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Warszawa, 28.08.2019

Review of the doctoral thesis of Teresa De Cicco, M.D., entitled.
"The role of the actin-remodeling proteins Cap2 and Cttn in the development of the neuromuscular synapse".

The dissertation submitted for review by Teresa De Cicco, was carried out under the supervision of Tomasz Prószyński, Ph.D., at the Łukasiewicz Research Network - PORT and the M. Nencki Institute of Experimental Biology in Warsaw.

The neuromuscular synapse (NMJ) is an extremely specialized type of synaptic connection through which it is possible to translate nerve impulses into movement of the entire organism. Thanks to the development of imaging methods, more and more is known about the structure and function of these extremely important synapses. However, we are still far from a thorough understanding of the molecular mechanisms that regulate their development. In her dissertation, Teresa De Cicco studied the physiological function of two proteins that regulate the actin cytoskeleton - Cyclase-associated protein 2 (CAP2) and Contractin (Cttn) - in the development of neuromuscular junctions. The results indicate an important role for these proteins in the formation of NMJ structure.

The dissertation has a classical structure and consists of an Introduction, a chapter describing Materials and Methods, Results and a Discussion. The bibliography contains 184 citations. The work is very well written, in clear, understandable language so that the reader has no problem following it.

In the **Introduction**, the author introduces the reader to the subject of the work starting with complete basics such as the structure of the nervous system and the types of cells that build it. The author devotes a separate very comprehensive chapter to the structure and postnatal development of the NMJ. Then we find an introduction on cytoskeletal proteins describing in detail their structure and the functions of specialized domains, which is very helpful for later reading of the results section. At the end of the introduction the author includes the research

hypothesis and preliminary results which were the basis of her further research. The introduction sufficiently introduces the reader to the problem described in the results and is enriched with clear and carefully elaborated diagrams.

The **Materials and Methods** section is sufficiently comprehensive, yet adequately concise, and I have no criticisms of it. The paper is methodically rich, and it is apparent that the author must have mastered advanced methods of molecular and cellular biology, imaging and also work with experimental animals to complete it.

The **Results** are described clearly and the experiments were planned with due care and concern for appropriate controls. The first part of this chapter describes studies on Cap2 KO mice, which, according to previous studies, showed motor coordination deficits. After careful imaging of the NMJ, the author showed an altered distribution of the size of synaptic connections which differed significantly from control mice and, in a similar manner, for the various muscles tested. In addition, the NMJs of CAP2 KO mice were more fragmented.

Perhaps it would be worthwhile to quantify the density of NMJs in a given tissue volume, particularly in the context of synaptic polyinnervation being increased in CAP2-KO mice, which is examined later in the paper?

Next, the author asks at what point in development do the observed differences in NMJ size and fragmentation arise. Mice at day 7 of postnatal development show only a slight difference in NMJ size, while by day 20 it is significant and virtually identical to the age of P30. Here, it would be advisable to study mice between P7 and P20 of age to accurately determine the time of onset of the observed changes.

Interestingly, re-expression of CAP2 protein in muscle does not improve the phenotype. This could indicate a role of CAP2 protein on the presynaptic side, i.e., on the side of the nerve fibers, which could be demonstrated using animals with selectively silenced expression in motoneurons. What other experiments could, according to the Author, confirm or disprove this hypothesis?

The second part of the results concerns the role of contractin, which localizes to the NMJ after electroporation of the expressing vector into the muscle. Using co-immunoprecipitation methods, the author of this paper demonstrated a direct interaction of contractin with aDystrobrevin1 and then confirmed that this interaction occurs within the MNJ. The description of the performed experiments on page 88 is a little chaotic and difficult to fallow. For example,

there is no mention of HEK293 cell line transfection with appropriate vectors and the author is only mentioning it in figure 4.13 title.

In Cttn-KO mice NMJ size was reduced in slow-twitching Soleus muscles but interestingly the difference was not observed in fast twitching muscles. In the last part of the paper, the author focused on the study of mice with muscle-specific deletion of contractin protein. It turned out that these animals showed no dysmorphology of NMJ or the general skeletal muscular integrity. Accordingly, they also expressed no locomotory defects in normal and intense exercise. So, is it possible that here again the effect could be presynaptic?

In the overall evaluation of the merits of the work, I would like to emphasize the good planning and execution of the experiments along with the appropriate amount of control. The analysis and discussion of the obtained results prove that the doctoral student is able not only to properly plan the experiments and use appropriate techniques to perform them, but also to correctly interpret the experimental data, compare it with the results obtained by others, and propose a probable mechanism for the studied phenomena.

I found the **Discussion** to be comprehensive and well-written. In the first section, the author discusses the role of the CAP2 protein in simple organisms, such as budding yeast, and in controlling the morphology of dendritic spines in vertebrate neurons, demonstrating its evolutionarily conserved importance in various organisms' species. Next, she is discussing the very interesting and attractive hypothesis that the KO CAP-2 phenotype associated with NMJ is a consequence of developmental delay or premature degeneration. She also proposes possible fallow-up experiments such as electrophysiological measurements of the functional capacity of MNs, which would complement the understanding of the role of CAP-2 in the neuromuscular system. In the chapter "Research limitations and future directions" chapter the author proposes the use of transgenic mice with tissue-specifically regulated expression of CAP-2 protein that that would allow for muscle-specific CAP-2 deletion at different developmental stages. This are a very good ideas that confirm authors understanding of the studied research problem.

The second part of the discussion highlights the Contractin part of the project and focuses on the differences observed in slow and fast contractile muscles in Cttn-KO mice. The discussion ends with a synthetic summary of the obtained results.

In conclusion, I find that the work submitted to me for evaluation by Teresa De Cicco meets all the conditions specified in Article 187 (1-4) of the Law on Higher Education and Science (Journal of Laws 2018, item 1668, as amended). Therefore, I wholeheartedly request Council of the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences to admit Teresa De Cicco to further stages of the doctoral program and I support the application to grant her the doctor of philosophy (PhD) degree.

dr hab. Magdalena Dziembowska

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