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REVIEW OF THE PHD THESIS "Developmental and epileptic encephalopathies as synaptopathie - assessment of the role of NMDA receptors in the etiopathogenesis of disease" BY RÓŻA SZLENDAK, MSC

Next generation sequencing technologies revolutionized molecular diagnosis of the diseases with genetic background improving the diagnostic success rate and widening the knowledge of the spectrum of genes involved in particular diseases. At the same time it generated new problems (or magnified some of already existing ones) like the amount of data to analyse, classification of variants found in patients, adjustment of the diagnostic methods (panel vs. WES vs. WGS) and the necessity to confirm the pathogenicity of the variants found. The PhD thesis of MSc Róża Szlendak refers to some aspects of all these problems conducting her research on the genetics of developmental and epileptic encephalopathies being severe and heterogenous group of diseases with their genetics far from being fully recognised and understood.

The thesis is presented as a 172-page manuscript in English taking the form classical for the theses in the field. The author decided to place some of the information typically placed in Materials and Methods (equipment used, PCR primer sequences, PCR conditions etc.) or Results section (the table gathering variants found in patients analysed (minor question – what do the asterisks in some of the places in the table mean?)) in the Attachments at the end of the manuscript what was a very good move facilitating reading of the thesis.

The Introduction presents comprehensive information on the state of the art. It begins with the description of the epilepsy as a disease and continues through its aetiology, known genetic background to finally extract the group of developmental and epileptic encephalopathies as a field of interest. Then, the author leads us, as using zoom function, to the synapse to concentrate on glutamate receptor, genes encoding its subunits (*GRIN* genes) and models and methods used to analyse its properties, including induced pluripotent stem cells. I highly appreciate the content of the Introduction. It is well written and complete. At this point I would like to praise the attractive

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and uniform graphical form of the manuscript. The drawings and figures are not only making easier understanding of the content of the Introduction, the workflows described in Materials and Methods etc. but are also just nice. Special thanks for the diagram showing which combinations of subunits are analysed on Figure 50.

The Materials and Methods are precisely described and allowing relatively easy repetition of performed experiments. Also the choice is appropriate. I am impressed by the number and variety of the methods used from classical molecular biology methods like PCR, Sanger sequencing or western blot to NGS and advanced cellular studies (patch clamp or iPSC generation and neuronal differentiation). I consider this part complete as well.

The PhD thesis covers two broad topics. The first one is the genetic analysis of almost 700 patients with early infantile epileptic encephalopathies and developmental and epileptic encephalopathies based on NGS gene panel. In about half of the group pathogenic, likely pathogenic or variants of unknown significance (VUS) were found and finally the molecular diagnosis was confirmed in about 20% of the subjects. Although the diagnostic success may not be overwhelming (Ms Róża Szlendak discusses it extensively in the proper chapter) the most valuable effect of this part of the study is learning the genetic structure of epileptic encephalopathies in Polish patients what is not only an answer to the basic scientific question but also enables quicker and more efficient molecular diagnosis in the future (this subject is also discussed later in the manuscript). In the genetic analysis the Author explores epileptic encephalopathies as synaptopathies – in consequence the second part of her studies is concentrated on detail functional analysis of the nonsense variant p.Glu839Ter in *GRIN2B* gene (encoding one of the two possible GRIN2 subunits of NMDA receptor). The choice of this particular variant made it possible to light up, at least a little bit, the role of C-terminal domain of GRIN2B protein as the consequence of this variant is the lack of it in the resultant protein. Using two model systems: HEK 293T cells transiently transfected with the number of constructs containing the variant and proper controls supplemented with the fragments encoding proteins enabling the evaluation of the interactions between NMDA receptor subunits (BRET system), and neurons differentiated from iPSCs created from patient fibroblasts Ms Szlendak has shown that:

- GRIN2B protein with the variant is produced but in the lesser amount than the wild type version
- GRIN2B protein with the variant interacts properly with GRIN1 and GRIN2A wild type subunits and may form the NMDA receptor containing all three types of subunits but the receptors containing this type of GRIN2B subunit are less prominent on the cell surface
- As whole cell patch clamp experiments show, the receptors containing GRIN2B protein with the variant are not fully functional
- The presence of the variant influences negatively the global NMDA-induced calcium influx.

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According to these results I have a question if the amount of mRNA of the mutant version of *GRIN2B* gene has been checked? It would be interesting to see whether transcription rate or mRNA stability are also influencing the phenotype.

Summarising this chapter: the results enhance our knowledge of genetic basis of epileptic encephalopathies, while the functional studies get us closer to understanding the function of C-terminal domain of GRINB2 protein and (not for the first time) confirm the utility of iPSC model.

The discussion being probably the most difficult part to write is at the same time the one really showing scientific level of Róża Szlendak. The results are presented in a very wide context and the Author shows directions for further studies as well as suggestions regarding the algorithm of genetic diagnosis of encephalopathic epilepsies. While NGS is used for diagnostic purposes for more than decade and multiple new variants and new genes were correlated with human diseases we are still far from heaving full picture of genetic basis of them. Róża Szlendak shows in the Discussion that she is aware of the problems with phenotype-genotype correlations, possible influence of multiple variants of numerous genes on the phenotype etc.

As some functional studies of p.Glu839Ter variant in *GRIN2B* gene have already been performed by other team basing on cellular models (Santos-Gómez et al. 2020) substantial part of Discussion is refering to them. Although the results of both studies are concordant at main points it is worth mentioning that this work goes much further by analysing the interaction between NMDA receptor subunits (WT and with the variant) and using the model mimicking better neuronal tissue – iPSCs differentiated to neurons.

In the Discussion Róża Szlendak gives pros and cons according to reporting VUSs in diagnostic context. I am curious what is her personal opinion on this subject? This question seems to be important especially in case of relatively new players, like GRIN genes in DEE because (as Ms Szlendak has noticed) due to limited data a significant number of variants is described as VUSs.

At the end of the thesis Ms Szlendak formulates 6 conclusions - not too detailed (a common mistake) and I can agree that these are the main points of her work. I have only a small remark to the first one: I would change "A group of synaptic genes is the common cause of DEEs" to „Pathogenic variants in a group of synaptic genes are the common cause of DEEs"

The lecture of this thesis shows that Róża Szlendak has the ability to plan and perform the experiments using multiple, advanced methods covering the field of DNA and DNA variant analysis and functional studies in cellular models. She also proved to be able to cautiously interpret the results but also to write and present data. Editing errors are rare and the ones I noticed are of

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minor importance. The text is well written both according to its structure and to ability to formulate thoughts.

In conclusion, this thesis brings us closer to understanding the molecular background of developmental end epileptic encephalopathies as well as the role of C-terminal domain in GRIN proteins. In the future it may allow development of dedicated therapeutic approach. Róża Szlendak has proven to be a skilful researcher who deserves PhD degree.

So I claim that the reviewed thesis fulfills the requirements necessary to obtain the PhD degree set out in the Act on Academic Degrees and Title *(Rozprawa doktorska spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce, tekst jednolity: Dz.U. z 2021 r. poz. 478)*, I recommend that the Scientific Board of the Institute of Mother and Child in Warsaw allows it to be defended in public.

In addition, appreciating multifaceted approach to the important scientific problems, the quality of the work and also the practical implications of the results obtained: influence on the diagnostic and therapeutic approaches I recommend awarding the thesis.

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