UNIWERSYTET IM. ADAMA MICKIEWICZA W POZNANIU
Wydział Biologii
Instytut Biologii Molekularnej i Biotechnologii

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Review of the doctoral dissertation of Ms Ayça Olcay, M.Sc. entitled
"Loss of Function Studies of Synapse-enriched circRNAs in Neurons"

The doctoral dissertation submitted by Ms Ayça Olcay was prepared at the Institute of Bioorganic Chemistry, Polish Academy of Sciences in Poznań, under the supervision of Dr hab. Monika Piwecka. It addresses a scientifically important and highly actual problem in the fields of molecular biology, RNA biology, and neurobiology, namely the role of synapse-enriched circular RNAs (circRNAs) in neuronal function. Circular RNAs are now recognized as abundant and dynamically regulated components of the nervous system transcriptome; however, the biological role of most synaptic circRNAs remains insufficiently understood. The topic of the dissertation is therefore both timely and well justified.

The dissertation presents an original experimental study aimed at identifying and functionally characterizing selected synapse-enriched circRNAs in mouse primary cortical neurons. The Candidate selected circRNA candidates based on RNA-seq datasets, validated their circularity and expression, analyzed their developmental dynamics, and performed loss-of-function studies using AAV9-mediated shRNA delivery. Among the tested candidates, the most important findings concern circGigyf2(4,5,6,7,8), whose knockdown led to a significant reduction in the expression of Dlg4, Cadm1, and Icam5 mRNAs, as well as a decrease in Synapsin-1 protein level. These findings indicate a potential role of this circRNA in the regulation of synaptic stability and synaptogenesis-related processes.

In addition to the experimental part, the thesis includes in silico analyses of evolutionary conservation, protein interaction propensity, miRNA binding, and coding potential of circGigyf2. These analyses support the hypothesis that circGigyf2 may act through interactions with RNA-binding proteins, rather than through miRNA sponging or circRNA translation.

In my opinion, the dissertation constitutes a valuable and original scientific contribution. It demonstrates that the Candidate is able to formulate a relevant research problem, design and perform experiments using appropriate methods, interpret the results critically, and discuss them in the context of the current literature.

The dissertation is generally written clearly and competently. The chapter composition is conventional and correct, with separate Materials and Methods, and distinct Discussion and Conclusions sections. The editorial side of the manuscript is satisfactory, although it contains a few formatting inconsistencies and minor stylistic or language errors. These are of editorial rather than substantive character and do not diminish the scientific value of the work.

It is also worth noting that the Candidate has demonstrated scientific activity beyond the dissertation itself, including a published paper, a manuscript in preparation, conference presentations, and scientific distinctions. This indicates a good level of academic maturity and scientific engagement.

The Introduction is comprehensive and well prepared. It presents the current state of knowledge concerning circRNA biogenesis, their expression in the brain, developmental dynamics, and possible roles in synaptic biology and neurological disorders. The Candidate also provides an informative overview of synapse formation and of selected synaptic molecules relevant to the functional analyses performed later in the dissertation, including SynCAM1, PSD-95, Synapsin-1, and telencephalin.

A clear strength of this chapter is the synthetic presentation of recent literature related to synapse-associated circRNAs. In particular, the introductory tables and figures effectively summarize the relevance of circRNAs in synaptic function and neuropsychiatric disease. This section shows that the Candidate has a good command of the literature and correctly identifies a meaningful gap in current knowledge.

The aims of the dissertation are clearly formulated. The main objective is coherent and is appropriately divided into specific aims, which are realistic, logically ordered, and well aligned with the methods used in the study.

The Materials and Methods sections are detailed and generally allow reproducibility of the work. The Candidate provides extensive information on reagents, plasmids, primers, antibodies, equipment, and protocols. The methodological approach is appropriate for the research question and includes a broad range of molecular, cellular, imaging, and computational techniques. Particular methodical strengths of the dissertation include the validation of circular RNAs by Sanger sequencing, RNase R treatment, and Northern blotting, as well as the use of AAV9-delivered shRNAs designed against back-splice junctions to achieve selective circRNA knockdown in primary neurons. The description

of statistical analyses is also adequate. Overall, the methodological part reflects solid laboratory competence and careful planning.

The Results chapter is the strongest and most important part of the dissertation. The Candidate first selected a group of circRNA candidates from available RNA-seq resources and validated their neuronal expression pattern. Three candidates, circGigyf2(4,5,6,7,8), circDtnb(5,6,7,8), and circMyst4(2), were chosen for further analysis on the basis of their high neuronal expression and predominance over corresponding linear transcripts. Their circularity was convincingly confirmed by several complementary approaches. An important result is the demonstration that the selected circRNAs are developmentally regulated and increase in expression during postnatal development, especially in the cortex. This observation is consistent with a potential role in synaptic maturation.

The most significant and novel findings concern the functional analysis of circGigyf2(4,5,6,7,8). Its knockdown caused a marked decrease in Cadm1, Dlg4, and Icam5 mRNAs, together with a reduction in Synapsin-1 puncta density. In contrast, knockdown of the two other circRNAs did not produce significant effects under the tested conditions. These results identify circGigyf2 as a particularly promising regulator of synaptic gene expression.

The in silico analyses further enrich the results by showing that circGigyf2 contains a conserved exonic core region and may interact with proteins, including FMRP and TDP-43 – known regulators of *Dlg4* mRNA. At the same time, the Candidate carefully notes that there is no strong support for a robust miRNA-based regulatory mechanism or for efficient circRNA translation. This restrained interpretation is a positive aspect of the dissertation.

The Discussion is well written and appropriately balanced. The Candidate places her findings in the context of contemporary scientific literature and discusses the possible molecular mechanisms with due caution. The potential involvement of RNA-binding proteins in mediating the effects of circGigyf2 is plausible and well reasoned, especially in light of the observed changes in synaptic transcripts known to be linked to post-transcriptional regulation.

The Conclusions are justified and consistent with the presented evidence. They appropriately emphasize the major contribution of the thesis, namely the identification of circGigyf2(4,5,6,7,8) as a developmentally regulated, synapse-enriched circRNA potentially involved in regulation of transcripts encoding PSD-95, SynCAM1, and telencephalin.

Despite my clearly positive evaluation of the dissertation, I would like to formulate several remarks of a critical and discussion-oriented nature. First, the Candidate should clarify in more detail the strategy used for the initial selection of circRNA candidates from RNA-seq datasets. According to the information provided, this selection appears to have relied on raw read counts, while one of the

selection criteria involved comparison between synaptosome and cytoplasmic or whole-brain fractions. In such cases, the issue of expression level normalization is important and should be explicitly discussed. Second, the presentation of Figure 10D could be improved. The notation that arrows indicate circRNA species and circles indicate linear mRNAs may be somewhat counterintuitive and confusing.
These comments, however, do not detract from the overall high scientific quality of the dissertation.

In connection with the public defense, I would like to ask the Candidate the following questions:

1. Is there an overlap between the conserved regions of circGigyf2(4,5,6,7,8) and the predicted protein interaction motifs, particularly those related to FMRP and TDP-43? If yes, how should this be interpreted functionally?
2. Were the predicted ORFs within circGigyf2(4,5,6,7,8) examined for evolutionary conservation or for the presence of recognizable protein domains? If not, how does the Candidate assess their probable biological relevance?
3. In Chapter 5.6.5, two different computational tools were used to analyze putative miRNA binding. Please clarify whether the IntaRNA analysis was performed using the entire circGigyf2 sequence, or only the candidate regions identified by miRDB.
4. While the functional effects of circGigyf2 knockdown on synaptic transcripts suggesting possible impairment of synapse formation are convincing, the biological interpretation would be further strengthened by additional structural analyses directly addressing synapse number or dendritic spine morphology. The available immunofluorescence data are valuable, but they do not fully resolve whether transcript-level changes correspond to a broader synaptogenic phenotype. Were there any changes in the phenotype observed in microscopy?

In conclusion, I state that the doctoral dissertation of Ms Ayça Olcay, M.Sc., entitled “Loss of Function Studies of Synapse-enriched circRNAs in Neurons”, represents an original and scientifically valuable study. The dissertation addresses an important research problem, is based on appropriate and well-executed methodology, and contains results that significantly extend current knowledge on the role of synapse-enriched circRNAs in neurons. The Candidate has demonstrated theoretical knowledge, practical laboratory competence, and the ability to conduct independent scientific research and to interpret the obtained results critically.

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Therefore, in my opinion the doctoral dissertation submitted for review meets the conditions set out in the Act of 20 July 2018 on Higher Education and Science (Journal of Laws of 2018, item 1668, as amended) and in the Procedure for awarding a doctoral degree at the Institute of Bioorganic Chemistry of the Polish Academy of Sciences in Poznań (Resolution of the Scientific Council of the Institute of Bioorganic Chemistry of the Polish Academy of Sciences No. 40/2025/Internet/RN_140 of 18 March 2025) and I apply to the Scientific Council of the Institute of Bioorganic Chemistry of the Polish Academy of Sciences to admit Ms Ayça Olcay, M.Sc., to the further stages of the procedure for awarding the doctoral degree.

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