Author's response to reviews

Title: Hydrogen sulfide and mild hypothermia activate the CREB signaling pathway and alleviate ischemia-reperfusion injury

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Author's response to reviews: see over
Dear editor and reviewers,

Thank you for carefully reviewing and providing feedback on the manuscript. We have made major revisions to our manuscripts. Specifically, we have sought help on translation and carefully re-interpreted our results. Herein, we have addressed your previous concerns. Please find below a point-by-point response to your questions or comments.

#note: Questions or comments are in gray and responses are in black.

Response to the editor's comments

Editor's Comment: Dear Dr. Dai, After reading your MS “Hydrogen sulfide and mild hypothermia can selectively activate synaptic NMDARs and trigger the CREB signaling pathway” as well as the comments by two reviewers, I am unable at this time to make a decision regarding the acceptability of your MS for publication. The reviewers expressed interest in your work and appreciated its findings. Nevertheless, a number of major issues requiring as a minimum a through rewriting of your MS and possibly additional experiments are necessary before any decision on its acceptability can be made. Before you address the issues raised by the reviewers, let me make a couple of summarizing suggestions. Firstly, even though it may appear as an excessive burden on non-native speakers, having your MS edited by someone really comfortable with English will: 1. make it more accessible to the audience, 2. make it easier to understand your findings and their interpretation and 3. reduce the frustration experienced by reviewers and editors with ambiguous or plainly erroneous formulations.

We have asked our bilingual colleague to review the manuscript, and we have revised the manuscript based on his suggestions.

For example the use of “the carotid atherosclerosis was removed” is nonsensical in your context, the sentence in L133/4 “after 6 hrs of reperfusion” probably is supposed to be “6 hrs following reperfusion”, the “immediately decapitated” is also ambiguous in this context (immediately after what?).

We have edited these confusing sentences. Please see the revised manuscript.

Secondly, please note that there are numerous spelling errors and inconsistencies (e.g. extrasynaptic vs. extra synaptic) which can be avoided simply by using a spell-check.

We have corrected the spelling errors based on the word spell-check and our colleague’s suggestions.

Thirdly, the literature you quote especially supporting a clear separation of NR2A vs. NR2B receptors into synaptic vs. extrasynaptic is somewhat outdated. To the best of my knowledge, this dichotomous separation does not stand up to more
recent scrutiny. Certainly, the discussion has to incorporate more recent reviews of this topic. We have updated this discussion and our paper no longer claim a strong separation between NR2A and NR2B receptors. We also added a recent review that refutes the dichotomous distinction.

I found a number of issues confusing that may or may not have also been raised by the reviewers:

a) It is unclear to me how the samples used for various analyses correlate with the number of animals in each group. There were 20 rats in each of the 5 groups. For each experiment (H2S concentration measurement, NR2A, NR2B, p-CREB western blotting, BDNF RT-PCR, HE staining), 4 out of the 5 samples from each group were used.

b) Does use of “NR2A/NR2B” always refer to the ratio between the receptor subtypes e.g. I 221? Correct, it refers to the ratio of NR2A and NR2B protein expression measured through western blotting.

c) I do not see support and quantification for your claim of shifting ratios (I 230) and the statistical significance of the differences. We carried out statistical tests and found that there were no significant differences between the ratios. We have retracted all statistical claims regarding the ratios.

d) Please also include the number of failing rats i.e. rats that you excluded from analysis because they did not meet behavioral criteria for each group. Moreover, please provide the rectal temperatures that were actually measured in the cooled group (e.g. showing it over the time course of the cooling).

We have added the success rate of generating mice that met the behavioral criteria to the manuscript. We did not have a record of the cooling time course. Throughout our procedure, most of the mice’s rectal temperature dropped below 33°C in 10 minutes as monitored. All animals in the mHT groups had rectal temperature between 32~33°C after 15 minutes of cooling.

e) Would it be possible to rename the groups in a more descriptive manner indicating the treatment e.g. Sh for sham, NT for normothermic, mHT and HT for mild the hypothermic groups with + HS indicating the addition of H2S? We have renamed the groups accordingly in our texts and figures.

f) Your use of the term “synergistic” which conventionally implies “more than additive”, is that what you imply? We misused this English term synergistic. The effect we observed was additive and we have corrected the wording.
f) Figure 3, while very impressive, is not useful unless you quantify the results or explain how you made sure that a representative as opposed to an arbitrary selected section is shown. This is an important point as you do not have behavioral tests to support your data with respect to outcome and therefore histological evidence must be solid.

Each image we showed was a representative section of the slides. We have also added table 2, where we quantified the number of irregularly shaped pyramidal cells under per 400x view.

g) An important point raised by Reviewer #1 is your claim to discovery / proof of mechanism. Your observations are certainly compatible with a role of NMDAR and H2S in ischemic injury but do not prove exclusivity and/or causality or the involvement of specific pathways. As a minimum, targeted use of antagonists of the beneficial pathways would be necessary to document their involvement.

We understand that our findings do not clarify some of the mechanistic details. We have toned down some of our claims on the causalities and mechanisms.

h) In general, the results section is excessively driven by teleologic interpretations mixed with results. Therefore, it is difficult to follow. Please present the results clearly and sequentially.

We have re-written the manuscript.

In summary, while there is interesting and important data contained in your MS, I cannot guarantee acceptance even of a revised version of this MS and even if you respond to or comply with all the requests / comments. Please note the suggestion of reviewer #1 and consider rewriting the MS as an observational instead of a causal mechanistic study.

We have re-written the manuscript with a more observational tone.

Response to reviewer 1’s comment

Title: Hydrogen sulfide and mild hypothermia can selectively activate synaptic NMDARs and trigger the CREB signaling pathway

Reviewer's report:
The authors of this study investigate the effects of mild hypothermia alone or in combination with NaHS administration in a rodent transient cerebral ischemia model. They measure expression levels of two glutamate receptor subtypes as well as pCREB and BDNF levels and use light microscopy to assess morphological changes in the pyramidal cell layer of the hippocampus.

Major Compulsory Revisions
Although experimental procedure of the transient cerebral ischemia and the other methodology seem to be well performed it is not very clear what question the
authors are pursuing. In addition data interpretation and conclusion seem long stretched at certain points as they claim to have found a mechanism by which hypothermia and NaHS "prevent" neuronal damage without providing data that can support that. Inhibitory experiments for example would have been helpful to support the role of H2S. In this case one cannot expect to solve a mechanism using just western blots.
1. Was spontaneous breathing of the rodents monitored by oximetry or capnometry? How can the authors rule out hypoxemia which can induce pre-stages of neuronal damage and which will affect all groups, especially the control group?
We did not monitor the breathing of the rodents through oximetry or capnometry. The four-vessel occlusion cerebral ischemia was an established model and thus we did not feel the need to further monitor it. Additionally, as seen in Figure 3A, the Sham operation group (Sh) did not show signs of neuronal damage.

2. Did groups I-III receive saline i.p.?
Yes, the control groups received saline i.p. as described in the manuscript.

3. The description of statistical tests are minimalistic. Please provide more information as to how the groups were tested (one-way, two way ANOVA, did they do any post tests? Did the authors assume gaussian distribution of the data (i.e. did it pass a normality test?)
We have added the name of each test to the manuscript's figure and table descriptions. Briefly, analysis between multiple groups was carried out by ANOVA using SPSS 13.0 software and any differences observed were further analyzed by least significant difference (LSD)-t test.

4. This sentence is convoluted, confusing and not correct:
   “…Whilst global cerebral ischemia-reperfusion, clearly caused a slight increase in H2S content, a significant increase in H2S content was seen in groups IV and V, which also had NaHS administered (p<0.05), whilst group III, which was subjected only to mild hypothermia showed an intermediate increase in the level of H2S compared to group II…”
According to this first half of the sentence there is only a slight increase in H2S which is NOT significant? And looking at the numbers in Table 1 the increase in H2S is actually larger in Groups II and III compared to IV and V (so the opposite of what the authors are claiming!) so how do they explain that?
We noticed that the last version of the Table 1 was inconsistent with our original documentation in Chinese due to miscommunication with the initial translator. We have provided the original documented number in this version, where H2S levels are higher in NaHS groups.

5. Line 220: “These broadly followed the same pattern as seen with H2S levels.”
no they actually don’t follow the patters seen in Table1. see previous comment.
This pattern hold true given the revised Table 1.
6. Line 226: What do the authors mean by 'basal level', are they referring to group 1? It is confusing as they mention relative levels in line 225, unless they levels are relative to beta-actin in which case the word relative is redundant as that should be clear from the methods.

We meant that the level of NR2A is higher than that of NR2B in Sh. We have revised the sentence.

7. Line 237. The authors repeatedly interchange and seem to not distinguish between activation and up regulation. These are two completely (yet not necessarily non-independent) processes. In fact the title is misleading as they do not really demonstrate 'activation' of the glutamate receptors, only an upregulation as measured in a western blot, which gives no functional information.

Our experiments supported up-regulation. We have revised the wording.

8. The statement of saying “…demonstrating the neuroprotective influence of H2S and mild hypothermia against ischemia related brain injury.” is a little fetched. In no way does that data suggest this statement. Instead something along the line of saying "...NaHS+hypothermia prevented ischemia induced pyknosis..." would be more appropriate.

We have revised this sentence.

9. Line 321. Again the authors mix up activation with up regulation! And as the authors do not look at recovery they should be more cautious making that statement.

We have changed the term.

10. Line 329. The authors say CREB was increased although they only blotted for phosphorylated CREB. I assume they are referring to pCREB, in which case both pCREB and BDNF are NOT increased with hypothermia in the NaHS groups (see Fig 2B and 2C)?

Yes, we were referring to p-CREB. Hypothermia alone increased p-CREB and BDNF levels. It is true that there is not a significant elevation of p-CREB and BDNF in NaHS groups induced by hypothermia.

11. Line 341. These “toxic products” are natural occurring essential neurotransmitters and hormones? Does that mean that they are bad although they are essential?

We have changed the wordings and described them as neurotransmitters. We believe excessive release of these neurotransmitters have cell-damaging effects.

12. Line 364. The authors mix up additive and synergistic (which they also mention on page 4). These are not the same!

The effect we observed were additive and we have revised the wording.
We have also revised our manuscript base on the reviewer 1’s comments in the minor revision section. Please see the updated manuscript for the corrections.

Response to reviewer 2’s comment

The manuscript by Dr. DAI Hai-binet al, entitled “Hydrogen sulfide and mild hypothermia can selectively activate synaptic NMDARs and trigger the CREB signaling pathway” showed the potential protective effect of hydrogen sulfide on transient cerebral ischemia. Although manuscript will add some information in present knowledge about these hydrogen sulfide and associated mechanism; authors needs to address some major issue to improve manuscript.

1. In the abstract result section statement made for Hippocampal H2S content was increased in all four test groups following ischemia-reperfusion is wrong, it should be decrease.

We did observe an increase in hippocampal H2S concentration following ischemia-reperfusion. As described above, we noticed that the last version of Table 1 was inconsistent with our original documentation in Chinese due to miscommunication with the initial translator. We have provided the originally documented number in this version, where H2S levels are higher in NaHS groups. Please see the updated Table 1.

2. In introduction first line Ischemia-reperfusion injury is one of the most common human pathologies is not correctly represented. Does reperfusion occurs in human?

Reperfusion means once the blood flow halt for a few time and then flow restored after some time.

We have revised the first sentence of the introduction. Yes, reperfusion can occur in human in situations like stroke or brain trauma.

3. What is the reason to keep rats for fasting and water deprivation for 12 h prior to the experiment?

Fasting and water deprivation is commonly applied before anesthesia treatment to ensure the typical dosage will be sufficient.

4. The rats were randomly divided into five groups (n=20). Please make clearer each group contains how many rat.

Each group contained 20 rats after excluding the failed models. Please see the updated text.

5. There should be image of TTC staining which can justify the animal has Ischemia injury.
In our knowledge, TTC staining is typically applied to focal ischemia, which is confined to a specific region of the brain, to evaluate the infarct size. Since we used a model of global ischemia (four-vessel occlusion cerebral ischemia as described by Pulsinelli et al), we did not need to measure infarct size and thus did not use TTC staining.

6. In line-77 Neuronal NOS should be represented as nNOS rather than NNOS. We have revised the wording accordingly.

7. Authors should use either H2S or H2S throughout the manuscript. We have changed all instances to H_{2}\text{S}.

8. Hematoxylin and eosin (HE) is not a Immunohistochemistry assay. Author wrongly presented it. We have changed the section name to histology.

9. Author should also label the blot properly which make clearer for reader. We have re-labeled the plots. Please see the updated figures.

10. Author stated that BDNF mRNA was separated by agarose gel electrophoresis but the blot is not there in data. While in line 2 and 30 they mentioned that, We further analyzed BDNF mRNA level by real-time PCR. It may be copy and paste error but should take care. We have corrected the error. BDNF mRNA level was measured through RT-PCR.

11. Authors should also label graph properly like mRNA level or protein label. We have re-labeled the BDNF mRNA expression levels in figure 2. Since we are showing protein expression levels in conjunction with the western blots we did not explicitly label those.