NEUROSCIENCE CENTER
HiLIFE UNIT

February 10, 2025

Re: Review of the PhD thesis manuscript of Diana Legutko-Bijoch, MSc.

To Whom It May Concern:

Thank you for the opportunity to comment on the manuscript intended as a PhD thesis by Diana Legutko-Bijoch. I would like to suggest the following comments on the manuscript.

Formal information on the thesis and Ph.D. student. Review of the PhD thesis of **Diana Legutko-Bijoch**, M.Sc., Laboratory of Neurobiology of the Nencki Institute of Experimental Biology Polish Academy of Sciences. Supervisors: Prof. Leszek Kaczmarek , Ph.D., D.Sc. and Dr. Ryohei Yasuda, Ph.D., D.Sc. Title of the thesis is "*Matrix Metalloproteinase 9 in dendritic spine plasticity*".

Evaluation of the scientific content:
The work described in the thesis has been generated at the labs of Prof. Leszek Kaczmarek and Dr. Ryohei Yasuda. The thesis consists of an Introduction of 24 pages, material and methods and results sections and discussion, altogether 101 pages, and it contains altogether 24 excellent and very clear figures. The manuscript is based on about 200 references. Altogether, the thesis is structured and presented in an excellent manner, it contains a lot of apparently unpublished data and the number of references, and their selection is satisfactory. The English language is fluent and essentially flawless.

The PhD student lists 8 **aims**:
1. Induction of sLTP: Induce sLTP under conditions excluding MMP-9 and analyse the timing of the resulting effects.
2. MMP-9 Release: Determine the precise timing of MMP-9 release induced by activation.
3. BDNF/TrkB Signalling: Investigate the role on MMP-9 and its activity in BDNF/TrkB signalling.
4. IGF1/IGF1R Signalling: Assess whether IGF1/IGF1R signalling is modulated by MMP-9 activity.
5. Role of IGFBPs: Validate the role of IGFBPs in sLTP and IGF1/IGF1R signalling pathways.
6. Dominant IGFBP Identification: Identify the dominant IGFBP expressed in the hippocampus.
7. IGFBP2 as an MMP-9 Substrate: Identify whether IGFBP2 serves as a substrate for MMP-9.
8. IGFBP2 Localization: Confirm the localization of IGFBP2 at synapses.

The aims are clearly defined and focus on elucidating the role of matrix metalloproteinase 9 (MMP-9) in structural synaptic plasticity. For all the aims, the student presents an impressive and consistent set of results and discusses them in a professional manner.

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The Introduction presents the themes to be investigated in a professional manner and it is well supported by the literature.

Materials and methods: The thesis employs diverse and appropriate methodologies for the study of synaptic plasticity, including genetic models, pharmacological inhibitors, advanced imaging techniques like two-photon microscopy, and biochemical assays. The integration of these methods demonstrates a solid and comprehensive approach to addressing the research questions. Methods used are presented clearly and with a sufficient detail. It was not quite clear from the manuscript whether the methods and results presented were all used and generated by the student herself, or whether results by other lab members or collaborators are presented; the manuscript that contains part of the results presented lists altogether 6 scientists. I got an impression that the author has herself used all the methods and generated all the results presented, but if results mainly generated by someone else are presented, it would have been good be explicitly mention this in the methods section, for example, when the particular method is being introduced.

The Results section presents an impressive set of data, most of which is apparently unpublished. The main findings of the aims 1-3 are included in a manuscript that has been posted in BioRxiv and apparently submitted to a journal, the student is the first author of the manuscript. I was surprised to find that a substantial number of useful control experiments included into the thesis were not part of the BioRxiv manuscript. Many of these unpublished data excellently support the findings, and I would like to encourage the authors if the BioRxiv manuscript to include these data as supplemental material into the manuscript when submitted. I paid attention to the variation in the maximal response between experiments shown in Figs 10C and 11 A, C, E (means between 200 and 350%, but this is appropriately clarified in the Discussion section. It was also not clear how the histograms in Figures 10 and 11 are derived from the curves presented. For example, in Fig. 10 C, the volume change between 1 and 3 minutes appears much higher than the about 200 presented in 10D. Nevertheless, these are very minor issues and do not diminish the value of the great amount of work done. Material in aims 4-8 was not included in any of the publications of the student and are probably now being written for a publication. I was impressed by the variety of methods applied, including expansion microscopy, detailed protein structure analysis to explore the localization and activity of MMP-9 and its substrates, and the creative use of in silico analysis, including utilization of Alpha-fold.

The conclusions are well-supported by the data and align with the stated research aims. They offer novel findings and insights into the role of MMP-9 in synaptic plasticity and its potential interactions with BDNF/TrkB and IGF1/IGF1R pathways.

The discussion contextualizes the findings within the current literature and explores potential implications and future directions. The discussion on the role of the IGFBP is perhaps a bit ambiguous, as it apparently has a dual role: to keep IGF-1 from binding to its receptor, but at the same time, concentrating IGF-1 in the vicinity of the receptor, thereby increasing signaling when IGF-1 is released. In the end, this issue is well discussed and the summary figure 24 clarifies it further. Altogether, the discussion highlights the role of MMP-9 in synaptic remodeling and the broader implications for understanding synaptic plasticity.

The manuscript is supported by over 200 citations that include a comprehensive range of relevant literature, demonstrating thorough knowledge of the field. The references are current and support the research context and discussion effectively. There is a very good balance between original papers and recent review articles.

Taken together, the thesis covers the subject area very well and I did not find any major mistakes in scientific reasoning or methodology. English language is fluent and nearly flawless. The dissertation provides several original findings and clearly demonstrates the role of MMP-9 in synaptic plasticity, providing novel insights into its interaction with the BDNF/TrkB and IGF1/IGF1R pathways. It significantly advances the understanding of how extracellular proteolysis is involved in regulating these pathways during synaptic plasticity.

The dissertation demonstrates that the Ph.D. student has obtained thorough theoretical knowledge of synaptic plasticity and related biological processes and technical skills to investigate these processes. The student's ability to design and conduct experiments, analyze data, and draw meaningful conclusions is evident, reflecting a capacity for independent scientific work.

Based on the evaluation criteria provided, this dissertation demonstrates high scientific quality, originality in addressing a complex biological question, and the Ph.D. student's competency in research. While minor issues could be improved upon, they do not significantly affect the overall excellent quality. Therefore, I would give this dissertation a grade of 'Outstanding', reflecting its substantial contributions to the field and the student's proficiency in conducting rigorous scientific research.

Sincerely,

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Krakow, 21 March 2025

Evaluation of the PhD thesis

„Matrix Metalloproteinase 9 in dendritic spine plasticity"

by Diana Legutko-Bijoch

prepared under the supervisors of Prof. Leszek Kaczmarek and Dr. Ryohei Yasuda

in the Laboratory of Neurobiology

of the Nencki Institute of Experimental Biology of the Polish Academy of Sciences

The PhD thesis of Diana Legutko-Bijoch focuses on the exploration of matrix metalloproteinase 9 (MMP-9) involvement in structural LTP (s LTP) on a single dendritic spine using in vitro approaches. The author examined the modulation of MMP-9 activity using chemical inhibitors and gene knockouts, and its interplay with neurotrophic factor signaling in organotypic hippocampal cultures (OHCs). Modern techniques of molecular biology and bioinformatics approaches have been implemented to identify the crucial substrates involved in the MMP-9 activity in the structural LTP. The topic addressed in the thesis is of broad interest in neuroscience and expands knowledge about the role of MMP-9 as a regulator of synaptic plasticity.

The thesis has been written in English as a standard monograph. The general title reflects the scientific content presented in the thesis. The structure of the dissertation is well-designed and informative. It follows a standard format, including Polish- and English-language *Abstracts* and five chapters (*Introduction, Material and Methods, Results, Discussion, Summary and Conclusion*). The total length of the thesis is 116 pages, including 13 pages of bibliographic references. On the last pages of the thesis, nine publications co-authored by the candidate have been listed. The work presented by Legutko-Bijoch was conducted in two laboratories, one in Poland (Nencki Institute of Experimental Biology in Warsaw) and one in the U.S. (Max Planck Florida Institute for Neuroscience).

The dissertation begins with an „*Introduction".* This chapter includes nine subsections: *Synapses, Synaptic plasticity, Long-term potentiation, NMDAR-dependent LTP, Dendritic spines, Structural LTP, Techniques for studying s LTP, Extracellular proteases and*

Neurotrophic factors in LTP. All sections are written clearly and well-grounded regarding the content related to the thesis topic. A significant part is focused on the specific molecular mechanisms involved in synaptic reorganization. The author describes the structure and types of synapses, the phenomenon of synaptic plasticity, and the mechanism of LTP, paying special attention to NMDAR-dependent LTP. This part of the dissertation contains nicely prepared figures, but the source/author of four of them has not been provided. Next, the author goes into a detailed description of dendritic spine architecture remodeling as a morphological hallmark of structural LTP as well as outlines techniques for studying the mechanisms of this process. The following part describes the role of proteolysis in LTP. The author included the most important reports related to the MMP-9 involvement in this process, pointed also molecular targets of MMP-9 and the crucial regulatory mechanisms of this metalloproteinase activity. Particularly interesting is the subsection concerning two neurotrophins identified to directly contribute to the synaptic plasticity. IGF1/IGF1R signaling is even more enjoyable. The "*Introduction*" chapter shows that the candidate has a good knowledge of the subject. However, as the PhD thesis concerns the involvement of the interaction between the IGF protein family with MMP-9, it is unfortunate that in this part, the information on IGF-2 in the context of this regulatory system is lacking.

The thesis aims to evaluate the role of MMP-9 activity in structural long-term potentiation on a single dendritic spine and to identify the key substrates involved in this process. The specific problems to be tackled to achieve this aim were divided into eight objectives, which are presented in a way that resembles a research plan.

The "*Materials and Methods*" chapter presents the experimental procedures, molecular approaches and the methods used for imaging by the author. The description of the protocols generally provides sufficient details to reproduce the experiments. A strength of the thesis is its multi-disciplinary character and the combination of various experimental approaches.
I have only a few minor comments. It is unclear why organotypic hippocampal cultures (OHCs) obtained from wild-type and genetically modified mice were used, whereas in the next set of experiments, the primary hippocampal neuronal culture prepared from Wistar rat pups was selected. The compound NBI-31722 applied in the research is a potent inhibitor of IGF/IGFBPs interaction, but it is also a non-peptide ligand that releases bioactive neurotrophic factor IGF1 from the IGF1/IGFBP3 complex, prolonging IGF-1 half-life and enhancing its biological effects via IGF1R. IGFBP-3 interacts with various components of the extracellular matrix and plasma membrane proteins, further modulating cell signaling pathways. Was this fact taken into account when interpreting the results? Last but not least, "experiments were repeated on OHCs from at least three different dissections" Please specify whether these were three replications of dissections from one culture or three independently conducted experiments?

The *Results* chapter is subdivided into eight major sections, consistently pursuing the goals of the dissertation. Generally, it was shown that dendrite spine-enlargement during s LTP was mediated by MMP-9 activity, vesicles carrying BDNF and MMP-9 were localized within the dendritic spines and they were released under stimulating condition, and there was a relationship between BDNF/TrkB pathways and MMP-9 activity in s LTP process. Next, the substantial role of MMP-9 in the activation of IGF1R in the course of s LTP was confirmed using the chemical inhibition or gene knockout approaches and the impact of this

metalloproteinase activity on the interactions of the IGFBP's/IGF1 complex were established. Finally, IGFBP2 expressed mainly on astrocytes and neurons in the hippocampus was selected as a key protein for MMP-9-mediated regulation of IGF1/IGF1R signal transduction pathway, and correctness of this selection was confirmed in multifaceted studies. Although this research requires continuation, the assessed dissertation is an original solution of a scientific problem and reveals crucial position of MMP-9 as a central regulator of synaptic plasticity.

The *Discussion* is presented on 11 pages. Experimental data are discussed on the basis of the cited references and author's own considerations. The following questions and comments should be noted:

- IGF1 R activation expressed in neuronal and glial cells may be modulated by several factors. Thus, can MMP-9-mediated activation of this receptor and cleavage of IGFBPs be affected in neurodegenerative diseases in the context of s LTP?
- The regulation of IGFBP-2 is influenced by endogenous and exogenous factors under physiological and pathological conditions. Among others, post-translational modifications play a significant role in the regulation of the interaction of IGFBP-2 with the IGF system. Phosphorylation, glycosylation, and proteolysis of IGFBP-2 and other IGF-binding proteins modulate their affinity for IGFs and their stability. Indeed the proteolytic cleavage of IGFBPs reduces their IGF-binding capacity, increasing the availability of free IGFs to interact with receptors and initiate signaling. This dynamic regulation may also be influenced by cytokines, such as TNF-α, IFN-γ, and other modulators, which are known to stimulate IGFBP-2 secretion, particularly in inflammatory conditions. Please comment briefly, in the context of the results presented in the thesis.
- IGFBP2 expression in the hippocampus fluctuates with age, which is of particular importance during neonatal development and is associated with learning and memory. Moreover, IGFBP2 enhances excitatory inputs onto CA1 pyramidal neurons, thereby facilitating intrinsic excitability and spike transmission, and regulates plasticity at excitatory synapses in a cell-type specific manner. Could the experimental procedure of obtaining and maintaining OHC cultures have an impact on the results obtained in this work?

In conclusion, the PhD thesis by Diana Legutko-Bijoch makes a significant contribution to the research on the role of MMP-9 activity in synaptic plasticity, highlighting its involvement in structural and molecular processes during LTP. The emerging multifaceted and time-dependent role of MMP-9 has been demonstrated as a crucial factor in inducing and maintaining spine growth and in activating trophic factor pathways. Moreover, the role of IGFBP2 in the mechanisms linking extracellular proteolysis to intracellular signaling during LTP has been established for the first time.

The thesis confirms that the candidate has general knowledge and can conduct research independently. The methodology of the analysis of MMP-9 activity in the single dendritic spine presented in the thesis is a valuable contribution to the field, and reinforced by research results on the targeted neurotrophic factor family proteins, represents an original solution to the research problem. The relevance of these studies is strengthened by the fact that PhD thesis has

been supported by the Polish National Science Centre MAESTRO grant, U.S. National Institutes of Health funds and the Polish National Agency for Academic Exchange Fellowship.

Therefore, I declare that the doctoral dissertation by Diana Legutko-Bijoch meets the requirements of the Polish law (Art. 187 of The Act on Higher Education and Science of 20 July, 2018. In view of the above, I request the Scientific Council of the Nencki Institute of Experimental Biology, Polish Academy of Sciences, to take further steps toward defending the thesis „ Matrix metalloproteinase 9 in dendritic spine plasticity."

Rozprawa doktorska spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2023 r. poz. 742 z późn. zm). W związku z powyższym wnioskuję do Rady Naukowej Instytutu Biologii Doświadczalnej o dopuszczenie mgr Diany Legutko-Bijoch do dalszych etapów postępowania o nadanie stopnia doktora.

Sincerely,

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GEORGETOWN UNIVERSITY MEDICAL CENTER

Department of Neuroscience

March 28, 2015

To Whom It May Concern:

The doctoral thesis “Matrix Metalloproteinase 9 in dendritic spine plasticity”, submitted by Diana Legutko-Bijoch with work surpervised by Professor Leszek Kaczmarek and Dr. Ryohei Yasuda, includes several novel and important findings regarding mechanisms that contribute to long-term potentiation (LTP) of synaptic activity.

Using a variety of complementary and cutting edge techniques, Legutko-Bijoch demonstrates that the protease MMP-9 is rapidly released from post synaptic sites following the stimulation of LTP and that MMP-9 is in turn able to stimulate BDNF/TrkB signaling as well as IGF1R activation that contribute to the structural underpinnings of sustained LTP. With respect to IGF1R activation, which is novel, doctoral work further shows that MMP-9 can cleave IGFBP2 which likely facilitates IGF1R activation.

The involvement of MMP-9 in structural LTP was studied using chemical inhibitors, constitutive and inducible MMP-9 knockout approaches, and a catalytic mutant. The complementary approaches increase rigor of the work.

All in all, sections of the thesis are well written and well referenced, including the introduction, aims, methods, results and discussion. In terms of comparison to other doctoral thesis works that I have read, which include well over 20, this is in the top 5%. In addition, the experimental work load and significance of the results obtained is well within the top 5%. My only suggestion, and it is minor and thus can be addressed at the author’s and mentors’ discretion, is that the introduction and/or discussion also mention phosphorylation of GluAs such as PKA dependent phosphorylation at serine 845 which is important to LTP, other MMP-9 mediated signaling events (ie integrin signaling) that may contribute, and important intracellular changes (ie phosphorylation of cofilin) that are important to sLTP.

My overall evaluation is positive and I recommend that Diana Legutko-Bijochbe admitted to subsequent stages of the doctoral defense. Since the work and dissertation are of an outstanding quality, I also recommend that the dissertation be distinguished.

Sincerely,

Katherine Conant M.D.
Professor of Neuroscience

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