Referee's Report on the Ph.D. thesis of Mgr. Miloš Rokić entitled:

"The functional role of disulphide bonding and extracellular vestibule in rat P2X4 receptor"

The Ph.D. thesis by Mgr. Miloš Rokić is focused on investigations of the molecular mechanisms of the purinergic P2X4 receptor channel activation and modulation. Particularly, the new results demonstrate the importance of conserved ectodomain cysteines and disulphide bonding in the channel functioning and identify amino acids in the extracellular vestibule that are important for channel gating, vestibule enlargement and receptor expression in the plasma membrane. The new results presented in this work are highly topical in the field and are based on two publications in refereed scientific journals (Physiological Research 59: 927-935, 2010 and PlosONE, 2013).

The Ph.D. thesis is written in English and is composed of Introduction in which the author presents the interest and novelty of the topic in the wider context of the purinergic signaling research (27 pages). The aims of the work are clearly formulated in two specific points, which include (i) the identification of critical disulfide bridges in the ectodomain and (ii) elucidating the role of extracellular vestibule residues in the P2X4 channel functioning. The Methods section (10 pages) is followed by the Results section (18 pages) which is logically structured into two subchapters. The Discussion (7 pages) very well and compactly covers the experimental results obtained from both interrelated studies. The thesis is well written and illustrated by relevant figures. There were some rare small inaccuracies and misprints that were identified and corrected immediately after the Ph.D. thesis internal defense.

The novelty of the obtained results consists in observation that the conserved ectodomain cysteines and disulphide bondings between specific cysteine pairs are important for ATP induced channel activation/deactivation kinetics. In addition, the results convincingly confirm the structural importance of six residues located in the extracellular vestibule of the P2X4 channel and characterize their role in ATP induced activation. Among the new key results that clearly deserve appreciation (beyond those already mentioned) is the very nice demonstration of the role of the glycine 325 as a gating hinge which is in consonance with the recently published crystal structure of the open state of the zebra fish P2X4 channel ortholog.

Beside the impressing amount of new experimental data that were obtained in this work, I value the way both the new results and the limitations of the experimental approach used to answer the experimental questions are discussed with the help of the most recent literature.

In conclusion this thesis contains a large amount of original material and is a well-polished piece of work. The clarity of the text is excellent and despite the underlying technical details about the effects of a great number of various mutations it is even absorbing during reading. The candidate has demonstrated a high degree of expertise in a variety of relatively sophisticated electrophysiological and molecular biological techniques.

Questions:

1. Page 44, the author states that patch electrodes were filled with intracellular solution containing 11 mM EGTA. Why exactly such concentration of EGTA has been used?
2. In the methods section of the accepted paper the authors state that: "…Hill coefficient was fixed to 1.3 in all experiments, a value obtained for the WT receptor by fitting." . In contrast, in the methods of this thesis it is stated that the concentration-response data were fitted by a three-parameter logistic equation. Which of these two approaches was used in this dissertation? Are the authors justified in using the fixed Hill coefficient if any of the mutations could affect the cooperativity of the channel activation?
3. Is it correct to say: "..the current amplitude was rescued by IVM.." (Page e.g. 51, 69)? What does it mean in terms of the channel function or in terms of positive allosteric modulation effects?
4. Figure 4.9, panel A, the correlation between the log EC_{50} values and the hydrophobicity for the F324 mutants does not seem to be perfect. How was the correlation coefficient determined (0.46)? How the author explains the observation that tryptophan at the position of F324 most effectively shifts the apparent affinity to the right even thought the hydrophobicity is well comparable to phenylalanine?

Conclusion:

In my opinion, Mgr. Miloš Rokić possesses the requisite qualifications, powers of original thought, talent for research, and has proved to be capable of efficiently solving new research problems. Parts of the Ph.D. thesis have been already published/accepted for publication in reputable impacted scientific journals and, in the form of abstracts, in several international conference materials. The thesis significantly contributes to the advancement of research in its specific area and fulfills the requirements for the degree of Ph.D.. Therefore, I recommend Ph.D. thesis submitted by Miloš Rokić for acceptance by the Committee.

Praha, 4th March 2013

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