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Review of the Doctoral Thesis of Ms. Paloma Álvarez Suárez, M. Sc.,

title: *Drebrin and myosin VI: Cytoskeletal regulators of the development of the postsynaptic machinery at the murine neuromuscular junction*

Supervisor: Prof. Maria Jolanta Rędowicz, Ph.D., D.Sc., The Laboratory of Molecular Basis of Cell Motility, Nencki Institute of Experimental Biology, Polish Academy of Sciences
Auxiliary supervisor: Dr. Marta Gawor, Ph.D.

Introduction

The dissertation of Ms. Paloma Álvarez Suárez contains 183 pages, 7 chapters, 52 figures, and 13 tables. Her research is based on a large number of references (343), related to the thesis topic. The dissertation has contemporary and modern aspects described by the research objectives. The thesis covers the problems of the roles of drebrin and myosin VI, two cytoskeletal protein, at the murine neuromuscular junction (NMJ). The PhD student hypothesizes that drebrin is a synaptic component of neuromuscular junctions, mediates cytoskeleton-dependent regulation of the postsynaptic machinery through actin stabilization and myosin VI plays a role in the mobility of the postsynaptic machinery. To verify these hypotheses, the student performed numerous experiments using the *in vitro* (C2C12, HEK293 cell lines and primary myogenic culture) and *in vivo* (mouse) methods.

Thesis' Review

The PhD thesis begins with an introduction to the subject-matter (chapter I). The dissertation of Ms. Paloma Álvarez Suárez is thematically homogeneous and begins with the presentation of the current state of knowledge related to the topic of the study. Chapter I presents the literature review and is logically constructed (from general to particular issues). The theoretical part of the thesis includes information concerning organization of peripheral nervous system, especially the neuromuscular junction system, skeletal muscle development, synaptogenesis, cytoskeletal dynamics in the nervous system, and contains information on two proteins being the subject of her research in the doctoral project: drebrin and myosin VI (MVI). To summarize the theoretical part of this thesis, it is worthwhile to note that the author has presented carefully the research subject with critical view, and used appropriate number of bibliography sources. It is evident that the PhD student gained a deep understanding of the

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theoretical knowledge and the discussed problems.

Chapter II presents the hypothesis and aims of study, which will be verified during the PhD project. This chapter has been written very precisely and clearly by the author. In the next part of the dissertation, Materials and Methods, the PhD student describes in details the methods used in the project. The methods range from the classical histology to sophisticated techniques used in molecular biology. This proves that the student is very well prepared to experimental work, and the methods she has used allowed her to obtain very interesting, novel data.

Chapter III, Results, was divided into subchapters related to the main objectives of the dissertation. Immunocytochemical analyses of whole-mount skeletal muscles from WT mice revealed that drebrin colocalized witch acetylcholine receptors (AChRs) at the crest of junction folds whereas myosin VI was present in AChR-free areas at the periphery of AChR branches. *In vitro* studies confirmed that drebrin and MVI are present in the vicinity of the postsynaptic machinery. Based on the knowledge that microtubules (MT) are crucial for AChR organization at NMJs and drebrin post-translational phosphorylation is relevant for its cross-linking function between actin cytoskeleton and MT network, the PhD student decided to test whether phosphorylation of drebrin affects its localization at NMJs. The data show that phospho-drebrin is required at the postsynaptic machinery, but its phosphorylation at Ser142 is not necessary for this recruitment. Drebrin was also found in skeletal muscle fibres where it colocalized with α-actinin. Drebrin also showed colocalization with Ryr1 channel receptor located at the SR membrane, and skeletal muscle myosin II in the sarcomere A-bands. This result is the first report of the drebrin presence in sarcomeres of mammalian skeletal muscles.

The role of drebrin in organization the postsynaptic machinery has been confirmed by loss-of-function studies and knockdown of drebrin expression with siRNA in C2C12 myotubes. The obtained results suggest that drebrin is involved in AChR organization *in vitro*, and might play a significant role in the NMJ formation. Another interesting result obtained by the PhD student was demonstration that drebrin participates in stability and maturation of AChRs through regulation of actin cytoskeleton rearrangements. Co-labelling of drebrin and podosomes markers revealed that drebrin was present at the core of synaptic podosomes. It has also been shown, by the use immunostaining and pull-down techniques, that drebrin depletion does not impair cell surface delivery of AChRs. Previous research revealed that MT recruitment

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to the NMJ postsynaptic machinery is important for AChR clustering and it depends on cytoskeletal regulators. The student decided to perform studies on a drebrin role in the cytoskeletal network. It has been shown that drebrin is involved in the MT recruitment. The effects of the drebrin depletion are a result of impaired MT capture specifically at the postsynaptic machinery. The PhD student has also shown that drebrin-mediated rearrangements of the actin cytoskeleton are necessary for MT recruitment at the postsynaptic machinery. Previous research has shown that actin-MT cross-linkers (MACF1 and α-actinin) directly bind to rapsin, and thanks to this, participate in the maintenance of AChR clusters. The student carried out tests whether rapsyn also interacted with actin- and MT binding proteins, drebrin and EB3. It has been shown that rapsyn could be involved in the drebrin-mediated regulation of AChR clustering and MT capture at the postsynaptic machinery. To fully demonstrate the drebrin function, the author used muscles of drebrin global knockout mice (DXKO), which lack drebrin isoforms. The data show that during postnatal development the drebrin absence mildly affects the size of NMJs in various muscle types. However, drebrin knockout does affect NMJ postnatal maturation since its absence could be compensated by other protein with similar function. Drebrin knockout also does not disrupt adult NMJ integrity that which was shown in DXKO mice.

PhD student also carried out *in vitro* and *in vivo* studies on MVI function in the NMJ maturation and maintenance. *In vitro* studies on MVI knockout mice (*SV*) demonstrated a reduction in the NMJ size and their plaque-to-pretzel transition. She also showed that the effects of the MVI knockout are not visible in all the examined muscles and are sex dependent. Muscle impairments were observed mainly in female individuals in their hind limb muscles. She also showed that only NMJs from fast-twitch *tibialis* (TA) muscles had decreased the AChR and endplate areas, but increased AChR perimeter. In MVI knockout mice in TA muscles, the PhD student noticed an increased number of perforated NMJs or with "holes" and increased number of NMJs with dispersed AChRs in the edges of NMJ branches.

In the last chapter, Discussion, the author summarizes all the results obtained during the implementation of the doctoral project. This chapter of the dissertation did not disappoint me either. The doctoral student precisely discussed all the results obtained in the project, drawing correct conclusions. The Discussion contains all the issues that the author has included for the

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purposes of the study and correctly interpreted on the basis of the available literature.

I have the following questions to be answered by the student during the thesis defence:

Question 1: What is the structure of the sarcomeres in drebrin knockout mice?

Question 2: What proteins can take over the function of drebrin?

Question 3. What is the structure of the sarcomeres in MVI knockout mice?

Conclusions

In summary, the main goals of the dissertation were fully realized by the PhD student. The results are original and valuable. The author of the doctoral dissertation precisely performed a series of well-thought-out experiments from which conclusions were logically drawn. The results obtained by the PhD student will certainly contribute to a better understanding of the development and maintenance of postsynaptic machinery in vertebrates.

Paloma Álvarez Suárez, M. Sc., doctoral dissertation entitled: *Drebrin and myosin VI: Cytoskeletal regulators of the development of the postsynaptic machinery at the murine neuromuscular junction*, meets the conditions specified in art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2021 r. poz. 478, 619 1630). On this basis, I recommend the Scientific Council of Nencki Institute for admission of the Ms. Paloma Álvarez Suárez, M.Sc., to further stages of the doctoral dissertation procedures.

Also, due to the large contribution of the PhD student's work, the importance and originality of the results obtained and her high level of scientific and methodological preparation, I propose the thesis of Ms. Alvarez-Suarez to be awarded with the distinction.

Prof. dr hab. Małgorzata Daczewska

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DEPARTMENT OF CELL BIOLOGY
PROF. ZBIGNIEW MADEJA, PH.D., DSC.

KRAKÓW, 20/07/22

Review of the Ph.D. dissertation of Paloma Álvarez Suárez, MSc
"Drebrin and myosin VI: Cytoskeletal regulators of the development of the postsynaptic machinery at the murine neuromuscular junction"

Completed in the Laboratory of Molecular Basis of Cell Motility, Nencki Institute of Experimental Biology, Polish Academy of Sciences
Under the supervision of Professor Maria Jolanta Rędowicz, Ph.D., DSc
and dr Marta Gawor, Ph.D. (Auxiliary supervisor)

It is widely accepted that the actin cytoskeleton determines the shape of the cell and is involved in cell division, movement of organelles, movement of the cell, and adhesion of the cell to other cells. However, it is also involved in a variety of other cellular processes. An example is the involvement of the actin cytoskeleton in postsynaptic-regulating processes, including local delivery and recycling of synaptic components, stabilization of postsynaptic complexes, and recruitment of other cytoskeletal filaments. As morphological and functional aberrations in these processes often lead to neuromuscular disorders, it is understandable that there is interest in fully understanding these processes. The proper functioning of the actin cytoskeleton in a cell depends on the proper interaction of many proteins. One of the interesting proteins of this group is drebrin. Although the role of this protein in postsynaptic regulation in the central nervous system has been described, the potential role of drebrin in neuromuscular junction formation is unclear. The second very interesting protein from the point of view of the mechanism of neuromuscular junction formation is myosin VI. Myosin VI is a very unusual myosin. It was the first myosin discovered to move toward the minus end of actin filaments. Myosin VI is involved in various cellular processes such as endocytosis, cell migration, maintenance of Golgi morphology, and cancer cell metastasis. Myosin VI is also involved in skeletal muscle differentiation and the

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organization of postsynaptic machinery, however, its role at the neuromuscular junction formation is not clear.

Mgr Paloma Álvarez Suárez focused in her thesis on the role of the actin-regulating proteins drebrin and myosin VI in the context of the murine neuromuscular junction's structure and function. In particular, she tried to verify three hypotheses: (i) drebrin is a synaptic component of neuromuscular junctions, (ii) drebrin mediates cytoskeleton-dependent regulation of the postsynaptic machinery through actin stabilization, (iii) myosin VI plays a role in the mobility of the postsynaptic machinery through endocytosis. The topic of the thesis seems to be very interesting from a scientific point of view. The rationale for the research and the reason the research was being carried out are well established. In addition, it should be mentioned that the doctoral dissertation of Mgr Paloma Álvarez Suárez was conducted within a team with extensive experience in this type of research.

The doctoral dissertation submitted for evaluation was written in English and consists of 183 typescript pages. There are 63 figures, 13 tables, and 343 references in the text. The dissertation has a typical layout for this type of study and consists of the following chapters: table of contents, summary in English and Polish, list of the Ph.D. student's publications, list of abbreviations, introduction, objectives, materials and methods, results, discussion, summary and conclusions, and references. In an extensive 32-page introduction, the author presented a carefully prepared set of information that helps the reader understand the work. In the first part of the introduction, the author discusses the organization of the peripheral nervous system and mouse skeletal muscle development. In the second part, divided into two chapters, the author presents information on cytoskeletal dynamics in the nervous system and the role of drebrin and myosin VI in this process. In my opinion, the introduction chapter is written clearly and well prepares the reader for the issues that are the subject of the doctoral dissertation. It is worth emphasizing the careful and extensive selection of the most important information, which means that the text is very informative, and it clearly leads to the presentation of the detailed goals of the work. I have only two minor comments on this part of the dissertation – (i) I think in the first sentence on page 18, it would be better to use the terms "to allow ions such as sodium (Na^+), potassium (K^+), and Ca^{2+} to pass through the membrane" instead of "leads to the influx of sodium (Na^+), potassium (K^+),

and Ca^{2+} ions"; (ii) How should the different colors of actin monomers be interpreted in Figure 1.9? If this is only to facilitate the visualization of the filament, why do we also have two classes of monomers marked with different colors before polymerization? Although I think I understand what the author intended to present in this drawing, in fact, the drawing may suggest that the conversion of ADP to ATP in actin monomers occurs after attachment to the filament.

In the chapter that describes the materials and methods used, the author provides comprehensive information on the biological material and the experimental methods used. Several biochemical, molecular biology, and cell biology methods, which are undoubtedly appropriate to solve the problem that is the subject of the doctoral dissertation, were presented. The description of the methods is clear and accurate, making it possible to repeat the described experiments. Clear diagrams make it easier to understand how the experiments were carried out. However, I would like to ask the author to clarify two points: to induce AChR cluster formation, two different protocols were used, depending on the experimental design (i) Neuron-derived agrin-mediated model and (ii) Laminin-mediated model. What was the reason for using two different models? And what is the reason for various efficiencies of cluster formation in these models (Fig.4.8 vs 4.9)? To study the insertion of new AChRs to preexisting clusters on the cell surface upon myosin VI knockdown, the author used immunofluorescence analysis of AChR turnover. If we use this method, can we be sure that BTX-AlexaFluor TM 555 does not dissociate from the receptor during a 6-hour incubation?

The next chapter focuses on presenting the results of the entire study. The main key points and research achievements can be divided into two parts.
In the first part the author presented that:
1/ drebrin is present at the muscle postsynaptic machinery, both *in vivo* and *in vitro*, and at the contractile machinery in mice
2/ drebrin is involved in the organization of postsynaptic machinery *in vitro*
3/ drebrin regulates microtubule recruitment under ACHR clusters *in vitro*
4/ drebrin loss mildly impairs postsynaptic machinery organization *in vivo*
5/ drebrin is upregulated in primary myotubes lacking myosin VI

In the second part, the author showed that myosin VI loss does not impair postsynaptic machinery organization *in vitro;* however, this protein is involved in the postsynaptic machinery organization *in vivo.*

The presented research results allowed the author to draw interesting and well-motivated conclusions. The most important conclusions from the work include the statement that drebrin is a novel component of murine postsynaptic machinery, synaptic podosomes in myotubes, and of sarcomeres in skeletal muscle. Furthermore, drebrin participates in the stability and maturation of AChRs *in vitro* through the regulation of actin cytoskeleton rearrangements and binds to the AChR anchoring protein rapsyn and the microtubule +TIP protein EB3 in order to stabilize the postsynaptic machinery by recruiting microtubules and their anchorage to the synaptic membrane. Equally interesting are the conclusions regarding the role of myosin VI in the processes studied. The author points out that myosin VI contributes to muscle contraction efficiency *in vivo* and loss of myosin VI affects the maturation and maintenance.

In summary, the author obtained original, interesting, and consistent results, convincingly proving that drebrin can be a novel regulator of postsynaptic machinery that cross-links two major cytoskeletal components involved in the stabilization of neurotransmitter receptor - microtubules and actin.

Reading this very interesting part of the work raises some questions:

1/ In Figure 4.5., the signal from the ‘phospho-dead’ drebrin E mutant seems to be slightly stronger than from the wild-type drebrin E. Can it have any biological significance?

2/ As far as I understood, in experiments on the effects of BTP2 on C2C12 myotubes (Fig 4.10), control cells were treated with the same concentration of DMSO as 5 μM BTP2 experimental cells (0.02%). I guess that at 10 μM BTP2, the concentration of DMSO was 0.04%. What was the effect of DMSO at this concentration on the area covered by the myotubes?

3/ Taking into the account the author's experience with the machine-learning-based algorithm, I would like to ask for her opinion on the reliability of this method.

4/ As some effects of myosin VI knockout were sex-dependent, what was the sex of the mice from which the cells were isolated for *in vitro* culture? Was it taken into account?

5/ Are the differences in Figure 4.33 really statistically significant? The differences seem to be very small with a relatively large SEM.

In the extensive 17-page chapter "Discussion", the author critically and carefully assesses her results against the background of literature data. The discussion is very well written. The author discusses all of the presented results in great detail and describes the possible directions of development of future research and proposes several additional experiments as a continuation of her investigations. The chapter "Summary and conclusions" presents the main research achievements and conclusions from the conducted research.

The comments and questions presented here do not in any way negate the most positive assessment of the entire doctoral dissertation. On the contrary, they prove that the work is read with great interest and the results obtained are an incentive to ask many new questions. My final evaluation of the doctoral dissertation is very positive.

The results of the Ph.D. thesis have already been published in three papers in peer-reviewed, indexed scientific journals. In two of them, the Ph.D. candidate is the first author. These works have already been cited 16 times in the literature. Moreover, she is co-author of two papers not directly related to the Ph.D., which were published during her Ph.D. studies in internationally recognized journals.

In summary, I highly rate both the academic achievements and the doctoral dissertation of mgr Paloma Alvarez Suarez. In my opinion, the author has obtained valuable scientific results that open up broad research perspectives for the future. The doctoral dissertation meets the conditions set out in Art. 187 of the Act of July 20, 2018 Law on Higher Education and Science (Journal of Laws of 2021, items 478, 619, 1630). Therefore, I am asking the Council of the Nencki Institute of Experimental Biology, Polish Academy of Sciences to admit Mgr Paloma Alvarez Suarez to the next stages of

her doctoral dissertation. In addition, as the results of the doctoral thesis have already been published in 3 papers, and considering the high scientific level of the dissertation, I would like to request that the dissertation be distinguished with an appropriate award.

(Zbigniew Madeja)

Krakow, August 24, 2022

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Review of the Ph.D. Thesis entitled „Drebrin and myosin VI: Cytoskeletal regulators of the development of the postsynaptic machinery at the murine neuromuscular junction" by Paloma Álvarez Suárez

Synapses and neuromuscular junctions are crucial elements of information transmission within the nervous system. Both structures are very complicated and involve many proteins on both pre- and postsynaptic sites. They also share some similar elements and processes as plasticity. Although synapses and neuromuscular junctions have already been studied for many years, still their structure, structural and physiological changes during development, stress, pathological conditions and aging are not fully recognized yet.

Paloma Álvarez Suárez analysed in her thesis functions of two proteins, drebrin and myosin VI in neuromuscular junctions. Drebrin consists of two major isoforms drebrin E and drebrin A which are localized in neuronal and non-neuronal cells in actin-rich region and play many functions including synaptic plasticity. The second studied protein is myosin VI, a minus end-directed actin motor protein which also plays many functions in cells. Both proteins are intensively studied now because of their importance in cell junctions of many cell types. Their loss is responsible for many pathological conditions and in the brain they have been postulated to be involved in Alzheimer's disease.

The thesis is well written and reports very interesting results. Paloma Álvarez Suárez found that drebrin is a novel component of the murine postsynaptic machinery, synaptic podosomes in myotubes and of sarcomeres

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in skeletal muscle. This protein is responsible for stability and maturation of acetylocholine receptors (AChR) by regulating the rearrangement of actin cytoskeleton. It also stabilizes the postsynaptic molecular assembly through binding to AChR-anchoring protein rapsyn and microtubule +TIP protein EB3. In case of myosin VI, Paloma Álvarez Suárez found that this protein contributes to muscle contraction efficiency and its loss impairs maturation and maintenance of neuromuscular junctions. These findings are new and important for neuroscience. Part of them has already been published in three papers in the following prestigious journals: International Journal of Molecular Sciences (IF 5.923), Cell (IF 6.6) and Seminars in Cell and Developmental Biology (IF 6.138). In two of them Paloma Álvarez Suárez is the first author. Paloma is also a co-author of two other papers which are not connected to her thesis. The thesis has been prepared under the supervision of Professor Dr. Maria Jolanta Rędowicz, a specialist in physiology and structure of muscle cells. The research reported in the thesis has been funded from two grants of the National Science Centre in Poland awarded to Dr. Marta Gawor, an Auxiliary Supervisor, and to Paloma Álvarez Suárez.

The thesis is written in English on 155 pages and is divided into the following chapters: Introduction, Research Hypotheses and Objectives, Materials and Methods, Results, Discussion and Summary and Conclusions. Each chapter begins with some sentences which are authored by famous women scientists, indicating that Paloma must be inspired by them in her scientific career. In addition the thesis contains: Table of contents, Acknowledgements, Abstract, Abstract in Polish (Streszczenie), List of Own Publication, List of Abbreviations. At the end of the thesis there is a list of **343** references. The form of the thesis is appropriate.

Introduction

Introduction (32 pages) is very well written and contains basic and more advanced recent knowledge about the peripheral nervous system, neuromuscular junctions, development of mouse skeletal muscle, skeletal muscle fibres in the adult mouse, synaptogenesis and NMJ maturation, postsynaptic development *in vitro*, cytoskeletal dynamics in the nervous

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system: actin and microtubule networks, cytoskeletal regulation of chemical synapses, cytoskeletal regulation of postsynaptic machinery formation and maintenance at the neuromuscular junction, drebrin as a cytoskeletal regulator of intercellular communication, myosin VI as a versatile unconventional actin-based motor. In my opinion the Introduction is a good compendium of knowledge to understand the subject of the thesis. It is solidly written, contains many appropriate citations and is illustrated by 16 good quality figures. The content of the Introduction indicates that Paloma Álvarez Suárez knows very well the literature related to the subject of her study and is able to clearly write about it. There is only a few mistakes in spelling and terminology. For example, page 17: instead of the extracellular fluid it should be the extracellular matrix, page 38: instead of "migrating neuronal cell body" it should be "migrating neurons where drebrin E appears but accumulates in the growth cones of axons and dendrites". I also disagree that" Dendritic spines constitute the postsynaptic specialization of the majority of excitatory synapses in the CNS", because inhibitory synapses can also be located on dendritic spines, and they are so-called double synapse spines (Jasinska et al., 2015).

In the Chapter 2: Research Hypotheses and Objectives, three clear hypotheses on the role of drebrin and myosin VI in neuromuscular junctions are formulated and three objectives to study localization of both proteins in neuromuscular junctions, mechanisms of the postsynaptic organization involving these proteins and to characterize the neuromuscular junctions in drebrin and myosin VI knockout mice.

Materials and Methods

In the Materials and Methods chapter (32 pages, 12 tables, 11 figures) all buffers and reagents are listed first in two tables followed by the description of the methods which have been used in experiments. Other chemicals used in the thesis are listed in 10 additional tables. Paloma Álvarez Suárez cultured cell lines and primary myoblasts to observe myoblasts differentiation and maturation of neuromuscular junctions and for protein analysis. She also used a SleepingBeauty system to generate stable cell line, and plasmid and siRNA transfections to genetically manipulate the expression of genes encoding

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protein studied and to label various proteins. All steps of experiments have been properly verified using immunofluorescence labelling, RT-qPCR, Western blotting, measurement of DNA/RNA concentrations and other methods. All genetic manipulations and genetic tool preparations are precisely and clearly described. All steps are illustrated by good figures. In addition to *in vitro* experiments, Paloma Álvarez Suárez also used animal models and carried out several experiments including behavioural tests to asses muscle strength of knockout mice. All methods used indicate that the PhD student has carried out intense *in vitro* and *in vivo* studies and enormous work to explain the role of drebrin and myosin IV in neuromuscular junctions (NMJs) in mice. All methods used are sufficient to verify the formulated earlier hypotheses, however, TEM study could help to visualise ultrastructural changes after genetic manipulations.

Results

The obtained results (42 pages) are interesting and clearly described. The Results are divided into 8 chapters and each chapter is divided into several subchapters. Titles of all chapters and subchapters are formulated as conclusions from results described in the chapters. All results are supported by good quality 35 figures containing confocal images, Western blots and/or graphs. Some blots have been provided by Dr. Marta Gawor.

In the Results, the PhD student reports that drebrin is present in both pre- and postsynaptic NMJ compartment but MVI is located in the postsynaptic compartment in AChR free areas. This was found in *in vivo* study but *in vitro* experiments showed that drebrin is in the postsynaptic machinery. Drebrin phosphorylation at Ser142 does not affect its localization. Moreover, drebrin is a component of sarcomeres in skeletal muscles. It organizes the NMJ postsynaptic element, rearranges F-actin, which was found after blocking this protein with its antagonist BT2 and organizes AChR as well as AChR cluster maturation in myotubes. Debrin is co-localized with synaptic podosome markers and *Dbn1* knockdown impairs AChR organization. However, this protein is not involved in synthesis and surface delivery of AChRs but regulates recruitment of microtubules under AChR clusters by interacting with EB3, a microtubule plus-end protein and rapsyn, a postsynaptic scaffolding

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protein. To learn about the function of debrin Paloma Álvarez Suárez has also used drebrin knockout mice (DXKO) and found that the size of NMJs, postnatal maturation of NMJs, its integrity and other parameters of NMJ are mildly or not affected by the lack of drebrin. How could be explained these differences in AChR parameters in various muscles of DXKO mice?
In case of myosin VI (MVI) *in vitro* study showed that drebrin is upregulated in myoblasts of MVI knockout mice (*SV*) but in *SV* mice the NMJ postsynaptic machinery and AChR cluster formation are not affected. The effect of MVI on the postsynaptic machinery organization was observed only *in vivo*. However in *SV* mice, Paloma has found that NMJs are smaller and their maturation is delayed in young (P10) mice comparing with the control but not in all muscle types. Surprisingly, measurements of the grip strength in middle-aged (one-year old) *SV* mice showed that the grip strength was impaired in females but not in males in hindlimb muscles but not in forelimb ones. In females the endplate surface was smaller in TA (fast-twitch fibers) but not in SOL (predominantly slow-twitch fibers). Small changes in NMJs were also observed in MVI knockout middle-age mice.

The obtained results clearly showed more pronounced changes in NMJs after knockout of drebrin or MVI in experiments *in vitro* than *in vivo*. The reason of that could be too small number of animals per experimental and control groups. This should be mentioned in the thesis.

Discussion

Discussion (20 pages) is divided into 5 chapters in which the PhD student describes, in the same order as in the Result chapters, the obtained results and discusses them with findings of other authors. The Discussion provides lots of information on NMJs and synapses in the CNS and I have read this chapter with a great interest. First of all, Paloma Álvarez Suárez underlines that drebrin at the vertebrate NMJ has not been studied yet, however, it is known that this protein regulates postsynaptic sites in neurons. In turn, the motor protein of microfilaments, myosin VI, has been studied by Prof. Jolanta Rędowicz group and other authors in muscle cells but Paloma's results showed that it is important for the postsynaptic machinery and is cross-linked by drebrin. In the Discussion, Figure 5.1 shows a model of drebrin role in the

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postsynaptic site of NMJ in the skeletal muscle. Moreover, Paloma Álvarez Suárez discusses her results in a broad evolutionary aspect and cites results obtained in other species, including *C. elegans* and *D. melanogaster*. There is also many hypotheses on additional functions of drebrin in NMJs and lots of directions for the future studies.

The role of drebrin in organising the actin-reach NMJ postsynaptic sites and in interactions with microtubules was nicely shown by *in vitro* study, however, in *vivo* the effect of lack of drebrin or MVI was not so pronounced. I would like to know the Author's opinion why *in vivo* models showed only mild or no effects of drebrin or MVI knockout. Although in the last chapters of the Discussion Paloma tries to explain these discrepancies, however, I would like to know, for example, if downregulation of drebrin influences expression of other actin-binding protein genes in NMJs. Another question is a role of glial cells, Schwann's cells, on NMJs since this element is not present in *in vitro* models and was not examined in the thesis.

Other comments and questions:

In summary, the Author has carried out many experiments which were technically challenging. It is important that Paloma Álvarez Suárez, studying the role of two proteins in NMJs, carried out *in vitro* and *in vivo* experiments. The obtained results are new and interesting, and the scientific content of the thesis is high. Moreover, the thesis has been prepared with a great care. There are only a few mistakes and the text is well written and clear. There is many figures in the thesis and all are in high quality. I also would like to indicate Paloma's broad knowledge of the scientific literature on her subject of study and on related topics.

In my opinion the Doctoral Thesis of Paloma Álvarez Suárez fulfils criteria defined in the article nr 187 of the Act of July 20, 2018 [Rozprawa doktorska spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2021 r. poz. 478, 619, 1630)].

I propose to the Faculty of the Nencki Institute of Experimental Biology of the Polish Academy in Science in Warsaw to proceed the Ph.D. procedure

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for Paloma Álvarez Suárez. Moreover, I postulate to distinct this thesis because of its high scientific value, methodology and writing.

Elżbieta Pyza

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