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**Evaluation of the Ph.D. thesis :
“Role of Stim2a protein in the neuroprotection in *Danio rerio*”**

**Submitted by: Rishikesh Kumar Gupta, MS
Supervisor: Professor Jacek Kuźnicki**

An increase in the incidence of neurodegenerative diseases is the growing problem of the modern world. The incidence of these diseases rises with age therefore the number of cases is expected to increase in the future as life spans in many countries continue to increase. Despite the progress in modern medicine, diseases such as Alzheimer's disease (AD), Parkinson's disease, or Amyotrophic Lateral Sclerosis, a major challenge for medicine and public health, currently have no cure but only care. Therefore the understanding of the mechanisms of these diseases and the involvement of cell processes in potentially neuroprotective actions are so important.

It is common knowledge that maintaining calcium ions (Ca^{2+}) homeostasis in the brain and regulation of Ca^{2+} signaling in neurons are part of neuroprotective mechanisms. However, cellular regulation of Ca^{2+} homeostasis is a complex process involving many components regulating Ca^{2+} entry into neurons, storage and removal.

In this thesis, Rishikesh Kumar Gupta investigated the role of one element of this complex intracellular Ca^{2+} regulation system, member of stromal interacting molecule (STIM) family that sense tiny changes in the levels of Ca^{2+} stored within the endoplasmic

reticulum lumen – STIM2a - in neuroprotection.

The thesis is presented as a manuscript created in a classic way, divided into five chapters (introduction, materials, and methods, results, discussion, and conclusions) followed by a list of references, list of author's publication, and Appendix containing four tables that present interesting and important additional information.

The main text is preceded by abstracts in Polish and English, a list of abbreviations.

The thesis' text is supported by diagrams, figures, and tables, which show in a concise form the methodology and the results of the experimental work. This way of thesis presentation gives a clear picture of the studied subject and allows to estimate the scientific value of each studied problem separately and also the thesis as an integrated investigation. However, I found many unexplained abbreviations and many type mistakes in the text that make the reading a bit difficult.

The Introduction gives a reader the compilation of basic knowledge concerning mechanisms of Ca^{2+} homeostasis in neurons supported by the diagram showing main pathways of calcium entry, storage and removal. However, the diagrams presenting the structure of STIM and the store-operated calcium entry (SOCE) signaling, in which STIM proteins are involved, seem to be indispensable for this part of the text.

In the Introduction, we can also find information concerning calcium signaling and the role of STIM2 in neurodegenerative diseases. This part of the text is however in my opinion too short and does not give the necessary information about the role of calcium in these diseases and the basic mechanism triggered by Ca^{2+} and leading to neurodegeneration.

The Methods chapter presents a really impressive wide spectrum of modern methods used in experiments presented in this thesis. Creation of mutant zebrafish and maintaining their genotype, behavioral methods, calcium imaging, and gene expression analysis carried out on samples obtained in different ways together with analysis using web databases indicate the author's good knowledge of modern methods that are used for this type of experiments. The methods description is complemented by several figures which make the understanding of used procedures much easier. However, the part dedicated to statistical analysis is not satisfying. It should describe all the tests used in the analysis of the results and indicate the p-value accepted as statistically significant.

The Results chapter presents interesting results that indicate a huge experimental work that was performed by the author. The results confirm that *stim2a*^{-/-} zebrafish mutant is a good material for performed experiments, something that is hard to achieve in similar rodent

mutants, and that the mutation did not change the expression of other components of a SOCE signaling system. Behavioral experiments indicate that *stim2a*^{-/-} mutants show higher thigmotaxis, hyperactivity, and lower phototaxis than wild-type animals.

The observed by the author changes in animals' behavior, especially hyperactivity, gave me an idea that this behavior resembles that observed in attention deficit hyperactivity disorder (ADHD). To my knowledge, the involvement of *stim2a* deficit in ADHD was never investigated, so maybe this idea has a future.

I have only one comment concerning the part describing visual-motor response. The author mentioned that glutamate present in the medium can infiltrate the zebrafish skin and reach the brain but does not explain how it is with pentyleneetetrazole. Does it also penetrate the skin? And if not, how does it affect the animal?

Calcium imaging showed that mutant zebrafish presented increased Ca²⁺ spike frequency in neurons indicating increased neuronal activity. This may be connected with changes in behavioral experiments.

The analysis of selected gene expression showed crucial differences between *stim2a*^{-/-} mutant zebrafish and WT. Several genes in which significant differences in their expression were found are part of the calcium-signaling toolkit, encoding proteins involved in Ca²⁺ signaling and homeostasis. This is an interesting finding showing the correlation between the presence of fully expressed *stim2a* and cell calcium homeostasis. Identification of the distinct cell clusters using single-cell RNA sequencing gave also interesting results indicating significant differences between WT and (*stim2a;stim2b*)^{-/-} mutants. What cut my attention in these results are two unidentified clusters. Did anybody try to analyze them closer?

The Results chapter presents results that are interesting and bring new knowledge in the field of regulation of calcium homeostasis in neurons. However, I have one comment – the author constantly repeats, both in the text and figure legends, information that was previously placed in the Methods part. This repeating is unnecessary and only distorts the description of the results.

The Discussion, although not easy to read, discusses the results in a comprehensive balanced way and the relevance of obtained results was assessed with caution that indicates an author's understanding of the subject. The Discussion also shows the ability of the author for critical appraisal of the results, supported by comparing presented results with results presented by other authors that are discussed in a competent way. We can find here a good justification for using the zebrafish mutant model and the connection of *stim2a* with neurodegenerative

diseases. The author presented also a logical connection between changes in selected genes expression observed in *stim2a*^{-/-} mutant with changes observed in these diseases.

The Conclusions placed at the end of the Discussion sum up observations resulting from the analysis of the results and set further directions for research. There is no doubt that the thesis presented for review is written in accordance with the art of writing scientific papers and that the results are not only interesting but also important. The fact that part of these results was published in an international journal with high IF confirms their importance.

It is also worth mentioning that Rishikesh Kumar Gupta is the first author or co-author in six papers published in very good journals.

In conclusion, the Ph.D. thesis "Role of Stim2a protein in the neuroprotection in *Danio rerio*" submitted by Rishikesh Kumar Gupta, MS, meets all the requirements of Polish government acts regarding conferring a doctoral degree. Therefore I recommend it for acceptance and to award the candidate the Ph.D. degree.

Przedstawiona do oceny rozprawa doktorska spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz. U. 2018 poz. 1668). Dlatego też, przedstawiam Radzie Dyscypliny Nauk Medycznych WUM wniosek o dopuszczenie mgr inż Rishikeshgo Kumar Gupty do dalszych etapów przewodu doktorskiego.

Z poważaniem

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