OPEN PEER REVIEW REPORT 1

Name of journal: Neural Regeneration Research
Manuscript NO: NRR-D-22-00134
Title: Cylophilin D-Induced Mitochondrial Impairment Confers Axonal Injury after Intracerebral Hemorrhage in Mice
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COMMENTS TO AUTHORS

The authors describe their study on the role of cylophilin D (CypD)-induced mitochondrial impairment in axonal injury in a mouse model of intracerebral hemorrhage and in a cultured cell model. They found that CypD was upregulated after intracerebral hemorrhage and both CypD deficiency and cyclosporin A (CsA) could mitigate mitochondrial damage (ICH), reduce the formation of retraction bulbs, and ameliorate functional deficits related to corticospinal tract injury. Although the study appears interesting with impressive photomicrographs, there are several major points that the authors should properly address.

Major concerns:

1) My first major concern is regarding the ICH models used. The authors injected autologous blood into the right "basal ganglia" and assessed axonal retraction bulbs, mitochondrial abnormality, and CypD expression 24 hours after injection. Since erythrocytes can remain morphological intact for several days in the brain parenchyma in vertebrates and they do not undergo hemolysis or release oxyhemoglobin until 2 days in vitro (J Cereb Blood Flow Metab. 2003, 23: 629-52), the cultured PC12 cells in which oxyhemoglobin was added to mimic ICH do not seem to be a model equivalent to the in vivo one. Although extravasated plasma may inflict a chemical insult to axons, tissue deformation resulting from accumulated blood cells could be the primary insult that directly compresses/stretches axons and breaks them.

2) Please describe the rational to use retraction bulbs as a readout of axonal degeneration. It is well known that retraction bulbs form at the proximal portion of severed axons acutely and persist for months to years in the central nervous systems. Proximal axons die back hundreds of micrometers with acute axonal degeneration within 30 min after injury, and then retraction bulbs form proximal to the died-back regions (Nat Med. 2005, 11: 572-7). Retraction bulbs are therefore considered unsuccessful attempt for axons to regenerate.

3) Critical information, which facilitates replication by others, is to be stated in experimental procedures. These include, but not limited to, the following: i) For injection of blood, please specify the speed of injection and the reference for 3 mm depth. The volume of 25 μl is huge given the size of the mouse striatum that was targeted. Was any anticoagulant used in the blood? ii) Details for the sham control. Was identical volume of saline injected? iii) Regimen of CsA treatment. Only one dose? iv) Details for tissue harvest should be described as to which brain region was actually taken for biochemical EM studies. For histomorphology data, low magnification views that cover the hematoma in each group would be informative and preferable. v) The schedule of oxyhemoglobin treatment in PC12 cells and the harvest of cells. vi) The criteria used to morphologically define retraction bulbs.

4) The retrograde tracing data is questionable to this reviewer. According to the mouse L2-L4 spinal atlas that I have at hand, the injection coordinates (0.2 mm laterally, 0.6 mm depth) do not seem to precisely target the dorsal corticospinal tract (CST). CSTs on both sides are too small and tightly close to each other. Precise targeting of unilateral CST is an extremely technical challenge for pressure injection. The authors should describe the technical details (for instance, the make and model of

syringe and needle, the size/gauge of the needle, the speed of injection, the stereotaxic equipment/instrument used, the name of the atlas referred), if they have faith in the validity of the tracing data. Please also check for certain the location of coronal sections showing the primary motor cortex; the coronal plane at -1.7 mm to Bregma (Page 16, Line 14) is not a correct one to show primary motor cortex.

5) Please justify the criteria for exclusion of mice from the analysis of behavioral data.

6) Please discriminate between biological and technical replicates, and properly treat them in statistical analysis. In Figures 2D and 6D, the technical but not biological (n=6 each) replicates are presented, which is incorrect.

7) A high resolution (>300 dpi, uncompressed) image of the whole gel with molecular weight markers for Western blots in Figure 4B should be submitted as a supplementary data for review. The CypD in Figure 4B does not seem to represent mitochondrial morphology or localization, especially when the morphology features in Figure 2A are used as a reference.

Minor points:

8) Supplementary Table 1: The number excluded for each condition should be stated. Twenty pregnant mice were used (Supplementary Table 1) but not described in the main text.

9) The dose of ketamine/xylazine should be stated as mg/kg body weight.

10) The rationale for CsA treatment should be described with citation in such a way that the credit is given to the original study.

11) Define abbreviations before using them, eg.,"DTI" (Page 16, Line 46). "10 μM/L" should be "10 μM".