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Report on the thesis manuscript of Ms Róża Szlendak to obtain the grade of Doctor of the University of Montpellier (FR) and Institute of Mother and Child (Warsaw, PL)

Ms Róża Szlendak presents a manuscript entitled: "Developmental and epileptic encephalopathies as synaptopathies – assessment of the role of NMDA receptors in the ethiopathogenesis od disease", under the supervision of Prof. Dorota Hoffman-Zacharska (Institut of Mother and Child) and Julie Perroy (IGF)

Ms Szlendak's thesis aims at identifying the molecular background of developmental and epileptic encephalopathies (DEEs) in a cohort of 694 patients from the Institute of Mother and Child (Warsaw, Poland), with the goal of determining the importance of synaptic genes in the pathogenesis of DEEs. She performed on this cohort targeted Next Generation Sequencing (tNGS) against a panel of 49 genes known to be causative for DEEs. Molecular diagnosis was established for ~18% of the patients, among which ~37% of the variants were on synaptic genes. With these results in hand, Ms Szlendak decided to focus on GRIN genes, which encode subunits of NMDA receptors (NMDARs). These receptors are part of the ionotropic glutamate receptor family, a class of post-synaptic ion channels that play key roles in synaptic transmission and plasticity. A growing number of studies have uncovered mutations of GRIN genes in patients suffering from several neurological diseases, including epilepsy and developmental encephalopathy. Ms Slendak's work focused on a particular mutation in the GRIN2B gene that results in a complete deletion of the C-terminal domain of the GluN2B subunit, p.Glu839Ter. This choice was motivated by the fact that, contrary to NMDAR extracellular domains, the role of NMDAR C-terminus in controlling receptor function is less known. Using BRET and Western blot analysis, Ms Szlendak showed that the truncated GluN2B839Ter subunit could still associate with the GluN1 subunit, either as di-heteromers containing two copies of the GluN2B839ter subunit, or as tri-heteromers together the a wild-type GluN2B subunit. However membrane expression of receptors containing GluN2B839ter was drastically decreased. Interestingly, GluN2B839ter also displayed increased magnesium sensitivity, which exacerbates the loss-of-function character of this mutation in physiological conditions. Finally, Ms Slendak developed cultures of iPSC neurons from skin samples of the patient bearing the GRIN2B p.839Ter mutation. She showed that in these derived neurons, which bear the endogenous genetic background of the patient, calcium influx was decreased compared to control neurons, further confirming the loss-of-function phenotype of the mutation. This work is in direct line with the booming number of studies uncovering the critical role of GRIN genes in the etiology of DEEs, which attests of its relevance. Compared to previous studies, the novelty of this work lies in exploring the functional role of this mutation in iPSC-derived neurons from the patient, which allows studying the functional effect of the GRIN2B p.839Ter mutation in its endogenous environment. **Overall, Ms Szlendak has obtained solid results of strong interest on the role of a GRIN2B mutation in the etiology of a patient's DEE. I have no doubt that these results will be published in a high impact journal in the near future.**

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Ms Szlendak's manuscript is overall well conducted and pleasant to read. It starts by an introduction on epilepsy and the definition of DEE. It is followed by a description of the discovery of the genetic origin of DEEs and the genes involved in this disease, with an interesting historical take. This part is well described and allows people outside of the field to comprehend well the molecular origins of DEEs. The introduction then describes glutamate receptors, a class of post-synaptic receptors expressed at excitatory synapses and playing major roles in synaptic transmission and plasticity, and especially NMDARs. Aside from a few inaccuracies (Magnesium binds extracellularly, not intracellulary), this part summarizes in a concise way all the aspects of iGluR and NMDAR properties and physiology necessary to comprehend Ms Szlendak's experimental work. However the references used in this part should be largely revised. In a separate document, I will indicate to Ms Szlendak where references should be changed/added. The part on glutamate receptors ends on a well-conducted review of the many GRIN variants already identified in the literature and their consequences in terms of pathologies. Finally, the introduction concludes with the description of the different methods used to study GRIN-related diseases and the use of iPSC-derived neurons as disease models.

After a clear description of the thesis objectives, the manuscript third part is a thorough description of the methods used during the thesis. This part is highly detailed and really well described. This manuscript will thus be a precious resource for future students/researchers working on similar projects.

The fourth part describes the results obtained by Ms Szlendak during her PhD. Ms Slendak first analyses the results of the tNGS sequencing of the cohort of 694 patients from the Mother and Child Institute with a focus on synaptic genes, i.e. genes expressed at the synapses. She then decided to focus her study on variants of GRIN genes, whose mutations were already found in patients suffering from DEEs. Among these, she chose one mutant of the GRIN2B gene, p.839Ter, that results in a complete deletion of the CTD of the GluN2B subunit. It is indeed interesting to know how a mutant that keeps the whole gating core (ABD + TMD) of the NMDAR intact but leaves out its intracellular domain can affect the overall receptor function. By combining techniques of BRET and Western blot on heterologously expressed NMDARs containing wt and mutant GluN2B subunits, she showed that NMDARs containing the GluN2B839Ter were expressed less at the plasma membrane, although association of the GluN2B839Ter subunit with a wt GluN2B subunit rescued some of its expression. I particularly appreciated the CODA-RET method, which is an elegant technique to probe for NMDAR heterodimerization. While the results of this part are very convincing, the next results on NMDAR induced cytotoxicity and electrophysiological characterization of the GluN1/GluN2B839Ter mutants are, to my opinion, the weakest part of the work and would benefit from additional experiments to strengthen the conclusions. Ms Szlendak shows that transfection of NMDARs containing the GluN2B839Ter subunit induces less cytotoxicity and much smaller NMDA currents. Expression and channel permeation properties are two confounding factors that could explain these functional effects. Since GluN2B839Ter-containing NMDARs have already been shown to express less, it is unclear if the decrease of cytotoxicity and NMDA currents is also mediated by a defect of the channel properties, as stated by Ms Szlendak. To fully conclude about that point, one should at least measure the open probability of the channels, either through single channel analysis or

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indirectly by measuring the kinetics of inhibition by MK-801, an open-channel blocker. In general I think the electrophysiological characterization of NMDARs containing the mutation is rather superficial. I would have expected dose-response curves of the agonists glutamate and glycine, as well as a fuller characterization of the effect of the mutant on magnesium sensitivity (dose-response curves at -60 mV and I-V curves), which is an interesting feature of this mutant.

In the last Results section, Ms Slendak used iPSC-derived neurons to study the effect of the mutation *in situ*. This is to me the most novel and exciting part of the work. Indeed, previous studies aiming at determining the functional effect of GRIN variants have mainly relied on heterologous expression of the variants. This approach, on the contrary, allows studying the mutation in the context of the genetic background of the patient with an expression profile that (hopefully) resembles the expression profile in the patient's brain. Ms Szlendak carefully describes the process leading to reprograming the patient's firbroblasts to obtain pluripotent stem cells and then to induce differenciation into neurons, with the many controls required to verify that these neurons are mature and functional. Using calcium imaging, she then shows that calcium influx in mutant neurons is lower than in control neurons, which is consistent with her results on HEK cells. Calcium influx between mutant and control neurons is evened out by application of ifenprodil, a GluN2B-specific antagonist, showing that the difference of calcium flux is most likely due to the presence of the mutated subunit in the patient's neurons. Given the potential of this model, I hope it will be further exploited by the lab to further investigate the expression and signaling properties of this mutant.

Finally, in her discussion Ms Szlendak undergoes a critical summary of her tNGS sequencing results and compares them to other screens performed in the literature. This part is very interesting and substantiated by many references. Similarly to the Results part, the section on the functional properties of GluN2B839Ter-containing mutants is the least convincing. While the discussion summarizes well the findings of the work, I would have expected Ms Szlendak to relate her results to the state of the art on the function of NMDAR CTDs. Indeed, functional studies on NMDARs with deleted CTDs already exist in the literature (see, for instance Maki et al., JBC 2012; Punnakal et al., Neuropharmacology 2012; Vissel et al., Mol. Pharmacol. 2001). I am furthermore surprised that there is no mention of the critical role of GluN2B CTD in mediating cytotoxicity, but also plasticity in the brain (reviewed in Shipton and Paulsen, Phil. Trans. R. Soc 2014; Papouin and Oliet, Phil. Trans. R. Soc 2014). This latter aspect is likely an important contributing factor to patient's symptoms.

In conclusion, the thesis work of Ms Szlendak is remarkable and novel. I am impressed by the multitude of techniques Ms Szlendak had to master during her PhD. All the presented results are convincing and the appropriate controls have been made, although complementary experiments are probably required to complete the present work. The concerns I have raised in this report are mild and should be easily fixed in a reasonable time frame. I have therefore no doubt that this work will be published in a high impact journal. **For all these reasons, I strongly support Ms Szlendak's defense at the University of Montpellier and Institute of Mother and Child.**

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