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PhD thesis review

Author of the thesis: Mgr. Pascal Michel Samir Jorratt Callejas
Thesis title: Effects of endogenous NMDA receptor modulators on neuronal morphology and synaptic plasticity

The present work focuses on the study of changes in morphology and protein expression in cultured cortical neurons and in selected behavioral parameters in mice after their exposure to substances with known modulatory effects on NMDA receptors. The thesis is divided into standard chapters: abstract in English and Czech (1 page each), list of abbreviations (3 pages), table of contents (2 pages), theoretical part (16 pages), objectives (1 page), material and methods (8 pages), results (14 pages), discussion (6 pages), conclusions (1 page), summary in English and Czech (1 page each), list of references (26 pages), list of publications of the author (1 page).

In the introductory theoretical part, the author summarizes the literature on the topics related to the experimental work, which are the structure, function and modulation of NMDA receptors. In addition, this section reviews the properties of several groups of endogenous NMDA receptor modulators and includes a brief chapter on pharmacological modulation of receptors in psychiatric conditions. The Materials and Methods section includes a description of the preparation of primary cortical cultures from rat embryos and the method of their incubation with four selected modulators - kynurenic acid (Kyna), pregnenolone sulfate (PS), spermidine and ZnCl2. In this section, the author also describes the methodological approaches used to assess the impact of modulators in vitro and in vivo. Specifically, cell viability assays, determination of glutamate concentration in culture medium, immunohistochemistry and morphometry, determination of protein levels in cell cultures by ELISA and WB, and behavioral assays performed in mice after intraperitoneal injection of PS. The list illustrates the wide range of experimental work carried out, but the description of the techniques used is rather brief and sometimes lacks important information. For example, there is no information on how to perform control experiments for the pharmacological application of modulators in vitro and the Immunohistochemistry part on page 37 does not mention the secondary antibodies used. The Results section includes three parts: 1. Study of the effect of modulators on excitatory

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neurons in vitro by monitoring viability, dendritic tree morphology, number of synapsin and PSD-95 clusters and expression of GluN1, PSD-95, synaptophysin, BDNF, TrkB and ERK1/2. 2. Study the effect of modulators on inhibitory neurons in vitro by monitoring intracellular and extracellular GABA levels, gephyrins and vGAT expression, and dendritic tree morphology of PV+ neurons. 3. Effect of PS on behavioral parameters in mice exposed to chronic despair model conditions. The parameters tested in this section were investigated using the open field test, the three-chamber test and the forced swim test. The description of the results documents the breadth of the author's experimental work, but it lacks in some parts information that would allow a proper understanding of the data. For example, the legends to Figure 9 and following do not contain a description of group "C" (it can only be assumed that this is a control group, but it would be useful to know its detailed specification for different types of experiments). The author also uses the somewhat non-standard terms "technical replicates" and "biological replicas" for the number of samples in the statistical evaluation. In my opinion, when describing experiments aimed at investigating the structure of the dendritic tree of excitatory and inhibitory neurons, it would be useful to indicate how the neurons were stained and how their subtypes were identified (using anti-MAP2 and anti-PV antibodies?). It is also not clear exactly how the density of puncta in the graphs in Figure 16 is defined and how a distinction was made between intracellular and extracellular GABA in determining their concentrations (Figure 19). The Discussion section is then divided into sections devoted to each part of the results described above. I have two comments on this part. Figure 25 on page 56 indicates the presence of gephyrin in the presynaptic terminal of glutamatergic neurons. I am not aware of any work showing this. In contrast, presynaptic GABA-A and glycine receptors do not form clusters but are diffusely distributed, consistent with the absence of gephyrins typically clustering these receptors in the postsynaptic membrane. Section 5.6 on page 55 would be better if it discussed more about the causes of PS failure in affecting behavior parameters. The only small effect was on the increase in center time during the open field test. However, the experiments were performed under very low illumination, <10 lux, which may reduce their conclusiveness regarding the anxiolytic effect of the applied substances. Some papers recommend using an intensity of 40 lux, a value close to that inside the breeding cages. Another aspect that would be useful to discuss is the passage of PS across the blood-brain barrier after its intraperitoneal administration in mice.

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Despite these shortcomings, this is an interesting work that may contribute to the elucidation of the mechanisms by which endogenous NMDA receptor modulators may be involved in the pathogenesis of neurological diseases. The dissertation is based on two first-authored publications. In addition, Mgr. Pascal Michel Samir Jorratt Callejas is co-author of 5 other articles published in journals with IF. In my opinion, he has thus demonstrated his ability to work independently as a scientist. I recommend his thesis for defense, where he will have the opportunity to clarify the ambiguities in the thesis and award him the PhD degree.

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Questions:

1. The main goal of the presented work is to study the effects of substances with previously described modulatory effects on NMDAR activity. However, it was shown that these substances also act in an NMDAR-independent manner, as also stated in the theoretical part of the thesis. Could the author estimate the contribution of NMDAR-dependent and -independent mechanisms to the observed effects of modulators on cortical cultures? The neurobasal medium used in the work generally contains MgCl2, which is a typical NMDAR inhibitor. Could its presence contribute to the negative results of experiments with modulators? To reveal the specific influence of NMDAR-dependent mechanisms, the effects of modulators should be compared with those of specific NMDA inhibitors. Have such attempts been made?
2. One of the results described in the dissertation is that NMDAR modulators do not significantly affect glutamate concentration in the culture medium after application of 50 mM K+. This is interpreted to mean that the modulators did not change the total number of glutamatergic synaptic connections and that

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presynaptic NMDAR activity was not altered. These conclusions do not seem to me to be fully supported by experimental data. I would like to ask the author to comment on the possibility of depolarization-evoked release of glutamate via non-synaptic pathways, e.g. by somatodendritic release from neurons and TBOA-insensitive reverse uptake from glia. Also, the contribution of presynaptic NMDARs to glutamate release from cortical cultures after their massive depolarization in the presence of high KCl will most likely be negligible. Can the author comment on that as well?

3. I would be interested in the reason why PS in excitatory neurons did not influence the length of dendrites and the number of their branches, while influencing the number of their intersections with circles in Scholl's analysis (p. 45). The author further states that "The data suggest that PS regulates dendritic arborization, but not branching." What is the difference between these terms?
4. Another aspect that should be considered when interpreting the results of behavioral experiments is the passage of PS through the blood-brain barrier after its intraperitoneal application in mice. The traditional idea, based on observations from older work, was that sulfated steroids do not cross the barrier. More recent work, on the other hand, has shown that labeled PS can pass if it first undergoes desulfation. As the author states in the theoretical part, unsulfated pregnenolone has little effect on NMDARs. Could this account for the mostly absent effect of PS in behavioral tests?

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