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OPEN PEER REVIEW

The Effects of Subchronic Methamphetamine Administration on the NLRP3 Inflammasome, Memory Function, and Hippocampal Morphology

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1 Original submission

3 comments · 10 Mar 2024 – 3 May 2024

Sayed Mehrdad Azimi Reviewer

MAJOR REVISION 10 Mar 2024

hello dear author.

I have carefully reviewed your manuscript and would like to provide you with some feedback and suggestions for further improvement.

best regards

Associate Editor

MAJOR REVISION 3 May 2024

Dear EIC,
Thanks to the authors and appreciation to the researchers. The manuscript is a valuable piece of research. But some revisions are needed. The authors should revise it based on the comments. Also, minor changes should be made to the abstract:
1- The study design should be mentioned in the first line of the methods section of the ABSTRACT.
2- A summary of the statistical analysis results should be mentioned in the results section of the ABSTRACT.

Editor-in-Chief

MAJOR REVISION 3 May 2024

Dear Author,
Thank you for submitting the article. It needs some revision. Please revise it based on the reviewers' and AE's comments.

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Revision 1

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Dear Editorial Board of Archives of Neuroscience,

We sincerely appreciate the time and effort you have dedicated to reviewing our manuscript. Your insightful comments and suggestions have been valuable in improving the quality and clarity of our work. We have carefully considered each of your points and have made the necessary revisions to address your concerns. Below, we provide detailed responses to each of your comments and outline the specific changes made in the manuscript.

Comments & Responses

1. The method states that 5 mg of methamphetamine was administered to groups B and 0.2 ml of normal saline to groups A. Given the repeated injections and evaluation of inflammation due to subchronic administration, the difference in the injected dose could induce variation in the duration of patient distress and injection-induced stress. How is this issue justified?

#1. Thank you for your insightful question. Your point about the potential impact of injection-induced stress and inflammation due to the varying doses is crucial. While we tried to design the study such that the corresponding control group (normal saline) effectively controls for the injection procedure, we acknowledge that differences in the nature of the administered substances (methamphetamine versus normal saline) and the cumulative dosage could indeed lead to fluctuations in stress and inflammation levels. It's important to note, however, that we maintained consistent frequency and volume of injections across all groups to minimize variability arising from the injection process itself. In response to this concern, we have incorporated a new section into the discussion, located in the first paragraph, which underscores the necessity for caution when interpreting the results.

2. How were pyroptosis and apoptosis differentiated in this study? The results section mentions "that might be resulted in pyramidal cell death in the hippocampus". Can speculative statements without logical justification be considered valid?

#2. Thank you for your insightful comments and for raising this important point regarding the differentiation of pyroptosis and apoptosis in our study. We appreciate the opportunity to address this matter and provide clarification and changes to the text of the manuscript.

You are correct in noting that our study did not directly differentiate between pyroptosis and apoptosis as the mediatory mechanisms in the observed hippocampal cell death, and we acknowledge the necessity for direct assessments to determine the specific roles of apoptosis and pyroptosis in hippocampal cell death. However, based on the currently existing literature, we would like to highlight that alterations in the expression of NLRP components “primarily” suggest a shift towards pyroptosis rather than apoptosis. The NLRP inflammasome primarily mediates pyroptosis and the release of pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β) and interleukin-18 (IL-18). This process is initiated through the cleavage and activation of caspase-1, which in turn cleaves gasdermin D, leading to the formation of pores in the cell membrane. Additionally, a recent study indicated that MA treatment induced the upregulation of cleaved GSDME-NT. While apoptosis is another form of

programmed cell death and is implicated in MA-induced toxicity and some NLRP inflammasome components, such as caspase-1, have been implicated in apoptotic pathways, it is typically induced by relatively different signaling pathways and in a and exhibits distinct morphological and biochemical features. Collectively due to the abundance of pyroptosis mechanisms in MA-induced toxicity (particularly regarding the most recent lines of evidence), our study focuses on the primary role of the NLRP inflammasome in promoting pyroptosis and inflammation.

Therefore, we deleted the specific sentence about the superiority of the pyroptosis pathway upon the apoptosis in the abstract and title of the work, as we have not measured it directly and decreased the emphasis of this note throughout the text of the manuscript**.**

Also, here are some relevant references:

1. Evavold CL, Ruan J, Tan Y, Xia S, Wu H, Kagan JC. The Pore-Forming Protein Gasdermin D Regulates Interleukin-1 Secretion from Living Macrophages. Immunity. 2018;48(1):35-44. e6. doi: 10.1016/j.immuni.2017.12.009

2. Man SM, Karki R, Kanneganti TD. Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. Immunol Rev. 2017;277(1):61-75. doi:10.1111/imr.12534

3. Shi J, Gao W, Shao F. Pyroptosis: Gasdermin-Mediated Programmed Necrotic Cell Death. Trends Biochem Sci. 2017;42(4):245-254. doi: 10.1016/j.tibs.2016.10.004

3. What was the rationale behind using a 5 mg/kg dose? It seems more appropriate to determine this dose based on an MTT assay using hippocampal neural cell lines and a series of serial doses. If a reference exists, please include the reason for selecting this dose.

#3. Thank you for your question. The selection of the 5 mg/kg dose of methamphetamine was based on previous studies demonstrating its efficacy in inducing neuroinflammation and cognitive effects in rodent models and we did not conduct cellular assessments for dose determination. Recent research has highlighted the dose-dependent effects of methamphetamine on neuronal morphology and function. Specifically, studies by Tu et al. (2019) and Liang et al. (2022) showed that MA (2 mg/kg) enhances synaptic density in the nucleus accumbens and hippocampus, respectively, while higher doses (30 mg/kg) induce striatal damage. We selected the 5 mg/kg dose to align with these findings and maintain consistency with existing literature. Our choice was entirely based on the findings of previous works. We have included a note regarding the rationale behind the selected dose in the final part of the introduction in the manuscript text and highlighted it. We appreciate your suggestion regarding dose optimization using cellular assays and will consider it for future studies.

4. What is the meaning of NIDA in the BACKGROUND section?

#4. Thank you for your question. NIDA stands for the National Institute on Drug Abuse, a federal research institute in the United States dedicated to supporting and conducting research on drug use and addiction, including its causes, prevention, and treatment. We included information from this institute in our manuscript; however, based on your feedback, we recognized it was not appropriately placed and have removed it.

5. The first paragraph of BACKGROUND states "Besides, there have been less noticed pathways, other than the merely direct transmitter-mediated ones, that strongly promote the MA effects", but the third paragraph refers to molecular pathways. How is this reconciled?

#5. Thank you for pointing out this issue. If we have understood your concern correctly, in the first paragraph, my intention was to highlight that there are additional pathways influencing methamphetamine (MA) effects beyond the commonly known neurotransmitter-based pathways. In all sections, we meant molecular pathways, but we wanted to emphasize the role of other molecular components, such as neuropeptides and neuroinflammatory pathways, which are supported by evidence in the literature. These pathways are discussed in our study and should be considered alongside neurotransmitter systems to fully explain the effects of methamphetamine.

6. It seems inappropriate to use the term "the therapeutic dosage" for the substance of interest in the second paragraph of BACKGROUND.

#6. Thank you. We accordingly corrected that and highlighted it.

7. Please avoid repeating the word "Methamphetamine" in the text. Use abbreviations such as MA or alternatives.

#7. Thank you. We accordingly corrected the words and highlighted.

8. What is the novelty of this study?

#8. Thank you for your inquiry regarding the novelty of our study. We appreciate the opportunity to highlight the knowledge gap addressed by our research.

Our investigation builds upon previous studies exploring the mechanisms underlying MA neurotoxicity, particularly focusing on the involvement of the NLRP3 inflammasome pathway. To the best of our knowledge, while existing research has provided valuable insights into the molecular mechanisms of NLRP3 activation in response to MA exposure, we identified a crucial gap in the literature related to the behavioral and cognitive consequences of NLRP3 modulation in this context. For instance, some studies have demonstrated MA-induced alterations in NLRP1 and NLRP3 activity, along with associated pyroptotic or apoptotic cell death pathways. However, these studies primarily focused on molecular signaling cascades. Furthermore, the applied MA doses varied widely, with some studies utilizing doses considered high or low, and for durations that may not fully capture long-term effects. Moreover, some studies with similar setting and scope have assessed the MA-induced toxicity in specific pathological contexts such as HIV or SARS. Hippocampal tissue was not the target of some of these studies a well that impede the accurate interpretation of cognitive functions, specifically memory. Our study aimed to bridge this gap by investigating the behavioral and cognitive outcomes of NLRP3 modulation in subchronic MA exposure. By utilizing a more multiscale approach, we aim to provide an understanding of the neuroinflammatory processes and cognitive impairments associated with MA abuse. In summary, our study introduces a novel perspective by integrating molecular insights with behavioral assessments with focus on the hippocampal parts to elucidate the impact of NLRP3 modulation on cognitive outcomes in subchronic MA exposure.

The most relevant references are provided here:

1. Du L, Shen K, Bai Y, Chao J, Hu G, Zhang Y, Yao H. Involvement of NLRP3 inflammasome in methamphetamine-induced microglial activation through miR-143/PUMA axis. Toxicol Lett. 2019 Feb; 301:53-63. doi: 10.1016/j.toxlet.2018.10.020. Epub 2018 Oct 28. Erratum in: Toxicol Lett. 2020 Oct 15;333:324-325. PMID: 30394308.

2. Fan R, Shen Y, Li X, Luo H, Zhang P, Liu Y, Si Z, Zhou W, Liu Y. The effect of the NLRP1 inflammasome on methamphetamine-induced cognitive impairment in rats. Drug Alcohol Depend. 2022 Aug 1;237:109537. doi: 10.1016/j.drugalcdep.2022.109537. Epub 2022 Jun 12. PMID: 35752024.

3. Ding, Jiuyang, et al. "Inflammasome inhibition prevents motor deficit and cerebellar degeneration induced by chronic methamphetamine administration." Frontiers in Molecular Neuroscience 15 (2022): 861340.

4. Dutta, Debashis, et al. "Methamphetamine enhancement of HIV-1 gp120-mediated NLRP3 inflammasome activation and resultant proinflammatory responses in rat microglial cultures." International Journal of Molecular Sciences 25.7 (2024): 3588.

5. Zheng, Ya, et al. "Methamphetamine augments HIV-1 gp120 inhibition of synaptic transmission and plasticity in rat hippocampal slices: Implications for methamphetamine exacerbation of HIV-associated neurocognitive disorders." Neurobiology of disease 168 (2022): 105712.

6. Qi, Yize, et al. "3'-Deoxyadenosin alleviates methamphetamine-induced aberrant synaptic plasticity and seeking behavior by inhibiting the NLRP3 inflammasome." Neural Regeneration Research 19.10 (2024): 2270-2280.

7. Xu, Enquan, et al. "Inflammasome activation by methamphetamine potentiates lipopolysaccharide stimulation of IL-1β production in microglia." Journal of Neuroimmune Pharmacology 13 (2018): 237-253.

8. Machhar, Janvhi Suresh, et al. "NLRP3 inflammasome activation underlies the neuro-oxidative stress associated with Methamphetamine (METH) and SARS-CoV2 induced co-morbidity in human microglia." NeuroImmune Pharmacology and Therapeutics 2.3 (2023): 245-252.

8. Edinoff, Amber N., et al. "Methamphetamine use: a narrative review of adverse effects and related toxicities." Health Psychology Research 10.3 (2022).

9. Please clarify the time intervals between behavioral tests in animals in the Materials section. At what time after injection were the animals subjected to behavioral testing?

#9. Thank you for bringing this to our attention. We would like to clarify the timing of the behavioral tests in the Materials section. For every behavioral cognitive assessment in each group of the study, we performed the test one day after completing the course of the study. Specifically, this means that we started the daily administration of MA or normal saline for the study groups, and after completing the specified time course of one, two, or three weeks for each group, we conducted the behavioral tests the following day. We will update the Materials section to clearly specify these time intervals to ensure clarity.

10. The Materials section mentions assessing RNA quality using a NanoDrop™ spectrophotometer. It seems appropriate to use gel electrophoresis to determine RNA quality accurately. If this method was used, please provide the corresponding images and include them in the Materials section.

#10. We appreciate your question. In our study, we utilized the NanoDrop™ spectrophotometer to assess RNA quality. While gel electrophoresis is indeed a valuable technique for determining RNA quality, we chose to use the NanoDrop™ spectrophotometer due to its rapidity and efficiency in quantifying nucleic acids, especially when working with small sample volumes. Furthermore, we would like to highlight that the quality of the extracted RNA was confirmed through subsequent RT-PCR analysis. The results of the RT-PCR assays provided us with insights into the integrity and suitability of the RNA samples for downstream applications.

11. Please discuss the software used for gene blasting and provide a complete table with gene information, including annealing temperatures and NCBI codes.

#11. Thank you for your question, we utilized the Primer-BLAST tool on the NCBI website for gene blasting, with an appropriate annealing temperature. Subsequently, we incorporated this information into the table within the manuscript, with gene information, including NCBI codes and annealing temperatures.

12. Was the quality of the genetic material assessed after RT-PCR? The second electrophoresis step should also be included in the Materials and Methods section.

#12. Thank you for your comment and valuable suggestions. Regarding the quality assessment of genetic material post-RT-PCR and the inclusion of a second electrophoresis step in the Materials and Methods section, we acknowledge that these steps were not performed in our study. However, we would like to highlight that the resulting melting curves from our RT-PCR analysis indicate the presence of the intended genes. Additionally, we conducted our processes with the corresponding negative controls, and the associated melting curves further confirmed the specificity and accuracy of our procedure and the yielded genetic material. Unfortunately, due to the limitations on the number of figures imposed by the journal, we were unable to include the melting curves of the RT-PCR process in our submission.

13. Please provide a more detailed description of the RT-PCR process in the Materials and Methods section and specify the type of SYBER used.

#13. Thank you for your inquiry. In our study, we performed RT-PCR using the MasterMix SYBR Green from BioFact brand without ROX. The instrument used for real-time PCR analysis was the Rotor-Gene real-time PCR machine from Qiagen brand. Accordingly, we added this information and elaborations to the methodology part. We hope this provides the additional information you were seeking regarding our RT-PCR methodology.

14. How were the data normalized?

#14. Thank you for your question regarding data normalization. In our study, we normalized the data using the reference gene B2M, as indicated in the primer file information and we tried to follow standard procedures for data normalization, ensuring consistency and reliability in our analyses.

15. Was GAPDH or B2M used as the reference gene?

#15. Thank you for your question. In our study, B2M was used as the reference gene (as provided in the table 1).

16. Four mice were sacrificed for gene expression analysis, while three were used for histology. What was the reason for excluding the remaining mouse?

#16. Thank you for highlighting this point. From each group (control or MA), we separated 7 rats for further genetic and histological assessments. Specifically, 3 rats were used for histological analysis, and the remaining 4 rats were used for gene expression analysis. This allocation was planned to ensure sufficient sample sizes for both types of analyses.

17. The number of study groups is not mentioned in the main section. According to the abstract, are there six groups? If so, considering 126 mice were included in the study, each group would have 21 mice, which is inconsistent with the basis of the manuscript. Please review and correct this.

#17. Thank you for your valuable feedback. We appreciate your careful review of our manuscript. You are correct that the number of study groups was not clearly mentioned in the main section. We designed the study to have three primary groups based on the timing of administration, as stated in the manuscript. Within each of these primary groups, the study animals were further divided into two subgroups: one receiving normal saline and the other receiving MA. Therefore, we indeed had six groups in total, with 21 rats in each group. This division aligns with the sample size of 126 rats mentioned in the manuscript.

To clarify:

Three primary groups based on the timing of administration.

Each primary group was subdivided into two groups (normal saline and MA).

This resulted in six groups (3 primary groups × 2 subgroups) with 21 rats per group, totaling 126 rats.

We will revise the main section of the manuscript to clearly specify the number and structure of the study groups to avoid any confusion.

18. In the first part of the results, on what basis is the Activity Level (AL) reported? The y-axis of the graph should include the unit.

#18. Thank you for your inquiry. We have addressed the concern regarding the reporting of Activity Level (AL) in the first part of the results. The values for AL are presented based on the counting derived from the formula AL = CS + IS + 2, where CS represents the number of times a rat enters a different arm in each of the three consecutive arm entries, and IS refers to the instances of a rat repeatedly entering the same arm previously visited within the three consecutive arm entries. We have taken corrective measures by explicitly mentioning in the caption of the corresponding figure that the values are presented as the number of counting. This ensures clarity regarding the basis on which AL is reported. Additionally, the y-axis of the graph now includes the appropriate unit, which is "Counting".

19. The graphs seem to exhibit a high error rate and significant data scattering. How is this justified?

#19. Thank you for your observation regarding the graphs. Several factors could contribute to this variability:

Biological Variability: The inherent biological differences among individual animals can lead to variability in their responses to treatments. This is especially true in behavioral and genetic studies, where individual variability is often pronounced. Small Sample Sizes: Although our sample sizes were statistically adequate, smaller sample sizes can result in greater variability and higher error rates. Increasing the sample size in future studies might help to reduce this variability. Environmental Factors:

Despite our efforts to maintain a controlled environment, slight differences in handling, housing, and other environmental factors can contribute to data scattering. Measurement Sensitivity: The sensitivity of the instruments and methods used for behavioral and gene expression analyses might also contribute to variability. Even minor deviations in measurement techniques can result in significant differences in data. Timing of Assessments: Variability in the exact timing of assessments relative to administration could introduce differences in the measured outcomes. Although we followed a strict protocol, slight variations in timing are inevitable in experimental settings. Stress Responses: The stress levels of animals, which can vary due to handling or environmental factors, might affect their behavior and physiological responses, leading to data scattering.

We will ensure to include these considerations in the discussion section of the manuscript to provide a more comprehensive understanding of the observed variability.

20. In the caption of Figure 5, please refrain from using the word "TREATED".

#20. Many thanks. We accordingly corrected the terms and highlighted them.

21. Please use a different color pointer to indicate blood vessels and neuronal cytoplasm in the hippocampal images.

#21. Many thanks for your attention. We have corrected this issue through the images.

22. Why are microglia cells in groups P1A, P2A, and P3A not labeled, while they are visible in other groups?

#22. Many thanks for your attention. We will correct this issue through the images.

23. The Materials and Methods section mentions the separation of brain tissue, but complete data and images for the whole brain and hippocampus have not been published.

#23. Many thanks, we incorporated this in the result section within the histological part.

24. The signaling pathway has not been investigated. Only the expression of a few genes has been studied, and the pathway has not been thoroughly examined, which is inconsistent with the novelty of the manuscript.

#24. Thank you for taking the time to provide feedback on this aspect. In response to your comment, we'd like to clarify the intent and novelty of our study. As we outlined in the last paragraph of our introduction, our study aims to traverse the breadth of understanding in the realm of methamphetamine neurotoxicity. Rather than delving deeply into each individual modality, we have endeavored to provide an overall evaluation across various domains, including behavioral, genetic, and histological assessments. Despite prior inconsistent findings on the impact of chronic low-to-moderate doses of MA on neurocognition and neuroinflammation, recent literature emphasizes the significant neurotoxicity of higher addictive doses, prompting a need for detailed investigations into the mechanisms and outcomes associated with MA use. Our approach, focusing on hippocampal inflammation and its functional consequences within a subchronic, low-dose regime, aims to shed light on the intricate interplay between MA-induced neuroinflammation and cognitive and histological outcomes. Through our multimodal evaluation,

including the examination of NLRP3 inflammasome activation and performance in memory tasks, we seek to explore the breadth of factors influencing MA-induced effects on the hippocampus and cognitive function.

We recognize the importance of thoroughly examining signaling pathways, and we agree that further investigation in this area would enrich our understanding.

25. The number of references appears to be insufficient for this manuscript.

#25. Thank you for your attention. We added more relevant references for different parts of the manuscript and highlighted them.

Associate Editor's Comments:

Associate Editor 1:

Dear EIC,

Thanks to the authors and appreciation to the researchers. The manuscript is valuable research. But some revisions are needed. Authors should revise it based on comments. Also, minor changes should be done in abstract:

1- Study design should be mentioned in first line of method section of ABSTRACT
2- Summary of Statistical analysis results should be mention in result section of ABSTRACT.

#1 & 2. Thank you for your insightful feedback. We have implemented the required revisions to the abstract section of the manuscript. Specifically, we have appropriately integrated the study design and employed statistical analyses into the method and results sections. These revisions have been highlighted in turquoise to enhance clarity.

2 Revision 1

2 comments · 26 Jun 2024 – 26 Jun 2024

Associate Editor

MINOR REVISION 26 Jun 2024

Dear EIC,
The great effort in improving the manuscript is highly appreciated. However, there is room for further improvement, which the authors should consider.

Editor-in-Chief

MINOR REVISION 26 Jun 2024

Dear Author,
Your manuscript still needs revision. Please revise it based on the reviewers' and AE's comments.

3

Revision 2

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Response to Reviewers-Second Round

We sincerely appreciate the time and effort that the editor and reviewers have invested in re-evaluating our manuscript. Their insightful comments and constructive feedback have been valuable in enhancing the quality of our work. We have carefully addressed each of their suggestions to further improve our study. Additionally, we have included a graphical abstract to visually illustrate the process. The changes are highlighted in turquoise in the manuscript.

Reviewer 1.

The way of writing the title should be corrected.

Thank you for this note. We accordingly corrected the title to be more neutral. The new title is:

The Effect of Subchronic Methamphetamine Administration on NLRP3 Inflammasome, Memory Functions, and Hippocampal Morphology

2. The working method is incomplete and incomprehensible. The comments in the work method are not corrected correctly.

Thank you for your feedback. We have thoroughly assessed your comments regarding the method part and have implemented more revisions across the method section. Specifically, we have made these updates within the text. It seemed that the main focus of the comments regarding the method part is about timing, grouping, and details of the RT-PCR. We have tried to revise these parts to address these concerns. The modifications include:

1. Revision of the first paragraph to offer a clearer explanation of our methodology, including grouping and timing considerations.

2. Revision of the part subtitled as “Quantitative Real-Time PCR (qRT-PCR)” to better represent the protocol used and also incorporate more details regarding the controlling the processes.

3 Revision 2

2 comments · 17 Jul 2024 – 17 Jul 2024

Associate Editor

ACCEPT 17 Jul 2024

Dear Editor in Chief,
Thanks for assigning me as an associate editor to handle manuscript No. 145644. The authors have successfully addressed the peer-review comments. This version of the manuscript is acceptable for publication in the Archives of Neuroscience journal.
Regards,

Editor-in-Chief

ACCEPT 17 Jul 2024

Dear Author,
Thank you for revising. The manuscript can be considered for publication now.