Dear Dr. Bajaj,

We have read and have, to the best of our ability, responded to every comment made by you and the reviewers. We hope these are enough to move forward. That notwithstanding, we are open to provide further material whenever necessary.

Yours faithfully,

Matthew Boyko, Ph.D.

**Editorial comments:**

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

*Done!*

2. Once all the comments of the reviewers are addressed, please ensure that the protocol is no more than 2.75 pages and the highlight is no more than 2.75 pages including headings and spacings.

*Done!*

3. Please proofread the manuscript well before submitting.

*Done!*

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

Now this is acceptable to print.

*Thank you!*

**Reviewer #2:**

Manuscript Summary:

The authors have incorporated all suggested changes. A few minor points remain to be considered.

Minor Concerns:

2.2 This should be first (2.1)

*Thank you for the remark. 2.2 is now 2.1.*

2.5 Ointment application is best to be done on prep table

*Thank you for the correction. This has been rectified.*

2.7 Bupivicaine is not necessary since the animals are anaesthetized.

*Thank you for the correction. This step has been removed from the protocol.*

2.8 The pack of the surgery should be already sterilized: cover mice with sterilized surgical drape.

*Thank you for calling our attention to that. It has been rectified.*

3.7 Cage or incubator?

*Thank you for that correction. Incubator it is.*

5 Describe the criteria for NSS scores 0-4.

*Instead of elaborating on this description, we have provided a reference for the detailed explanation of the procedure: “Ref 32. Menzies, S.A., Hoff, J.T. & Betz, A.L. (1992) Middle cerebral artery occlusion in rats: a neurological and pathological evaluation of a reproducible model. Neurosurgery, 31, 100–106; discussion 106–107.”*

6.1.3 Wash brains in saline 0.9% not water.

*Thank you for the correction. Water has been replaced with 0.9% saline.*

8.4.1 Height is not 1000 cm (10 m); please correct. Also correct in Table of Materials.

*Thank you for the correction. This has been corrected in the manuscript and table of materials.*

l. 381: Change "model is illustrated in the fact that the ECA and its branches, the occipital artery" to: model is illustrated by the fact that the ECA and its branches, including the occipital artery

*Thank you for the correction. This change has been effected, and the manuscript as a whole has been proofread for grammatical errors.*

Table 1 and 2: include the N value; which values were significantly different from sham?

*The N values for MCAO and Sham have been added to the legends of both tables.*

**Reviewer #4:**

Manuscript Summary:

This manuscript provides procedures for a new surgical technique for inducing MCAO. The strengths of this technique, such as decreased mortality and lower variability in weight, edema, and infarct volume have been previously established. The authors seek to document the procedure and validate it by demonstrating that it produces a significant deficit in forced swim and sucrose preference tests, which are common models of PSD. This contribution establishes that the technique can be used to assess PSD, but does not establish that it produces PSD with any higher efficacy or advantage over the traditional model. This manuscript is much improved from its former version. It still needs some work, including an improvement in the language and grammar. However, it makes a significant contribution to the literature, and the new procedure is ideally transmitted via videotaped demonstration.

Major Concerns:

First concern: The introduction states that "The data obtained with this protocol show that this model of MCAO could be a more effective way of inducing PSD in rats and could potentially lead to a better understanding of the pathophysiology and the future development of new drugs and other neuroprotective agents." This is simply not true - it is neither supported by the data nor by previous data. If there is no comparison MCAO group utilizing the traditional MCAO procedure with which the requisite comparison can be made, the claim simply cannot be made that this procedure "more effectively" produces PSD in the model.

I suggest the following: in abstracts, introduction, and discussion, de-empathize PSD as the major advantage of the method since there is no evidence that there is any such advantage. Instead, summarize advantages that have previously been established in other studies that did compare to traditional MCAO (I think it was implied that mortality, variability of infarct volume and weight, etc. may be such variables). Then, instead of stating that "The primary objective of this protocol is to outline the steps for inducing PSD in rats by occluding the MCA via the ICA" State that the objective is to validate that this new MCAO method (which can be utilized along with any endpoint, not just PSD), has some impact (NOT a better, worse or similar impact compared to other procedures) on an important behavioral paradigm: PSD.

This would require some minor restructuring mainly of the introduction, abstracts, and a bit in the discussion.

*Thank you for your observation. However, we have removed the word more, giving the entire manuscript the intended meaning. As can be seen from the manuscript, we do not make comparisons between different models that cause the development of PSD. The purpose of the work remains unchanged. “The primary objective of this protocol is to outline the steps for inducing PSD in rats by occluding the MCA via the ICA". The changes you suggested require a fundamental review of the article, which will include: change of the main goal that comes with changes to the methods, results, discussion, and conclusions. In other words, the article should be radically changed, and it will have other objectives, methods, results, discussion, and perhaps even a title. This means complete rewriting.*

Second concern: Reference in methods to striatum and cortex has been removed, and this improves the clarity of the methods greatly. However, the striatum and cortex are still mentioned in results, which is confusing since there is no method to match those results. These procedures should either be clarified or the results removed.

*We agree with this assertion. We might have just forgotten to redo the results last time. We have now removed the words ‘striatum’ and ‘cortex’ from the protocol and table 1. The protocol will contain only the value of the total brain, which is convenient since we measured total brain only.*

Minor Concerns:

The measurement methods for the two hemisphere sizes should be specified. On line 269, the formula makes sense only so long as the ipsilateral hemisphere is measured in entirety (not just the healthy tissue), but I could not verify that this is what they did.

*Thank you for this observation. In our protocol, we applied the method previously described in an article published with JoVE (Reference 35 “Poinsatte, K. et al. Quantification of neurovascular protection following repetitive hypoxic preconditioning and transient middle cerebral artery occlusion in mice. Journal of visualized experiments. (99), e52675 (2015).”*

That said, this equation will undercorrect the infarct size since the swelling in and around the stroke will be higher than in healthy tissue. Even if this remains the best method for assessing edema, it will systematically produce an inaccuracy, which is a point that should be mentioned so that measurements can be properly qualified.

*We used the conventional and the best method for measuring the cerebral edema described in the guidelines in our investigation and have provided a reference to back that up: “ref 37. Liu, S., Zhen, G., Meloni, B.P., Campbell, K., Winn, H.R. Rodent stroke model guidelines for preclinical stroke trials. Journal of Experimental Stroke & Translational Medicine.* ***2*** *(2), 227 (2009).”*

Figure 3 legend - the authors should not refer to learned helplessness if they're not measuring learned helplessness directly. The phenomenon may factor into the swim test, but since they never establish a scenario in which kicking would be efficacious for escape, they're not directly assessing it.

*Thank you for this correction. The figure legend has been rewritten to remove ‘learned helplessness.’*

Lines 53, 80, and 84 - the term "variability" is used without mentioning what measure shows higher variability in this model compared to the traditional one. Those variables should be listed and references cited of course.

*Line 53 is in the abstract where it is usually not customary to provide citations. The necessary reference to this term is given in line 80.*

*Line 80 has a reference at the end of the phrase (reference 28), and*

*Line 84 - the term "variability" was used without citation to avoid repetition given that ref 28 had been cited earlier in line 80. Nevertheless, at the request of the reviewer, we have again added this citation to line 84.*