
Major Revision - JCBFM-0783-20-ORIG

Journal of Cerebral Blood Flow and Metabolism <onbehalf@manuscriptcentral.com> Wed, Mar 31, 2021 at 6:09 PM

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31-Mar-2021

Dear Dr. Farkas,

Your manuscript, JCBFM-0783-20-ORIG, Malignant astrocyte swelling and impaired glutamate clearance drive the expansion of injurious spreading depolarization foci, has been carefully reviewed by two (2) experts in the field. Although they found merit in your study, they have raised a number of concerns that preclude its acceptance in the present form. However, we invite you to respond to the reviewers' comments and revise your manuscript accordingly. The revised version will be re-evaluated by the original referees. Please be aware that this invitation does not guarantee acceptance of your manuscript. You may view the referees' comments at the end of this e-mail.

There are three new requirements recently added to the journal's Submission Guidelines:

1. At the revision stage, authors of manuscripts that contain cropped gels/blots must submit the original, uncropped image of each cropped gel/blot appearing in the manuscript. Each unedited gel/blot should be clearly labeled as "Full unedited gel/blot for Figure X", and the lanes of the unedited gel/blot that appear in the cropped image in the manuscript should be highlighted. All blots should contain markers denoting the location of molecular weight standards (units in kDa). The original gel/blot images should be submitted in a single PDF file and uploaded with the file designation "Supplemental Files". The file of the unedited images will be made available in the online supplementary material if the manuscript is accepted.
2. Where statistical analyses are described, please specify which formal tests for normality were used to assess data distribution. All data should be subject to tests for normality. Data that do not exhibit a normal/Gaussian distribution should be analyzed via a non-parametric equivalent.
3. For bar graph presentations, please show exact data points (e.g. dot plots) rather than bar plots throughout the manuscript and supplemental files.

In addition, ARRIVE guidelines could be cited in the Methods. PERCIE DU SERT, N., HURST, V., AHLUWALIA, A., ALAM, S., AVEY, M. T., BAKER, M., BROWNE, W. J., CLARK, A., CUTHILL, I. C., DIRNAGL, U., EMERSON, M., GARNER, P., HOLGATE, S. T., HOWELLS, D. W., KARP, N. A., LAZIC, S. E., LIDSTER, K., MACCALLUM, C. J., MACLEOD, M., PEARL, E. J., PETERSEN, O. H., RAWLE, F., REYNOLDS, P., ROONEY, K., SENA, E. S., SILBERBERG, S. D., STECKLER, T. & WURBEL, H. 2020. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *J Cereb Blood Flow Metab*, 40, 1769-1777, doi:10.1177/0271678X20943823. PMID: 32663096.

You should submit your revised manuscript promptly, as manuscripts received more than two months after first review are treated as new manuscripts. The revised manuscript should be accompanied by a check sheet identifying how you have responded to each of the suggestions of the referees and include a copy of the revised manuscript with the changes highlighted, as well as a clean copy.

Due to time differences around the world, the submission deadline given in our manuscript tracking system might fall earlier in your time zone, so timely submission is strongly advised.

We will inform you of our final decision on the acceptability of your manuscript once we have studied your revised manuscript.

Thank you.

Sincerely,

Dr. Guohua Xi
Associate Editor

Referee : 1

Comments to the Corresponding Author

Mounting evidence point to deleterious effects of SD to exacerbate tissue damage in ischaemia, thus controlling SD would be a potential target for neuroprotection. The paper has explored potential pathophysiological mechanisms of spreading depression (SD) in ischaemia with special reference to roles of astrocytes, cell swelling and extracellular glutamate. The study has used extensive and comprehensive methodology in several preclinical models and ex vivo preparations for SD. The results presented show that SDs as well as simultaneous SDs (SiD) take place following 2-vessel occlusion with anoxia in rats setting the scene for detailed experiments to explore effects of several ischaemia-related factors, such as those above. Both in vivo and ex vivo rat brain preparations were used to these ends where osmotic and pharmacological manipulations were used to complement ischaemia/anoxia. Histology techniques were applied to assess degree of tissue damage. In addition to substantial preclinical data, a Figure containing recordings from human brains is included. It is concluded that astrocyte swelling together with extracellular glutamate and elevated intracellular Ca^{2+} are crucial to elicit SD and SiD. The data support the claim that prevention of swelling could be a potential target to intervention to limit SD-induced tissue damage.

Specific points

1. Relevance of some models and manipulations to 'cerebral ischaemia'. The key difference with several of the models used here and ischaemia is that the latter has hallmarks of severe energy failure and high intracellular Ca^{2+} , to mention just a few of the key factors, both these not evident in water intoxication in vivo and some ex vivo models used in the study. This raises a question, how well several of the models and manipulations mimic pathophysiology of ischaemic stroke in vivo. The authors need to consider their preclinical models in the context of ischaemia in vivo.
2. Abstract and body of the text. The study is designed to address potential mechanisms of SD in ischaemic stroke. 'Stroke' is commonly understood as a focal brain ischaemia, yet none of the animal preparations used in the study are akin to focal ischaemia. Rather, global ischaemia/anoxia models and ex vivo brain slice preparations were used which may only partially mimic pathophysiology of stroke. It would be appropriate to refer to 'cerebral ischaemia' instead of stroke in this regard.
3. Abstract. Line 21 'paralysis of glial metabolism', a very clumsy expression, use 'inhibition of glial metabolism'.
4. Physiological monitoring. The procedures appear to be adequate, however, so essential data should be given. These include blood pressure, PaO_2 in control and anoxic conditions.
5. 'Live brain slices'. This is a rather unorthodox expression for ex vivo slice preparations and may just indicate the opposite to 'dead brain slices'. It is pertinent to stick on 'ex vivo brain slice preparation' inks.
6. Use of 'brain glutamate' in text. Extracellular glutamate was assayed both in vivo and ex vivo. Yet, the text gives rather ambiguous expressions for the data referring is to 'brain glutamate', 'tissue glutamate accumulation' etc. This is severely misleading, no evidence for tissue glutamate measurements is given, neither measurements of 'glutamate accumulation', when extracellular glutamate is monitored. One must use consistently 'extracellular glutamate', and 'glutamate release' to make sure the readers know that data are pertinent to glutamate outside neural cells.
7. P6, Line 10; 'electropolymerization (20 mns)', what units are abbreviated as 'mns'? Line 14. 'D-serine'. Is D-serine added to test sensitivity or specificity of glutamate assay? Line 43, PBS is abbreviated already earlier.
8. P7, water intoxication. Is there evidence that the total brain weight increases as a result of water accumulation into the brain?
9. P10, line 50. 'Fifty minutes later', does this value represent a single animal or is it an average of many? Line 57, 20.2 vs 45.6, statistics missing.
10. P11, line 52, 317 vs 197, add statistical significance.
11. P13, lines 39-48. Multiple abbreviation in text without explanations.
12. P13, line 58. 'SiD phenotype', what is the meaning of this concept?
13. P15, line 19, 2422 vs 1220, statistics missing.
14. P15, lines 29-31, 'water intoxication model of cytotoxic edema'. While water intoxication may cause tissue swelling, it doesn't result in energy failure that is the hallmark of cytotoxic oedema due to ischaemia, not to undermine

increase in intracellular Ca^{2+} . In this regard the water intoxication is not a model of cytotoxic oedema in stroke. This should be clearly indicated.

15. Figure 1C. CBF %, what was the baseline CBF? D1 and D2: what do the numbers in each subpanel refer to?

16. Fig 2 and so forth. Are the numbers of experiments referring to data from independent ex vivo slice preparations or values obtained from the same slice in multiple measurements? This has a significance when choosing appropriate statistics.

17. Fig. 4. Mixing data from ex vivo and in vivo experiments is rather unorthodox, justification is needed.

18. Fig. 6. The data from human brain are rather loose in the context of the paper. The Figure legend states that methods for the experimentation have been published previously, but no reference to any of these is given under Methods section. Similarly, Fig 6 data are neither adequately presented nor discussed in the body of text. A mentioning of Fig. 6 is found in Discussion, P17, line 38, yet, the observations described here have much different time relationship to ischaemia than what are evident from the preclinical experiments and therefore, inference of Fig. 6 data is not straightforward. Therefore, the relevance of Figure 6 to this study is questionable.

Referee : 2

Comments to the Corresponding Author

This study characterizes the phenomenon of simultaneous depolarization (SiD) of a cortical gray matter that may occur under pathological conditions of brain edema. The striking difference between clinically-relevant spreading depolarization (SD) events with defined punctate 'focus' of origin (or in some cases multiple punctual foci) is that SiD origin encompasses a very large volume of tissue from which SD spreads. Notably, SiD-like events were recently reported in patients during dying in the wake of circulatory arrest. These observations suggested that SiD is a clinically relevant phenomenon. This provides the rationale for the experimental characterization of SiD in this manuscript. There are a lot of good experiments in this study, especially that phenomenon of SiD was reproduced in vivo in the rat cerebral cortex with ischemia/anoxia combination. The central hypothesis is that "acute tissue swelling may drive the expansion of the SD focus" to become a SiD event is plausible. Nevertheless, my major concern is that to test this hypothesis, brain slices were exposed to highly unphysiological hypoosmotic stress. In many experiments (HM60), osmolarity was dropped by 140 mosM! There is no doubt that astrocytes under such conditions will be massively swollen and release gliotransmitters, including glutamate, coinciding with SiD occurrence. It's entirely possible that similar results could be obtained with more pathophysiological osmotic reductions. This concern should be addressed if not by additional experiments but then by an appropriate section in the Discussion. In this regard, there is no clinical evidence that low osmolarity induces SD in patients. The first line of the Discussion that cerebral edema is a sufficient condition for spreading depolarization to occur spontaneously should be modified. There should be a discussion on how these findings can be translated to an actual penumbral region.

Below is the list of additional concerns:

There are a lot of references to the Supplemental Material section. The Supplemental Materials should be carefully checked because Supplementary Methods contain unfinished sentences and other inconsistencies. In some places, there is no correspondence between Materials and Methods in the manuscript and the Supplementary Materials (e.g., size of the cranial window; IOS - a halogen lamp or LEDs? etc.)

P7L53. 1 $\mu\text{m}/\text{pixel}$ spatial resolution for multiphoton imaging of astrocytes seems inadequate to quantify volume changes (too low resolution). Please, comment. What was the NA of objective used?

P9L29. Bumetanide is a specific inhibitor of NKCC1 at a concentration of 10 μM . I doubt that it will retain its specificity at 100 times higher concentration (1 mM) used in the study.

P.11L45. There is no Fig. 7A.

P12.L7. Add that it was under extreme osmotic stress.

P12. L19 There is nothing about dendritic injury in Ref. 35.

P12. L29 "The cortical area of SiD origin in a brain slice revealed neuronal necrosis in close proximity to swollen astrocytes (Fig. 3B)". Was it rigorously measured? Please, provide quantifications or remove this statement.

P12. L33 "Golgi-Cox staining identified extensively swollen astrocyte somata and vestigial processes (Fig. 3D1)". Please, provide a control image and quantifications.

"These astrocytes displayed prominent soma and perivascular endfeet swelling, and fragmented intracellular organelles (Fig. 3B&D2)." What are these fragmented organelles? None can be seen in the images. Please, provide control images as well. As regarding Fig 3B1; How far from the cut slice surface are these degenerating neurons? Neuropil preservation around these neurons is terrible. Are there any better images? How were astrocytes in Fig. 3B2 identified? How can one know that astrocytes in Fig 3D2 were swollen? This is a single image. Please, quantify with stereology to make this statement and provide control images.

P12L50 "Nuclear localization of CC3 was associated with fragmented GFAP fibrils in astrocytes (Fig. 3F2)." I do not see a fragmentation of GFAP. Please indicate with an arrow in the Figure.

P12.L55. "Our histological results together suggest that astrocytic edema and the linked SiD seriously jeopardize the survival of neurons and astrocytes alike and impose significant damage to the nervous tissue." In 9 slices (P11.L35), anoxia just caused propagating rSD (not SiD). What are the histological results for this case? Was SiD more damaging in comparison?

P13. L8. "Astrocytes appeared substantially swollen after HM exposure; both the perinuclear plasma and processes increased in size (Fig. 4A1-A2). This swelling progressed further after the passage of SD1 (Suppl. Fig. 4A)." Was it quantified? How does the same EM image of astrocyte in Fig. 4A and Suppl. Fig. 4A suppose to demonstrate additional swelling?

Why is there a decrease in the IOS signal in Fig. 4B and 5A during HM when astrocytes suppose to swell? The same is for Suppl. Fig 4D4.

P15L55 "The maximum surface area of selected astrocyte somata was significantly greater in the DW group (160.47 ± 12.29 vs. 134.85 %, water intoxication vs. control) (Fig. 5F2). The maximum swelling of astrocytes reported in the literature is about 60%. 160% should rupture the cell membrane, I guess. Was control (baseline) 100% instead? Is this a typo in the manuscript? In any case, I hardly see swelling of SR101 astrocytes in Fig. 5. Please, check the literature on the degree of astroglial swelling. Traces in Fig5E suggesting that swelling was ~60%.

P16 L3 This should be Fig. 5F3 (not Fig. 6F1).

As regarding FC, please describe the Supplementary Materials how FC was prepared. It is not trivial. Also, was an FC application affecting neurons? Was it checked?

P14L3 "...cortical astrocytes became swollen (Suppl. Fig. 5A)" with FC application. The single EM section does not confirm anything. It should be quantified with stereology to state it as the Result.

Fig. 6A. Why trace from -0.5 mV to -5 mV is called depolarization? It seems like hyperpolarization to me (I agree 6D is depolarization). Was Y-axis mislabeled?

P18L41 "Our results that preventive hyperosmotic intervention reduced the excitability of the nervous tissue and most importantly, averted SiD, provide pathophysiological insight into this empirical clinical strategy for the first time, and emphasize the need to invent new ways of preventive osmotherapy in the treatment of acute brain injury." It would be nice to add mannitol experiments to in vivo water intoxication model to emphasize this point.