

Manuscript NO.: 54803:

Round-1

Title: Acupuncture accelerates neural regeneration and synaptophysin production after neural stem cells transplantation

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We would like to thank all the reviewers for their explicit review and constructive comments. We have made all the changes as suggested, and incorporated them in the revised manuscript. Listed below are our point-by-point responses to their comments.

Response to Comments of Reviewer #1:

Critique #1: Comments on the manuscript: “Acupuncture accelerates neural regeneration and synaptophysin production after neural stem cells transplantation” Numerous people worldwide are living with dementia, and the number will increase in the future. The purpose of this study was to observe the effects of acupuncture on nerve regeneration and synapse production after the regulation of the microenvironment of neural cells injected into mouse embryos. To do this, the authors studied the effect of acupuncture in mice accelerated by senescence that received exogenous neural stem cells. This consistent study brings interesting results, and it will certainly be useful in developing treatments for the prevention of demence. However, This manuscript needs several improvements before to consider it for publication. I have several remarks. Title : specify that the experiment was carried out on mice.

Response: Thank you for your advice. We have rewritten the Title as “Acupuncture accelerates neural regeneration and synaptophysin production after neural stem cells transplantation in mice”.

Critique #2: Introduction : The use of mice for current experimentation is not indicated at the end of the introduction: add a sentence indicating this use (although it is written that previous work used experimental mice).

Response: We have added the use of mice for current experimentation at the end of Introduction according to your suggestion.

Critique #3: *Material and methods : In general, observational methods are very little described, with basic details missing. Check this part carefully and complete.*

Response: We have checked the observational methods and rewritten the part of Material and methods.

Critique #4: *NSCs transplantation : specify what samR1 mice are (accelerated Senescence R1 pregnant mice, I guess, as it is written in the methods given in the abstract, or in the discussion). What were the characteristics of centrifugation (number of g? duration?)*

Response: We have redescrbed SAM, SAMP8, and SAMR1 in detail of NSCs transplantation in Material and methods. And we also added the characteristics of centrifugation.

Critique #5: *The authors have written: “The positive rate of Nestin staining, the proliferation of NSCs, and the differentiation to neurons and neurogliocytes were observed (immunocytochemical positive staining of anti-nuclear antibody, NeuN and glial fibrillary acidic protein, and GFAP, respectively)” Give some details on the antibodies used (supplier, characteristics...) to detect NeuN and GFAP. What was the type of reactions used: direct or indirect immunocytochemistry, use of fluorescent dye or enzyme coloration? Which one? Have you used amplification methods? If so, what methods have you done? How were the negative controls confirmed?*

Response: We are sorry we did not identify this part clearly. We have now supplemented the details such as antibodies and staining methods in the part of NSCs transplantation in Material and methods.

Critique #6: *I have the same questions for the immunocytochemical detection of BrdU: what is the anti-BrdU used? What is the chromophore used (giving a blue coloration). Have you used an indirect method with or without amplification? What were the negative controls? These specifications must be written in the paragraph "hematoxyline-eosine (HE) and*

immunohistochemical coloration." Also give some details on the coloration of hematoxylin-eosin:

What was the inclusion medium (paraffin, plastic wax ...?), the thickness of the section?

Response: We have redescribed the relevant experimental reagents, detection methods and staining methods of Hematoxylin-eosin (HE) and immunofluorescence staining in Material and methods. In response to another reviewer's suggestion that the magnification of HE staining and immunohistochemical images maybe not enough to illustrate the legends, we have supplemented HE staining figures of mouse hippocampal tissue, and carried out BrdU immunofluorescence detection at the same time. Now we have replaced the previous immunohistochemistry picture with immunofluorescence pictures, in order to better illustrate the results of our study. Thanks again for your valuable comments.

Critique #7: Figures Figure 3 : in this figure, BrdU was detected with an immunocytochemical reaction. It would be better to write "Positively labeled cells with an anti-BrdU were observed and counted" instead "The brdU positive cells were observed and counted". Specify in the legend the chromophore used to detect the anti BrdU by giving a blue coloration. It would be useful to show negative control in an insert.

Response: We have revised the content in Figure3 llegend, redescribed in detail the staining and anti-BrdU application in the immunofluorescence experiments, and rewritten the contents in Material and methods accordingly.

Response to Comments of Reviewer #2:

Critique #1: Relevance, significance and potential impact of the manuscript is moderate. The topic is appropriate and of general interest. The manuscript is novel and interesting and adds to the knowledge base. The presentation should be improved. Grammar and style should have a revision. Insert line numbers. Without page and line numbers, it is difficult to give specific comments to the spotted weaknesses in the text. Citation need to be inserted into several parts of the text. Avoid repetition.

Response: Thank you for your advice. We have carefully checked the English, and corrected some grammar mistakes. We have inserted line numbers and checked the citation as you suggested.

Critique #2: Add information about software used for Morris water maze recording.

Response: We have added the Morris software version information in the section of Morris water maze in Material and methods.

Critique #3: Add information regarding HE and immunocytochemical staining - cross-sectional thickness, cutter, concentrations, dilutions and producers of the used chemical.

Response: We have redescrbed the relevant experimental reagents, detection methods and staining methods of Hematoxylin-eosin (HE) and immunofluorescence staining in Material and methods. In response to another reviewer's suggestion that the magnification of HE staining and immunohistochemical images maybe not enough to illustrate the legends, we have supplemented HE staining figures of mouse hippocampal tissue, and carried out BrdU immunofluorescence detection at the same time. Now we have replaced the previous immunohistochemistry picture with immunofluorescence pictures, in order to better illustrate the results of our study. Thanks again for your valuable comments.

Critique #4: Add conclusion.

Response: We have added Conclusion.

Response to Comments of Reviewer #3:

Critique #1: This paper aimed to explore the anti-dementia mechanism of acupuncture by regulating the microenvironment of exogenous neural stem cells (NSC) isolated from senescence-accelerated R1 pregnant mice (SAM), labeled with BrdU, and injected into the hippocampus of SAMP8. Synaptophysin plays a key role in synaptic development and synaptic

plasticity of neurons and is closely related to the cognitive process of Alzheimer's disease (AD) patients. Sanjiao acupuncture was administered. Morris water maze showed significant cognitive impairment of learning and memory in 8-month SAMP8, which improved in all the NSC transplantation groups. The behavioral change in the PTA group (acupoint, NSCs transplantation) was stronger than the other two groups ($P < 0.05$). Flow cytometry showed that after co-culture of NSCs with hippocampal slices in vitro, the synaptophysin expression of PC decreased in comparison to the RC SAMR1 group, that in PT, PTA, and PTN groups increased as compared to the PC group, and that in the PTA group increased significantly as compared to the PTN group with acupoint-related specificity ($P < 0.05$). The authors conclude that Sanjiao acupuncture may promote nerve regeneration and synaptogenesis in SAMP8 mice by regulating the microenvironment of NSCs transplantation to improve the nerve activity and promote the recovery of AD-damaged cells. The title reflects the main subject of the manuscript, the abstract and key words reflect the main topics of the entire text. The results are discussed in detail using 54 references for the previous 5 years. There are comments on the article. Based on histopathological data, the authors state that the hippocampal structure was clear, the cell arrangement was dense and orderly, and the necrosis of cells in CA1 and CA3 areas was significantly reduced in the PTA group when compared with the PC SAMP8 group. The BrdU-positive proliferating cells were found in NSC hippocampal transplantation groups, and the number increased significantly in the PTA group than that in the PT NSCs transplantation and PTN non-acupoint and NSCs transplantation groups ($P < 0.05$). However, low quality photomicrographs are at too low magnification to illustrate what their legends say and not even allow the identification of individual cells. The article does not indicate what type of microscope was used. I suggest replacing the photomicrographs with those of good quality that confirm the authors' conclusions. After corrections, the article can be accepted for publication.

Response: Thank you for your advice. We have redescribed the relevant experimental reagents, detection methods and staining methods of Hematoxylin-eosin (HE) and immunofluorescence staining in Material and methods. We have supplemented HE staining figures of mouse hippocampal tissue, and carried out BrdU immunofluorescence detection at the same time. Now we have replaced the previous immunohistochemistry picture with immunofluorescence pictures, in order to better illustrate the results of our study. Now the Figures have a higher magnification

and we can clearly observe the pathological changes of HE staining and NSCs proliferation labeled by BrdU of immunofluorescence staining. We have also added the experimental details such as microscopes and antibody application in Material and methods. We hope it will be suitable for publication. Thanks again for your valuable comments.

Response to Comments of Reviewer #4:

Critique #1: In this manuscript the authors establish that the positive effects of hippocampal NSC transplantation followed by accupuncture enhance its effects on neuroregeneration in mice. The methods and controls are appropriate. The study is of interest and I support its publication.

Response: Thank you for your affirmation of our work.

Round-2

Thanks for the reviewers' advice. We have revised our manuscript according to the second-round review report. Response to Comments of Reviewer #1: Critique #1: Comments on the revised manuscript : " Acupuncture accelerates neural regeneration and synaptophysin production after neural stem cells transplantation in mice" This manuscript about an interesting and useful study has been improved. I have just a minor remark : page 7, lines 5 -6 : give the speed of centrifugation in number of g, which is more universal than the rpm, which is related to the model of the centrifuge used. This article can be published after this minor correction. Response: Thank you for your advice. We have corrected the speed of centrifugation in number of g in page 7, lines 5 -6. Response to Comments of Reviewer #2: Critique #1: My only comment on this article was that the photomicrographs were not illustrative enough. Now this flaw has been corrected, and I recommend the article for publication. Response: Thank you for your affirmation of our work.