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Review of the doctoral dissertation by MSc. Olga Katarzyna Doszyń titled "Mechanisms underlying TSC-associated neuropsychiatric disorders in the zebrafish model"

The subject of the review is the doctoral dissertation of MSc. Olga Katarzyna Doszyń, entitled *"Mechanisms underlying TSC-associated neuropsychiatric disorders in the zebrafish model"* which was prepared at the Laboratory of Developmental Neurobiology International Institute of Molecular Mechanisms and Machines, Polish Academy of Sciences and Laboratory of Molecular and Cellular Neurobiology, International Institute of Molecular and Cell Biology in Warsaw under the supervision of Dr. hab. Justyna Zmorzyńska. The title clearly indicates the study of mechanisms underlying neuropsychiatric disorders associated with Tuberous Sclerosis Complex (TSC) in the zebrafish model. It is appropriate for the content of the dissertation, which focuses on this issue, though it covers a wide range of topics from theoretical introduction, through literature reviews and research protocols, to original experimental results.

The dissertation consists of nine chapters: starting with a general introduction (Chapter 1) and definition of the aims of the work (Chapter 2), through four review or methodological chapters (Chapters 3, 4, 6, 7), two chapters containing the author's original research results (Chapters 5 and 8), and concluding with Chapter 9 encompassing summary, discussion, and future perspectives. This layout combining published review papers, research protocols, and original experimental articles is unconventional but increasingly common in dissertations based on a cycle of publications. The structure of the work is generally logical, but the presence of extensive review chapters (3 and 4) and protocol-based ones (6 and 7) raises some doubts about their necessity and contribution to the originality of the dissertation. These chapters, although valuable as a compendium of knowledge and methods, contribute less to the presentation of new discoveries by the doctoral candidate. From a structural standpoint, one could expect a stronger emphasis on the author's own research results, which are concentrated mainly in two chapters (5 and 8). Nonetheless, the entire dissertation is thematically coherent. All parts concern the zebrafish model in the context of TSC and neuropsychiatric disorders and together provide a comprehensive view of the topic.

Chapter 1: General Introduction

The first chapter is a theoretical introduction to the subject matter presenting Tuberous Sclerosis Complex (TSC), the characteristics of TSC-associated neuropsychiatric disorders (TAND), known molecular mechanisms underlying TSC, and a review of existing treatment strategies. This chapter is well-prepared and comprehensive. The author clearly outlines the biological and medical background of the problem, demonstrating familiarity with current literature and an ability to synthesize information.

Subchapter 1.5, concerning animal models of TSC (including the significance of zebrafish as a model organism), smoothly leads the reader to the justification for using *Danio rerio* in subsequent research. The introduction is therefore sufficiently thorough and provides a solid foundation for the following sections of the dissertation.

Chapter 2: Objectives and Scope of the Work

The second chapter defines the main goal and scope of the dissertation, but it does so in a fragmented and not entirely convincing way. The only clearly formulated objective appears in the first sentence of the chapter and refers broadly to the mechanisms of neuropsychiatric disorders in the TSC zebrafish model. The rest of the text in this chapter does not develop scientific goals but rather describes the actions taken. There is a lack of clearly defined sub-goals. Such as goals for the described protocols or the included textbook chapter, which raises questions about the rationale for their inclusion in the dissertation and the role they play in the structure of the research project. In its current form, it is difficult to determine the scientific objective of these parts and how they fit into the overarching research question of the doctoral dissertation. This lack of clarity in goal construction limits the coherence of the work and hinders its straightforward interpretation in the context of a doctoral project. Additionally, it should be noted that the literature cited in this chapter should rather be included in Chapter 1, which serves as the theoretical introduction. Its current placement is inappropriate and may lead to confusion about the structure of the work.

Chapter 3: The zebrafish model of Tuberous Sclerosis Complex to study epilepsy

This chapter is a review of the zebrafish model in studies of epilepsy associated with TSC. As noted in the text and editorial note, it is a full chapter prepared for the "Handbook of Animal Models in Neurological Disorders." This fact alone raises questions about the justification for including it in such an extensive form in the dissertation. Chapter 3 is interesting as a literature review and practical guide to using the zebrafish model to study epilepsy. However, its scientific value and contribution to the originality of the thesis are questionable. It is difficult to identify a clear contribution from the author to advancing knowledge, aside from compiling and presenting existing information. In a doctoral dissertation, literature reviews are expected to serve primarily as a background to one's own experiments, not to function as a stand-alone "book chapter" with limited novelty.

Aside from the above strategic concerns, the substantive quality of Chapter 3 raises some reservations. It looks like a collection of practical tips ("how to raise and study TSC fish") and personal observations that are not always properly supported by literature. For example, the author claims that "TSC fish are sensitive to hypoxic conditions and thus water should be exchanged daily." It is not clear on what basis this recommendation is made. Whether from literature, personal experiments, or anecdotal husbandry experience. Such statements should be supported by a source or mechanistic explanation (why would TSC mutants be particularly hypoxia-sensitive?). The lack of such clarification suggests an anecdotal character.

There are also some inaccuracies and omissions. Illustrations in this chapter are described too briefly, missing explanations of what they show, the meaning of symbols, arrows, or abbreviations, and the source of the data. Are they the author's own, literature-derived, or illustrative schemes? This should be clarified in figure legends to ensure transparency.

The description of seizure-related behavior in zebrafish larvae is mostly limited to increased locomotion ("hyperactivity"), which oversimplifies the complex behavioral phenotype of seizures. The literature commonly includes parameters such as seizure duration, intensity, and movement patterns. The author omits a well-known seizure behavior in zebrafish larvae referred to as "whirlpool-like behavior" (rapid, circular swimming), despite citing studies that report this phenotype (e.g., Afrikanova et al. 2013). It would be important to explain whether this was not observed in the TSC model or why it was omitted.

Minor errors also appear, such as the awkward phrase "embryos were born," which is biologically inaccurate for oviparous species like zebrafish. Furthermore, the author repeatedly uses the term "frontbrain," which is not a valid neuroanatomical term. The correct terminology should refer to the forebrain or its subdivisions (telencephalon, diencephalon). While "frontbrain" occasionally appears

in publications, mostly from the author's own research group. It is not formally accepted. Introducing non-standard nomenclature can create conceptual confusion, especially among early-career neuroscientists. As a PhD candidate in neurobiology, the author should use accurate anatomical terminology. This usage suggests knowledge gaps in this area.

Other anatomical inaccuracies include: “Because the zebrafish brain is smaller and contains fewer neurons, some structures of the human brain do not have a direct zebrafish homolog.” This explanation is misleading. The absence of homologous structures is due to evolutionary divergence, not brain size. The statement “Nonetheless, zebrafish do lack major portions of the human cortex” is also inaccurate. Zebrafish lack the neocortex altogether, not just “portions” of it. While the pallium in zebrafish performs partially analogous functions, it is histologically distinct. These examples suggest that the author occasionally relies on personal interpretations of neuroanatomy rather than established facts. Although this does not directly affect the results of the dissertation, it signals theoretical weaknesses, which will be revisited later in the review.

In summary, Chapter 3 is a useful guide to the zebrafish model and reflects the author's hands-on experience with this system. However, in the context of a doctoral dissertation, its scientific originality is limited, and it contains simplifications and errors. It serves as an extensive review that may benefit readers unfamiliar with the zebrafish model but does not contribute novel findings or breakthrough hypotheses.

Chapter 4: Diving into the zebrafish brain: exploring neuroscience frontiers with genetic tools, imaging techniques, and behavioral insights

Chapter 4 is another review, this time broader in scope. It discusses various modern research techniques in zebrafish neurobiology (genetics, imaging, behavior). It was published as a review article in *Frontiers in Molecular Neuroscience*. The ambition to summarize and assess methodologies used in zebrafish neuroscience is commendable which is typically done by experienced researchers, but the author has taken up this task to demonstrate her broad methodological awareness.

The content of Chapter 4 is solid, though necessarily selective. The author describes chosen tools (e.g., transgenic lines, optogenetic indicators, brain imaging, behavioral assays) in a somewhat subjective manner, and not all described methods are directly tied to the doctoral thesis objectives. Still, the chapter reflects a wide-angle view on the field. As it was peer-reviewed and published, a detailed critique of every statement is unnecessary. However, one claim in the discussion is questionable: “Zebrafish have a relatively short lifespan compared to some other model organisms.” It is unclear which organisms are being compared. Basic zoological knowledge indicates that zebrafish have a relatively long lifespan among small animal models living 5-6 years, while mice and rats live about 2-3.5 years. Even larger models like rabbits live 8-10 years. This example suggests the author should approach such generalizations more critically. While minor, it serves as a cautionary note.

To summarize, Chapter 4 is a well-executed review. It does not present novel research findings but demonstrates the candidate’s competence in modern neurobiological tools. In the context of the dissertation, it broadens the methodological background. One could debate whether early-career scientists should publish such reviews (typically authored by experts), but the fact that it was published confirms its acceptance by the scientific community.

Chapter 5: Hyperactive mTORC1 disrupts habenula function and light preference in zebrafish model of Tuberous Sclerosis Complex

Chapter 5 is one of the key components of the dissertation, presenting the doctoral candidate’s original research findings. It is formatted as a scientific article (already published) and focuses on the role of hyperactive mTORC1 signaling in habenula dysfunction and altered light preference in the TSC zebrafish model. This chapter is evaluated very highly. The publication of these findings itself attests to their quality and innovative character.

The content of Chapter 5 demonstrates that in tsc2 mutants, excessive activation of mTORC1 affects habenula function, a brain structure involved in processing aversive stimuli and regulating light avoidance behavior. The study combined behavioral analysis (light/dark preference test) with

microscopy techniques (e.g., p-Rps6 staining as a marker of mTORC1 activity, habenula imaging). The results showed that mTORC1 hyperactivity disrupts habenula function, which manifests as altered behavior in the light preference test. These data are significant because the habenula is implicated in mood and anxiety regulation. Its dysfunction may help explain neuropsychiatric symptoms such as anhedonia or abnormal sensory processing observed in TSC. Moreover, this study suggests that modulation of mTORC1 (e.g., via rapamycin) influences these phenotypes, indicating potential therapeutic directions. Thus, the scientific value of this chapter is high. It provides new insights into the role of mTOR signaling in the habenular system and its behavioral consequences, effectively linking molecular biology with excellent knowledge of the zebrafish model.

From an experimental and data presentation perspective, Chapter 5 is of high quality. The methods are appropriate and modern, the statistical analyses are correct, and the discussion is well-reasoned and situated in the context of current literature. I find no major flaws or errors in this part. It constitutes a strong foundation for the dissertation and demonstrates the candidate's research competence.

Chapter 6: Protocol for visualization of pRps6-positive cells in larval zebrafish brains using whole-mount immunofluorescence and lightsheet microscopy

Chapter 6 is a methodological publication (protocol) published in the journal *STAR Protocols*. It presents a procedure developed (or adapted) by the author for visualizing cells positive for phosphorylated Rps6 (p-Rps6) in zebrafish larval brains, using whole-mount immunofluorescence and light-sheet microscopy. Such protocols are valuable technical contributions that facilitate reproducibility and adoption by other researchers. However, the scientific originality of Chapter 6 is limited, as it does not contain new biological discoveries but rather the optimization of existing methods.

The practical value of this chapter is moderate: it provides a step-by-step description of p-Rps6 staining, a marker for mTORC1 activity. Since the author used this method in her experiments (e.g., in Chapters 5 and 8 to assess neuronal activity), including the protocol in the dissertation demonstrates her laboratory proficiency and commitment to standardizing procedures. Unfortunately, this protocol is not free from inaccuracies. For example, the author writes that the *casper* line (a transparent zebrafish strain used for imaging) is transgenic. In fact, "*casper*" is a mutant line obtained by crossing *nacre* and *roy* mutations, resulting in a lack of pigmentation. It is not transgenic in the sense of harboring foreign genes but rather a combination of genetic mutations. This is a minor terminological error, but important for precision. Additionally, the protocol states that "the effect of PTU on brain morphology and function is unknown." This statement is incorrect. Many studies have demonstrated PTU's negative effects on development and physiological function. For instance, PTU disrupts thyroid hormone synthesis, which is essential for proper nervous system development. Scientific protocols are expected to reflect up-to-date knowledge; if uncertain, the author could have written that "PTU may affect nervous system development, and its use requires caution," rather than suggesting complete ignorance.

Aside from the above, Chapter 6 raises no major concerns, though it is rather basic. Similar protocols exist in the literature, so the novelty here lies primarily in the combination of the TSC fish model, p-Rps6 antibody, and light-sheet microscopy. For the evaluation of the candidate, it is important that she not only performed these stainings but also formalized the procedure and published it. This reflects her strong technical skills. Nevertheless, this chapter has less scientific weight than the results chapters and should be viewed as a methodological supplement to the dissertation.

Chapter 7: Protocol for microinjection of rapamycin into the zebrafish habenula

Chapter 7 is another protocol published in *STAR Protocols*, this time describing rapamycin microinjection into the zebrafish habenula. Again, it provides a valuable technical description, precise drug delivery directly into a defined brain structure is highly relevant for functional studies. This chapter further demonstrates the candidate's practical skills and her contribution to the development of methods used in the zebrafish model.

While the scientific value of Chapter 7 is limited (as it describes a method rather than a result), the protocol itself is valuable. It likely contributed to the data presented in Chapters 5 and 8 (where the

effects of rapamycin in the habenula are analyzed). However, there is a significant issue in this chapter: it concerns the anatomical schematic (Figure 2), which shows the larval zebrafish brain. The figure contains neuroanatomical errors. In the side view, the optic tectum is labeled in a way that suggests it occupies the entire mesencephalon and diencephalon. In other words, structures such as the midbrain tegmentum and even the hypothalamus appear to be incorrectly labeled as part of the optic tectum. This is a substantive error indicating some deficiencies in detailed knowledge of zebrafish neuroanatomy. While the dorsal view was acceptable, the side view is misleading. Publishing incorrect anatomical diagrams is problematic, as it promotes terminological confusion and spreads misinformation among readers. As a PhD candidate in neurobiology, the author should pay particular attention to accurate labeling of brain structures.

Aside from this issue, Chapter 7 fulfills its role as a methodological supplement. The procedures described here demonstrate that the doctoral candidate has mastered advanced microsurgical techniques and can communicate them effectively in publication form. However, as with Chapter 6, this section does not provide original scientific discoveries. It is a technical component of the dissertation.

Chapter 8: Evolutionary perspectives on anxiety: telencephalic circuitry and the anxiogenic role of TrkB signaling in Tuberous Sclerosis Complex

Chapter eight is the second fundamental chapter (along with chapter five) that contains original research results. This is a manuscript (currently available as a preprint on bioRxiv) focusing on the evolutionary aspects of anxiety: investigating telencephalic circuits involved in anxiety responses and the role of TrkB receptor (BDNF receptor) signaling as an anxiogenic factor in the TSC model. The data presented in this chapter are very interesting and valuable. They provide deeper insight into how TSC mutations affect anxiety-like behaviors in developing zebrafish larvae and the underlying neural mechanisms.

It is commendable that the doctoral candidate explored anxiety from the perspective of evolutionary conservation of mechanisms. The zebrafish telencephalon performs functions analogous to limbic structures in mammals (e.g., the amygdala, septum), so understanding its role in anxiety-like behaviors in zebrafish may shed light on anxiety mechanisms in TSC patients. Furthermore, investigating TrkB/BDNF signaling is justified, as BDNF is a key factor in neuronal plasticity, and its disruptions have been associated with anxiety and ASD. The candidate tested pharmacological interventions, e.g., using the TrkB antagonist ANA-12 and rapamycin, to assess their effects on behavior and neuronal parameters in TSC zebrafish.

However, it must be noted that chapter 8 (being a preprint and therefore not yet fully peer-reviewed) has several methodological and interpretative shortcomings. The behavioral protocol description is imprecise: the Materials and Methods section lacks crucial details. For example, it states, "All stock solutions of drugs used in this study were prepared in E3 medium […] or DMSO". This is unclear: which solutions were prepared in DMSO? Does this imply that some substances required DMSO to be dissolved? Another example: "bathing medium, containing up to 50 dechorionated larvae". What was the volume of the medium? In what plate were the 50 larvae placed? Such details are essential for reproducibility. Also: "following a 15-minute period of habituation to the behavioral testing room". The conditions of this habituation are not specified: what was the temperature? Were the larvae kept in darkness or under baseline lighting? These conditions may affect behavioral test outcomes, especially anxiety-related tests. The protocol states, "The plate was uniformly illuminated with bottom light set to 80% intensity". 80% of what? The lamp's maximum power? This is not a measurable unit. Light intensity should be reported in standard units (e.g., lux), or at least the light source should be described. Such lack of precision hampers interpretation and comparison with other studies.

In the results section, the candidate describes larval behavior in the light-dark test. She uses phrases like "Relative time next to the walls" in graphs, which is imprecise. The behavior of staying close to the walls is known as thigmotaxis. However, this term does not appear even once in the results description. In scientific work, established terminology should be used; a better phrasing would be, for example, "time spent in the outer zone, a measure of thigmotaxis." This reflects some stylistic and terminological shortcomings. Moreover, the candidate misinterprets "freezing behavior." She notes that TSC mutants (or certain drug-treated groups) exhibit reduced locomotor activity in the light phase of the test. She labels this as "freezing", which appears to be a misapplication of the term. Freezing typically

refers to a brief, fear-induced immobility in response to a sudden stimulus. In contrast, the candidate's light phase lasted 10 minutes, and the reduced activity persisted over a longer period. In fact, the graphs show that after the light was turned on, larvae initially displayed a spike in activity, followed by a drop to a lower level than in the dark phase. This suggests that light onset did not cause classical freezing (no immediate immobility, on the contrary, there was a increase of activity).

Another important issue is the acclimatization phase. The candidate mentions a 15-minute habituation in the testing room, but we don't know whether the larvae had a prior acclimation phase in light or dark and whether their activity was recorded then. The lack of an activity graph during acclimation complicates interpretation. If activity gradually declined during adaptation and remained similar during light, it would suggest a typical lighting response rather than anxiety-induced freezing. In summary, labeling 10 minutes of low activity as "freezing" is inappropriate. This conceptual error should be corrected to avoid misleading conclusions.

In Figure 1H, the same colors are used to denote two different groups, making it difficult to distinguish curves for ANA-12 and rapamycin-treated groups. This technical error should be fixed before final publication.

There are also numerous concerns regarding the immunohistochemical analyses. The candidate did not provide specific antibody identifiers (e.g., catalog numbers, species specificity). In zebrafish studies, antibodies against mammalian proteins are commonly used. They are effective if the immunogen used to generate the antibody is identical or highly similar, but this needs verification. The candidate briefly mentions antibody selection challenges in chapter 6, but we don’t know how specificity was verified in chapter 8. For instance, an anti-calretinin antibody was used, but zebrafish have two calretinin genes (calb2a and calb2b). Which protein was detected? Both? Similarly, the ELISA kit for TrkB was designed for human protein. Does it reliably detect the zebrafish homolog? These issues are not clarified, raising concerns about the reliability of some immunofluorescence/ELISA results.

Furthermore, it was stated that “Integrated density was chosen as a proxy for the number of parvalbumin-positive cells due to the low optic resolution in the medial subpallium, making it difficult to distinguish single cells.” This decision is questionable. If single cells can’t be distinguish, the likely cause is either inadequate resolution or poor staining quality (e.g., insufficient antibody penetration or lack of antibody specificity to zebrafish antigen).

In Figures 2F, it is visually apparent that the $tsc2^{-/-}$ group has more p-Rps6+ cells than controls. Despite this, the candidate did not count the cells but instead measured intensity in five randomly chosen cells. This is suboptimal. If there are visibly more cells, they should be counted and statistically analyzed. Measuring intensity in a few cells might miss significant results: for instance, a mutant may have more active cells with lower individual intensity, resulting in an averaged signal similar to controls. Cell counting or measuring total fluorescence across the region (not just in 5 cells) would give a more complete picture. This oversight should be addressed.

Additionally, many images in Figures 4-8B are of insufficient quality. Expected structures are hard to discern (e.g., VGlut1 signal doesn’t resemble the zebrafish brain atlas referenced by the candidate). Some stainings appear nonspecific, possibly due to improper antibody use (see above). Moreover, figure and text descriptions are inadequate: e.g., in Figures 4-8C, quantitative data are presented, but details on how they were obtained are missing. How many animals (N), how many slices or sections per animal, what quantification method was used? Without this, it's difficult to assess the reliability of the findings. Such information is necessary for scientific transparency. Furthermore, regions on these graphs are labeled (e.g., "medial," "central," "lateral" part of a layer) without defining what these mean. A diagram or indication on the figure would help. Some images include annotations like "(soma)" or "(fibers)," while others don’t. Does this mean perikarya and nerve fibers were analyzed separately? If so, this should be clearly explained. Even the notation of quantities (e.g., using “-” vs “--” in legends) is unclear. All these shortcomings indicate that the manuscript needs refinement before publication.

Additionally, there’s inconsistency in one data set, the candidate counts cells for one marker (calretinin), reports intensity for another (parvalbumin), and provides no quantitative analysis for others. Why were some markers omitted from quantification? Perhaps the results were insignificant or difficult to analyze, but then this should be stated in the discussion, not omitted.

Lastly, the anatomical scheme in Figure 9c is problematic. It is impossible to determine whether the signal from one marker (e.g., NPY) overlaps with others (e.g., VGlut1), since stainings spatially overlap.

In summary, chapter 8 provides very interesting findings but also requires the most revisions. Nonetheless, it should be emphasized that the scale and difficulty of the experiments are impressive. The candidate tackled a complex neurobiological problem, mapping anxiety-related circuits in zebrafish and manipulating a specific signaling pathway (TrkB) in a disease model. The data suggest that excessive TrkB signaling (perhaps due to the loss of TSC's inhibitory influence) has anxiogenic effects in fish, potentially analogous to anxiety symptoms in TSC patients. This is a novel contribution, suggesting that modulating BDNF/TrkB signaling may alleviate anxiety symptoms (potentially opening paths for pharmacotherapy, e.g., with TrkB antagonists like ANA-12, although developmental context should be considered).

The candidate also contextualizes her results evolutionarily, which is valuable — comparing zebrafish telencephalon regions to mammalian amygdala or septum. However, caution is needed here too: in the thesis conclusion (chapter 9), issues of nomenclature reappear .

Chapter 9: Conclusions, General Discussion and Future Perspectives

The final chapter serves as a summary of the dissertation's findings, their synthetic discussion in a broader context, and proposals for future research directions. The author integrates the conclusions from the experimental chapters (5 and 8), relating them to the study's objectives and the literature. She highlights, for instance, the role of disrupted light stimulus processing in *tsc2* mutants (habenula) and maps the potential basis of anxiety in the developing zebrafish brain (telencephalon), which may be relevant for understanding anxiety and autism in TSC. Further research is also proposed, such as more precise identification of homologous limbic structures in fish and testing of new therapies.

This chapter fulfills its synthetic role well, but significant issues with nomenclature and neuroanatomical interpretation in the general discussion need attention. The author uses ambiguous terms like *"the most dorsal layer of the pallium"*. The pallium (the dorsal telencephalon of the fish brain) is not layered like the mammalian cortex, so referring to a "layer" of the pallium is misleading. Perhaps she means the *superficial neuropil* or a specific zone. Such imprecise analogies pose a risk of misinterpretation. The reader might assume that the fish pallium has layers like the cortex. Furthermore, the doctoral candidate seems to equate certain fish structures with their mammalian counterparts without full awareness of the differences. For example, she suggests the presence of a "pallidum" in zebrafish, whereas in fish morphological nomenclature, there is no separate entity called the "pallidum". Parts of the ventral telencephalon (subpallium) may be functionally homologous to the mammalian pallidum, but morphologically, such a nucleus is not distinguished. It appears that the author may not fully realize this. Similarly, the author attempts to identify amygdala equivalents in fish. This is a controversial topic in comparative neuroanatomy. Fish have amygdala-like structures (parts of the lateral/anterior pallium and subpallium), but there is no consensus on exact homologies. Freely using the term "amygdala" in fish without clear definition could cause more confusion than clarity.

To summarize Chapter 9: The author undoubtedly makes insightful observations about the implications of her research. However, greater caution and precision in neuroanatomical interpretations are necessary. A young researcher, eager to connect findings with the "big comparative picture," must also recognize limitations. For example, not to hastily overlay the mammalian brain map onto that of a fish. Despite these comments, Chapter 9 fulfills its role: the conclusions are consistent with the presented data (assuming proper interpretation), and the tone of the discussion is cautiously optimistic about the translational potential of the research for understanding TSC in humans.

Bibliography Used

The literature cited in the dissertation is extensive and up to date. The author cites both classic publications (e.g., TSC criteria, mTOR mechanisms) and the latest studies (including publications from 2023 and 2024), indicating an updated knowledge base. In the review chapters, the number of cited sources is impressive, confirming a significant effort to compile information. In the original results

chapters, the bibliography also seems appropriate, combining sources from developmental neurobiology, epileptology, behavioral biology, and methodology, consistent with the interdisciplinary nature of the work.

Aim of the Study and Its Execution

The aim of the study, to investigate the mechanisms underlying TSC-associated neuropsychiatric disorders in the zebrafish model was largely achieved. In my view, the doctoral candidate fulfilled the main goal. Her research demonstrated that the TSC animal model exhibits measurable changes in brain function that translate into behavior, and that these changes are linked to specific molecular mechanisms. Of course, not all aspects were addressed (as this is a very broad topic), for example, other neuropsychiatric symptoms of TSC (such as social or cognitive deficits) were not analyzed. However, the scope of the dissertation was already extensive, so focusing on selected, well-measurable aspects (anxiety, light stimulus processing) was reasonable.

The study's goal was ambitious and multifaceted. During its execution, the author not only obtained new results but also created tools (protocols) that contributed to achieving the goal. This reflects a thoughtful approach and independence in solving experimental problems.

Research Methods Used

The set of methods used in the dissertation is impressive and modern. I assess that the choice of methods was adequate to the hypotheses posed. Particularly noteworthy is the integration of multiple levels of analysis: from whole-organism behavior, through organ-level (function of specific brain structures), to the molecular level (activation of signaling pathways). Such a multidisciplinary approach is a major asset of the candidate. The range of methods mastered by the candidate reflects her excellent experimental training.

Discussion and Interpretation of Results

The discussion of the results is largely sound and demonstrates the doctoral candidate's understanding of the subject. Generally, the author draws appropriate conclusions from her results. She is also effective at placing her findings within the scientific context, linking her observations with known phenomena. For example, she discusses how the habenula is often overactive or dysfunctional in models of epilepsy and depression, thus her findings support this. She refers to the role of BDNF/TrkB in plasticity and how its excess may lead to hyperexcitability, consistent with the TSC phenotype (epilepsy, anxiety). Also, the evolutionary angle: that certain mechanisms of anxiety are conserved from fish to mammals is an interesting point. However, as discussed above, the nomenclature and analogies to mammalian structures should be used with greater caution. The author is eager to claim that she "found something like an amygdala in fish responsible for anxiety." This is tempting, but such claims require strong neuroanatomical evidence, which is currently lacking. I am concerned that introducing such analogies without precision may cause more confusion than benefit.

Overall, the discussion of results is extensive and written with engagement. The author demonstrates critical thinking, though this is more often applied to the literature than to her own results.

Potential Practical Applications of the Results

The research presented in the dissertation is of a basic nature, but several potential long-term practical applications can be identified. For example, the TSC zebrafish model described by the author, with clear behavioral phenotypes (epilepsy, anxiety-like responses), could be used to test new therapies. Results from Chapter 5 suggest that rapamycin corrects certain symptoms, supporting its continued use in TSC. Chapter 8 points to the TrkB antagonist (ANA-12) as a compound that alleviates anxiety responses. Although ANA-12 is not a clinical drug, the mechanism (TrkB inhibition) may be a target for future treatments of anxiety or autism in TSC. In other words, the dissertation identifies mTOR and BDNF pathways as potential pharmacological targets for alleviating TANDs.

The author also describes potential markers of neural changes that could be neuropsychiatric biomarkers, such as habenular activity as an indicator of system overactivation or specific changes in the telencephalon as a correlate of anxiety. If these findings are translated to higher organisms in the future, they could assist in identifying, for instance, imaging biomarkers or stimulation targets. These are distant implications, but such studies lay the groundwork for such ideas.

Practically, the protocols from Chapters 6 and 7 are useful in themselves. Other labs may use the pRps6 staining protocol to assess mTORC1 activity in various mutants or conditions. The habenula microinjection protocol could also be adapted for research in other disease contexts where the habenula plays a role. This contributes to the toolkit of developmental neurobiology.

It should be stated clearly: the dissertation does not have immediate clinical application, which was never its goal. However, it provides new translational insights.

The practical value of the data from Chapters 5 and 8 lies mainly in deepening the understanding of the disease, a necessary step toward therapy.

Originality of the Scientific Contribution

The originality of the dissertation should be assessed in terms of the new findings and hypotheses it brings to the field. In this work, the innovative core lies in Chapters 5 and 8. Taken as a whole, the dissertation contains a significant original component, even though it is concentrated in approximately two out of nine chapters. The remaining sections serve as background and complementary material. In my opinion, this structure is acceptable: the doctoral candidate completed two major research projects, published one, prepared the other for publication, and additionally undertook the effort of writing reviews and protocols. That is a considerable achievement for a single PhD.

Some critics might argue that they would have preferred more chapters featuring “genuinely new experiments” instead of, for instance, a 50-page review. However, I understand that the author pursued the doctoral path based on publications, hence the inclusion of everything she published on the topic. Personally, I believe that the key measure of originality should be the quality of the new results, and these (with some adjustments) are solid and interesting.

The new scientific solutions proposed by the author (e.g., TrkB modulation as a potential therapy, the use of zebrafish to study TANDs) are relevant to the research community working on TSC and animal models of neurological diseases. Therefore, I evaluate the originality of the dissertation positively.

Theoretical Knowledge of the Doctoral Candidate

Analysis of the dissertation allows me to assess Olga Doszyń’s theoretical knowledge as extensive, though not fully established in all areas.

On one hand, the author demonstrates excellent expertise in fields such as the molecular biology of TSC (mTOR, signaling pathways), fish behavioral science, neuroimaging techniques, and genetics, as evidenced by the richness of the review sections and the skillful planning of experiments integrating various levels of analysis. She undoubtedly possesses strong knowledge of the literature: she cites hundreds of publications, is aware of the latest trends (e.g., optogenetics, activity indicators), and understands complex biological relationships. This places her at a high level of scientific preparation.
On the other hand, as pointed out in the review, there are areas where the doctoral candidate’s theoretical knowledge appears to have gaps or be superficial, for example, in the neuroanatomy of zebrafish and comparative neuroanatomy. Despite these remarks, I believe that the overall level of her theoretical knowledge is high. A PhD dissertation is a learning process and it is evident that the author has learned a great deal during this work, though there are still some details to refine.

I am particularly pleased with her interdisciplinary knowledge. She connects neurobiological and psychiatric issues (TAND is a complex topic requiring understanding of clinical aspects), molecular and behavioral aspects. Such a broad perspective required acquiring knowledge from multiple disciplines, which she has successfully achieved.

Independence and Scientific Maturity of the Doctoral Candidate

The dissertation demonstrate a high level of commitment and a large degree of independent work by Olga Doszyń. Her achievements include a review article, a book chapter, two experimental protocols, one published research paper, and one preprint, which is a significant accomplishment for a PhD program. This scope indicates that she was able to conduct several projects simultaneously and carry them through to publication, evidencing strong planning and scientific project management skills. Although she certainly benefited from the support of her advisor and collaborators, the main burden of work must have rested on her shoulders.

The complexity of the conducted research, combining various techniques, developing protocols, introducing new methods, confirms that the doctoral candidate not only applied ready-made solutions but also actively created new tools when needed. Examples include the development of a microinjection technique for the habenula and the optimization of larval zebrafish brain imaging, which required advanced experimental skills. All of this points to significant independence and innovation in addressing research challenges.

The writing style and structure of the dissertation demonstrate the candidate's ability to construct logical arguments, formulate research questions, and develop them effectively. Despite certain shortcomings in neuroanatomical interpretations and nomenclature, her capacity for scientific thinking and her drive to understand the broader context are evident. The work also includes signs of research self-awareness. The doctoral candidate acknowledges the limitations of her methods and shares her developed protocols, reflecting maturity and adherence to open science principles.

Her readiness for independent research is further demonstrated by the decision to publicly share the results of Chapter 8 in the form of a preprint, an expression of openness to critique and a desire to improve the work before formal publication. Moreover, there have been no signs of unethical behavior (such as data manipulation or plagiarism), which indicates an understanding of and compliance with ethical standards in science.

In conclusion, Olga Doszyń has shown a high level of independence and scientific maturity. Her work reflects well-established motivation, the ability to conduct independent research, and maturity in her approach to scientific challenges. After refining certain substantive aspects and filling in some knowledge gaps, the doctoral candidate will be fully prepared for a career as an independent researcher.

Final Summary and Conclusion

The doctoral dissertation of Olga Katarzyna Doszyń, titled *"Mechanisms underlying TSC-associated neuropsychiatric disorders in the zebrafish model"*, is a comprehensive, multifaceted, and valuable piece of work. The doctoral candidate addressed a challenging interdisciplinary topic at the intersection of neurobiology and developmental psychiatry, focusing on an important clinical issue - neuropsychiatric disorders associated with Tuberous Sclerosis Complex (TSC). By using an innovative zebrafish model, she obtained new insights into the mechanisms of epilepsy and anxiety, particularly highlighting the role of the mTORC1 pathway and TrkB/BDNF signaling in the pathophysiology of these phenomena. Despite numerous critical remarks and identified shortcomings, I must state clearly that none of the above issues undermine the main results or the originality of the dissertation. They merely require refinements in presentation and more thorough argumentation of some claims.

Despite the critical remarks presented in this review (intended to highlight areas for improvement and future development), my overall assessment of the dissertation is positive. I believe the conclusions drawn from the work make an important contribution to the understanding of TSC and animal models of neurological disorders, and that the dissertation has laid a solid foundation for the author's further scientific career.

I, the undersigned, hereby state that the doctoral dissertation of Olga Katarzyna Doszyń 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 Olga Katarzyna Doszyń 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. Due to the originality of the

results, the interdisciplinary approach, the advanced methodological skills, and the significant independence of the doctoral candidate, I also recommend that the dissertation be awarded with distinction.

Signed by / Podpisano przez:

Piotr Michał Podlasz
Uniwersytet Warmińsko-Mazurski w Olsztynie

Date / Data: 2025-11-10 15:50