Name of journal: Neural Regeneration Research
Manuscript NO: NRR-D-21-00139
Title: Optogenetic stimulation of the contralesional anterior lateral motor cortex improved the functional recovery after middle cerebral artery occlusion
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Reviewer's country: UK

COMMENTS TO AUTHORS
I. OVERALL SUMMARY
Aims – To determine whether the activity of the anterior lateral motor cortex (ALM) in the contralesional hemisphere effect the extent of repair to the ipsilesional infarct area (primary motor cortex) and motor recovery of mice that had undergone middle cerebral artery occlusion (MCAO stroke model).
Results – Motor function improvement was significantly greater for stroke model mice undergoing inhibition of contralesional ALM (SOI group). Although NSS-R score improvement was similar for SOI and the MCAO mice undergoing activation of contralesional ALM (SOA group). At the neurological level, neuroplasticity appeared to be enhanced for both SOI and SOA group (increased dendritic length, increased number of intersections, perforated synapses and multiple synaptic boutons) compared to sham stimulated MCAO mice.
Conclusions - In mouse stroke model, circuit repairs to the ipsilesional infarct area appeared to be enhanced by stimulation of the contralesional ALM. These neuroplasticity changes were greatest for the SOI group. This was reflected in the greater motor function recovery in SOI group. These findings could help in the development of stroke recovery therapies.

II. OVERALL STRENGTHS
The authors provided complementary methods to robustly show the neuroplasticity changes in the ipsilesional primary motor cortex of stroke model mice undergoing optogenetic inhibition and activation in the contralesional anterior lateral motor cortex. The neuroplasticity changes measured were markers of neuronal recovery in the primary motor cortex. This recovery was in the most part greater in the stroke model group undergoing optogenetic inhibition (SOI) relative to activation group (SOA) and sham group. However, behavioral effects would be improved by a “before and after surgery” comparison (i.e healthy mice vs mice post MCAO). Nonetheless, this behavioural work provided an additional level of understanding of the impact the neurological changes in the primary motor cortex had on improvements in motor performance.

III. OVERALL IMPACT
In general terms of the authors provided complementary methodology to address their hypothesis. If the authors could place this data more clearly among the current literature, and discuss novel aspects of their study directly, the impact could be improved.

IV. MAJOR POINTS
INTRODUCTION
Introduction, paragraph 1, page 13. Please could the authors explain the clinical definition of a stroke further? In particular in terms of occlusion of blood flow to the brain (introduce term of infarction), immediate symptoms and then short term clinical profile and long term recovery stats.
Introduction, paragraph 1, page 14. Please could the authors clarify their hypothesis? Did they predict
whether both inhibition and activation of contralesional ALM would have the same beneficial effect on the ipsilesional primary mortex cortex? Or would inhibition or activation have different effects?

Introduction, paragraph 1, page 14. Please can the authors define and expand upon “preparatory activity”. How are they going to test this behaviour in their model?

Introduction. The introduction would benefit from a clearer rationale of why targeting the contralesional ALM activity; both inhibiting the cALM and activating the cALM would have an impact on the ipsilateral mortex cortex.

The introduction would benefit from a clearer understanding of what novel question is being answered by this study.

METHODS

Experimental design. Please can the authors clarify the sham group. For example, are the sham mice those that received MCAO and the viral injection to express either ChR2 or NPHR, but not stimulated?

Or are the sham mice those that received MCAO but no viral injection, and then stimulated.

Experimental design. Can the authors clarify how many mice per group were used for each experimental procedure.

Page 16, “Stimulation Paradigm” paragraph. How many mice per group were excluded from the analysis due to “an absence of ChR2 or NPHR gene expression”?

Page 18/19, “RNA-seq” paragraph. The authors need to provide a lot more detail on RNA-sequencing experiment procedure. For example, the methods should inform the reader on the following: what brain region was the RNA from? How many independent brain tissue samples were used for each bulk RNA library? What was the read depth of sequencing?

Page 18/19, “RNA-seq” paragraph. The authors need to provide a lot more detail on RNA-sequencing analysis steps. For example: What was the reference genome used for mapping? What quality control checks were carried out postsequencing – i.e. what was the % mapped reads, how many protein coding genes were detected per sample? What was the filtering criteria of the samples for inclusion in the analysis? How were the differentially expressed genes determined? How was gene ontology enrichment analysis performed? Define rich factor. How has the heat map been generated? Describe how “Log2FC from edgeR’s LogFC” (see Results page 21) were calculated.

Page 18/19, “RNA-seq” paragraph. Are the authors able to give the raw gene expression data matrix (i.e. samples, genes and their transcripts per million) in the supplementary materials?

Page 19, “Data analysis” paragraph. Please can the authors correct the type of post-hoc test used in their statistical analysis. For example, in the figure legends, Dunnett post hoc test is referenced not Tukey’s multiple comparisons post-test.

FIGURES

Abstract Figure. This figure would benefit from some extra labels. The key details of mouse stroke model should also be shown. As well as minor changes to the left-hand schematic of the mouse brain (label viral injection, label red vs green connection tracts). For the results displayed, the authors should always put the results for both activation and inhibition groups. The terms “moving activity” could be changed to something more relating to recovery.

All figures. Can the authors re-order figures so that the first main text figure discussed (i.e in Methods, page 15, line 8) is titled Figure 1A. The figures should be labelled 1A, 1B, 1C… according to their chronological appearance in the text. In this case Fig1B should be re-titled Fig1A as Fig1B is referenced first.

All figure legends. Please can the authors ensure that all abbreviated terms in the figure/figure legend are given in full in their figure legend. A suitable way to ensure abbreviations are clarified in the figure legend is to have the final sentence of all figure legends as an abbreviation list of all used terms. i.e.

“Abbreviation: anterior lateral motor cortex, ALM ; control group with sham

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stimulation, Group S; ...” All figure legends. Please can the authors add the number of mice per group for each experimental procedure.

All figures of post-mortem tissue. Please can the authors clearly show the scale bar with size labelled (i.e. 5 μm) on all the images. Figure 2, page 11. From the experimental timeline provided in Figure 1, the authors recorded the NSS-R score in mice before stimulation. Please can figure 2A/2B/2C/2D be updated with the before stimulation baseline values from day 6?

Figure 2, page 11. From a control group point of view, behavioural measures from before and after the MCAO surgery are needed to fully understand the degree of recovery achieved through optogenetic manipulation. Please can figure 2A/2B/2C/2D be updated with the before MCAO surgery baseline values? If the authors did not collect this data, this limitation needs to be addressed in the discussion. Are the authors able to cite wild type C57BL/6J adult male mice, 10-12 weeks old, (no MCAO surgery) behavioral results from the literature?

RESULTS

Various sections, Results. In general the Results section would benefit from authors quoting numerical values from graphs to give a more descriptive account of the results.

Various sections, Results. Can the authors ensure that each time statistical reference is made to a figure, the figure is immediately cited after. For example, sentence on page 21 has figure citation missing: “Besides, Group SOA had more perforated synapses than Group S(p<0.05).”

Results, page 21, line 7/8. Please can the authors clarify the definition of what perforated synapses are.

The current wording is confusing. The definition from Kim et al. 2018 may be of help (DOI: 10.1523/JNEUROSCI.1295-17.2017): “Perforated synapses, a subtype of synapses with discontinuous postsynaptic densities are known to have abundant AMPA receptors as well as a larger overall postsynaptic density area, indicating a heightened level of functional efficacy and maturation.”

Results, page 21, line 8-10. Please can the authors clarify the definition of what multiple synaptic boutons are. The current wording is confusing. The definition from Kim et al. 2018 may be of help (DOI: 10.1523/JNEUROSCI.1295-17.2017): “Boutons forming synaptic contacts with more than one postsynaptic element.” Results, page 20, paragraph titled “Optogenetic cALM inhibition promotes dendritic morphology in ipsilateral M1 after MCAO”. The title is misleading as both SOA and SOI groups showed dendritic morphology changes compared to sham group. Please can the authors change the paragraph title to address this? Could the change in dendritic morphology also be added to the title (i.e. promotes more complex or mature dendritic morphology)?

Results, page 21, paragraph titled “Effects of optogenetic cALM stimulation on synaptic connection after MCAO”. For consistency can the authors change the title of the paragraph to a result (i.e “Optogenetic cALM inhibition and activation showed differential effects on synaptic rewiring after MCAO”.)

Results, page 21, paragraph titled “Optogenetic cALM inhibition and activation showed differentially expressed genes in ipsilateral M1 after MCAO”. The author needs to be provided a much deeper discussion of Figure 6A, 6B, 6C and 6D.

Results, page 21, paragraph titled “Optogenetic cALM inhibition and activation showed differentially expressed genes in ipsilateral M1 after MCAO”. Please can the authors provide a definition of the GO term “positive regulation of cell activation”. Where is this GO term from, as this GO term is not shown in Figure 6C.

DISCUSSION

Various sections, Discussion. In several places the authors fail to add a supporting reference to points that they are citing. Please can the authors ensure any statement about independent studies is referenced.

Discussion point. Are the authors able to find reference to optogenetic stimulation in the anterior lateral motor cortex of control, healthy mice and the outcome to add to their discussion of what we know of
the anterior lateral motor cortex activity on behavior.

Discussion point. Are the authors able to cite references that show the innocuous nature of this in vivo expression of ChR2/NPHR when non-stimulated? Are the authors able to cite references that show blue/yellow light stimulation in vivo in wild type mice is harmless/no consequence on behaviour?

Discussion, page 23. Future explanation needed. Please can the author use independent studies in the literature to justify why specific morphological changes were selected in their analysis. i.e Why did the author select to look at multiple synaptic boutons as a measure of neuroplasticity?

Discussion, page 23. Elaboration needed. Please can the authors extend their discussion on clinical application of their findings and limitations/concerns. In particular extend the discussion of: “Indeed, reasons of concern include the multi-targeted and multicellular effects exerted by cALM inhibition.” Are there any clinical trials using neuromodulation technology in the clinic that can be discussed?

Discussion. Please can the author provide a discussion of the limitations of their study. For example, behavioural improvements are weak as there was no before MCAO surgery behaviour recorded on the mice to compare to.

Discussion. Please can the author provide extra discussion of their findings in reference to other studies that have investigated the effect on contralesional brain region activity on neuroplasticity in region of infarction.

V. MINOR POINTS

TITLE
Long title. The authors could change the title slightly to add the following details: species, stroke model, optogenetic inhibition, neuroplasticity changes.

Short title. The title would be better without any abbreviations used.

ABSTRACT
Abstract. If possible within the word count, please could the authors add results from the SOA group into the discussion. This would give a more complete account of the article.

INTRODUCTION
Various sections. Please can the authors ensure that the species (mouse, rat, human) is stated when referencing independent studies.

Page 13, introduction, paragraph 2. Is ipsilateral brain the right term to use in the following sentence, or would “area of infarction” be better: “reconstruction of neurological function in ipsilateral brain during…”?

Page 13, introduction, paragraph 3. For clarity the author may want to define “contralesional” and “ipsilesional”

Page 14, introduction, paragraph 1. Re-wording of this sentence is needed to improve understanding. “To clarify how brain plasticity change when activated or inhibited contralesional PMC area directly…”

Page 14, introduction, paragraph 1. Further introduction needed on ChR2 and provide full name (i.e. channelrhodopsin 2)

Page 14, introduction, paragraph 1. Further introduction needed on NpHR and provide full name (i.e. halorhodopsin)

Page 14, introduction, paragraph 1. Why was the MCAO mouse model used? Can the author find supporting references to the usefulness of MCAO mouse model as a stroke model?

METHOD
Page 17, “Golgi staining” paragraph. Please can the authors clarify the fixing procedure for the brain tissue timing. In particular the line: “…then stored in the dark at 22°C-25°C WITHIN TWO weeks…”. Are the tissues stored “FOR” two weeks or “UP TO” two weeks?
Page 17, “Golgi staining” paragraph. Why was Layer V of the primary motor cortex selected? Page 18, “The revised neurological severity score (NSS-R)” paragraph. Please could the authors elaborate on what the NSS-R evaluation entailed (i.e. more detail on what motor tests were carried out, what reflexes were tested…)
Page 18, “The revised neurological severity score (NSS-R)” paragraph. Please can the authors add when this behavioral testing was carried out. Did the authors take these NSS-R recordings from mice before MCAO, as well as after?
Page 18, “The revised neurological severity score (NSS-R)” paragraph. Please can the authors add whether the photostimulation was happening simultaneously during this NSS-R behavioral testing?
Page 18, “Open field test” paragraph. Please can the authors add when this behavioral testing was carried out. Did the authors take these recordings from mice before MCAO, as well as after? Page 18, “Open field test” paragraph. Why did the authors decide to carry out the photostimulation simultaneously during the behavioural testing? Is there data available for the open field test when mice were not being photostimulated?

SUPPLEMENTARY MATERIALS
Supplementary materials. To provide a complete story/comparison, please could the authors provide corresponding videos for animals in all groups. For example, for supplementary material 1 could a video be given of a mouse moving before and after MCAO surgery. For example, for supplementary material 2 could a video of mice from each group (SOI, SOA and S) be provided?
Supplementary materials. Can an excel spread sheet of the raw RNA-sequencing gene expression data matrix be provided?

FIGURES
Figure 1A. For improved accuracy, the authors may find it useful to change the following three labels: “stroke” to “Performed middle cerebral artery occlusion procedure”; “Stimulation” to “Optogenetic Stimulation”; “Sac for analysis” to “Post-mortem brain tissue analysis”
Figure 1C. Please can the authors label the photo to enhance information delivery. At the present the fact that this is a photo of the viral injection procedure is not clear. To improve this, please can the authors label the apparatus (emphasis on viral injection set up needed), the brain region targeted, the mouse.
Figure 1D. Please can the authors provide additional labels to the schematic. For example label type of cross sectional view of the mouse brain presented. The following questions also remain unclear from the figure/figure legend: what the grey vs white area represents, what does the black dot represent, what do the red vs green connections represent, what do the abbreviations ALM and CST stand for.
Please can the figure and figure legend be updated to clarify these questions?
Figure 1D. Please can the authors label the post-mortem brain tissue image. Possibly add small white arrow heads to clearly mark the red positive cells on the image for the ease of the reader. Can the authors also ensure that the following questions are made clear from the figure/figure legend: what are the red cells; what experimental group (group SOI or SOA?) is this brain tissue from; what area of the brain is this section from?
Figure 2A. Can the authors correct the y-axis label to “NSS-R score”
Figure 3A. Please can the authors label the post-mortem brain tissue image to emphasise the differences between group S, SOI and SOA. Possibly add small white arrow heads to clearly mark the neurons showing dendritic branching pattern/morphology typical of that group. Figure 3C. Is it possible to add the error bars to the plot so the reader can see the degree of variation from mean?
Figure 3D. Please can the authors label the post-mortem brain tissue image to emphasise the differences between group S, SOI and SOA. Possibly add small white arrow heads to clearly mark the dendritic spines.
Figure 3E. The authors might consider updating the graph to bar graph with standard error bars instead
of box-and-whisker plot.
Figure 4A. Please can the authors label the schematic and the electron microscope image in general (i.e. synaptic cleft, synaptic vesicles, pre synaptic neurons, postsynaptic neuron) and explain what the dark black regions are.
Figure 4B/4C/4D. Please can the authors label the electron microscopy image to emphasise the differences between group S, SOI and SOA. Possibly add small white arrow heads to clearly mark the perforated synapses.
Figure 5A. Please can the authors label the schematic and the electron microscope image in general (i.e. synaptic cleft, synaptic vesicles, pre synaptic neurons, postsynaptic neuron) and explain what the dark black regions are.
Figure 5B/5C/5D. Please can the authors label the electron microscopy image to emphasise the differences between group S, SOI and SOA. Possibly add small white arrow heads to clearly mark the multisynaptic bouton.
Figure 6 (ALL). To allow the reader to fully understand the figures, please can the author add the following information to the figure legend. At what time point along the experimental paradigm was the samples for RNA seq taken? From what brain region were the samples from?
Figure 6A. Please can the authors clarify the details of the heat map on the figure and in the figure legend. For example is the -2 to +2 scale a normalized correlation (Z-score)? What does each row and column on the heat map represent (i.e. each row represents a gene and each column a sample)? What does the branch sidebar on the left and top of the heat map mean? What does the colour coding of this left-hand side bar mean? Could the authors add GO terms corresponding to the gene list clusters onto the heat map?
Figure 6B. Is this plot needed? From the readers point of view very little information comes from this plot.
Figure 6C. The figure legend description does not seem to correspond to the actual figure (i.e “The orange bar graph represents -Log(Padjust),The blue line graph represents the quantity”).
Figure 6C. Please can the authors clarify on the plot what these GO terms refer to (i.e. what group comparison has been made, and are the terms enriched in, SOA or SOI relative to the other?).
Figure 6D. To clarify the direction of the effect (either increase or decrease in SOI relative to SOA) can the authors add additional labels to the plot. For example to the right hand side of the graph, authors can add two arrows. One pointing vertically up (from 0 up on y-axis), label higher expression in SOI relative to SOA. Second arrow pointing vertically down (from 0 to minus values on y-axis) label lower expression in SOI relative to SOA.

RESULTS
Results, page 20, paragraph titled “Optogenetic cALM inhibition promotes on neurobehavioral changes after MCAO”. Authors may find it useful to discuss the open field test results in terms of where the mice were traveling within the field (i.e centre or corners). To clarify the difference weren’t in terms of where the mice spent their time.
Results, page 20, paragraph titled “Optogenetic cALM inhibition promotes dendritic morphology in ipsilateral M1 after MCAO”. Please can the authors define the meaning of the term shell in “…intersections per shell as…”
Results, page 20/21, paragraph titled “Optogenetic cALM inhibition promotes dendritic morphology in ipsilateral M1 after MCAO”. Please can the authors add a description of the changes to the spine number at the basal side.

DISCUSSION
Discussion, page 22. Can the authors elaborate on this ambiguous statement: “We demonstrated that cALM stimulation altered neurobehavorial.”
Discussion, page 22. Overstatement. The suggestion by the authors: “all three groups showed transient
deficits in the Day 14 after MCAO in the open field test, possibly reflects post-stroke anhedonia, or loss of incentive motivation/“loss of interest”…” would need further backing to explain why the open field test is not simply a reflection of motor deficit. If this effect of MCAO on mood was going to be investigated, what tests would be performed in the future and why? 

Discussion, page 23. Overstatement. The following statement by the authors is too strong, especially when the authors do not provide behavioural results from control, healthy mouse to compare to their SOA/SOI results: “…current findings strongly demonstrate that cALM inhibition rather than activation can contribute to persistent synaptic plasticity gene expression and rescue neurobehavioral defects. These findings contribute to a novel mechanism of cALM that leads to stroke recovery.” Please can the authors rephrase this statement