Fundação
Champalimaud

Report from Dr. Michael Orger, Lisbon, 28 October 2025.

Report on the doctoral dissertation presented by Olga Katarzyna Doszyń.

Introduction

The presented thesis is very impressive and scholarly body of work that deals with zebrafish models of an important human disease, TSC. The thesis encompasses 6 published works and preprints, including 2 primary research papers (one peer-reviewed, one preprint), two published review articles/ book chapters and two published experimental protocols. These manuscripts are bookended by thorough and thoughtful introductory and concluding sections.
The work spans from molecular and genetic manipulations to studies of behavior and 3D brain imaging with light-sheet microscopy. As such, it is an exceptionally impressive body of work for a student which shows mastery of the subject on both an intellectual and experimental level, and an impressive breadth of knowledge and range of skills. As I have indicated in the conclusions section below, this dissertation, in my opinion, amply meets the criteria to be considered for the **distinguished** grade.

Review of individual chapters

Chapter 1: Introduction. This chapter provides a well researched and logically constructed introduction to Tuberous Sclerosis Complex (TSC), and its pathology, treatment and the state of research. Beginning by introducing the disease and its clinical manifestation and known causes, it proceeds to introduce the association with other neuropsychiatric disorders and symptoms, before discussing what is known about molecular mechanisms and pathways known to be affected in TSC. This sets up very well a discussion of the effectiveness of current treatment strategies, and the state of the art of animal models, focused on mouse and zebrafish. The candidate has shown excellent scholarship in summarizing a large and complex literature, and providing concise distillations of the main findings as well as critical evaluation of the results. Furthermore, it establishes very well the key knowledge required to understand the motivation for the work described in the thesis. While the introduction is very good, it would be better for the reader if the topic of

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the thesis was introduced first with an abstract or by reversing the order of Chapters 1 and 2, so that the reader is prepared for what is to come when absorbing the introduction.

Chapter 2. Scope of the thesis. This chapter provides a brief overview of the thesis, with descriptions of each subsequent chapter, and is very clear. Six of the chapters have been published as peer reviewed papers, protocols or preprints, and I think it would therefore be useful to list the publications arising from the thesis here.

Chapter 3. This chapter, also published as a book chapter, provides a review of the state of the art regarding the use of zebrafish models of TSC to study epilepsy with a careful elaboration of the methods available, detailed technical and experimental considerations and recent results. While there is especial focus on the authors own work in this area, there is also ample discussion and review of other approaches. The chapter has a broad scope, ranging from genotyping details, to in vivo calcium imaging and behavioral profiling. Lastly, some time is taken at the end of the chapter to discuss more broadly the benefits of using the zebrafish model to study diseases such as epilepsy, and to review models other that TSC for this purpose. Detailed glossaries and tables of references provide a comprehensive introduction to the research area. Taken together, this chapter us a very useful and accessible introduction that should be invaluable to researchers seeking to understand or join this field.
I have a some minor suggestions;
'frontbrains' is an unusual term. Does it refer to forebrains, or the front part of the brain more generally?
The heading 'Other Model Systems' is confusing as it suggests different animal models. 'Alternative zebrafish epilepsy models' would be a better heading.

Chapter 4. This chapter also takes the form of a review, published in Frontiers in Molecular Neuroscience, which highlights the advantages of using the zebrafish model for neuroscience research, and discusses the latest tools for studying the brain and behaviour. Section 2 describes genetic tools used to label different neuronal populations such as the GAL4 system. This section is clear and well explained, but it would be interesting to expand the discussion to evaluate a wider range of alternatives such as the QF system. A detailed overview is given of photo-activatable and photoconvertible proteins. I note that kaede is described as 'produced by accident' but photoconvertible kaede exists in nature, and it is discovery of this colour-changing property that was *discovered* by chance. In the remainder of Section 2, several methods for probing neuronal circuit connectivity are described, including trans-TANGO for synaptic tracing and single neuron laser ablations. While this section is necessarily selective, given the wide range of techniques available, an interesting and relevant set of examples have been chosen. The principles involved are succinctly described and the benefits and limitations of the methods are critically considered. Section 3 deals with methods for probing neuronal circuit function. First an excellent and interesting overview is given of the development of the GCaMP indicator family. The many variations on this theme that have been applied successfully in

neuroscience studies are presented, including the use of spectral variants and activity integrators. Again, I would only take issue with the description of GCaMP as 'discovered' rather than engineered. In discussing ratiometric indicators in the context of the brain and behaviour, it is also perhaps worth mentioning the benefits for avoiding motion artefacts. A comprehensive survey is also given of the state of development of voltage and neurotransmitter indicators. It could be useful here to add some discussion of the different response kinetics, and how that might affect their application. The review of virtual reality techniques and their application, together with different forms of volumetric imaging, to study the brain during behaviour, is very well researched and does an impressive job of synthesizing the main advances in this rapidly developing area. Lastly, there is an exceptionally thorough treatment of the history and latest developments in the use of optogenetics and electrophysiology in zebrafish.
Once again, the candidate has demonstrated the ability to critically evaluate a large and complex literature, and present the results in a clear and accessible form that will be of great benefit to anyone looking for an introduction to the field.

Chapter 5. Another published manuscript is presented, this time presenting new experimental results. This work deals with the molecular and neuronal mechanisms underlying a newly discovered behavioural phenotype in a TSC model. First they show that a TSC2 mutant shows abnormal light-dark preference and that this is due to hyperactivity in the mTorC1 pathway. Using light-sheet imaging they show that there is increased activity in the habenula, a critical brain region involved in light responses, in the mutants in response to light, and that this difference is abolished by suppressing mTorC1 activation with rapamycin. Finally they investigate inputs to the habenula at the anatomical level, finding a reduction in the size of the habenular commissure that is also reversed by rapamycin treatment.
This is an impressive, integrative study that uses a variety of state-of-the-art techniques to draw powerful connections between molecular functions, neuronal circuits and behaviour in an important disease model. All of the results are very clear, with a large number of animals tested and both the raw and summary data is presented in a very transparent fashion. I did have some small confusion with the labeling of the axes in Figure 3D and F and I wonder if there is a typographical mistake there?

Chapter 6 presents a protocol for visualization of mTorC1 activation in zebrafish larvae, by labeling phosphorylated Rps6. This protocol has been published as a STAR Protocol, and is very clearly presented with troubleshooting tips and pitfalls carefully explained. Clear illustrations and step by step instructions make the method very easy to reproduce, and even the image analysis steps are explained in detail. This is a very useful resource for those wanting to use this technique.

Chapter 7 presents a protocol for injection of rapamycin into the habenula of zebrafish larvae, although the method as presented could be useful for any pharmacological interventions targeting the habenula. This protocol has been published as a STAR Protocol,

and, as for the work in Chapter 6, is exceptionally thorough and detailed, including photographs of the workflow, which will make the method very straightforward to reproduce. Together with the previous chapter, these two methodological advances make an important contribution to the field.

Chapter 8 is based on a second primary research manuscript which is submitted as a preprint to the biorxiv preprint server, with the candidate as sole first author. In this work the anxiety phenotype in a TSC zebrafish model, which is reversed by application of TrkB antagonists, is examined using a combination of behavioral assays and the measurement of forebrain neural activity with calcium imaging. First, the phenotype is examined with a number of new automated behavioural assays, reproducing the thigmotaxis phenotype in an open field assay and additionally revealing a hyperactivity on light onset, which is also blocked by ANA-12. I would be interested to see more discussion of the details of the behavioural phenotypes and reflection on the terms used. For example, the terms freezing and thigmotaxis have very specific definitions in the behavioural literature although they are frequently used with broader meanings.
Next, activity changes in the mutant with and without drug treatment are measured systematically using phosphorylation of ERK as a proxy for activity. Whole-brain registration allows activity changes to be evaluated across populations of individuals, with high spatial specificity. After initially focusing on the habenula, replicating earlier findings, they extend this work to the forebrain more widely, co-staining with several markers to identify different anatomical domains in the pallium and subpallial regions. As a minor comment, I found that the figure legends in this section were not completely clear on whether representative data from individual fish, or the pooled cohort was being shown, and how many fish were included for each image. Aside from this minor comment, these experiments therefore these experiments performed and analyzed to a very high standard. This region of the larval brain is still relatively unexplored, and therefore these results are extremely interesting and will have an important impact beyond the immediate questions addressed in this work. Several populations of interest are identified where activity changes are observed in the mutant and are sensitive to ANA-12 treatment. These findings should be a springboard to many interesting new lines of investigation. Beyond providing important insights into this TSC model, the work also extends significantly our understanding of forebrain organization in the zebrafish larvae, and functional circuits involved in anxiety-like behaviours, which are an important factor in many neurological disorders. I expect that this preprint, like the previous work, will be successful in being published in an international peer-reviewed journal.

Chapter 9. Conclusions. The work of the whole thesis is critically evaluated, with consideration of how the results might impact thinking about the mechanisms of human disease. Again, the candidate shows that they can put their work in the context of a large and complex literature, and they can see their work in the context of the bigger picture of research in the field across different models. They are also able to identify and elaborate on future directions for the research and interesting unexplored observations.

Potential points for further discussion during the defense

1. *Outlook for applications to human disease*. The experiments described here have teased out distinct behavioural phenotypes in a zebrafish model of a human disease, and have identified some of the neural and molecular pathways that underlie them. What new possibilities does this work now open up in terms of developing or improving treatments for the disease using the zebrafish model? To what extent - and in what ways - do you see that having a better understanding of the specific neural circuits affected in this model will lead to refinements in these approaches, and ultimately better outcomes?
2. *Methods for recording neural activity.* In the early chapters of your thesis, you review many methods for recording neural activity, and ultimately in this thesis make use of two: light-sheet imaging of GCaMP in vivo, and pERK staining. I am interested first in how much we know about the relationship between neural activity and pERK levels, and second what considerations went into choosing the methods that were used in the two studies? What are the benefits and limitations of each approach, compared to each other, or other possible methods such as the integrator Campari?
3. *The role of the habenula in the light dark preference phenotype and beyond.* The focus on the habenula in Chapter 5 is justified in the text by previous studies that implicate it in light-dark preference. This leaves open some questions: first, how much do we know about the neural circuit basis of light-dark preference, and the role that the habenula might be playing? Second, the habenula is something of an enigmatic structure that, even in the zebrafish larva, has been implicated in a diverse array of behaviours. In light of this, and given that the habenula appears to be affected in this disease model, what other behaviours might be interesting to explore?

Final Conclusions

Combining original research of the highest quality in a clinically important area, with thoughtful reviews of the literature, and methodological and conceptual advances, this doctoral thesis clearly makes an original and important contribution to the authors field of study, which is further emphasized by the multiple publications. Having demonstrated deep knowledge of the literature, and mastery of a diverse array of cutting-edge techniques, **Olga Katarzyna Doszyń** has established that she is an independent and knowledgeable expert in this field and deserving of the PhD degree and the additional consideration of a **distinguished dissertation**.

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. Furthermore, it is my opinion that, in light of the breadth and deep scholarship of the dissertation, the outstanding publication record and the important contributions to their field, this doctoral degree should be awarded **with distinction**.

Sincerely yours,

Michael Orger, PhD.

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