1. Explanations of GFP and RFP, and the Principle of RFP-GFP-LC3 Labeling:

(1) GFP (Green Fluorescent Protein): Used to label the LC3 protein, GFP can detect green fluorescence punctate signals at the early stage of autophagosome formation. However, GFP is prone to quenching in the acidic lysosomal environment, and the green signal will weaken or disappear after the fusion of autophagosomes with lysosomes.

(2) RFP (Red Fluorescent Protein): Co-expressed with GFP, RFP has stronger acid resistance and can continuously emit red fluorescence in autolysosomes, thereby

distinguishing autophagosomes (GFP+/RFP+) and autolysosomes (GFP-/RFP+) through the co-localization or separation of red and green fluorescence.

(3) Autophagosomes (not fused with lysosomes): Display GFP+RFP+ double-positive yellow fluorescence (i.e., aggregation of LC3-II on the autophagosome membrane).

(4) Autolysosomes (fused): GFP is quenched in the acidic environment ($\text{pH} < 5.0$), leaving only RFP single-positive red fluorescence.

Therefore, this system can dynamically distinguish early autophagosomes (yellow punctate signals) and late autolysosomes (red punctate signals) to assess the integrity of autophagic flux.

2. Regarding the detection of LC3 punctate signals, we have added local magnification images (Figure 5G) to more clearly display faint punctate signals. These high-magnification images can more clearly show the distribution of LC3 in autophagosomes, thereby verifying our experimental results. The specific details are as follows:

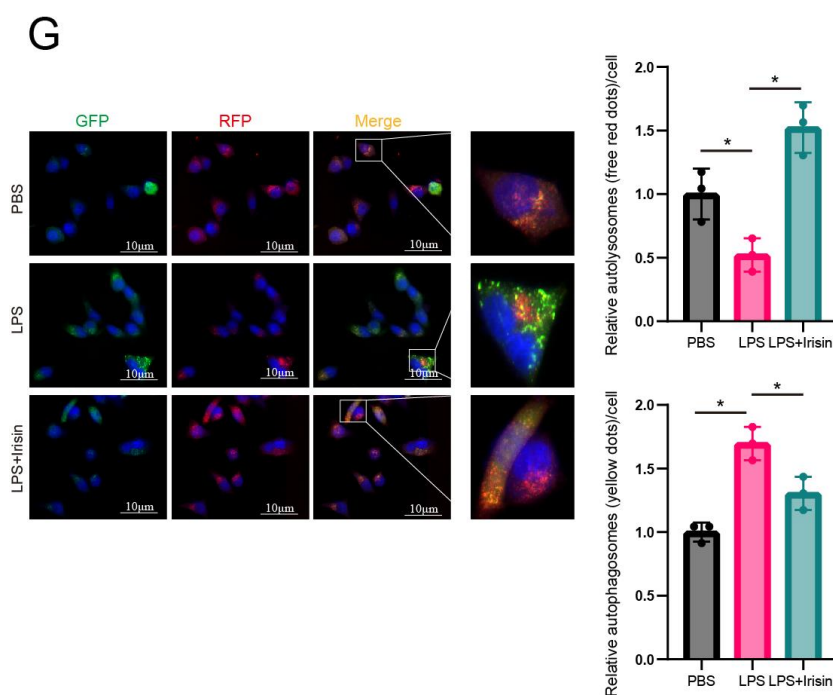


Figure 5. G: Restoration of autophagic flux in BV2 cells by irisin after labeling autophagosomes with RFP-GFP-LC3. Scale bars: 10 μm . PBS vs LPS, $*p < 0.05$; LPS + Irisin vs LPS, $*p < 0.05$.

We believe that these additions and revisions will make our research results clearer and more convincing. Thank you again for your valuable comments. We will continue to strive to improve the quality and transparency of our research.

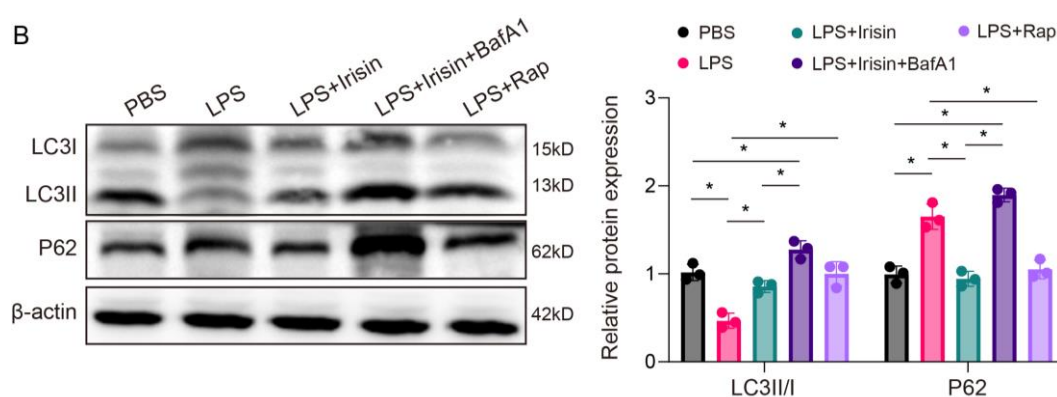
Question 6. Supplementary Fig. 3B: Why was only LC3 increased in Sup 3B bufA + LPS + Irisin?

Reply: We appreciate your attention to our study and the thorough review provided. Regarding the question you raised, we have given it considerable attention and offer a detailed explanation as follows:

Bafilomycin A1 (BafA1), an inhibitor of the V-ATPase, inhibits autophagy flux by blocking the fusion of autophagosomes with lysosomes. This blockage leads to the accumulation of autophagosomes, preventing the normal degradation of LC3-II; hence, we observed a significant increase in LC3 protein levels in the LPS + Irisin + BafA1 group.

In this experiment, the purpose of using BafA1 was to ascertain whether autophagy flux plays a role in the inhibition of microglial cell inflammation mediated by irisin. When BafA1, LPS, and irisin were co-administered, BafA1 blocked the fusion of autophagosomes with lysosomes, preventing the degradation of autophagosomes. This resulted in the accumulation of LC3-II (i.e., the detected LC3) due to impaired degradation, which manifested as an increased LC3 level. The results indicate that BafA1 can reverse the inhibitory effects of Irisin on pro-inflammatory and anti-inflammatory markers, further confirming the critical role of autophagy flux in the anti-inflammatory action of irisin on BV2 cells.

We are grateful once again for your valuable feedback and look forward to your continued guidance.



Supplementary Figure 3. B: WB analysis of LC3 and P62 protein levels in BV2 cells treated with Rapamycin (Rap, an autophagy activator) or Bafilomycin A1 (BafA1, an autophagic flux inhibitor). The effects of these treatments on autophagy were confirmed by changes in LC3 and P62 levels. PBS vs LPS, $*p < 0.05$; LPS vs LPS + Irisin, $*p < 0.05$; LPS + Irisin vs LPS + Irisin + BafA1, $*p < 0.05$; PBS vs LPS + Irisin + BafA1, $*p < 0.05$; LPS vs LPS + Rap, $*p < 0.05$.

Question 7. Fig. 7F-H: β -actin misspelled. Line 578-580: Is Irisin actin? Misspelling?

Reply: We are grateful for your valuable comments on our manuscript. Regarding the misspelling of β -actin in Figures 7F-H, we sincerely apologize for this oversight. We will carefully review and correct this error in subsequent revisions to ensure accurate spelling.

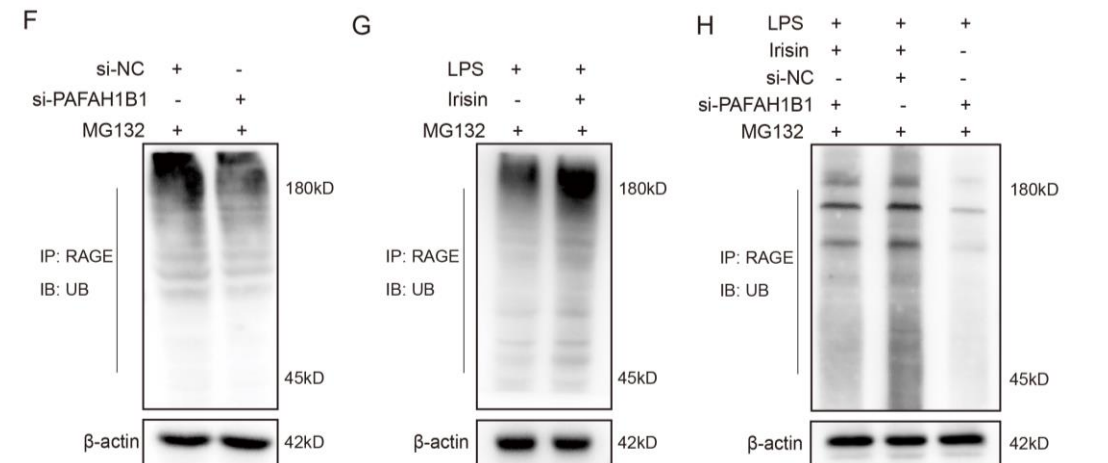


Figure 7. F: Ubiquitination level of RAGE upon co-treatment with si-PAFAH1B1 and MG132. G: Increase in the ubiquitination level of RAGE in BV2 cells after irisin addition. H: Reversal of irisin-induced ubiquitination of RAGE by combining irisin with si-PAFAH1B1.

As for the statements on lines 578 to 580, we apologize for any misunderstanding caused. There was an error in our original text that led you to believe we were referring to irisin as actin. Irisin is a myokine derived from the proteolytic cleavage of the FNDC5 protein [1], and although it is associated with actin (as it originates from FNDC5 containing an actin-like domain), it is not actin itself. We intended to emphasize that irisin, as a molecule with actin-related properties, may play a role in the pathogenesis of Parkinson's disease by inhibiting immune-inflammatory responses. We will revise this passage to more clearly and accurately convey our meaning, as detailed below:

“We hypothesized that Irisin, as an actin-associated myokine with anti-inflammatory and anti-apoptotic effects, may play a role in the development of PD by inhibiting immune inflammatory responses.” Page 31 in the manuscript.

References:

[1] Boström P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, Rasbach KA, Boström EA, Choi JH, Long JZ, Kajimura S, Zingaretti MC, Vind BF, Tu H, Cinti S, Højlund K, Gygi SP, Spiegelman BM.

A PGC1- α -dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature*. 2012 Jan 11;481(7382):463-8. doi: 10.1038/nature10777.

Reviewer 3:

This manuscript by Lijun Cai et al presents a comprehensive and well-structured investigation into the therapeutic potential of Irisin in Parkinson's disease (PD), integrating clinical data, in vivo mouse models, and in vitro mechanistic studies. The mechanistic focus on autophagy enhancement and RAGE ubiquitination via the PAFAH1B1 axis, along with TFEB-NLRP3 signaling, significantly contributes to the current understanding of immunomodulatory strategies in PD. The general purpose of this study is clear, and the experiments appear to be of interest. However, several critical aspects require further clarification. The statistical validity of the key finding-namely, the decreased serum Irisin levels in PD patients-should be substantiated with detailed statistical data. In addition, considering the experimental design, the use of one-way ANOVA for Figure 7E appears to be insufficient. In my opinion, therefore, this manuscript is not recommended for publication in its present form.

Question 1. Fig. 1C: In the main text, it is stated that “We further measured serum Irisin levels in the peripheral blood of PD patients. The results indicated that serum Irisin levels in PD patients were significantly lower than those in controls ($p < 0.05$) (Figure 1C).” However, based on the variability and error bars shown in the graph, the difference does not appear to be statistically significant. As this is a critical point, I kindly request that the authors provide detailed statistical values (e.g., exact p-value, effect size, confidence intervals) to support the claim of significance.

Reply: Regarding the statistical issue of irisin levels in the peripheral serum of Parkinson's disease (PD) patients that you have raised, we take this very seriously. In the original text, we only briefly stated that the serum irisin levels in PD patients were significantly lower than those in the control group ($p < 0.05$), and indeed, we did not provide more detailed statistical values, which might lead readers to question the reliability of the results. In fact, during the statistical analysis, we used the Unpaired t-test to compare the differences between the two groups. The specific statistical values are as follows (Figure R1):

1. Exact p-value: $p = 0.0435$, which is less than 0.05, indicating that the difference between the two groups is statistically significant.
2. Confidence interval: For the difference in irisin levels, the 95% confidence interval is -5654 to -84.65, further supporting our conclusion.

未配对 t 检验 表结果	
1 分析的表	Figure 1C
2	
3 列 B	数据集-B
4 vs	vs
5 列 A	数据集-A
6	
7 未配对 t 检验	
8 P 值	0.0435
9 P 值摘要	*
10 显著不同 (P < 0.05)?	是
11 单尾或双尾 P 值?	双尾
12 t, df	t=2.036, df=148
13	
14 差异有多大?	
15 平均值列A	16799
16 平均值列B	13930
17 平均值差异 (B - A) ± 标准误	-2870 ± 1409
18 95% 置信区间	-5654 到 -84.65
19 R 平方 (eta 平方)	0.02725
20	
21 用于比较方差的 F 检验	
22 F, DFn, Dfd	2.271, 78, 70
23 P 值	0.0006
24 P 值摘要	***
25 显著不同 (P < 0.05)?	是
26	
27 分析的数据	
28 样本大小, 列A	79
29 样本大小, 列B	71

Figure R1. The detailed statistical analysis information of Figure 1C.

We will include these detailed statistical data in the revised manuscript to enhance the transparency and credibility of the results. Thank you once again for your suggestion, and we believe this will help improve the quality of our research.

Question 2. Fig4: Interleukin-6 (IL-6) is a pleiotropic cytokine that can exhibit both pro-inflammatory and anti-inflammatory properties depending on the context. It may play roles in stimulating immune responses as well as regulating local and systemic acute-phase inflammatory reactions. In this manuscript, the authors appear to classify IL-6 as an anti-inflammatory cytokine. If this is the intended interpretation, I kindly request that the authors provide supporting evidence or citations to justify this classification within the context of their study.

Reply: Thank you for your attention to our study and the valuable comments you have provided. Regarding the classification of IL-6 that you have mentioned, we would like to provide a detailed explanation here.

In our study, we chose IL-6 as an anti-inflammatory factor primarily because of its close association with the polarization of M2 macrophages. IL-6 can induce the polarization of macrophages towards the M2 phenotype through the activation of the STAT3 signaling pathway in the tumor microenvironment [1-3]. These studies indicate that IL-6 can function as an anti-inflammatory factor in the tumor microenvironment by modulating the polarization state of macrophages, thereby influencing tumor

progression.

However, we also acknowledge that IL-6 is a pleiotropic cytokine that may exhibit both pro-inflammatory and anti-inflammatory properties under different pathophysiological conditions. In our research, the detection of IL-6 was not the focus, but rather as a potential anti-inflammatory marker for assessment. While IL-6 may demonstrate certain anti-inflammatory effects in specific contexts, this is not a universal characteristic of IL-6. To avoid potential confusion for readers, we have decided to remove the data related to IL-6 and focus on more definitive anti-inflammatory markers, such as IL-4 and IL-10, to elaborate on the anti-inflammatory effects of irisin.

We appreciate your valuable feedback and believe that these revisions will make our study more rigorous and complete.

References:

- [1] Fu XL, Duan W, Su CY, Mao FY, Lv YP, Teng YS, Yu PW, Zhuang Y, Zhao YL. Interleukin 6 induces M2 macrophage differentiation by STAT3 activation that correlates with gastric cancer progression. *Cancer Immunol Immunother*. 2017 Dec;66(12):1597-1608. doi: 10.1007/s00262-017-2052-5.
- [2] Zhao C, Zeng N, Zhou X, Tan Y, Wang Y, Zhang J, Wu Y, Zhang Q. CAA-derived IL-6 induced M2 macrophage polarization by activating STAT3. *BMC Cancer*. 2023 May 1;23(1):392. doi: 10.1186/s12885-023-10826-1.
- [3] Wu X, Lu W, Xu C, Jiang C, Zhuo Z, Wang R, Zhang D, Cui Y, Chang L, Zuo X, Wang Y, Mei H, Zhang W, Zhang M, Li C. Macrophages Phenotype Regulated by IL-6 Are Associated with the Prognosis of Platinum-Resistant Serous Ovarian Cancer: Integrated Analysis of Clinical Trial and Omics. *J Immunol Res*. 2023 Apr 19;2023:6455704. doi: 10.1155/2023/6455704.

Question 3. Statistical analysis in Fig 7: The manuscript states that pain thresholds were analyzed using one-way ANOVA. However, for Figure 7E, a two-way ANOVA may be more appropriate given the presence of multiple factors. I recommend that the authors clearly specify the factors involved and reanalyze the data using two-way ANOVA to provide a more accurate statistical interpretation.

Reply: We are grateful for your professional and meticulous review comments, and we highly value the suggestions you have made regarding the statistical analysis method for Figure 7E, which are invaluable for enhancing the quality of our manuscript.

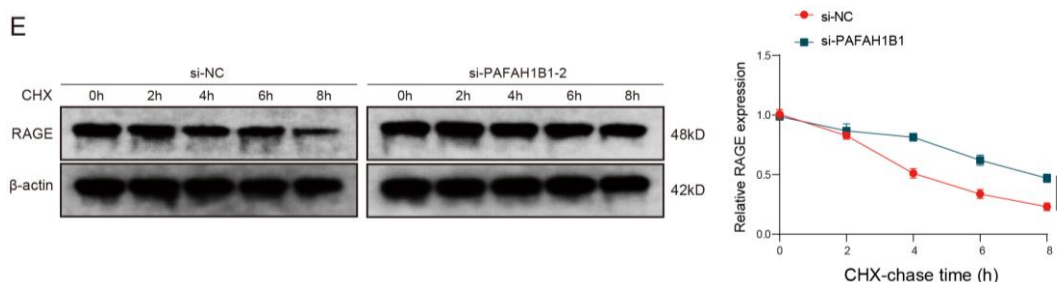


Figure 7. E: Protein half-life analysis of RAGE when PAFAH1B1 is inhibited.

In response to your comments on Figure 7E, we have carefully reviewed the factors involved in the experimental design, which include two key elements: the interference conditions (si-NC, si-PAFAH1B1) and the time course of CHX treatment (0h, 2h, 4h, 6h, 8h). Following your advice, we have conducted a re-analysis of the data in Figure 7E using Two-way ANOVA. The specific details are as follows:

“For Comparisons between the control group and the model group in vitro were conducted using an independent samples t-test. For comparisons among three groups, one-way ANOVA and two-way ANOVA were performed, followed by pairwise comparisons using the LSD method.” Page 17 in the manuscript.

The new analysis results provide a more precise dissection of the interactive effects of different interference conditions and time factors on RAGE protein expression, making the statistical interpretation more aligned with the logical design of the experiment and more scientifically accurate. We have replaced the original one-way ANOVA results with the re-analyzed statistical data and corresponding explanations. This improvement enhances the rationality of the data statistics and increases the reliability of the conclusions drawn in our manuscript.

Question 4. Discussion: The manuscript suggests that irisin suppresses the increase of pro-inflammatory cytokines and prevents the decrease of anti-inflammatory cytokines. Could the authors clarify which of these effects is considered the primary mechanism of irisin’s anti-inflammatory action? Alternatively, is the dual regulation of both cytokine types essential to its function? Please discuss this non-selective activity in greater detail based on your data and supporting literature.

Reply: We are grateful for your attention to our research and for raising such valuable questions. Your inquiries regarding the primary mechanism of Irisin’s anti-inflammatory action and the importance of its dual regulation of pro-inflammatory and anti-inflammatory cytokines are of great significance for us to further understand the mechanisms underlying Irisin's effects. Here are our detailed responses to these issues:

1. Primary Mechanism of Irisin's Anti-inflammatory Action: In our study, Irisin significantly impacted both pro-inflammatory and anti-inflammatory cytokines in the Parkinson's disease (PD) model. Specifically, Irisin inhibited the increase of pro-inflammatory cytokines such as TNF- α and IL-1 β (Figure 4A, Figure 5C, Figure 8H, Figure 9J, Figure S3E, and Figure S7F), while preventing the decrease of anti-inflammatory cytokines such as IL-4 and IL-10 (Figure 4B, Figure 5D, Figure 8H, Figure 9J, Figure S3E, and Figure S7G). Both of these effects play crucial roles in Irisin's anti-inflammatory action, making it difficult to regard either as the primary mechanism. Mechanistically, Irisin activates PAFAH1B1-RAGE ubiquitination and TFEB-dependent autophagy, thereby modulating intracellular inflammatory signaling pathways. This process not only suppresses the production of pro-inflammatory cytokines but also promotes the secretion of anti-inflammatory cytokines. For instance, TFEB-dependent autophagy can clear damaged mitochondria and abnormal protein aggregates within cells, thereby reducing factors that trigger inflammatory responses. Additionally, the autophagy process can regulate the intracellular metabolic state, facilitating the synthesis and secretion of anti-inflammatory cytokines.

2. Importance of Dual Regulation of Pro-Inflammatory and Anti-Inflammatory Cytokines: The dual regulation of pro-inflammatory and anti-inflammatory cytokines by Irisin is essential for its anti-inflammatory function. This dual regulation mechanism enables Irisin to maintain immune balance in complex inflammatory environments. In neurodegenerative diseases such as Parkinson's disease, the overactivation and persistence of inflammatory responses are key factors leading to neuronal damage and disease progression. By inhibiting the excessive production of pro-inflammatory cytokines, Irisin can effectively reduce the intensity of inflammatory responses. Meanwhile, by promoting the secretion of anti-inflammatory cytokines, Irisin can enhance the body's anti-inflammatory capacity, facilitating the resolution of inflammation and tissue repair. In our experiments, we observed that after Irisin treatment, the levels of pro-inflammatory cytokines TNF- α and IL-1 β in the brain tissue of PD model mice were significantly reduced, while the levels of anti-inflammatory cytokines IL-4 and IL-10 were significantly increased. This dual regulation not only alleviated the inflammatory response but also promoted neuronal survival and functional recovery. Therefore, we speculate that Irisin's anti-inflammatory action relies on its dual regulation of pro-inflammatory and anti-inflammatory cytokines. This regulatory mechanism helps maintain the dynamic balance of inflammatory responses, thereby exerting its anti-inflammatory and neuroprotective effects.

3. Support from Related Literature: There are also studies in the related literature that support the importance of this dual regulatory mechanism. For example, in the

regulation of microglial polarization, Irisin can inhibit the polarization of microglia towards the pro-inflammatory M1 phenotype while promoting their polarization towards the anti-inflammatory M2 phenotype, thereby achieving fine-tuned control of inflammatory responses [1]. In an acute lung injury model, Irisin can reduce the levels of pro-inflammatory cytokines such as TNF- α and IL-6, while increasing the expression of the anti-inflammatory cytokine IL-10, thereby alleviating inflammatory damage to lung tissue [2]. In a cerebral ischemia-reperfusion injury model, Irisin reduced infarct size and neuronal damage in brain tissue and improved neurological deficits by dually regulating cytokines [1].

4. Detailed Analysis and Updated References: We have added a detailed analysis of these contents in the discussion section and supplemented the latest relevant references, hoping that these additions will better elucidate the mechanisms of action of Irisin. The specific details are as follows:

“In addition, the results of this study indicate that irisin exerts its anti-inflammatory effects through the synergistic regulation of the pro-inflammatory and anti-inflammatory cytokine network. The experimental data show that irisin significantly inhibits the expression of pro-inflammatory cytokines such as TNF- α , IL-1 α , and IL-1 β , while maintaining the levels of anti-inflammatory cytokines such as IL-10 and TGF- β . This dual regulatory mechanism is highly consistent with the findings in various disease models. In neuroinflammatory models, irisin regulates the polarization balance of microglia, suppressing the pro-inflammatory M1 phenotype and promoting the transformation of the anti-inflammatory M2 phenotype ³⁰. In acute lung injury, irisin downregulates the expression of TNF- α and IL-6 while upregulating the expression of IL-10 ³¹. This “biphasic regulation” enables irisin to control the inflammatory response process more comprehensively: on the one hand, it reduces tissue damage by inhibiting pro-inflammatory cytokines, and on the other hand, it promotes tissue repair by maintaining anti-inflammatory cytokines. This synergistic mode of action may be the key mechanism underlying the effective therapeutic outcomes of irisin in different inflammatory diseases, providing an important theoretical basis for the development of novel anti-inflammatory drugs.” Page 33 in the manuscript.

In summary, we believe that Irisin’s anti-inflammatory action relies on its dual regulation of pro-inflammatory and anti-inflammatory cytokines. This dual regulatory mechanism not only effectively alleviates inflammatory responses but also maintains immune balance, promotes tissue repair, and provides neuroprotection. In future experiments, we will further investigate the regulatory mechanisms of Irisin on inflammatory signaling pathways and the role of this dual regulatory mechanism in different inflammatory diseases.

References:

- [1] Jia Z, Yue W, Zhang X, Xue B, He J. Erianin alleviates cerebral ischemia-reperfusion injury by inhibiting microglial cell polarization and inflammation via the PI3K/AKT and NF- κ B pathways. *Int Immunopharmacol.* 2024 Nov 15;141:112915. doi: 10.1016/j.intimp.2024.112915.
- [2] Han Z, Ma J, Han Y, Yuan G, Jiao R, Meng A. Irisin attenuates acute lung injury by suppressing the pyroptosis of alveolar macrophages. *Int J Mol Med.* 2023 Apr;51(4):32. doi: 10.3892/ijmm.2023.5235.

Question 5. Discussion: Additionally, I encourage the authors to elaborate on the potential effects of irisin on microglial polarization, if possible. Any insights into whether irisin promotes M2 (anti-inflammatory) phenotypes or suppresses M1 (pro-inflammatory) activation would strengthen the interpretation. Furthermore, please consider discussing the possible influence of irisin on other glial cell types, such as astrocytes, within the context of neuroinflammation or neuroprotection.

Reply: We are grateful for your valuable comments, and we have provided a further detailed discussion on the effects of irisin on microglial polarization and related glial cells.

1. Effects of Irisin on Microglial Polarization: Irisin promotes the transition of microglia from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype. Studies have found that pretreatment with irisin significantly reduces the number of LPS-induced M1 microglia while increasing the number of M2 microglia [1]. The mechanism mainly involves the activation of the STAT6 signaling pathway to inhibit nuclear translocation of NF- κ B1 (p50/p65), reducing the activation of M1 microglia, and thereby promoting the expression of anti-inflammatory factors [1]. Additionally, irisin can also promote the polarization of microglia to the M2 phenotype through the Sirt3-mediated autophagy pathway [2]. In research on cerebral ischemia/reperfusion injury (CI/RI), Jia et al. confirmed through in vitro and in vivo experiments that irisin treatment significantly alleviates neurological deficits, reduces infarct size, and mitigates neuronal damage [3]. Mechanistic studies found that irisin inhibits the polarization of microglia to the pro-inflammatory M1 phenotype, such as by decreasing the expression of M1-specific markers like inducible nitric oxide synthase (iNOS), while enhancing the production of anti-inflammatory factors Arg1, CD206, IL-4, and IL-10, promoting the transition of microglia to the anti-inflammatory M2 phenotype [3].

2. Effects of Irisin on Other Glial Cells: In addition to its effects on microglia, irisin also has certain impacts on astrocytes. Studies have found that pretreatment of astrocyte

cultures with irisin effectively protects astrocytes from A β -induced cytotoxic damage, reduces the levels of IL-6 and IL-1 β released by astrocytes, and decreases the activation of NF- κ B by inhibiting the phosphorylation of IKK α , thereby downregulating the expression of cyclooxygenase-2 (COX-2) [4]. This indicates that irisin has inhibitory effects on the activation of astrocytes and neuroinflammation, thereby exerting neuroprotective effects. Furthermore, Kim et al. found that irisin can significantly reduce A β pathology by increasing the release of A β -degrading enzyme neprilysin (NEP) from astrocytes. This process is mediated by the downregulation of the ERK-STAT3 signaling pathway, which is of great significance in the prevention and treatment of Alzheimer's disease [5].

In summary, irisin plays an important role in regulating microglial polarization and affecting other glial cells, exerting anti-inflammatory and neuroprotective effects through various signaling pathways. Currently, our research focuses mainly on the effects of irisin on microglial polarization, but in future studies, we hope to validate this mechanism in other types of glial cells (such as astrocytes) and explore its potential clinical application value in depth.

3. We have added a detailed analysis of this content in the discussion section and supplemented it with the latest relevant references. We hope that these additions will better clarify the possible effects of irisin on microglial polarization and other glial cell types (such as astrocytes) in the context of neuroinflammation or neuroprotection. The specific details are as follows:

“We focus on the potential impact of irisin on microglial polarization and neuroglial cells. Current studies have demonstrated that irisin can promote the transformation of microglia from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype. The mechanism involves the activation of the STAT6 signaling pathway to inhibit the nuclear translocation of NF- κ B1 and the autophagy pathway mediated by Sirt3^{32,33}. Irisin also has significant neuroprotective effects in cerebral ischemia/reperfusion injury³¹. Moreover, irisin has an impact on astrocytes. It can protect them from A β -induced damage, inhibit the release of inflammatory factors, downregulate the expression of COX-2, and reduce A β pathology by increasing the release of NEP^{34,35}. These findings suggest that irisin has great potential in modulating the function of neuroglial cells, suppressing neuroinflammation, and exerting neuroprotective effects. In future research, we aspire to validate this mechanism in other types of glial cells, such as astrocytes, and to delve into its potential clinical application value.” Pages 33-34 in the manuscript.

References:

[1] Wang J, Gao S, Fu S, Li Y, Su L, Li X, Wu G, Jiang J, Zhao Z, Yang C, Wang X, Cui K, Sun X,

Qi X, Wang C, Sun H, Shao S, Tian Y, Gong T, Luo J, Zheng J, Cui S, Liao F, Liu F, Wang D, Wong CCL, Yi M, Wan Y. Irisin reprograms microglia through activation of STAT6 and prevents cognitive dysfunction after surgery in mice. *Brain Behav Immun*. 2025 Mar;125:68-91. doi: 10.1016/j.bbi.2024.12.019.

[2] Li C, Cong Y, Song W, Wu Y, Gou X, Qi S. Irisin Regulates Microglia M1/M2 Polarization and Promotes Autophagy Through the Sirt3 Pathway to Alleviate POCD. *J Neuroimmune Pharmacol*. 2025 Apr 11;20(1):34. doi: 10.1007/s11481-025-10201-w.

[3] Jia Z, Yue W, Zhang X, Xue B, He J. Erianin alleviates cerebral ischemia-reperfusion injury by inhibiting microglial cell polarization and inflammation via the PI3K/AKT and NF- κ B pathways. *Int Immunopharmacol*. 2024 Nov 15;141:112915. doi: 10.1016/j.intimp.2024.112915.

[4] Wang K, Li H, Wang H, Wang JH, Song F, Sun Y. Irisin Exerts Neuroprotective Effects on Cultured Neurons by Regulating Astrocytes. *Mediators Inflamm*. 2018 Sep 26;2018:9070341. doi: 10.1155/2018/90703417474.

[5] Kim E, Kim H, Jedrychowski MP, Bakiasi G, Park J, Kruskop J, Choi Y, Kwak SS, Quinti L, Kim DY, Wrann CD, Spiegelman BM, Tanzi RE, Choi SH. Irisin reduces amyloid- β by inducing the release of neprilysin from astrocytes following downregulation of ERK-STAT3 signaling. *Neuron*. 2023 Nov 15;111(22):3619-3633.e8. doi: 10.1016/j.neuron.2023.08.012.

Question 6. Abbreviations: Abbreviations used should be defined once the first time they appear in the text. Once a word is abbreviated, the abbreviation should be used consistently throughout the manuscript.

Ex Line57 Alzheimer's Disease $\Rightarrow$ Alzheimer's Disease (AD)

Line75 DA $\Rightarrow$ Dopamine?

Line94 TNF- α 、IL-1 β 、IL-6 $\Rightarrow$ please spell out.

Reply: Thank you for your feedback. We have made the necessary revisions to the manuscript following your suggestions. We have defined the abbreviations upon their first appearance in the text and have consistently used these abbreviations throughout the manuscript. The details are as follows:

"Currently, PD ranks as the second most prevalent neurodegenerative disorder, following Alzheimer's disease (AD)." pages 3-4 in the manuscript.

"...may ultimately lead to the death of dopamine neurons (DA)." page 4 in the manuscript.

"...by downregulating inflammatory cytokines tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) in the hippocampus..." page 5 in the manuscript.

"...the serum levels of TNF- α , interleukin-1 α (IL-1 α), and IL-1 β in PD mice...interleukin-4 (IL-4), IL-6, and interleukin-10 (IL-10)..." page 21 in the

manuscript.

Additionally, we have meticulously reviewed the entire manuscript to ensure that all abbreviations have been correctly defined and properly utilized.

Reviewer #3 (Remarks to the Author):

The revised manuscript presents a comprehensive and well-structured study on the therapeutic potential of Irisin in Parkinson's disease, supported by consistent in vivo, in vitro, and clinical evidence. The authors have addressed the previous concerns with diligence and have significantly improved the clarity and scientific rigor of the manuscript.

However, the Discussion section remains somewhat lengthy and occasionally repetitive. The key conclusions regarding Irisin's neuroprotective and anti-inflammatory effects—particularly its ability to cross the blood-brain barrier, regulate microglial polarization, and modulate cytokine balance—are reiterated multiple times throughout the text. In addition, the mechanistic discussion of the PAFAH1B1–RAGE–TFEB–NLRP3 axis, while valuable, is at times over-explained across several paragraphs. Streamlining the narrative and removing redundancy would enhance the clarity and impact of the discussion.

Furthermore, the section on gender differences and hormonal regulation, although interesting, could be more concisely integrated as a future direction rather than occupying a substantial portion of the main discussion.

In conclusion, I believe the study is scientifically sound and presents valuable findings. However, I recommend minor revision prior to acceptance to improve the organization and conciseness of the Discussion section.

Re: Dear Reviewer, First and all, thank you sincerely for your positive feedback on our research. Based on the responses we provided to previous reviewers' comments, we have indeed expanded the discussion section. However, we sincerely apologize for any redundancy and repetition that may have occurred as a result. Thank you very much for bringing this matter to our attention. In response to your suggestion, we have removed a significant portion of the content related to gender discussions and

condensed the parts concerning the immune microenvironment and the PAFAH1B-RAGE-TFEB-NLRP3 axis. Additionally, we have carefully reviewed and adjusted the manuscript format in accordance with the editor's guidelines. We hope that this revised version meets your expectations. Once again, we deeply appreciate your time and effort in reviewing our work.

Thank you for the opportunity to peer review. The authors elucidated the protective effects of Irisin against inflammatory in PD. This discovery could lead to the potential development of therapeutic drugs and functional supplements. Thus, this research is very important for the creation of a sustainable society in the future. Although the study is great, I have provided a few comments and suggestion that make the manuscript better. We appreciate the efforts of the authors in conducting this important study and look forward to seeing the final version of the manuscript.

Fig. 1: How did you detect the protein in human serum, ELIZA? Fig. 1C unit (pg/ml*1000) meant fg/mL?

Fig. 2I: It is interesting that the concentration of Irisin was different in each brain region. Is Irisin produced in the brain? If so, what are the producing cells?

Fig. 3A,3B The quantitative data should be submitted because only typical images did not express the decrease in HE and Nissl stained neuronal cells.

Fig. 5G: Since LPS damage is cell density dependent, areas of equal cell density should be taken. It would be helpful to indicate what GFP and RFP represent. Also, since the LC3 dot-like signal was not seen, the cell processing method should be reviewed and images taken at higher magnification should be added.

supplementary Fig. 3B: Why was only LC3 increased in Sup 3B bufA+LPS+Irisin?

Fig. 7F-H: β -actin misspelled.

Line 578-580: Is Irisin actin? Misspelling?