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Review of the Ph.D. Thesis entitled „Role of Stim2a protein in the neuroprotection in *Danio rerio*" by Rishikesh Kumar Gupta

Calcium signalling and homeostasis are important processes in cells, particularly in neurons and glial cells. The concentration of calcium ions in cytoplasm is very low 0.1 μm while in the extracellular matrix is about 1 mM, so influx of calcium ions has to be precisely regulated. It is known that Ca^{2+} concentration increases after release of ions from internal stores, mainly from endoplasmic reticulum but also after opening voltage and ligand-gated channels in the plasma membrane. After many calcium-dependent processes, calcium ions have to be efficiently removed back to the internal stores and pump out to the extracellular matrix. Mechanisms of both processes are still not fully recognised yet, however, they are currently studied in many labs. One of them is the lab of Prof. Jacek Kuźnicki who is a supervisor of R. K. Gupta and who is an expert in calcium signalling and homeostasis.

The study by R. K. Gupta described in his thesis is important and timely. The Author has tried to describe the role of Stim2a protein in neurons of the zebrafish brain at early stages of development. This protein belongs to STIM proteins of the store-operated calcium entry (SOCE) system which detects low level of Ca^{2+} in ER, activate Orai/TRP channels and SERCA calcium pump. Since the concentration of Ca^{2+} in the cytoplasm is critical for calcium signalling and cell survival, the role of STIM proteins seem to be very important. They have already been studied in mice and their functions seem to be very important in many processes in neurons, so the lack one of them, Stim2a, is lethal in mice. It seems that using another model species, zebrafish,

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is a good choice to study the role of Stim2a in neurons. Zebrafish is an important model species to study various processes, especially during development because of its transparent body and a possibility of generating many mutants. Zebrafish has two isoforms of Stim2, Stim2a and Stim2b encoded by *stim2a* and *stim2b* genes, respectively.

In the thesis the Author used *stim2a* mutant obtained using CRISPR/Cas9 technique and tested behaviour, Ca^{2+} concentration changes in neurons and gene expression in various group of neurons and single cells.

R.K. Gupta found that *stim2a* mutants are hyperactive, their thigmotaxis is increased but phototaxis is decreased. In neurons of the optic tectum, Ca^{2+} fluorescence oscillations, using GCaMP5G sensor, had higher frequency than in wild-type (WT) fish, which was further increased after exposure to glutamate. Gene expression study showed that in the mutant 86% of genes out of 392 was upregulated but 14% downregulated. In turn single-cell RNA sequencing showed 13 different cell clusters in WT but 15 in the double mutant *stim2a:stim2b*$^{-/-}$.

The thesis is written on 102 pages, however, most pages contain tables (29 pages) following the main text and references (15). The thesis is written in English and is divided into 5 chapters: Introduction, Material and Methods, Results, Discussion and Conclusion and future directions. The Introduction is preceded by Acknowledgements, Table of contents, List of figures, List of tables, List of abbreviations, Abstract in English, Abstract in Polish. At the end of thesis there is a list 167 references, publications of R.K. Gupta and conferences which he has attended. In the Appendix there are five tables with name of genes which expression has been detected in various experiments and function of CaTK proteins encoded by genes in WT and (*stim2a:stim2b*)$^{-/-}$.

Introduction is very short, only 6 and half pages. The Author described calcium signalling and Ca^{2+} homeostasis inside the cell via SOCE, calcium signalling in neurodegenerative diseases and the role of Stim2 in neurodegenerative diseases, and Stim2 in zebrafish. Nothing is written about zebrafish as a model species and about the role of glia in Ca^{2+} signalling and homeostasis. Even other Stim proteins are not described in the Introduction. Calcium signalling in neurodegenerative diseases is summarised in 10 lines of the text. There is one good figure (Figure 1.1) but this figure is not prepared

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by the Author. I hope that the Author has a permission from the publisher to use this figure in his thesis.

In the Material and Methods section (10 pages, 5 figures, 4 tables) R. K. Gupta described methods and next listed materials in several tables. Usually materials are presented first following by methods. In this section there is no information about strains used in the study. The methods are adequate but they are described in a confusing way, for example: "PCR combined with enzyme restriction digestion (RD) was used to determine the WT and *stim2a-/-* fish. What was it determined. Embryos were collected in a Petri dish (50 larvae/dish)….". Embryos or larvae were collected. Transgenic lines used in the study are not described, as well as other abbreviations: TE buffer, dpf embryos etc.
The Author used *stim2a*$^{-/-}$ mutant created using CRISPR/Cas9 technique, one line with a calcium sensor and the double mutant (*stim2a:stim2b*)$^{-/-}$, however, it is not explained how the double mutant was obtained and why it was used only in one experiment. Using the mutant *stim2a*$^{-/-}$ the Author carried out behavioural experiments testing motor activity in a normal medium (E3) and after exposure to glutamate or PTZ, thigmotaxis in an open field test, dark/light preference test and visual motor response test in the normal medium (E3) as well as after exposure to glutamate or PTZ solution. In the visual-motor response test larvae were tested in 0%, 70% and again 0% light intensities. What does it mean 70% light intensity? How was it obtained?
After behavioural tests the Author carried out *in vivo* Ca^{2+} imaging in cerebellum, habenula and optic tectum. The method is illustrated in Figure 2.4 but images are too small, dark to find out what the Author was measuring. According to the Author: "The number of cells that were analysed 392 cells from seven *stim2a*$^{-/-}$ animals and 168 cells from three Tg animals". It is not clear which cells were analysed in the mutant and the control. I assume that Tg are control animals but the genotype of Tg should be provided.
Finally the Author described qRT-PCR gene expression analyses of 30 larvae per a sample. It looks that only the SOCE genes were analysed. Another methods a single-cell RNA sequencing was used on larval heads and sorting out cells with the GCAMP5G fluorescent marker.

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After describing the methods, the Author listed chemicals used in one table, and in the following tables: cell culture media, buffers, equipment and software.

The in the Results (22 pages) the mutant *stim2a*$^{-/-}$ was described in the first chapter. It looks that the mutant survived better than WT at the age of 96 h. How should it be explained? What is the mutant and WT longevity?

Next the Author analysed SOCE transcripts in the mutant and WT and did not detect any significant changes. In the behavioural studies R. K. Gupta detected more differences between the mutant and WT, however, surprisingly both lines were not so different in the applied tests. The mutant showed higher mobility, however, when larvae were tested in the third phase of 15 min. test, there were no differences between the mutant and WT.
The mutant also showed lower phototaxis than WT. It is possible that in the mutant the lack of Stim2a protein in photoreceptors may affect the process of phototransduction. It would be worth to test it. In turn in the visual-motor response (VMR), the Author concludes that no significant difference were detected in mobility between *stim2a*$^{-/-}$ mutant zebrafish larvae and WT in the baseline in the first stage of the experiment. According to the previous experiment (open field test) the mutant should show higher mobility.
In turn during the light period of VMR, both the mutant and WT had low activity with slightly higher activity observed in the mutant. How could the effect of light be explained in all behavioural experiments? In another experiment the same test VMR was repeated after exposure to glutamate or pentylenetetrazol (PTZ). Why were these chemicals used and how was the concentration established? In the thesis there is no information that PTZ is a $GABA_A$ receptor antagonist. It looks that after glutamate or PTZ fish could not distinguished between darkness and light and similar effects of both chemicals were in the mutant and WT. How could it be explained?

Calcium imaging in the brain showed higher frequency but low amplitude of oscillations in the mutant comparing with WT but after glutamate exposure only slightly higher frequency of Ca^{2+} oscillations were observed but not changes in the amplitude.

Gene expression analysis by next-generation RNA sequencing showed mostly upregulated genes, 336 out of 392 in the mutant. Again nowhere is

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written in the thesis that in zebrafish, 26 206 coding protein genes have been identified. In the next step the Author reported single-cell RNA seq analysis from cells of neuronal origin in WT and in the double mutant (*stim2a:stim2b*)$^{-/-}$ to identified cell clusters and Calcium toolkit (CaTK) genes. Why were glial cells identified in 13 cell clusters in WT if a neuronal marker was used to select cells for analyses? For comparison of CaTK gene expression between WT and the double mutant only 6 clusters of 13 identified earlier in WT were used. Why was this procedure applied? This experiment showed that 77 genes were common in the both WT and double mutant, only 11 in WT and 25 in the double mutant.

My general comment for all results obtained in the thesis is that the results are interesting but I do not understand why for different experiments larvae of different age were used. It is also possible that differences between WT and the mutant *stim2a*$^{-/-}$ were small or not observed because of a compensation by *stim2b* gene. The results related to gene expression are quite confusing and I think this study has to be continued.

Discussion and Conclusions and future directions takes 10 pages. In the Discussion the Author concludes that zebrafish can be used as a model species to study calcium homeostasis. It is already known from other studies. In the next chapter of the Discussion R. K. Gupta describes several genes which were highly upregulated and one downregulated in the mutant with their possible functions, however, their functions were not examined in the present study. Behavioural experiments are discussed next, but some explanations, for example the reduced phototaxis in the mutant are rather speculative. The Author writes: "the reason for such irregularity in phototaxis might be that the brain network connectivity controlling such behavior might have altered due to the absence of Stim2a isoform".

In the chapter about calcium oscillations of the Discussion, it is concluded that in the mutant there was higher frequency and higher amplitude of Ca^{2+} oscillations in neurons than in WT while in the Results the higher frequency is shown but the amplitude was smaller in the mutant.

Other comments and questions:

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The title of the thesis does not reflect its content. The aim of this study, according to the Author, "was to identify the new candidate genes involved in Ca^{2+} signalling in all types of zebrafish neuronal origin cells". However, later in the text there are also mentioned glial cells and glial precursors. In my opinion the thesis is about the role of Stim2a protein in zebrafish. Regarding Stim proteins, nowhere is written that Stim means stromal interacting molecule.

In the table with abbreviations not all abbreviations used in the thesis are listed.

There are many terms which are incorrect, for example: 1) *stim2a* deletion – this gene was not deleted but its fragment (Abstract), 2) higher activity in the low activity phase in the visual-motor response test (everywhere in the text). Instead of neuronal junctions it is better to use the term synapses and instead of spines of neurons it is better to use dendritic spines.

There are sentences in the thesis which are difficult to follow, for example: "The SOCE channels are mainly mediate the Orai proteins and transient receptor potential (TRP) channels.." (p.7, para1, l. 3); Since Ca^{2+} is known to control memory formation… and during the developmental state of neurons…".

"The mushroom spines in postsynaptic hippocampal neurons were less in the amyloid precursor knock-in mouse AD model" (P. 9, para 2., l.11).

Names of proteins and genes are not written consistently. The Author uses both capital and small letters for names of proteins and genes.

In summary, the Author has carried out many experiments which were technically challenging. The obtained results are new and interesting, however, they should be continued because the observed changes in gene expression involved in calcium homeostasis are difficult to explain now.

In my opinion the Doctoral Thesis of Rishikesh Kumar Gupta fulfils criteria defined in the article of the 13th Act of March 4, 2003 regarding degrees and title in science and arts in Poland [Rozprawa doktorska spełnia warunki określone w art. 13 Ustawy z dnia 14 marca 2003 ro. O stopniach naukowych I tytule naukowym oraz o stopniach I tytule w zakresie sztuki (Dz. U. z 2016 r. poz. 882)].

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I propose to the Faculty of the Warsaw Medical University to proceed the Ph.D. procedure for Rishikesh Kumar Gupta.

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