Tomasz Wójtowicz, PhD DSc
Laboratory of Cell Biophysics
Nencki Institute of Experimental Biology
Polish Academy of Sciences
t.wojtowicz@nencki.edu.pl

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**Review of the Doctoral Dissertation of Ewelina Latoszek entitled**

**"Characteristics of generated human iPSC-derived models of Huntington's disease, identification of calcium signaling dysregulation and dendritic spine dysfunction"**

To the Honorable Members of the Doctoral Committee,

Huntington's disease (HD) is a genetic and incurable neurodegenerative disorder that affects populations worldwide, with a prevalence ranging from 4 to 13 cases per 100,000 individuals. As an autosomal dominant disorder, HD is characterized by progressive motor dysfunction, cognitive decline, and psychiatric disturbances, making it a devastating condition for affected individuals and their families. Despite decades of research, there remains no cure or disease-modifying treatment, underscoring the urgent need for innovative experimental models that accurately reflect the disease's molecular and cellular mechanisms. HD is caused by a mutation in exon 1 of the huntingtin gene (HTT), leading to the formation of toxic, insoluble aggregates of mutant huntingtin protein (mHTT). The hallmark of HD at the molecular level is the aggregation of mHTT in medium spiny neurons (MSNs) of the striatum, a region critically involved in motor and cognitive functions. HD symptoms can appear at any age, ranging from 2 to 87 years old, which poses a challenge in understanding the pathomechanisms underlying both juvenile and adult-onset forms of the disease.

The doctoral dissertation submitted for my review was conducted by Mrs. Ewelina Latoszek under the supervision of Dr. hab. Magdalena Czeredys and was co-financed by the NCN OPUS 17 project 2019/33/B/NZ3/02889 (to M. Czeredys). Traditional animal models and immortalized cell lines have provided valuable insights into HD pathology; however, they fail to fully mimic human-specific disease mechanisms, particularly the complex interactions within the human brain. This is where human-induced pluripotent stem cells (hiPSCs) and organoid models serve as revolutionary tools in HD research. Mrs. Latoszek leveraged a hiPSC-derived medium spiny neuron (MSN) model to study the disease in a patient-specific context, offering a platform to investigate early molecular changes, calcium signaling dysregulation, and synaptic dysfunction. Additionally, she incorporated 3D brain organoids, which provide a unique opportunity to model cell-cell interactions, neurodevelopmental abnormalities, and the impact of HD on neural circuits—aspects that cannot be fully captured in conventional 2D cultures.

The research presented in this dissertation suggests that pathological changes observed in HD MSNs result from the cumulative effects of multiple perturbations in [redacted] This doctoral thesis represents an outstanding contribution to the field of neurodegenerative disease research, particularly in the study of Huntington's disease. The application of hiPSCs and organoid models demonstrates a cutting-edge, patient-relevant approach that overcomes key limitations of traditional HD models.

The dissertation is written in English and comprises 190 pages, including abstracts in both Polish and English, 31 pages of introduction, 60 pages of results, 30 pages of discussion, and a comprehensive literature section. Additionally, it includes sections on key conclusions, research limitations, and potential future research

directions, as well as a list of publications authored by Mrs. Latoszek. **The only atypical structural aspect of this dissertation is the placement of research objectives - which consist of six main points - after the materials and methods section. In my opinion, the dissertation would benefit from restructuring this section, with the Research Aim positioned immediately after the introduction to Huntington's disease and the discussion of experimental models (hiPSCs and human striatal organoids).** Figures are presented in a standard format, placed within the same chapter and under the text where they are first mentioned. Additionally, the dissertation includes a dedicated chapter discussing the scientific limitations of the project and potential directions for future research (six key points), which greatly enhances the overall quality of the work. The list of publications included in the dissertation comprises four papers, in which Mrs. Latoszek is the first or second author, and which contain results presented in the dissertation. Additionally, two other publications are included, where she is either the first author or a co-author. In total, this amounts to six scientific publications produced during the doctoral research period, further highlighting the scientific merit and productivity of this work.

The literature used in the dissertation is applied correctly and is comprehensive (396 references). It is worth emphasizing that the doctoral candidate provides the reader with a glossary of complex medical terms, presented in Table 1.1, which significantly facilitates the understanding of Huntington's disease symptoms. Additionally, Table 1.2 is highly useful, as it offers a characterization of iPSCs and hSOs, along with the limitations of using these models in scientific research.

In the Introduction section, Mrs. Ewelina Latoszek extensively presents the current state of knowledge in the field of Huntington's disease, including the molecular mechanism. She presents in vitro models and the limitations of the experimental approach. The text is aided by three color figures. This section is written with great competence and breadth. It also introduces key terms and explains strategies used further in the research presented in the thesis. It is evident that the doctoral candidate has an excellent understanding of current publications in this field and navigates them with ease. **It would be beneficial to include a few concluding sentences at the end of each subsection or chapter to provide a summary. The language used in the Introduction is highly scientific and detailed, and due to the dense citations and molecular biology details, it may be challenging for the reader to grasp the broader picture.** For example, the section on organoids and their application in disease modeling is written in a very accessible, almost popular science style, making it easy to follow. Similarly, the chapter on the limitations of using organoids (pages 41–43) is also very well written and engaging.

Following the introduction, the candidate presents the research objectives (six main points). The research objectives are ambitious, numerous and valid. They include observation of the role of SIP protein in mHTT aggregation in HD cellular model and generation of hiPSC-derived MSNs and human organoid models. In these models from juvenile and adult onset HD patients, author studied gene expression and calcium signaling. Experiments were planned correctly, and the results allow for answering the research objectives. The author showed that SIP overexpression promoted the degradation of huntingtin protein via the ubiquitin-dependent pathway. The author also analyzed how increased SIP dimerization may affect mutant HTT ubiquitination. Mrs. Latoszek showed that mutations stabilizing the SIP dimerization domain diminish the ubiquitination of mHTT. This also led to decreased anti-aggregation activity against mHTT. The author also verified the hypothesis that the [redacted] However, this had no effect on the levels of the products of these genes (Fig. 5.16). The author successfully differentiated hiPSCs into MSNs that express typical cellular markers (*GAD65*, *DARPP32*). [redacted]

Nencki Institute of Experimental Biology, Polish Academy of Sciences
Pasteur 3, 02-093 Warsaw
phone.: (+48 22) 58 92 207, email: dyrekcja@nencki.edu.pl
www.nencki.edu.pl

[redacted] Mrs Latoszek also used an organoid model to verify whether similar observations to those made in hiPSC neurons could be observed at a higher organizational level of neuronal tissue. [redacted] **The research methods used by Mrs. Ewelina Latoszek are very difficult to handle and require precision, patience, and dexterity. In addition, skills to culture hiPSC and organoids are currently in very high demand. Therefore, in my opinion, this proves the candidate has very good laboratory skills.**

In the Discussion section, the author refers to all results sequentially and ends the chapter with conclusions. It is very helpful that the author decided to summarize the most important results relevant to HD cellular models in a separate chapter (5.5.0), thus enabling the reader to better understand the discoveries she made. The Discussion is very well written. **I only find the discussion is lacking a word on the role of the SIP monomer in regulating HTT protein function in cells (since the monomer was mainly detected in MSNs). In addition, the Discussion section would benefit if the author explained how future manipulations of SIP could be applied in the therapy of HD (Gene therapy? Pharmacological treatment to modify protein activity or monomer/dimer formation?).**

A great advantage of this study was the comparison of juvenile- vs. adult-onset HD *in vitro* models. While there are plenty of results, in my opinion, the author could discuss more or better summarize the differences between the two [redacted] Currently, Figs. 6.3 and 6.4 provide some graphical summary, but more graphical summaries would be helpful.

[redacted] This could be discussed. As suggested by the author, in the future, patch-clamp electrophysiological recordings from individual neurons of differentiated MSNs or hSOs could be beneficial to address this issue. In summary, the Results and Discussion sections are very well written and demonstrate an enormous amount of work in the laboratory. In this doctoral thesis, the candidate investigated several genes. It would be helpful to compile all the main results into a table or infographic to summarize the findings. Currently, the description of results and discussion spans over 60 pages.

The loss of MSNs is the most severe hallmark of HD, which is reflected in the motor impairments of the patient. However, most studies of HD MSNs are conducted either in mouse models or postmortem brain tissue samples. The author developed and described the features of four human fibroblast-derived hiPSC lines, differentiated into NPCs, MSNs, and organoids. She characterized hSO cultures and confirmed that they serve as an intermediate model for the formation of assembloids for further examination of HD pathology. The development

of these tools enables the study of pharmacological treatments for HD. In this regard, two main original discoveries reported in this thesis are that SIP protein could be considered a potential target for anti-HD therapy during the early stages of HD pathology. [redacted] **Neural progenitor cells (NPCs) derived from hiPSCs by Mrs. Ewelina Latoszek could be valuable for further research at the Institute, particularly in studies on neurological mitochondrial disorders, Fragile X syndrome, metachromatic leukodystrophy, and neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). The cellular models established by Mrs. Latoszek could be used for basic cell biology experiments and for identifying new drug targets in the future.**

In the Discussion, the author raises an important issue regarding the process of obtaining hiPSC lines from patient-derived fibroblasts or blood cells. She highlights that validating and characterizing these lines for publication acceptance is a time-consuming and costly process compared to purchasing commercial lines from biobanks. However, instead of using commercial sources, the author applied the lentiviral reprogramming method to develop various cell lines that are not currently available commercially. This approach allowed her to gain deeper insights into pluripotent cell biology and explore how different hiPSC lines behave. GABA is critical to striatal function, as MSNs are GABAergic, forming inhibitory synapses on neighboring neurons via collateral axons. The GABAergic system and neurotransmission are also impaired in the HD brain, and its concentration was decreased in the brains of HD patients, as reported by Mrs. Latoszek in the Discussion section. [redacted] A significant positive aspect is the analysis of the limitations of the doctoral candidate's research. This demonstrates scientific maturity, a deep reflection on the results obtained, and provides a solid foundation for planning future studies in this field. In summary, in my opinion, the doctoral dissertation constitutes an original solution to a scientific problem.

Below, I raise questions for open discussion and list suggestions at the end of the document that could improve the communication of the thesis to a the readership:

1. I don't understand why the author used Student's one-sample t-test to compare the effect of SIP overexpression on cell cultures expressing a plasmid with 72 glutamine repeats instead of using, for instance, an unpaired Student's t-test. A Student's one-sample t-test is used to compare the mean of a single sample to a known or expected population mean value to determine if there is a significant difference between them. This test is commonly used in situations where the population standard deviation is unknown (i.e., Fig. 5.3). The same issue occurs with Figure 5.14 data. A Student's one-sample t-test is typically used to compare the mean of a single sample to a known reference value (e.g., a theoretical mean or expected value). However, in the case of comparing [redacted] a two-sample (independent) t-test or Mann-Whitney U test (if the data is not normally distributed) would be more appropriate. The same issue occurs in Fig. 5.23 and many others. Importantly, sometimes the author compares only data within a given model (e.g., juvenile HD), but for other data, she used One-Way ANOVA and compared all models and treatments (e.g., Fig. 5.29).

[redacted]

[redacted]

In summary, the candidate clearly has theoretical knowledge in the discipline and can perform scientific research. She is a first author of a review article on molecular components of store-operated calcium channels in the regulation of neural stem cell physiology, neurogenesis, and the pathology of Huntington's disease (published 2021 Fron Cell Dev Biol). She is also the key author in 4 other research articles in which data presented in this doctoral thesis have been encapsulated. In summary, the assumptions and goals of the study were clearly defined and described thoroughly. **The planned experiments were adequate to resolve the scientific problem and met top standards. The conclusions were correctly drawn and supported by the results. The PhD thesis proved the candidate's general theoretical knowledge in a discipline and the ability to independently conduct scientific work. Also, the subject of the PhD thesis is an original solution to a scientific problem.**

I, the undersigned, hereby state that the doctoral dissertation of Ewelina Latoszek meets the requirements specified in Article 187 of the Act of July 20, 2018 – Law on Higher Education and Science (c.t., Journal of Laws of 2024, item 1571, as amended). I hereby recommend to the Doctoral Committee of the International Institute of Molecular and Cell Biology in Warsaw to admit Ewelina Latoszek to the subsequent stages of the procedure for the conferment of the doctoral degree in the field of natural sciences, in the discipline of biological sciences.

In addition, considering the level of difficulty to perform experiments described in the dissertation, complementarity and advancement of the research models, the importance of the research problem and the number of research articles published by Mrs Ewelina Latoszek, **I believe this doctoral dissertation is beyond average and deserves distinction.**

Dokument
podpisany przez
Tomasz Wójtowicz
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Nencki Institute of Experimental Biology, Polish Academy of Sciences
Pasteur 3, 02-093 Warsaw
phone.: (+48 22) 58 92 207, email: dyrekcja@nencki.edu.pl
www.nencki.edu.pl

**Minor comment and suggestions for corrections:**

1. In the first experiment, author presents exemplary pictures of huntingting aggregates in HEK293 cells transfected with plasmid coding either exon 1 with 25 glutamine repeats or 72 repeats. However, fig. 5.1 show only quantification of the second condition. This is particularly needed as in the next figure author shows that SIP overexpression does affect huntingtin level by promoting degradation in cells expressing 25 glutamine repeats (Fig. 5.2.).
2. Font size in figures could be larger, it is sometimes difficult to read what X/Y axis show (Fig. 5.2).
3. In Fig. 5.1 it is shown N=15 but in figure legend it says 5 independent experiments. So it is not clear what n in figure stands for. Same is in fig. 5.5 n=75 vs n=3 in figure legend. Same problem is in Fig. 5.29, values n=293-401 most likely represent individual region of interests (i.e. neuronal somata) analyzed but it is not clear and the reader must guess it.
4. How come, control in panel D fig. 5.5 is not significantly different from mutants? Only SIP WT is considered for statistical analysis. Same in panel C. I don't know why some data have been compared and significant/ns is shown for others not (same panels). For instance we don't know from the figure or text whether there is significant difference between effect of SP K21W and SIP T30R_S33E mutants.
5. Figure 5.7 panel D and figure in general is difficult to read, quality and font size should be fixed.
6. Fig. 5.13 does not have any statistical analysis, how were conclusions drawn?
7. On Page 90, author declare successful reprogramming of jHD-V1 cell line to NPC. But I can't find data supporting this (unlike M-T1 cell line).
8. Fig. 5.14. Exemplary Pictures show expression of neuroectodermal marker nestin in adult and juvenile onset HD hiPSC-derived NPC lines. However, quantification of rosettes' area is shown only for juvenile NPC.
9. In Fig. 5.35, Top panels lack A,B descriptions, matching figure caption.
10. Figure 5.36 seems to lack median or standard error values in some of the presented datasets. Why?
11. Fig. 5.41 lacks statistical analysis (multiple histograms, no information, or table with results of multiple comparisons).
12. Some statements would require citation. For instance page 147 "Dendritic spines that did not contain postsynaptic protein PSD95 were shown to survive no longer than one day. "
13. In the Methods chapter, the manufacturer is listed as Sigma (page 46), but the correct name is Sigma-Aldrich, which is now part of Merck. Similarly, Tocris should be referred to as Tocris Bioscience, part of Bio-Techne. I could not find a company named ChemCruz selling puromycin—it is likely referring to ChemCruz Biochemicals, a brand of Santa Cruz Biotechnology (USA). Thermo Scientific is a brand, while the actual company name is Thermo Fisher Scientific. Therefore, it might be more accurate to list brands instead of company names in the table to ensure consistency. Roth should be referred to as Carl Roth. The fluorescent calcium ion indicator Fura-2 is sold by Thermo Fisher Scientific under the Invitrogen brand, yet in the dissertation, the company name was used instead of the brand. Conversely, for the DAPI reagent (page 48), the opposite logic was applied. The full name of VWR is VWR International Sp. z o.o. On page 49, Expredia is listed as a manufacturer, but it is likely referring to the Epredia™ SuperFrost Plus™ brand, which operates under Thermo Fisher Scientific. In Table 2.8 (page 51), the column title "Company # Catalog number" is incorrect, as it contains only the company names. A similar inconsistency appears in Table 2.9.
14. It is unclear why single-digit numbering was used for chapters, while three-digit numbering was applied to subchapters. Tables and figures, on the other hand, are labeled with two-digit numbering. Additionally, starting from page 30, in subchapter 1.2.3, the doctoral candidate switches to bold font to indicate subsequent subchapters (paragraphs) instead of continuing with numerical labeling (e.g., 1.2.3.1, etc.), as used earlier.

15. The authors of the free ImageJ (Fiji) software used in this dissertation have not been cited (Johannes Schindelin et al., 2012, Nature Methods, doi:10.1038/nmeth.2019).

Imprecise formulations that could be corrected to better communicate the discoveries made by the Author:
1. In many places in the text, the Author mixed past tense and present tense. It is a custom to use one tense while reporting literaturę. (typically past tense or past perfect tense). Sometimes tenses are mixed in neighbouring sentences, i.e. page 34: [redacted] Also there are grammatical error and typos which could be corrected. Examples are liste at the end of the review.
2. Grammatical errors or style problem, sentences should be rephrased or corrected:
- as shown by postmortem studies compared to healthy individuals, which indicated the regional heterogeneity of a vulnerability (could read "regional heterogeneity in vulnerability").
- Page 23: "Also observed changes in striatal shape and morphology such as deformation of the caudate nucleus and putamen, as well as changes in the connectivity patterns of the striatum with other brain regions" (should read "It is also observed")
- Page 32 "Also, abnormal synapses containing synaptic vesicles that varied in size or formed defectively were observed" could read "were formed defectively, were also observed."
- Page 32 "For this purpose first, they [Authors], used to transfect chemically", better "the used, applied".
- Page 87 "Dorsomorphin was used to stronger inhibition of the BMP signaling pathway" rather "to enhance inhibition"
- Page 100 "Using transduction at 76 DIV of lentivirus with GFP expression, performed visualization of single neurons with dendritic spines in a very dense and complex adult"
- Page 100 "According to (226), it was possible also distinguish" should read "also to distinguish"
- Page 139 "we had to opportunity to confront," rather " the opportunity to"
- In many places in the text, the Author mixes past tense and present tense. It is a custom to use one tense while reporting literaturę. (typically past tense or past perfect tense). Sometimes tenses are mixed in neighbouring sentences, i.e. page 34: [redacted]
- Similarly, page 72: "Previous results of Czeredys and co-workers show"
- Sentence „the HEK293T cells were transfected N1 plasmids" should read "were transfected with N1 plasmid".
- Page 92: " HAP1, TRPC1, or STIM2 was not change" it think author meant "did not change"?
- Page 114: "Moreover, hiPSC-derived MSNs characterized by a fast response to high concentrations of K + , which results in the opening of voltage-gated channels (VGCC) and a rapid influx of Ca 2+ into the cytosol". This sentence should be rephrased.
- Another problem with grammar is in Discussion page 135: " Impact of SIP on aggregation and HTT protein level in respect to literature" should read "Impact of SIP on aggregation and HTT protein levels with respect to the literature."
- Discussion Page 140" While the correction of the HD mutation in the isogenic lines rescues the impairment in neural rosette formation". This sentence should be rephrased, since it has some intrinsic error.
- Aim of the study has some grammatical error i.e." Obtaining and characterization human fibroblast-derived iPSC lines".

- Page 79 “HD onset is manifest mainly” maybe “manifested”?

3. Typos

- Degeneracją (page 13), uncontrol (uncontrolled, page 16), loner median (longer median, page 17) protein that may (remove may, page 19), which not directly (which does not directly, page 20), reprogramed (reprogrammed, page 26), in vitro (in in vitro, page 26),

4. Other issues:

- The witch hunt and the Salem trials took place in colonial North America during the period of 1692-1693, not as stated in the 1962 dissertation. (page 15)
- In Figure 1.1, symbols such as nSOCE are used but are not explained in the text or the figure description. Figure 1.1 has panels A and B, but their content is not described in the figure caption, leaving it up to the reader's interpretation. In contrast, Figure 1.2 is well-described and explained, including abbreviations and symbols.
- Sentence like in Discussion section” no change in SIP protein levels was observed between both HD onsets and control” is not grammatically correct. The phrase "between both" is redundant because "both" already implies two groups.
- In page 124, the sentence “ There was statistically non-significant difference in” is ackward. I think it could be rephrased due to logic.

Nencki Institute of Experimental Biology, Polish Academy of Sciences
Pasteur 3, 02-093 Warsaw
phone.: (+48 22) 58 92 207, email: dyrekcja@nencki.edu.pl
www.nencki.edu.pl