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Review of the doctoral dissertation:
"Loss of Function Studies of Synapse-enriched circRNAs in Neurons" by Ayça Olcay.

The PhD dissertation, being the subject of this review, was prepared under the supervision of dr hab. Monika Piwecka, in the Department of Non-coding RNAs at the Institute of Bioorganic Chemistry, Polish Academy of Sciences in Poznan.

The research described in the thesis concerns circular RNAs, as a distinct class of stable, non-coding RNAs generated by backsplicing, whose abundance and developmental regulation in the brain suggest important roles in neuronal function. Circular RNA research remains an active and expanding field, gradually shifting from descriptive studies toward functional and translational investigation. While circRNAs are increasingly implicated in neuronal processes and brain disorders, their precise functional roles, particularly at synapses, remain incompletely understood. With this in mind, the topic of this dissertation, investigating the potential function of several synapse-enriched circRNAs, is certainly novel and original.

The Author selected 18 circRNA candidates based on the mouse RNAseq analysis previously performed on synaptoneurosomes, cortical primary cultures, and frontal cortex. Next, those that showed higher expression in neurons compared to astrocytes, and higher expression compared to their mRNA counterparts, the Author focused on three molecules: circDtnb(5,6,7,8), circGigyf2(4,5,6,7,8), and circMyst4(2). The expression of the candidate circRNAs was confirmed in the cortical cultures at DIV21 and in the mouse forebrain. Their levels in the cortex showed an increase throughout development. The knockdown, loss-of-function analysis showed that one of them - circGigyf2(4,5,6,7,8) led to a decrease in mRNAs encoding PSD-95, SynCam1 and

telencephalin (and potentially Synapsin-1 and Neuroligin-1). Based on imaging results of immunolabeled PSD-95 and Synapsin-1 puncta, knockdown of circGigyf2(4,5,6,7,8) leads to a decrease in the density of these proteins. The Author then moves to the computational analysis of the circGigyf2(4,5,6,7,8) architecture and identifies a possible interaction with FMRP and TDP-43 proteins that could potentially link the circGigyf2(4,5,6,7,8) with the PSD-95 regulation on the transcriptional level.

The thesis follows a classical format, with the Introduction, Materials, Methods, Results, and Discussion sections. The work is written in clear, comprehensible language. Minor linguistic and editorial issues do not undermine the scientific content.

The Introduction outlines current knowledge on circRNA biogenesis, brain- and synapse-enriched expression, and their proposed involvement in synaptic plasticity, learning, memory, stress-related behaviours, and neuropsychiatric or neurological disorders. The description of circRNAs in synapses and neurological disorders is very thorough. The Author points out that although many circRNAs have been identified and some functional examples have been described, the functions of most synapse-enriched circRNAs remain unknown.

The second part of the introduction somewhat shifts to synapse formation and reviews core molecular components involved in synaptogenesis, with particular emphasis on adhesion and scaffold molecules such as neurexins, neuroligins, SynCAM1, NGL-3, Synapsin-1, PSD-95, and telencephalin.

The structure of the introduction is logical, but some sections are longer than necessary and sometimes repetitive. For example, similar points about various circRNAs being abundant in the brain, implicated in various disorders, recur in multiple places. Shortening and consolidation would improve clarity. Perhaps focusing more on those identified as playing a role in development (which is more relevant to the study) than on neurological disorders would suffice.

The Materials and Methods section is thoroughly described. I have only one comment about the use of the ENCODE database, which does not appear in this section. Which brain structures were used for comparisons and at what developmental stage?

The Results are clearly described, and the rationale for the consecutive experiments is mostly well outlined. I only have a few critical comments:

1) In Figure 4, the description “mouse brain” is very vague. “Frontal cortex” would be more informative.

2) In Figure 5, what was the rationale for using neurons at DIV21 and astrocytes at DIV30? Would older astrocytic cultures contain higher levels of circRNAs, thus making the selection of the neuronal candidates more stringent?

3) In Figure 6, why was the whole brain extract used instead of the cerebellum, as it is done in the following experiments?

4) In Figure 12, panel B, the legend is missing, indicating which bars are circular and which are mRNA results.

5) In Figure 15, was it possible to analyze the protrusions (visible more in the images with synapsin-1 staining, compared to PSD-95)? By analyzing the number of protrusions, one could infer the dendritic spine density.

The discussion section is relatively short and to the point. The Author correctly interprets the obtained results in the context of current literature and appropriately acknowledges methodological limitations (shRNA method, rescue experiments, *in vitro* model, lack of follow-up experiments assessing spine morphology and electrophysiological properties). The most important achievement of the dissertation is the identification of circGigyf2(4,5,6,7,8) as a novel circRNA that may be involved in regulating synapse-related transcripts. The possible interaction with FMRP is particularly interesting. Given the known link between FMRP and PSD-95 mRNA translation, disruption of FMRP-dependent post-transcriptional pathways may lead to abnormal spine density and persistence of immature spines. Thus, as the Author noticed, spine density analysis would significantly strengthen the thesis. Testing the effects of circGigyf2(4,5,6,7,8) in the Fragile X Syndrome mouse model would also be very interesting.

Although the precise molecular mechanism circGigyf2(4,5,6,7,8) remains unresolved and functional synaptic consequences require further confirmation, the results constitute an **original** and **valuable** contribution to the developing field of circRNA neuroscience.

In summary, I conclude that the doctoral dissertation submitted to me for evaluation by M.Sc. **Ayça Olcay** demonstrates the general theoretical knowledge expected of a candidate applying for the doctoral degree in the discipline of Biological Sciences.

The results obtained by the Author are original and identify circGigyf2(4,5,6,7,8) as a novel candidate regulator of synapse-related gene networks and provide a basis for future *in vivo* and mechanistic studies examining its relevance to neuronal physiology and disease. Therefore, in my opinion, the **submitted doctoral dissertation fulfills the criterion of providing an original solution to a scientific problem**.

Moreover, on the basis of the research results presented in the dissertation, I can state that the Doctoral Candidate demonstrates the ability to conduct independent scientific research.

The doctoral dissertation submitted for review **meets the requirements** specified in the Act of 20 July 2018 – Law on Higher Education and Science (Journal of Laws of 2018, item 1668, as amended), as well as in the Procedure for Awarding the Doctoral Degree at the Institute of Bioorganic Chemistry, Polish Academy of Sciences (Resolution of the Scientific Council of the Institute of Bioorganic Chemistry PAS No. 40/2025/Internet/RN_140 of 18 March 2025). I therefore request that the Scientific Council of the Institute of Bioorganic Chemistry PAS admit M.Sc **Ayça Olcay** to the further stages of the proceedings for the **award of the doctoral degree**.

Z poważaniem

Anna Beroun

Dr hab. Anna Beroun

Podpis jest prawidłowy
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