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Review of the Doctoral Dissertation of Razieh Amini

The doctoral dissertation "Genetic and Physical Regulation of Ventricular Development and Reissner Fiber Formation in Zebrafish" by Razieh Amini was prepared under the supervision of Dr hab. Vladimir Korzh in the Laboratory of Neurodegeneration. Razieh Amini was enrolled in the Warsaw PhD School in Natural and BioMedical Sciences, and the dissertation was submitted to the Doctoral Committee at the International Institute of Molecular and Cell Biology in Warsaw.

The main goal of the work was to understand how molecular regulation and physical constraints together govern formation of the Reissner fiber (RF) and morphogenesis of the brain ventricular system (BVS) in the zebrafish embryo. The Candidate addressed three related questions: how the voltage-gated potassium channel Kv2.1, composed of the electrically active subunit Kcnb1 and the modulatory subunit Kcng4b, influences secretion of SCO-spondin (Sspo) and assembly of the anterior RF; whether cholesterol-dependent membrane organisation and developmental signalling contribute to these processes; and whether gravity-dependent mechanics, tested by changing the orientation of the embryo, affect anterior RF formation. The title of the dissertation fits its topic, which is relevant to developmental neurobiology and to the discipline of biological sciences as a whole.

The dissertation is in the form of a collection of four manuscripts, accompanied by an Introduction, a statement of the research questions, a Discussion organised by manuscript, a General Conclusion, Materials and Methods, and a bibliography. Manuscript 1 (Frontiers in Cell and Developmental Biology, 2026) and Manuscript 4 (Developmental Biology, 2024) are published. Manuscript 2 has been submitted to npj Microgravity, and Manuscript 3 is available as a preprint and is under review at the Journal of Physiology. The Candidate designates Manuscripts 1 and 2 as her major contribution and Manuscripts 3 and 4 as minor contributions, and she is first author on the two core manuscripts. The dissertation is written in English, and the literature cited is extensive and appropriate.

The experimental work is extensive and technically sound. The Candidate combines confocal and light-sheet imaging with whole-mount AFRU immunostaining and the *scospondin-GFP^{ut24}* knock-in line, makes careful use of several alleles affecting the two Kv2.1 subunits, and introduces an original and a paradigm in which embryos are held in defined body orientations under normogravity. Several findings are new and conceptually interesting. The RF is shown to form in stages: a transient posterior RF derived from the floor plate serves as a template for the anterior RF secreted by the subcommissural organ (SCO), and the flexural organ (FO) acts as the junction between the two. Mutations in the two Kv2.1 subunits have opposing effects on anterior RF formation, and initiation of the anterior RF is highly sensitive to the orientation of the embryo. I find the Manuscript 2 particularly intriguing. Using a simple and elegant experimental design, it links the formation of an extracellular structure in the brain ventricles to the direction of

gravity. It opens a new perspective on how physical forces acting on the whole embryo can shape brain development, and it may have implications for development under altered gravity, as well as for the evolution of the RF system. Together with the unpublished data on Wnt/ β -catenin and Hedgehog signalling, cholesterol availability, oxidative stress and ciliation, this work demonstrates an excellent theoretical and practical background of the Candidate in developmental biology.

My main concern is not with the data but with the way they are presented. A doctoral dissertation serves a different purpose from a journal article. A paper may be compressed and may assume a specialist reader, whereas a thesis should demonstrate that the Candidate can build a logical argument from first principles and describe it clearly, precisely and consistently for an educated reader outside the immediate field. Judged by this criterion, I recommend to improve the dissertation as follows.

The Introduction relies heavily on anatomical terms and abbreviations, including RF, aRF, pRF, SCO, FO, MHB, MDB, CSF and CSF-cNs, as well as the roof plate, floor plate, cerebral aqueduct, third ventricle and ampulla terminalis. Many of these structures are never shown in a figure before they are discussed, and a list of abbreviations is missing. Section 1.3, for example, describes the origins of the SCO and the FO in a single sentence that reads as if the SCO arose from the floor plate. A schematic of the embryonic zebrafish brain and anterior neural tube, in lateral and ideally also dorsal view, on which these structures are labelled with exactly the abbreviations used in the text, would make the thesis far easier to follow. It would help further to include a simple timeline from approximately 16 to 72 hpf, showing when each structure appears, when *sspo* expression shifts from the floor plate to the SCO, and when the body-orientation experiments were performed.

In my opinion, the schematic in Figure 4 of Manuscript 1, which depicts the posterior RF derived from the floor plate and FO, the anterior RF derived from the SCO, their fusion at the FO and the disassembly of the fiber in the ampulla terminalis, is the conceptual core of the thesis. A simplified version of this schematic could be introduced early as the working framework, and each part of the thesis could state explicitly which step of the model it addresses: *Sspo* synthesis, secretion, extracellular assembly, fusion at the FO or disassembly. The General Conclusion, which currently summarises the findings in words only, would then close with an expanded version of the same figure showing where each regulator examined in the thesis is thought to act, and distinguishing mechanisms supported by data from links that remain hypothetical. As such, the three research questions in Section 1.9 would be easier to follow if the reader were told which manuscript or dataset answers each of them. The unpublished data in Section 3.3, which directly answer the second research question, are placed within the Discussion; they would be better presented as a separate results section, and the corresponding entry in the Table of Contents ("Development and validation of a stable HG reporter line") does not match the content of that section.

A related difficulty is that the thesis often does not state clearly what was already known before this work and what the Candidate has newly shown. For example, Manuscript 1 reports that the floor-plate-associated AFRU signal splits into two lines, noting that "this phenomenon was not reported previously", and proposes that the floor-plate-derived posterior RF serves as a template for the SCO-derived anterior RF. In Section 3.1, however, the statement that "RF formation is a staged developmental process involving transient floor plate-derived and later SCO-derived components" is supported only by earlier literature (Shih and Fraser, 1996; Halpern et al., 1997; Strähle et al., 2004; Lehmann and Naumann, 2005; Meiniel et al., 2008), so the reader cannot

tell which part of this model is new. Similarly, the opposing roles of the two Kv2.1 subunits in BVS development were reported earlier (Shen et al., 2016; Jędrychowska et al., 2024), and the novelty of Manuscript 1 lies in extending this antagonism to RF formation; yet the Discussion cites the Candidate's own Manuscript 1 ("Amini et al., 2026") alongside these earlier studies without distinguishing them. The origin of the *kcng4b* alleles is also presented inconsistently: Manuscript 4 states that "we generated two novel Kcng4b mutant alleles", whereas the Materials and Methods of Manuscript 1 describe them as "described previously" and Section 3.5 attributes them to Jędrychowska et al. (2021) and Gasanov et al. (2021). In each section of the Discussion, I recommend stating explicitly what was known before, citing it, and then what the Candidate has added, for example by writing "In Manuscript 1, I showed that..." rather than citing her own work in the same way as that of others. This would also make the Candidate's personal contribution easier to judge.

Inconsistent nomenclature is the obstacle to understanding. The term "Kv2.1" is used in some places for the Kcnb1 subunit and in others for the heterotetrameric Kcnb1/Kcng4b channel, and "Kv6.4", "Kcng4" and "Kcng4b" are used interchangeably. The same *kcng4b* alleles are called *kcng4b^{waw304}* and *kcng4b^{waw305}* in Manuscript 1 but *kcng4b-C1* and *kcng4b-C2* in Manuscript 4. Each term should be defined once and used consistently, and a single table giving, for each allele, the molecular lesion, its effect on the protein, the predicted effect on channel activity and the resulting BVS, RF and ear phenotypes would greatly help the reader. I also recommend a clearer separation between what was measured and what was inferred. The conclusion that Kv2.1 negatively regulates RF formation rests mainly on whole-embryo qRT-PCR and correlative morphology, the CSF-flow model in Section 3.1 is not based on direct measurements of flow, and the proposal that *chl1a/camel* acts as a gravisensor remains speculative. A short paragraph on limitations, including tissue specificity, the possible effects of agarose immobilisation and the small sample sizes (n = 6) in the morphometric analyses, would be appropriate.

There are also a number of formal errors. Section 3.2 cites Lang et al. (2006), a study of femoral adaptation after spaceflight, in support of a developmental window for anterior RF initiation, and the legend of Figure 5 in Section 3.3.4 cites Rodriguez et al. (1990), which is absent from the reference list. Figure numbering in the Discussion restarts within each subsection, and Figure 4 in Section 3.3.3 refers to panels K–L that are not described in its legend. In the Materials and Methods, the numbering jumps from Section 5.5 to Section 5.7, the atorvastatin protocol is described twice, and the Wnt3:GFP and KillerRed lines are attributed to "Amini et al., 2024" rather than to their original descriptions. In Section 1.6, "neurogenesis" presumably should read "RF formation". The names Sspo, SCO-spondin and Scospondin, and the distinction between *sspo* (gene) and Sspo (protein), should be used consistently. These points do not detract from the scientific value of the work, but together with the comments above they should be addressed as minor revisions in the final version of the dissertation.

I have some questions and suggestions to which the Candidate may respond during the defence:

1. The conclusion that body orientation alters Hippo/Yap signalling (Manuscript 2) is based on increased mRNA levels of *yap1*, *lats1*, *nf2a* and *mst1* in whole embryos, and *yap1* is described as "well established as a gravisensor". Since Yap is regulated mainly post-translationally, through Lats1/2-dependent phosphorylation and nuclear exclusion, how does the Candidate interpret an increase in *yap1* transcript levels? Does the concurrent increase in *lats1* indicate stronger inhibition of Yap, or could it reflect negative feedback following

increased Yap–TEAD activity? How could Yap activity itself be measured, for example with a set of conserved Yap/Taz–TEAD target genes such as *ccn2a/ctgf*, *ccn1/cyr61*, *ankrd1a* and *amotl2*, with the Yap–TEAD reporter line 4xGTIIC:d2GFP, or by the nuclear localisation of Yap, and in which tissues would the Candidate expect Yap activity to change? I suggest that these points be considered in the revision of the submitted Manuscript 2.

2. The Abstract states that the RF is found in vertebrates with a horizontal body posture. The RF is present in most mammals, including rodents, but is absent in adult humans, in whom the SCO regresses after birth and the *SSPO* gene has been annotated as a pseudogene. In the light of her finding that anterior RF initiation is sensitive to body orientation, could the Candidate speculate whether the transition to an upright posture and bipedal locomotion might have relaxed the need for an RF-dependent system of axial proprioception and body-axis straightening? How does the gravitational situation of an embryo developing in water, or in amniotic fluid, differ from the postural loads experienced after birth, and how could such an evolutionary hypothesis be tested, for example by comparing the integrity of *SSPO* and the RF across primates and other mammals with different postures?
3. Manuscript 4 concludes that the *kcng4b-C2* (*waw305*) allele acts in a dominant-negative manner and reduces the Kv2.1 current, whereas Section 3.1 associates increased Kv2.1 activity with loss of the anterior RF, which is the phenotype of *waw305*, and Section 3.5 describes this allele as "loss-of-function-like". Could the Candidate explain what the evidence indicates about channel activity in each allele, and how the opposing RF phenotypes of the *kcnb1* and *kcng4b* mutants fit her model?

I evaluate this PhD dissertation positively. The Candidate presents an original and technically sound approach to each of the research questions, and the results are of clear relevance to developmental neurobiology and to our understanding of how the brain ventricular system and the Reissner fiber are formed. The revisions recommended above concern the written presentation and are minor in nature.

I, the undersigned, hereby state that the doctoral dissertation of Razieh Amini meets the requirements specified in Article 187 of the Act of July 20, 2018 – Law on Higher Education and Science (consolidated text, 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 Razieh Amini 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.

Date 26.9.2026

Signature



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