Dr hab. Paweł Pomorski, prof. Instytutu
Instytutu Biologii Doświadczalnej
im. Marcelego Nenckiego,
Polska Akademia Nauk

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Review Report on the PhD thesis

submitted to Medical University of Warsaw,
Author: Rishikesh Kumar Gupta, MSc
Title: "Role of Stim2a protein in the neuroprotection in Danio rerio"
Scientific Supervisor: Prof. dr hab. Jacek Kuźnicki

The presented review report is organized in the following sections: project background, general description of the thesis, specific comments followed by a final evaluation statement.

Project Background

Free calcium ions are one of the most widespread regulatory elements in the living world. The changes of those ion concentration in the cell cytoplasm evolved as a common and important way the signal is transmitted in the cell and between the cells. The term "calcium signal" is understood as the rapid rise of the cytoplasmic concentration of the free calcium ions from very low, nanomolar level to the micromolar concentration, which activates a plethora of calcium binding proteins. There are two sources of calcium ions which may enter the cytoplasm: extracellular environment and cisternae of endoplasmic reticulum, known as the "intracellular calcium store". In the second case, the signal leads to the emptying of aforementioned cisternae, and further influx of ions from the extracellular environment is needed to restore the store calcium content. For years it was unknown how the fact of store emptying could be transmitted to the cell membrane to open ion channels and replenish calcium ions. In 2005 this mechanism was finally uncovered and the calcium sensor STIM became the key factor in making the cell ready to transmit calcium signal. There are however two basic types of the STIM proteins, STIM1 and STIM2, and if we have a well crystalized view, how STIM1 protein works, there are still controversies what is the role of STIM2 protein, especially in excitatory cells. This is why further work on

the STIM2 biology is so important and why Rishikesh Kumar Gupta has chosen STIM2 protein as the object of his study.

General Description of the Thesis

The PhD thesis submitted by Rishikesh Kumar Gupta has a classical layout. It contains four canonical parts: Introduction, Materials and Methods, Results and Result Discussion, followed by a summary of the results, totaling to 65 pages. The separate part of the thesis is a list of the cited literature, containing 167 positions. At the end of the thesis, author places an extensive set of tables, containing detailed results of the RNA sequencing. Additionally, the thesis is preceded by a bilingual abstract, provided both in Polish and English and very elaborate Acknowledgements.

Introduction is a short description of the cell calcium signaling with the emphasis put on the pathways present in the neuronal cells. The most important proteins engaged in this complex mechanism are enumerated and main pathways described. The concept of calcium homeostasis is also introduced and Capacitative Calcium Entry, as calcium stores restoration process explained. In this context, STIM2 is introduced and its function in neurons sketched. Finally the context of neurodegenerative diseases is overlaid on the STIM2 physiology. The chapter ends with a detailed description of the current knowledge about STIM2 in zebrafish biology.

The Materials and Methods description is very extensive and concise at the same time. Methods are described properly and allow the reader to fully comprehend results presented in the next chapter. The range of described methods is impressing and shows how much work Rishikesh Kumar Gupta put into the thesis preparation. First, preparation of the mutants was described, CRISPR/Cas9 knockout of *stim2a* gene, as well as preparation of (*stim2a*/*stm2b*)$^{-/-}$ double mutant by crossing of this mutant with *stim2b* negative fish prepared by another PhD student. Then animal husbandry and behavioral experiments were described, followed by calcium measurement protocol. Since calcium measurements were performed in the live fishes, the protocol, even if the author applied non-ratiometric GCaMP5G probe, was difficult and required extensive image processing. Finally, the whole process of RNA sequencing was described, both bulk sequencing as the single cell sorting and sequencing in the case of the double mutant. All that on the twelve pages.

The heart of the thesis is the Results chapter, consisting of the three main parts. First part describes differences between behavior of the larvae of wild fish and *stim2a*$^{-/-}$ mutant . As thigmotaxis test shows, *stim2a*$^{-/-}$ mutant answers much with stronger anxiety to the change of environment. That difference is statistically significant on the level of $p<0.001$ so the effect of mutation is very strong. On the other hand, the difference of larvae behavior in dark and light was strongly reduced in the *stim2a*$^{-/-}$ mutant as shown by dark/light preference test. However, in the visual-motor response test mutant larvae showed a strong response to the illumination, even if weaker than wild type larvae. Finally, the author tested the reaction of larvae to pentylenetetrazol and glutamate, to test the influence of the *stim2a*$^{-/-}$ mutation on the effect of chemical behavior modulators. In both cases the reaction of *stim2a*$^{-/-}$ mutant was similar to that of the wild fish. The second group of experiments showed the

frequency and amplitude of calcium transients associated with neuronal activity. The presence of *stim2a*$^{-/-}$ mutation led to the rise in the frequency and fall of the amplitude of observed calcium oscillations. Use of glutamate diminished the difference in amplitude to an insignificant level. The third part, the largest, presents an effect of RNA sequencing. First, the author has found profound differences in the RNA present in wild type zebrafish larvae and in *stim2a*$^{-/-}$ mutant larvae. Then, the author performed similar analysis on (*stim2a/stm2b*)$^{-/-}$ double mutant, this time sorting brain cells before single cell sequencing. This time he found differences not only between wild type and mutant larvae but also those differences were influenced by the type of studied cells.

Finally, on the nine pages Rishikesh Kumar Gupta discussed his results with the literature. After discussion we find a short summary of conclusions, concentrating on the changes in gene expression pattern caused by *stim2a* mutation.

Specific Comments

Presented thesis impresses with the amount of data it includes. It is also impressive from a methodological point of view. For such large work, the thesis is however very compact and it has a price.

The first missing part appears in the introduction. Author describes a vast number of channels, however completely ignores metabotropic calcium signaling, where G-protein coupled receptors evoke calcium signal via activation of phospholipase C and production of inositol trisphosphate (IP3). When IP3 opens IP3 receptor channels on endoplasmic reticulum, the calcium signals appear and at the same time Capacitative Calcium Entry begins. The fact that the author has not mentioned metabotropic signaling in the introduction is strange, since it is common in the neurons.

Author also tends to make shorthands and thought abbreviation, eg. in the same introduction, he states that voltage gated calcium channels open when "membrane potential develops", while voltage gated mean, that those channels are closed by membrane potential and open when it depolarize, in the very first phase of action potential. Has the author intended to write "action potential develops"?

Author also tends to ignore any need for justification in the experiment design. Certainly, studying such complicated phenomena, on so many organizational levels, some choices have very practical reasons: the availability of equipment or transgenic animals. But not all, for sure. It would be interesting to know, why those behavioral experiments were used and why visual-motor response test was used to study the neuronal activity chemical modulation?

It would be also interesting to check the influence of stim2a mutant on the basal level of calcium in the endoplasmic reticulum. There are hypotheses that this is the main role of STIM2 protein. The use of a ratiometric probe (chameleon-type) with already mastered image processing should be possible and it should reveal possible changes in the basal cytoplasmic concentration. Is this possible?

It would be also very interesting to perform exactly the same sequencing on the double mutant and single one and show side by side before performing the single cell analysis. This would place the final analysis in the proper perspective. In this place also one more concern appears, difficult to cope with, I am afraid. Before sorting, cells from the zebrafish larvae have been dissociated. In the case of neurons, basically cell bodies survive such procedure. How could it influence RNA sequencing, since we do know that vast amounts of RNA is present in neuron processi? What control is possible for this potential problem?

Final Statement

All those concerns are, however, the effect of the immense amount the author decided to describe in the thesis, and some flows are inevitable if somebody tries to face such a large amount of data. As Joseph Conrad said, "It's only those who do nothing that make no mistakes". Summarizing, the results are well presented and their interpretation is at the highest scientific level. This thesis is ready to be defended orally and certainly meets the requirements laid down for the degree of Ph.D. by the statutes in the Journal of Laws of the Republic of Poland (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)).

Paweł Pomorski