Official opponent's report

of assessment scientific and practical value of the PhD thesis

by Mrs **Olesia Lunko**,

titled "**Modulation of Large conductance cationic channels of the inner nuclear membrane of rat cerebellar Purkinje neurons.**"

for candidate's degree by speciality – 03.00.02 – biophysics

Relevance of the research.

The PhD thesis of Olesia Lunko is devoted to investigations of large conductance cationic channels (LCC channels) localised in the inner nuclear membrane. The channels seem to be prominent cell actors, acting on neuron electrical dynamics operating different conformations states. Therefore, it is critical to focus on the channel's physiological role, structure, and amino acid sequence. Thus, it is essential to survey the kinetics of single LCC channels with new blockers that are undoubtedly an urgent task. The patch-clamp registrations of single-channel kinetics provide crucial information related to the conformational states. Using a rather non-trivial approach, beta-distribution analysis, the author has suggested the number and duration of conformational states for native LCC channels. The chosen method estimated the time scale of the states in the microseconds range. The proposed kinetic model of the channel was used to determine the mechanism of action of pharmacological agents and opens broad prospects for further studies of LCC channels' structure and functions.

Dr. Leonid Savchenko

University College London, Gower Street, London WC1E 6BT

Tel: +44 (0)20 7679 2000

leonid.savtchenko@ucl.ac.uk

www.ucl.ac.uk

Scientific novelty of the obtained results, their theoretical and practical significance.

In the thesis, the author presents several actual results. For the first time, the author recorded LCC channels' kinetic properties and proposed a relevant kinetic model. Also, the author invented tubocurarine as a blocker of the LCC channel and recognised gadolinium's effect. Additionally, using an appropriate kinetic model, the properties and the blocking mechanism of the non-depolarising agent gallamine have been identified.

The submitted thesis presents a deep and rich analysis of single-channel data containing a recording of numerous conformational transitions, including fast events lasting in microsecond time range and events associated with various sublevels and intermediate states. Original experimental and theoretical results demonstrate the importance of high-quality single-channel analysis to calculate fast blocker effects and estimate pharmacodynamic parameters.

The obtained results are helpful to study molecular interactions between LCC channels and different chemical modulators. The description of the mechanism of gallamine action using the kinetic model with five states leads to a suggestion about some LCC channel structure features.

The validity and reliability of scientific statements and conclusions of the dissertation.

The reliability of the scientific provisions submitted for defence is based on a large array of patch-clamp recordings of single channels currents in the configuration inside-out, which fully meets the purpose and objectives.

Connection of work with scientific programs, plans, themes.

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www.ucl.ac.uk

The dissertation was performed following the research topics of the Bogomoletz Institute of Physiology of National Academy of Sciences of Ukraine "Cellular and molecular mechanisms of functioning of brain neurons in normal and pathology."

The structure and scope of the dissertation, assessment of its completion and compliance with established requirements.

The dissertation of Olesia Lunko corresponds to the generally accepted form under the Ministry of Education and Science of Ukraine requirements. It consists of an introduction, a review of the literature, materials and research methods, a presentation of the results of own research, a discussion of the results and conclusions. The list of references contains 305 links. The dissertation is presented on 155 pages, includes 5 tables and is illustrated with 47 figures, which give a complete picture of the scope and quality of research.

The **introduction** explains the importance of nuclear ion channels for the cell functioning. The author outlines the current state of research and emphasises the significance of filling the gap in knowledge relating to LCC channels' kinetics and importance in determining their pharmacological profile for studying processes occurring in the nucleus of living cells.

Section 1 (Literature review) consists of two main parts, which cover general information on the selected topic.

The first part describes the structure of the nuclear envelope and the transport systems of the nucleus. The second part of the thesis outlines the types of modulation and the various mechanisms of blocking LCC channels. All the issues considered are entirely consistent with the main areas of research in this work. The literature for this part is carefully

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www.ucl.ac.uk

referenced, and a comprehensive bibliography contains all the relevant papers for the discussed field. Most of the references are relatively new, showing the problem of the scientific area. This part is well written and presents an excellent background for following the rest of the thesis.

Section 2 (Materials and methods) explains the procedure for isolating Purkinje neurons' nuclei to obtain access to an inner membrane for patch-clamp current recordings. Data of recordings were carefully analysed via step-by-step instructions for beta distribution analysis with corresponding illustrations. The author demonstrated a high ability to choose appropriate research methods and apply them to reach the thesis's goals.

Section 3 (Research results) consists of three parts. The first part presents the results of examining substances that can potentially block transmembrane currents of LCC channels. Venoms, toxins and anaesthetics, as well as metal ions, were tested. As a result, the first found blocker for LCC channels was tubocurarine. This compound's effect was characterised by fast blocking (flicker-block) of LCC channel, which led to a voltage-dependent decrease of the apparent amplitude of the current. The modulating effect of trivalent gadolinium ions was also found.

Another blocker proposed in this work, gallamine, had an even more pronounced flicker-blocking effect than tubocurarine. This effect leads to significant difficulties for an adequate assessment of the pharmacodynamic parameters of blocking; therefore, it was necessary to construct a proper kinetic model for native LCC channels.

In the second part of the work, β-distribution analysis was used to characterise the rapid events in the registration of LCC channel currents under control conditions to build a

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corresponding kinetic model. The steady and non-steady state characteristics of LCC channels were also investigated, and the kinetic parameters of slow events were evaluated to fully describe the transitions between the primary conformational states of LCC channels. The channel was found to have one open state associated with three closed states (slow, medium and slow) and one sublevel. It was shown that the conductance of LCC channels is stable in the range of membrane potential from -80 mV to +80 mV. The rate constants for transition between the open state and all other states connected with the open state exhibited a clear dependence on the voltage.

In the third, final part of this work, the effectiveness of the established kinetic model together with the use of the β-distribution analysis was demonstrated on the example of the study of the blocking mechanism of the fast LCC channel blocker - gallamine. Rapid blocking was characterized by the dependence of the rate of association of blockers on the concentration of gallamine. Also, the parameters of the sensitivity of the blocker to the applied voltage were set. The chosen method for analyzing the kinetics of LCC channels made it possible to establish that gallamine acts as a classical blocker of the ion channel.

The analysis revealed that the proposed kinetic model for native LCC channels and β-distribution analysis are powerful analytical tools to define mechanisms and kinetic parameters of single-channel current modulation.

Section 4 discusses the research results; the most important contributions are highlighted and compared with previous studies on this issue.

The conclusions of the work, in general, correspond to the tasks and based on the obtained results.

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www.ucl.ac.uk

The results presented in the dissertation were published in 6 articles and 10 abstracts and reported at scientific conferences.

The thesis is written well, with occasional minor grammatical and spelling mistakes. Some figures are composed of parts in different directions, and axes signatures have different positions. Moreover, units of measurements for rate constants are missed in figure 3.18. The exponent is written in various forms in mathematical formulas. These are, however, minor issues that can be easily corrected.

During the review of the thesis, I had the following questions:

1. The relaxation time at transitions between channel states in the opposite directions of membrane voltage modification is not the same. I would appreciate any idea what this may be related to this observation? Are there similar examples among other ion channels?

2. To select the kinetic model, you analysed only those most common events in registering single LCC-channels current. Is it possible to include rare events in the data analysis?

3. Calcium and lanthanide ions such as gadolinium have very close radii. How do you think about why they have a different effect on LCC channels?

4. Which frequency bins did you use in your data analyses? How does the bin width affect amplitude histograms?

5. Did you validate the method of beta-distribution analysis? How can you prove that rate constants are calculated correctly?

Conclusion:

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The present manuscript is a structured and independently written PhD thesis with the identifiable contribution of the author. The results are well presented, and their interpretation is at a pretty high scientific level. The relevance, scientific novelty, high methodological level, theoretical and practical significance, conclusions, prospects of the scientific and practical application of the submitted thesis meets the general requirements for this type of academic works according to paragraphs 9, 11, 12, 13 of the "Procedure for awarding scientific degrees", approved by the Cabinet of Ministers of Ukraine №567 of July 24, 2013 (as amended).

For the reasons named above, I recommend for acceptance the PhD thesis, entitled "Modulation of Large conductance cationic channels of the inner nuclear membrane of rat cerebellar Purkinje neurons" and to award Olesia Lunko the candidate's degree in Biology by specialty – 03.00.02 – biophysics.

Official opponent

Senior Researcher at the Department of Clinical and Experimental Epilepsy
Institute of Neurology, University College London.

Dr. Biol. Sci., PhD. Leonid P. Savchenko

25/03/2021

Dr. Leonid Savchenko

University College London, Gower Street, London WC1E 6BT

Tel: +44 (0)20 7679 2000

leonid.savtchenko@ucl.ac.uk

www.ucl.ac.uk