



Genetic and Physical Regulation of Ventricular Development and Reissner Fiber Formation in Zebrafish

by

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Doctoral dissertation

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ABSTRACT

The vertebrate brain develops around fluid-filled cavities whose growth and patterning depend on epithelial secretion, membrane organisation, and mechanical forces transmitted through cerebrospinal fluid (CSF). Within this environment, the Reissner fiber (RF) is an evolutionarily conserved extracellular structure found in vertebrates with horizontal body posture. RF is assembled largely from the glycoprotein SCO-spondin (Sspo), which is secreted by the subcommissural organ (SCO; anterior RF) and floor plate (posterior RF). RF acts in proprioception during straightening of the body axis and its maintenance, as well as in ventricular homeostasis. In this thesis, I used zebrafish to test how molecular regulation and physical constraints jointly shaped ventricular development and contributed to the initiation of the anterior RF.

I addressed three related questions. First, I examined how the voltage-gated potassium channel Kv2.1, which consists of the electrically active subunit Kcnb1 and the modulatory subunit Kcng4b, influenced Sspo secretion and anterior RF assembly. Mutations in Kv2.1 subunits produced strong, opposing anterior RF phenotypes. These phenotypes coincide with changes in RF-associated transcript levels, Sspo localization and secretion, and differences in SCO apical architecture. They are consistent with an effect of Kv2.1 on membrane potential and Sspo secretion.

Second, I tested whether membrane composition contributed to these defects. Manipulating cholesterol availability disrupted the conditions required for Sspo secretion and anterior RF initiation. These findings support a model in which cholesterol-dependent membrane organisation shaped signalling competence and polarised secretion in ventricular epithelia.

Third, I tested whether gravity-dependent mechanics influenced anterior RF formation. Sustained changes in embryo orientation during a defined developmental window reduced anterior RF and were accompanied by altered ventricular morphology and transcriptional responses in mechanosensory genes expressed in CSF-contacting neurons (CSF-cNs).

Overall, my results supported the view that RF formation depends on SCO secretion, membrane potential, and body position during early brain morphogenesis.

STRESZCZENIE

Rozwój mózgu kręgowców zachodzi wokół komór wypełnionych płynem, których wzrost i wzorcowanie zależą od wydzielania nabłonkowego, organizacji błon komórkowych oraz sił mechanicznych przenoszonych przez płyn mózgowo-rdzeniowy (CSF). W tym środowisku włókno Reissnera (RF) stanowi ewolucyjnie konserwowaną strukturę zewnątrzkomórkową, występującą u kręgowców o poziomej postawie ciała. RF powstaje głównie z glikoproteiny SCO-spondyny (Sspo), wydzielanej przez narząd podkomisuralny (SCO; przednia część RF) oraz płytkę podłogową (część tylna RF). RF odgrywa rolę w propriocepcji podczas prostowania osi ciała i utrzymania jej prawidłowej postawy, a także w homeostazie układu komorowego. W niniejszej pracy doktorskiej wykorzystałam danio pręgowanego jako model do zbadania, w jaki sposób regulacja molekularna oraz ograniczenia fizyczne wspólnie kształtują rozwój układu komorowego oraz przyczyniają się do inicjacji przedniej części RF.

W pracy tej podjęto trzy powiązane zagadnienia. Po pierwsze, zbadano, w jaki sposób napięciозależny kanał potasowy Kv2.1, składający się z aktywnej elektrycznie podjednostki Kcnb1 oraz modulującej podjednostki Kcng4b, wpływa na sekrecję Sspo oraz tworzenie przedniej części RF. Mutacje w podjednostkach Kv2.1 prowadziły do silnych, przeciwstawnych fenotypów przedniej części RF. Zjawiska te korelowały ze zmianami poziomu transkryptów związanych z RF, zaburzoną lokalizacją i sekrecją Sspo oraz różnicami w architekturze apikalnej SCO. Są one zgodne z wpływem Kv2.1 na potencjał błonowy i wydzielanie Sspo.

Po drugie, sprawdzono, czy skład błony komórkowej przyczynia się do obserwowanych zaburzeń. Manipulacja dostępnością cholesterolu zakłócała warunki niezbędne do sekrecji Sspo i inicjacji przedniej części RF, wspierając model, w którym zależna od cholesterolu organizacja błony kształtuje kompetencję sygnalizacyjną oraz spolaryzowaną sekrecję w nabłonkach komorowych.

Po trzecie, oceniono wpływ mechaniki zależnej od grawitacji na powstawanie przedniej części RF. Utrzymujące się zmiany orientacji zarodków w określonym oknie rozwojowym prowadziły do ograniczenia inicjacji przedniej części RF, czemu towarzyszyły zmiany morfologii układu komorowego oraz odpowiedzi transkrypcyjne genów mechanosensorycznych ekspresowanych w neuronach kontaktujących się z płynem mózgowo-rdzeniowym (CSF-cNs).

Podsumowując, uzyskane wyniki wskazują, że powstawanie RF zależy od sekrecji SCO, potencjału błonowego oraz pozycji ciała podczas wczesnej morfogenezy mózgu.

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1 INTRODUCTION

The development of the vertebrate central nervous system involves more than the generation of neurons and glia. From early stages onward, brain morphogenesis depends on the formation of ventricles and the CSF that fills them. CSF has long been viewed as a protective and nutritive medium. Accumulating evidence shows that CSF actively participates in developmental processes. Through the transport of signaling molecules and the transmission of mechanical cues, CSF influences ventricular epithelial organisation, tissue patterning, and the growth of the developing brain (Lowery & Sive, 2009; Fame & Lehtinen, 2020). When CSF dynamics or ventricular architecture are perturbed, the consequences are often severe, leading to conditions such as hydrocephalus, neural patterning defects, or abnormalities of the body axis, manifested as scoliosis (Lehtinen et al., 2011). However, how ventricular epithelia coordinate CSF composition with the assembly of CSF-borne extracellular structures during development remains poorly understood.

1.1 The Brain Ventricular System

The brain ventricular system (BVS) is a conserved network of interconnected cavities that in mammals includes the two lateral ventricles, third, and fourth ventricles, linked by narrow channels such as the interventricular foramina and the cerebral aqueduct. These spaces are lined by neuroepithelial and ependymal cells, whose behavior determines both the shape of the ventricles and the properties of the CSF-filled lumen (Lowery & Sive, 2009; Fame & Lehtinen, 2020).

Formation of the BVS is a progressive process that involves neuroepithelial expansion, morphogen-dependent patterning, and ventricular lumen inflation. Disruption of any of these steps can compromise brain morphogenesis and give rise to developmental abnormalities, including hydrocephalus and neural tube closure defects. CSF circulation, driven by cardiac activity, ciliary beating, and secretion from the ventricular epithelium initially and from the choroid plexus later on, contributes to ventricular shaping and provides a route for distributing signaling factors that regulate neural progenitor proliferation and differentiation (Lehtinen et al., 2011; Silva-Vargas et al., 2016).

Studies in zebrafish and other vertebrate models have shown that CSF flow itself can act as a developmental cue. By linking hydrodynamic forces to epithelial organisation and tissue polarity, CSF flow integrates physical and molecular aspects of brain development (Olstad et

al., 2019; Reiten et al., 2017). In this context, the BVS functions not simply as the CSF conduit but as an active environment that participates in shaping the developing nervous system.

1.2 Structure and function of the Reissner Fiber

Within the ventricular system of vertebrates with horizontal body posture, one of the most distinctive CSF-associated structures is the RF, a glycoprotein-rich filament. It extends along the ventricular cavities and into the spinal central canal and remains largely suspended within the CSF. Its principal component is SCO-spondin (Sspo), a large, multidomain glycoprotein of about 500–600 kDa. In adults, the SCO secretes Sspo apically into the ventricular lumen. Sspo is a member of the thrombospondin family. It serves as a matricellular protein, interacting with various molecules in the CSF to support neural development and influence neuronal growth, differentiation, and guidance (Rodríguez et al., 1998; Meiniel et al., 2008; Muñoz et al., 2019). Following secretion, Sspo polymerizes and associates with extracellular partners, including Clusterin (Clu) and Galectin-1, forming the mature fiber (Muñoz et al., 2019). The properties of the RF are closely linked to the molecular architecture of SCO-spondin, which contains multiple adhesive and matrix-binding domains that support filament assembly and stability while enabling interactions with the surrounding ventricular environment (Rodríguez et al., 1998; Meiniel et al., 2008). Rather than behaving as a static structure, the RF shows regulated stability and turnover over developmental time (Muñoz et al., 2019).

Functional studies across vertebrate species have demonstrated that an intact RF is required for neural tube patterning, body-axis alignment, and coordinated locomotion, whereas disruption or fragmentation of the fiber is associated with hydrocephalus and spinal deformities, including scoliosis (Cantaut-Belarif et al., 2018; Troutwine et al., 2020; Xu et al., 2023). The RF interacts with CSF-cNs, which are sensitive to flow and mechanical strain, suggesting that the fiber integrates structural support with mechanosensory signaling (Böhm et al., 2016; Cantaut-Belarif et al., 2020; Orts-Del'Immagine et al., 2020). Assembly and maintenance of the fiber depend on cilia-driven CSF flow, which promotes filament elongation and alignment and provides a hydrodynamic context in which extracellular matrix organisation and neural activity can be coordinated (Olstad et al., 2019; Thouvenin et al., 2020; Fame & Lehtinen, 2020).

Together, these observations suggest that RF assembly depends on specialized cell types capable of sustained, polarized secretion into the ventricular lumen.

1.3 The Subcommissural Organ and its Secretory Role

The SCO is a specialized circumventricular organ located at the dorsal midline of the brain, at the entrance from the third ventricle to the cerebral aqueduct. Unlike some circumventricular organs (organum vasculosum of lamina terminalis, subfornical organ, area postrema), which are characterized by permeable vasculature and primarily chemosensory functions, the SCO maintains a tight blood–brain barrier and serves predominantly as a secretory epithelium (Rodríguez et al., 1998; Grondona et al., 2020; Aboitiz & Montiel, 2021). It is composed of radial glia-like ependymal cells lining the roof of the third ventricle, which exhibit pronounced apical–basal polarity and tight junctions typical of regulated secretory tissues (Muñoz et al., 2019).

The SCO is the only site of Sspo synthesis in adult animals. After processing in the endoplasmic reticulum, Sspo is packaged into apical vesicles and released into the CSF via regulated exocytosis. Once secreted, the protein self-assembles into filaments that form the RF and associates with additional extracellular matrix components that influence filament adhesion and stability (Rodríguez et al., 1998; Gobron et al., 1999; Sepúlveda et al., 2021).

During vertebrate development, the SCO originates from the floor plate, giving rise to the flexural organ, followed by the anterior roof plate of the neural tube, which gives rise to the SCO. In zebrafish and other model systems, SCO cell differentiation coincides with the onset of apical secretion into the cerebral aqueduct (Lehmann & Naumann, 2005; Meiniel et al., 2008). The secretory activity of SCO cells depends on the organisation of the apical membrane, which is enriched in lipid-raft domains and ion channels, including members of the voltage-gated potassium channel (Kv) family. Through this organisation, membrane properties and bioelectrical regulation are coupled to vesicle docking, trafficking, and release (Tamkun et al., 2007; Deutsch et al., 2012; Johnson et al., 2019).

These features place the SCO among epithelial systems that coordinate epithelial morphogenesis, extracellular matrix production, and CSF dynamics.

1.4 Regulation of Reissner Fiber assembly

Although Sspo provides the structural core of the RF, additional molecular factors influence how the polymer assembles and stabilizes during development. Proteins such as Clu, Chl1a/Camel, and galectins contribute to epithelial organisation, apical secretion, and filament consolidation, modulating interactions between the RF and the ventricular epithelium (Thijssen et al., 2006; Muñoz et al., 2019; Yang et al., 2021). Post-translational modifications of Sspo

further affect polymer stability and its association with cellular membranes, supporting the view of the RF as a composite extracellular matrix (Muñoz et al., 2019).

In addition to these influences, the secretory activity of SCO and roof plate is also shaped by midline patterning pathways. BMP and Wnt/ β -catenin signaling characterizes the roof plate and the SCO, while Sonic Hedgehog (Shh) signaling contributes to floor plate identity and ventricular patterning (Nusse, 2003; Beachy et al., 1997; Porter et al., 1996; Bond et al., 2013).

Membrane composition also plays an important role in RF biogenesis. Cholesterol is the principal component of plasma membrane. It is required for Shh maturation, Wnt secretion, and formation of apical membrane microdomains involved in vesicle docking and polarized exocytosis (Beachy et al., 1997; Porter et al., 1996; Nusse, 2003; Ng et al., 2016; Veerapathiran et al., 2020). Together, developmental signaling pathways and membrane properties regulate apical secretion within the ventricular system.

1.5 Body orientation and physical constraints during ventricular development

Physical forces are an unavoidable part of embryonic development. Gravity constantly acts on body fluids and shapes the mechanical conditions tissues experience. Under standard laboratory conditions, we often treat this as background. However, changes in body orientation can shift CSF distribution. This can alter the mechanical environment of fluid-filled structures such as the BVS (Lowery & Sive, 2009; Fame & Lehtinen, 2020). Over the past few years, work across vertebrate models increasingly supports the view that CSF and ventricular biomechanics actively contribute to neural development, rather than serving only a passive, protective role (Lehtinen et al., 2011; Olstad et al., 2019).

This becomes especially relevant during early development, when CSF circulation is still limited and depends largely on immature cilia. During this period, the zebrafish embryo periodically contracts and changes orientation while remaining largely horizontal. In this developmental window, ventricular morphology is sensitive to physical constraints and fluid redistribution (Fame et al., 2016; Olstad et al., 2019). Alterations in embryo orientation may affect subsequent developmental outcomes. These outcomes depend on a stable and well-organised CSF environment. This environment is determined by specific ventricular geometry and CSF flow patterns.

For these reasons, I manipulated the embryo's body orientation under normogravity. Such an approach provided a practical and controlled way to examine how physical forces such as gravity intersect with CSF dynamics during development. I tested the contribution of gravity-

dependent fluid redistribution to ventricular morphogenesis and its downstream effects. I also compared these findings with those of genetic and molecular analyses.

1.6 Voltage-gated potassium channels (Kv2.1/Kcnb1 and Kcng4b) in epithelial organization and development

Voltage-gated potassium channels of the Kv2 family are best known for their role in regulating membrane excitability. However, evidence from multiple systems indicates that these channels also participate in intracellular trafficking. Kv2.1 channels are composed of electrically active Kcnb1 subunits and four classes of modulatory (silent) subunits, such as, for example, Kcng4, which together influence channel localization and functional output, as schematically illustrated in Figure 1 (Bocksteins & Snyders, 2012; Vacher et al., 2008). The relative balance between these subunits, rather than current amplitude alone, appears to shape cellular behavior.

This functional diversity is further supported by a recent study in mice, which found that the silent subunit Kv6.4 (encoded by *Kcng4*) forms complexes with Kv2 channels in PV interneurons and alters spike shape and short-term synaptic plasticity during high-frequency firing (Ganesh et al., 2026). This result highlights that silent Kcng subunits can tune Kv2 function without simply changing current amplitude, reinforcing the importance of subunit composition.

At the cellular level, Kv2.1 channels form clusters at the plasma membrane that are associated with endoplasmic reticulum–plasma membrane junctions. These specialized regions regulate membrane organization, protein trafficking, and vesicle docking and fusion (Tamkun et al., 2007; Deutsch et al., 2012; Johnson et al., 2019). Through non-conductive functions, Kv2.1 channels contribute to membrane architecture and secretory capacity, particularly in polarized epithelial cells. This dual functionality arises from the modular domain organization of Kv2.1 channels, which supports both conductive and non-conductive roles.

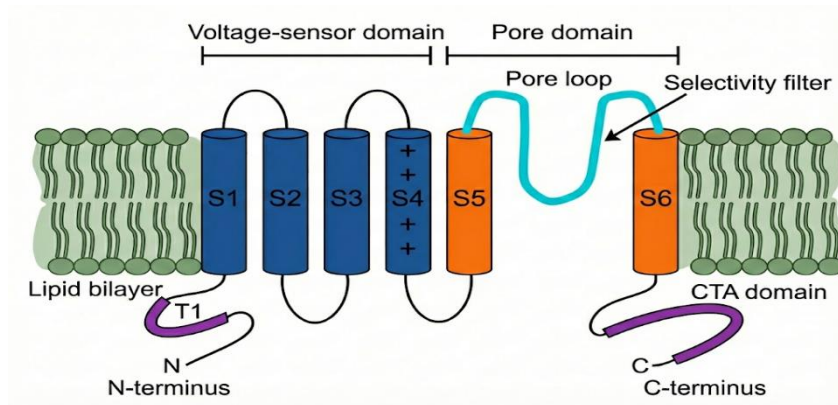


Fig. 1: Scheme of domain organisation in the single Kv channel subunit.

The S1–S4 domain forms the voltage sensor, while the S5–S6 domains and intervening P-loop form the ion-conducting pore. The N-terminal T1 domain mediates subunit assembly, and the C-terminal tail contributes to channel regulation and clustering at the plasma membrane.

In zebrafish, disruption of Kv2.1 subunits results in tissue-specific developmental phenotypes. Here Kcng4 is encoded by two genes, *kcng4a* and *kcng4b*. Loss of *Kcnb1* or *Kcng4b* affects hollow organs such as the BVS and the inner ear, revealing antagonistic roles for these subunits during formation of these structures (Shen et al., 2016; Jędrychowska et al., 2021; Jędrychowska et al., 2024). Whereas *kcnb1* mutants show defects in ventricular and otic development, such as microcephaly and reduction of the otic vesicle, *kcng4b* mutants develop enlarged brain (hydrocephalus) and otic vesicle phenotypes. Transport of *Kcng4b* from the endoplasmic reticulum to the plasma membrane requires *Kcnb1*, linking Kv2.1 subunit composition to membrane organization and secretory processes (Bocksteins et al., 2014; Jędrychowska et al., 2024).

These findings suggest that the *Kcnb1/Kcng4b* axis contributes to tissue integrity and secretion during development. Because neurogenesis depends on sustained, polarized secretion of Sspo by SCO, the Kv2.1 channel likely plays a coordinating role, linking tissue physiology to the controlled release of Sspo and the assembly of RF within the BVS.

1.7 Zebrafish as a model system

Zebrafish (*Danio rerio* H.) is a well-established vertebrate model for studying early development. Its externally developing, transparent embryos facilitate direct in vivo observation of organogenesis and tissue morphogenesis, allowing researchers to correlate

morphological changes with gene activity. Furthermore, the species' rapid development and large clutch sizes enable robust experimental analyses (Kimmel et al., 1995; Choi et al., 2021; Hwang & Chou, 2013; Lieschke & Currie, 2007; Shehwana & Konu, 2019; Srivastava et al., 2025; Teame et al., 2019).

In addition to these advantages, the zebrafish exhibits extensive genetic tractability through transgenesis and CRISPR/Cas9-based genome editing, enabling targeted manipulation of gene function during early neurodevelopment (Lin et al., 2025; Ono et al., 2025; Uribe-Salazar et al., 2022; Varshney & Burgess, 2025). Consequently, the zebrafish is particularly well-suited for investigating ventricular morphogenesis, CSF dynamics, and the development of CSF-associated structures such as the RF (Lowery & Sive, 2009; Fame et al., 2016; Olstad et al., 2019; Fame & Lehtinen, 2020; Muñoz et al., 2019).

1.8 Evolutionary context and translational relevance

The RF is conserved across most vertebrates. However, species-specific differences exist in CSF regulation, ventricular architecture, and circumventricular organ function (García-Lecea et al., 2017; Kiecker, 2018; Korzh & Kondrychyn, 2020). Despite the absence of RF in humans, the role of soluble SSPO in neurogenesis remains debatable. Studies in model animals have shown that disruption of pathways associated with SCO and RF development has been linked to congenital hydrocephalus and scoliosis. Therefore, understanding RF assembly and function is crucial. This knowledge helps us understand the mechanisms of these disorders and supports the development of targeted therapeutic strategies and potential clinical interventions.

1.9 Research Objectives

This PhD project aims to explore how potassium channels and physical constraints contribute to RF formation and the morphogenesis of the BVS in zebrafish. My work aimed to examine how voltage-gated potassium channel Kv2.1, developmental signaling pathways (Wnt, Shh), and mechanical forces (gravity) acting on the CSF intersect to influence the secretion of Sspo and the assembly of CSF-associated extracellular structures.

My PhD focused on three key questions:

- 1) How does the voltage-gated potassium channel Kv2.1, composed of the Kcnb1 and Kcng4b subunits, affect RF formation?

2) How do Shh and Wnt/ β -catenin signaling pathways interact with cholesterol-dependent membrane organisation to regulate secretory activity in the roof and floor plate, and how do changes in membrane composition impact ventricular morphology and RF integrity?

3) How does the embryo's body orientation and the gravity-dependent redistribution of CSF influence RF morphogenesis, ventricular system architecture, and the expression of genes regulating the mechano-elastic properties of cells?

By addressing these questions, this thesis advances our understanding of the regulation of Sspo secretion and RF assembly, the integration of signaling pathways and membrane properties during RF formation, and the effects of mechanical forces, such as gravity, on the development of brain ventricles and RF formation.

2 RESEARCH ARTICLES IN THE COLLECTION

2.1 Manuscript 1

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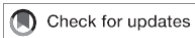
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I hereby declare that I, Razieh Amini, made a substantive contribution to the development of this publication. My contribution consisted of:

- conducting experiments,
- data analysis,
- writing sections of the manuscript

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Kv2.1 negatively regulates Reissner fiber development

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Introduction: The potassium voltage-gated channel Kv2.1 plays a crucial role in the development of the brain's ventricular system. Defects in the development of this system affect the formation of the Reissner fiber, a rope-like structure produced by the flexural and subcommissural organs that secrete Scospondin. **Methods:** The development of the Reissner fiber has been studied during normal development and in zebrafish mutants deficient in activity of the two Kv2.1 subunits – Kcnb1 and Kcng4b using a combination of immunohistochemistry and transgenic lines expressing EGFP in the subcommissural organ and floor plate.

Results: The Reissner fiber develops in stages. First, the midline floor plate cells, originating from the embryonic organizer, secrete Scospondin, forming the posterior Reissner fiber. This allows us to define the posterior Reissner fiber as the acellular derivative of the embryonic organizer. The fiber separates from the floor plate, beginning in the hindbrain and extends through the neural tube, from the most anterior floor plate (i.e. the flexural organ) anteriorly to the ampulla terminalis. Second, the subcommissural organ, which is derived from the anterior roof plate, begins secreting Scospondin. This forms the anterior Reissner fiber, which spans the cerebral aqueduct. Third, the anterior Reissner fiber connects to the flexural organ, where the two fibers fuse. Fourth, after the floor plate ceases to express Scospondin, the Reissner fiber derived from the subcommissural organ replaces the transient posterior fiber derived from the floor plate. Like the subcommissural organ, the flexural organ is an attachment point for the Reissner fiber. Reissner fiber assembly involves the formation of individual microfilaments that fuse in several steps to form the single fiber.

Discussion: Analysis of zebrafish mutants of Kv2.1 subunits (Kcnb1 and Kcng4b) revealed that Kv2.1 negatively regulates Scospondin production at several levels. These mutations have opposing effects on the transcript levels of several genes involved in Reissner fiber development (*sspo*, *lgals2*, and *chl1a/camel*), affect the subcommissural organ and microfilament formation, and impact Reissner fiber assembly.

KEYWORDS

ampulla terminalis, CD133/prom1a, flexural organ, KV2.1, midline floor plate, roof plate, Scospondin, subcommissural organ

Introduction

The basic structure of the brain ventricular system (BVS) has remained largely unchanged throughout vertebrate evolution although the number of brain ventricles varies (Lowery and Sive, 2009). It increases in parallel with increase of brain complexity, from hemichordates to mammals (Fame et al., 2020). The BVS consists of a series

of interconnected cavities filled with cerebrospinal fluid (CSF) and surrounded by neuroepithelial ependymal cells. It plays a crucial role in neurogenesis and brain homeostasis by regulating the flow of CSF. The neuroepithelial lining of the BVS consists of several cell types, including tanycytes, ependymocytes, CSF-contacting neurons, macrophage-like supraependymal cells, and radial glia (Del Bigio, 2024). These cells express ion and water channels, as well as cell adhesion extracellular matrix proteins and components. During embryogenesis, the roof and floor plates are two opposing signaling centers along the dorsal and ventral midlines of the BVS. The anterior region of the roof plate develops into the subcommissural organ (SCO), and the anterior floor plate forms the flexural organ (FO) (García-Lecea et al., 2017; Kiecker, 2018). The midline floor plate originates from the embryonic shield, also known as the zebrafish organizer (Shih and Fraser, 1996). Both the SCO and FO express Scospondin (Sspo), a giant matricellular protein that comprises the Reissner fiber. The Reissner fiber is an acellular filamentous structure found along the neural tube from the third ventricle to the end of the spinal cord. Several other genes (*clu*, *lgals1*, *chl1a/camel*, *cyc*, and *oep*), which are expressed by the ependyma, roof plate, and floor plate, as well as low density lipoproteins from CSF have been associated with RF formation (Cantaut-Belarif et al., 2018; Driever, 2018; Lehmann and Naumann, 2005; Meiniel et al., 2008; Muñoz et al., 2019; Sepúlveda et al., 2021; Troutwine et al., 2020; Vera et al., 2015; Yang et al., 2021). Despite this progress, the complete molecular composition of the RF and the RF self-assembly mechanism remain unresolved (Muñoz et al., 2019).

In zebrafish, BVS is formed by the cavitation of the neural tube due to the input of ion channels causing water influx (Lowery and Sive, 2009; 2005). Dysregulation of these channels can result in neurodegenerative diseases, including microcephalia and hydrocephalus (Sakka et al., 2011; Tully and Dobyns, 2014; Zhang et al., 2006). Animal models of BVS defects have been employed to investigate disturbances in CSF circulation and alterations in neuroepithelial integrity (Chae et al., 2004; Chang et al., 2012; Jiménez et al., 2001).

The voltage-gated potassium channel Kv2.1 is a tetramer consisting of two subunits with opposing functions. Kcnb1 is electrically active, while Kcng4 is modulatory (Bocksteins and Snyders, 2012; Vacher et al., 2008). An appropriate balance of these activities is necessary for channel function, including intracellular protein transport; the formation of endoplasmic reticulum (ER)

- plasma membrane junctions; the maintenance of the plasma membrane potential; the formation of cholesterol-enriched lipid rafts; and interaction with integrins (Bortolami et al., 2023; Deutsch et al., 2012; Forzisi and Sesti, 2022; Johnson et al., 2019; Tamkun et al., 2007). Due to its connection to the intracellular skeleton, Kv2.1 activity is mechanosensitive to the physiological perturbation of the cell membrane. This is consistent with the idea that membrane tension regulates ion flow through the channel (Schmidt et al., 2012).

Kv2.1 heterozygous mutations have been associated with epileptic encephalopathy in humans (Bar et al., 2020; Jędrychowska and Korzh, 2019; Srivastava et al., 2014; Torkamani et al., 2014). Developmental defects affecting Kv2.1 subunits in homozygous mutants have been studied in mice and zebrafish. Studies in mammals have suggested roles for Kcnb1 in insulin secretion and behavior and for Kcng4 in spermatogenesis (Jacobson et al., 2007;

Regnier et al., 2017; Specia et al., 2014). Zebrafish studies have shown an antagonistic role for Kv2.1 subunits in the development of hollow structures, such as the brain and ear (Jędrychowska et al., 2021; Jędrychowska et al., 2024; Shen et al., 2016).

The *kcnb1*^{sq301} mutant phenotype in zebrafish is incompletely

penetrant. Some mutant embryos exhibit delayed epiboly and gastrulation failure, while others undergo gastrulation and display the late phenotype of reduced hollow organs, including the brain ventricles and ears (Jędrychowska et al., 2021; Jędrychowska et al., 2024; Shen et al., 2016). In contrast, the *kcnb1*^{sq301} zebrafish mutant develops hydrocephalus, which closely resembles the phenotype of *hyh* mice lacking α -SNAP, a component of intracellular protein trafficking mechanism (Pérez-Figares et al., 1998; Shen et al., 2016; Wagner et al., 2003). Kcnb1 transports Kcng4 from endoplasmic reticulum to plasma membrane, i.e., similar to α -SNAP Kcnb1 is involved in intracellular protein trafficking (Bocksteins et al., 2014). Previous studies have shown that BVS defects in *hyh* mice are associated with abnormal RF development (Pérez-Figares et al., 1998; Wagner et al., 2003). Therefore, we expanded our analysis of BVS in Kcnb1-Kcng4b mutants to focus on the roles of SCO and FO in RF development.

Materials and methods

Animals

Zebrafish (*Danio rerio*) were maintained according to established protocols (Westerfield, 2007) in the Zebrafish Core Facility at the International Institute of Molecular and Cell Biology in Warsaw. This facility is licensed for breeding and research (PL14656251, registry of the District Veterinary Inspectorate in Warsaw; 064 and 051: registry of the Ministry of Science and Higher Education). The experiments involving zebrafish embryos, larvae, and adults were conducted in accordance with the European Communities Council Directive (63/2010/EEC). The developmental stages in hours post-fertilization (hpf) are based on (Kimmel et al., 1995). The *kcnb1*^{sq301} (also referred to as *kcnb1*^{-/-}) mutant has been previously described (Shen et al., 2016). *kcnb1*^{sq301} and *kcnb1*^{sq301} mutants have been described previously (Gasanov et al., 2021; Jędrychowska et al., 2024). The reduced fertility of *kcnb1*^{sq301} mutants has prevented the analysis of gene expression level.

The transgenic lines used in this study served as *in vivo* markers: the ET33-mi2A transgenic line (sqet33mi2Aet) carries the transposon Tol2 insertion in *CD133/prom1a* position. It expresses cytosolic GFP in the RP, SCO, ear, etc. The ET33-E20 (sqet33e20Et) transgenic line carries the transposon insertion in between *zic3* and *zic6* genes and expresses cytosolic GFP in the choroid plexus, etc. The ET33-10 (sqet3310Et) transgenic line carries the transposon insertion in the *grip2b* intron (García-Lecea et al., 2017; Parinov et al., 2004). The *scospondin-GFP*^{ut24} transgenic line is a knock-in allele, where the C-terminal portion of the *scospondin* gene was precisely tagged with the GFP coding sequence (Troutwine et al., 2020).

Microscopy

Live imaging: Zebrafish embryos were raised in E3 medium (2.5 mM NaCl, 0.1 mM KCl, 0.16 mM CaCl₂, and 0.43 mM MgCl₂) with the addition of 0.2 mM 1-phenyl-2-thiourea (PTU, Merck, Germany) to block pigmentation. At selected developmental stages, the embryos were manually dechorionated and anesthetized with 0.02% tricaine (Sigma-Aldrich, United States). Then, they were oriented after embedding in 2% methylcellulose (Merck, Germany) on the glass slides. A research stereomicroscope SMZ25 (Nikon, Japan) was used for imaging. The light-sheet fluorescent microscopy was performed as previously described (Jedrychowska et al., 2021). Zeiss Lightsheet Z. 1 microscope with W Plan-Apochromat objective (20x/1.0 UV-VIS) was used.

For fixed specimens, 0.8% low-melting agarose in PBS was used instead of E3 medium with 0.02% tricaine. Zeiss Lightsheet Z. 1 microscope with W Plan-Apochromat objective (40x/1.0 UV-VIS or 63x/1.0 UV-VIS) was used. Transmitted LED light was used to obtain high-resolution bright-field images. The data were saved in the LSM or CZI format and processed using ZEN (Zeiss) or ImageJ 1.51n (Fiji) software. Maximum intensity or sum slice projections were generated for each z-stack. Brightness and contrast adjustments, as well as resizing were performed using FastStone viewer 7.4 (FastStone Soft). SCO area in Figure 3 was normalized to the head–MHB distance to correct for embryo size variation.

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde prior to staining. Embryos were stained using two-color immunohistochemistry for RF with a polyclonal rabbit AFRU antibody (1:1000), a gift of Drs. J. Grondona [Malaga, Spain], E. Rodriguez, and M. Guerra [Valdivia, Chile]) according to the described protocol (Korzh et al., 1998). The detected GFP was expressed in transgenic embryos.

Image quantification

Embryos were scored for the shape of the SCO apical surface in the unified analysis cohort (n = 15 embryos per genotype, except *kcnq4b^{waw305}*) used for Figure 5; Table 1. SCO openings were classified as concave, flat, convex. Frequencies were recorded as counts and percentages. Statistical analysis was performed using a Chi-square test of independence across genotypes, followed by pairwise Fisher’s exact tests.

Anterior RF length was measured in Fiji using the segmented line tool, from the SCO apical margin to the anterior tip of FO. Values were expressed as mean ± SD. n = 6 embryos per genotype were analyzed, selected from the imaging cohort based on QC criteria requiring clear lateral views with the entire SCO–FO span visible. Group differences were tested by one-way ANOVA.

SCO area was normalized to the head–MHB distance to correct for embryo size variation. Fusion node density was calculated as the number of junctions per 50 μm filament length between the SCO and FO. The SCO–fusion distance was measured as the linear span from the SCO apical surface to the nearest fusion node. Six embryos per genotype were analyzed, chosen for high-quality dorsal

TABLE 1 qRT-PCR Primers.

N	Genes	qRT-PCR primers, 5'- > 3'
1	<i>chl1</i>	TGCAGCATTCGTGCTCAACGT AACAGCCGATGAGGACAAGCA
2	<i>clusterin</i>	TCGCAAGTTGGTGAGAAATAC GGAGGCTGTGAAACAAGGAGT
3	<i>foxa</i>	AGACAGAATCAGCAGAGATGG CGGAGTCAGGGTGTAAGC
4	<i>foxa3</i>	ATGTCCAACGAGCAGAAGATG GTAGTAGGCACCAGAGTCAATG
5	<i>gli1</i>	AGCAGCAGCAGCATTCAG AGGTGTTCTAAGGCATTGGAC
6	<i>lgals2a</i>	GGAGCACAGAGACAACAAC ATCCTGACTTCGCCTTCG
7	<i>lgals2b</i>	CAACAACCTCAGCAAGATGG TGAAGTGAAGAGCGATGG
8	<i>shha</i>	GAGAGTGCTGCTGGTGTC CTGCTTGTAGGCGAGAGG
9	<i>spn1a</i>	GTGACCAAGAGGAGGATTATGCT TGTCATGAATTGCGTGTCTTCCT
10	<i>sspo</i>	CAGTGTGGATTGTGATGTG CAGAGCAGAAGCGATACC

views that permitted accurate quantification. Values were reported as mean ± SD. Correlations between node density and SCO–fusion distance were evaluated using Pearson’s correlation coefficient.

AFRU + signal in the ampulla terminalis was extracted using ImageJ following thresholding of representative lateral projections. The AFRU-positive area (μm²) was measured for each embryo and expressed as mean ± SD. n = 6 embryos per genotype were included, selected for consistent lateral orientation and sufficient staining quality. Statistical analysis was performed using one-way ANOVA followed by Tukey’s *post hoc* test. Quantification reflects the total AFRU-positive area (μm²) within the ampulla terminalis, not integrated fluorescence intensity. Measurements capture spatial distribution rather than overall brightness of the AFRU signal.

Cryosectioning

The embryos were cryo-sectioned, and stained immunohistochemically as described previously (Korzh et al., 1993) using anti-rabbit AFRU antibody (1:1000; gift of Drs. J. Grondona (Malaga, Spain), E. Rodriguez, and M. Guerra (Valdivia, Chile), secondary Alexa Fluor 488 tagged donkey anti-rabbit antibody (1:1000; Invitrogen, United States) and Alexa Fluor 594 phalloidin (1:200; Life Technologies, United States) for F-actin staining. Confocal imaging was performed using the LSM 800 microscope (Zeiss, Germany).

qRT-PCR

qRT-PCR was performed according to the manufacturer's instructions using the SsoAdvanced Universal SYBR Green Supermix and CFX Connect real-time PCR system (Bio-Rad, United States). Total RNA was extracted from 20 to 50 zebrafish embryos (except *kcng4b*^{waw305} homozygotes due to low fertility) at 28, 48, and 72 hpf using the TRIzol-chloroform RNA extraction protocol (Sigma-Aldrich, United States). RNA was quantified using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, United States), and cDNA was synthesized from 1 µg of the total RNA using the protocol provided with the iScript™ Reverse Transcription kit (Bio-Rad, United States). Gene-specific primers for *eef1a111* (a housekeeping gene) and primers specific for other genes (www.zfin.org) (Table 1) were used to amplify the mRNA of interest, which were selected based on the results of the RNA-seq analysis detailed above.

Bio-Rad CFX Maestro software automatically determined the threshold cycle (Ct) of each target and reference gene amplification in control and mutant embryos. Normalized expression values were obtained directly from the software using the $\Delta\Delta C_t$ method, with wild-type samples serving as the reference condition. Expression values were normalized to wild-type controls at each developmental stage to account for shifts in tissue composition between 24 hpf and 72 hpf, when *sspo* expression transitions from the floor plate to the SCO. This stage-specific normalization ensures that mutant versus control comparisons remain biologically meaningful. To clearly represent the data, all qPCR graphs were provided with a dotted line at the 1.0 ratio, which corresponds to wild-type expression. The data were analyzed at the normalized expression values without further transformations (e.g., $2^{(-\Delta\Delta C_t)}$). Statistical significance was

determined using a one-way ANOVA with Tukey's *post hoc* test for multiple comparisons; each time point was compared to the wild-type control. Comparisons between experimental groups and wild-type controls at the relevant time point are indicated by statistical significance markers (***, **, *, n.s.). Error bars show the standard error of the mean (SEM) from biological replicates. To ensure that the efficiency values remained within the ideal range of 90%–110%, the reaction efficiency (E-value) was determined independently for each gene examined. Melting curve analysis and agarose gel electrophoresis were used to verify product specificity.

Results

The formation of Reissner fiber is a multistep process

Recent improvements in scientific research methods have prompted the reevaluation of previously studied biological processes (Muñoz et al., 2019). Combining whole-mount immunohistochemistry with light-sheet microscopy improves the analysis of macromolecular complexes during development, both *in vitro* and *in vivo*. We used this approach to reanalyze RF development in zebrafish. An early AFRU + signal was detected at 16 hpf in association with the anterior floor plate (not shown), which is known to form the FO. At 18 hpf, the intensity of this signal increased, as did the size of the area (Figure 1A). At 30 hpf, the

floor plate-associated AFRU + signal in the hindbrain enlarged anteriorly and split into two lines posteriorly (Figures 1B–D; Figure 2A; Supplementary Figures S1B, C). This phenomenon was not reported previously.

Scospondin-GFP^{ut24} transgenics enable imaging of RF formation *in vivo* (Troutwine et al., 2020). Two signals in the hindbrain were detected in *scospondin-GFP^{ut24}* transgenic fish also (Figures 2B,C). The analysis revealed that the ventral signal is associated with the apical surface of the hindbrain floor plate, which bends ventrad (see Figures 2B–D). The dorsal signal represents the RF. During development, the anterior neural tube bends, while it continues to secrete pre-RF AFRU + material. The splitting of the signal is likely due to the ventral bending of the floor plate. During this process the deposited RF likely separates from the apical surface of the floor plate, which continues to secrete pre-RF AFRU + material that forms the additional signal.

During development, the anterior portion of AFRU + signal begins to stretch along the dorsal-ventral (D - V) axis (Figure 2A) (Placzek and Briscoe, 2005). As a result of the floor plate bending posterior to the midbrain-hindbrain boundary (MHB), the FO acquired the hook-like shape at 72 hpf (Figure 2D). High-resolution imaging of the hindbrain revealed the presence of the multiple GFP-positive threads between the two main signals (Figure 2B). These could be the result of AFRU + material deposition by the floor plate during continuous bending or individual RF microfilaments/groups of microfilaments that have separated during the floor plate bending. During the 30–36 hpf period, the diencephalic-mesencephalic roof plate forms the SCO. Initially, cells from the most anterior region of the roof plate extend along the D - V axis (Figures 1C,D; Figures 3A,B). This conclusion is supported by the analysis of the SCO elongation index (Figure 3E). As a result, the SCO reshapes into a triangular pocket (Figures 1C,D; Figures 3C,D) and its area increases significantly (Figure 3F). These measurements demonstrate that the SCO undergoes progressive enlargement and elongation between 36 and 72 hpf. Roof plate cells became AFRU

+ at 30 hpf (Figure 1B). By 36 hpf, the SCO-derived AFRU + thread connected the SCO and FO, illustrating the fusion of the anterior and posterior RF at the FO (Figure 1C). Anterior RF staining intensity increased during the 30–72 hpf period (Figures 1B–D). At 72 hpf, multiple SCO-derived branches fused at different anteroposterior (A-P) levels between the SCO and FO (Figure 1D).

These results support an idea that RF forms step-by-step by the midline floor plate and SCO, respectively (Lehmann and Naumann, 2005; Meiniel et al., 2008). The hindbrain RF lost contact with the floor plate soon after being formed (Figures 4A,B). In contrast, the FO remained in contact with both the anterior and posterior RF (Figure 4B). Thus, the FO appears to function as a connecting hub for the two independently derived parts of the RF. The posterior RF, which is generated by the midline floor plate, is a transient structure that functions as a guide for the anterior RF, which is generated by the SCO. Thus, during RF development there is a transition from the ventral midline-derived posterior RF to the dorsal midline-derived anterior RF.

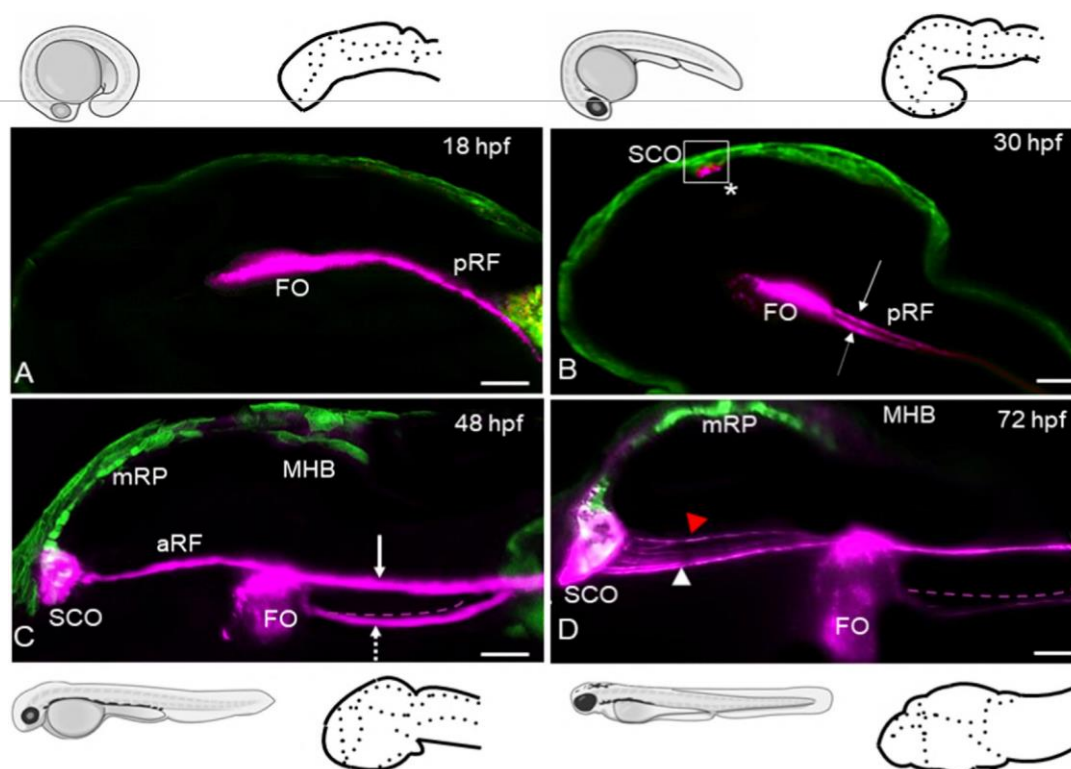


FIGURE 1

RF develops in three major steps. First, posterior RF is formed by the midline floor plate. Second, anterior RF is formed by SCO. Third, posterior RF and anterior RF fuse at FO. (A) In the 18 hpf embryo, anterior FP developed anti-AFRU antigenicity (magenta). (B) At 30 hpf, SCO developed the anti-AFRU antigenicity (asterisk). The duplicated signal was present at the level of the anterior hindbrain. (C) At 36 hpf, the FO acquired its characteristic shape with most of the staining dorsally. The separation of the two signals continued posterior to the FO. Additional staining corresponding to the anterior RF was detected between the SCO and FO. The signal was more intense anterior to the FO, while the region adjacent to the SCO was weakly positive for AFRU staining. (D) At 72 hpf, the intensity of anterior RF staining increased; several SCO-derived branches of the anterior RF formed; and the SCO and FO became elongated. For 48 hpf wild-type control, see A, A'. All images are in lateral view. The cartoons illustrate the stage of development and schematics of the brain. Solid arrows indicate trajectories or branches of the aRF; dashed arrows indicate duplicated AFRU⁺ signals posterior to the FO. Abbreviations: aRF, anterior Reissner fiber; FO, flexural organ; MHB, mid-hindbrain boundary; mRP, midbrain roof plate; pRF, posterior Reissner fiber. N = 6. Scale bar = 20 μ m.

The Kv2.1 channel regulates the development of RF

Mutations affecting the Kv2.1 subunits, *Kcnc1* and *Kcng4b*, resulted in reduced or enlarged BVS, respectively (Shen et al., 2016). Since such changes affect RF development in mammals, the question arose as to whether these developmental defects affect RF formation in zebrafish and whether the circumventricular organs involved in RF formation, the SCO and FO, are affected. These organs originate from the signaling centers of the neural tube, i.e., the roof plate and midline floor plate. *kcnb1^{sq301}* and *kcnq4b^{waw304}* and *kcnq4b^{waw305}* mutants were crossed with ET33-mi2a transgenics that express green fluorescent protein (GFP) in the SCO and FO. In the 48-hour-post-fertilization (hpf) wild-type controls, the SCO and FO were connected by a single line of anterior RF (Figure 5A) with the two parallel signals extending in the hindbrain posterior to the FO (Figure 5A, arrows). A dorsal view of the 48-hpf control embryo revealed a complex pattern of RF assembly from individual microfilaments. These microfilaments originated

from the SCO apical surface (Figure 5A). The surface shape varied between concave (67%) and flat (33%; Table 1). The filaments fused at least twice before forming the single anterior RF. The staining also showed the complex radial distribution pattern of AFRU + material inside the SCO (Figure 5A'). To quantitatively define the microfilament fusion pattern, the SCO-FO distance (Figure 5E), fusion node density and distance between the SCO and the first fusion point (SCO-fusion) were calculated. Fusion node density was calculated as the number of junctions per 50 μ m of filament length between the SCO and FO. In the controls this value was $49.0 \pm 33.6 \mu$ m (Figure 5F), with an SCO-fusion distance of $18.6 \pm 9.7 \mu$ m (Figures 5E,F).

In *kcnb1^{sq301}* homozygotes at 48 hpf, the size of the midbrain cavity (i.e., the cerebral aqueduct) was smaller than in the control group (Korzh, 2018; Shen et al., 2016). In this mutant, the two branches of the anterior RF appeared in a significant proportion of embryos with ectopic anterior RF in the dorsal position (lateral view, Figure 5B, arrowhead) or ventral position (Supplementary Figure S2A). This phenotype resembled that caused

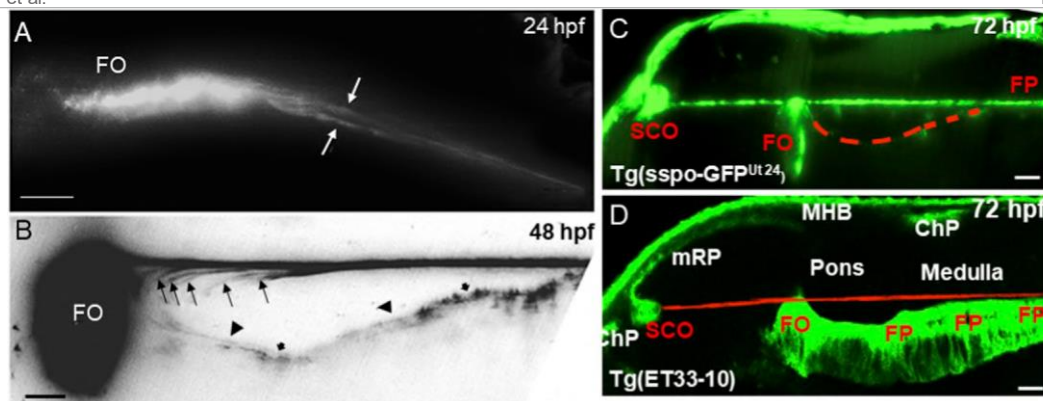


FIGURE 2

RF is connected to SCO and FO but separated from the hindbrain floor plate. (A) The AFRU-positive signal (shown in white color) is duplicated at the level of anterior hindbrain (24 hpf, white arrows). (B) The multiple fine layers of GFP-positive material (shown in black color; black arrows; the *scospondin-GFP^{ut24}* transgenic line) were detected posterior to the FO at the level of anterior hindbrain (48 hpf; black arrowheads indicate the apical surface of the FP). The RF projects towards the central canal in a straight line. The hindbrain RF is disconnected from the apical surface of the FP. (C) Tg (Sspo-GFP), the white arrows show the weak GFP signal at the hindbrain FP apical surface (red dashed line); (D) ET33-10 (modified from [García-Lecea et al., 2017](#); green - GFP; red - projected RF trajectory). In (A,B) The GFP signal is converted to black-and-white. Abbreviations: ChP—choroid plexus; E, epiphysis; FO, flexural organ; FP, floor plate; MHB, midbrain-hindbrain boundary; mRP, midbrain roof plate; SCO, subcommissural organ. N = 6 (A–C). Scale bar, (A,B) = 20 μ m; (C,D) = 10 μ m.

by *Chl1a/Camel* gain-of-function ([Yang et al., 2021](#)). The complex pattern of AFRU + material distribution in SCO was detected in these mutants. Posterior to the FO, the two parallel signals were present ([Figure 5B](#), arrows). In a dorsal view, the four groups of two to four primary microfilaments originated from the largely flat apical surface of the SCO ($n = 12/15$; [Figure 5J](#)). The single thread of the anterior RF formed caudal to the SCO after at least three rounds of microfilament fusion. The SCO-FO distance was reduced compared with controls ([Figure 5E](#)). The fusion node density in these mutants ($77.8 \pm 31.5 \mu\text{m}$) was significantly higher than in controls, and the SCO-fusion distance was similar to that in controls ($18.0 \pm 7.8 \mu\text{m}$) ([Figures 5F,G](#)). *kcnb1^{sq301}* mutants show increased fusion node density compared with wild type and changed curvature of the SCO's apical surface ([Figure 5J](#)). These quantitative trends support the general observations ([Figures 5A,B](#)).

After observing the effects of *kcnb1^{sq301}* on RF development, we investigated whether this mutation affects the transcript levels of genes associated with RF. Although the change in activity of this ion channel may have an indirect effect on transcription, this analysis suggests the potential avenues for the follow-up investigations and provides qualitative evidence to support the morphological data. At 24 hpf, *sspo* is only expressed in the midline floor plate ([Meiniel et al., 2008](#)). At this stage, *sspo* transcript level declined in the *kcnb1^{sq301}* mutant compared to the wild-type control ([Figure 5H](#)). At 48 and 72 hpf, *sspo* expression in the floor plate declined during normal development, while increasing in the SCO ([Meiniel et al., 2008](#)). *sspo* expression levels in the *kcnb1^{sq301}* mutant increased during this period compared to the wild-type controls ([Figure 5H](#)). This mutation appears to affect *sspo* transcript level differently in the floor plate and SCO. The levels of the *chl1a/camel*, *spon1a*, and *clu* transcripts have changed in the mutant, in a manner resembling the changes of *sspo* transcript level, with a significant increase at 72 hpf ([Figure 5H](#)). Galectin-1 acts as a partner of Scospondin during RF assembly in

mammals ([Muñoz et al., 2019](#)). The expression pattern of galectin-1 (*lgals1*) has not been studied in developing zebrafish; however, two related genes (*lgals2a* and *lgals2b*) are expressed in the developing ependyma ([Thijssen et al., 2006](#)) and may be involved in BVS development. The *lgals2a* transcript level was significantly lower in the *kcnb1^{sq301}* homozygotes than in the wild type controls at 24–72 hpf ([Figure 5H](#)).

In the *kcnb1^{waw304}* mutant, which represents a hypomorphic

Kcng4b loss-of-function allele ([Jędrychowska et al., 2024](#)), the BVS was enlarged compared to the wild-type control. The SCO-associated AFRU + filamentous network, as seen in the dorsal view, was less complex in this mutant compared to the wild-type control and *kcnb1^{sq301}* homozygotes ([Figure 5C, A' and B'](#)). The anterior RF was present, but it formed after the fusion of two microfilaments derived from the predominantly concave apical surface of the SCO ($n = 13/15$; [Table 1](#)). The fusion node density in these mutants was lower than in controls ($36.9 \pm 11.1 \mu\text{m}$) as was the SCO-fusion distance ($14.2 \pm 5.0 \mu\text{m}$) ([Figures 5F,G](#)). The complex pattern of SCO staining was preserved in the *kcnb1^{waw304}* mutant ([Figure 5C](#)). Quantitative analysis confirmed a measurable reduction in fusion node density and SCO-fusion distance in *kcnb1^{waw304}* mutants compared with wild-type controls ([Figures 5F,G](#)). The phenotype of the *kcnb1^{waw305}* allele ([Jędrychowska et al., 2024](#)) was more severe. The anterior RF was absent in some samples. This correlated with the significant reduction of the complex pattern of SCO staining and the convex apical surface of the SCO ([Figures 5D, D', J](#)). This phenotype resembles that caused by *Chl1a/Camel* loss of function ([Yang et al., 2021](#)). Yet the phenotype in majority of *kcnb1^{waw305}* homozygotes was like *kcnb1^{waw304}*, i.e., with convex apical surface ([Figure 5J](#)). In contrast to the hypomorphic *kcnb1^{waw304}*, *kcnb1^{waw305}* homozygotes exhibit reduced fertility and poor survival. Therefore, there was not enough material to perform the gene expression analysis on this line (see, Material and Methods).

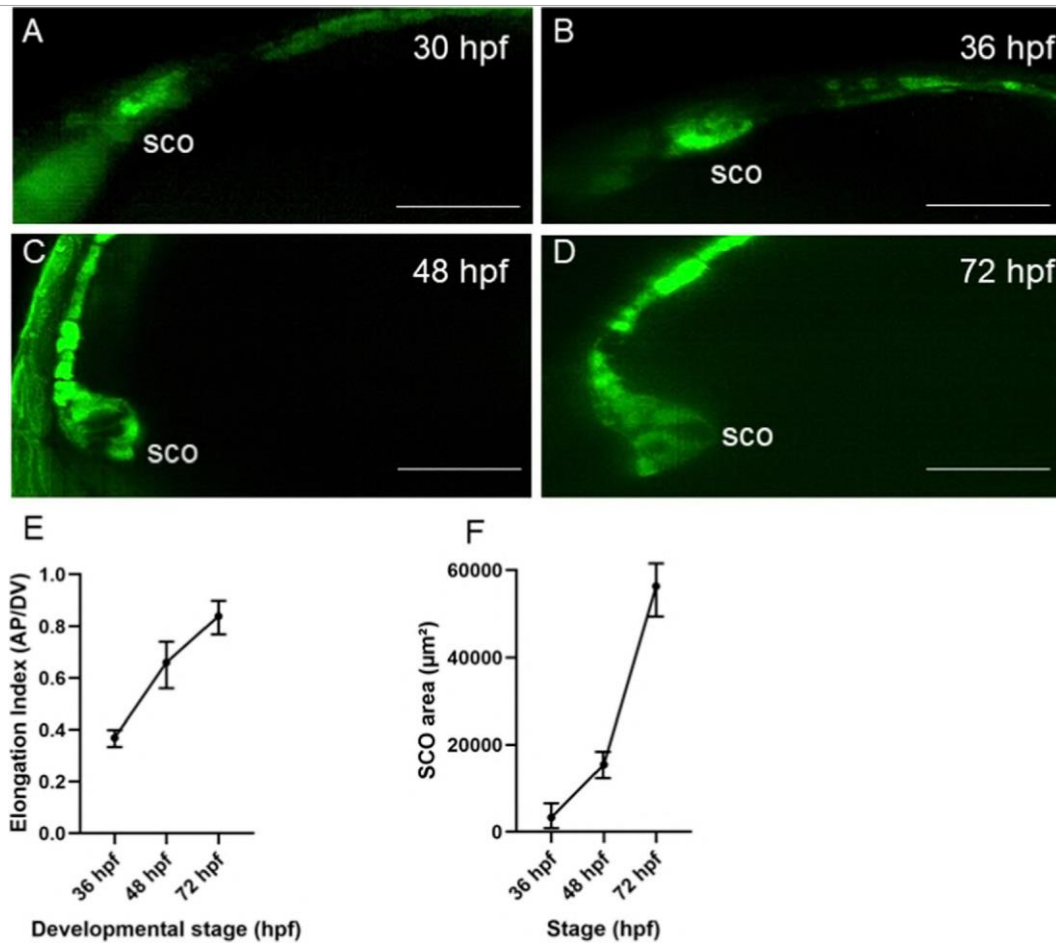


FIGURE 3 SCO morphogenesis in ET33-mi2a transgenics. (A) 30 hpf, (B) 36 hpf, (C) 48 hpf, (D) 72 hpf. All images are in the lateral view. (E) SCO elongation during development; (F) SCO area enlargement. For each embryo, the SCO area (μm^2) was measured, and the antero-posterior (A-P) length and dorso-ventral (D-V) height were recorded to calculate the elongation index (A-P/D-V ratio). Values are presented as mean $\pm$ SD ($n = 6$ embryos per stage). MHB—midbrain-hindbrain boundary. Scale bar = 60 μm .

The reduction of the filamentous network of anterior RF in the *kcng4b^{waw304}* mutant correlated with the robust reduction in *sspo* transcript levels during the 48–72 hpf period, following a transient increase at 24 hpf (Figure 5I). While the *lgals2a* transcript level increased to 24 hpf, it decreased significantly by 48 and 72 hpf (Figure 5I). The level of the *chl1a/camel* transcripts significantly declined in contrast to that of *spon1a* and *clu* that were much less affected (Figure 5I). Thus, the transcript levels of *sspo* and *chl1a/camel* changed oppositely in the mutants affecting the two Kv2.1 subunits. At 24 hpf, the floor plate is the only source of *sspo*; however, by 72 hpf, *sspo* is predominantly expressed in the SCO (Meiniel et al., 2008). Therefore, the 24 hpf stage could be useful for evaluating the effect of Kv2.1 on the posterior RF using molecular markers expressed in the floor plate. The shortage of embryos prevented analysis of gene expression in homozygotes of the *kcng4b^{waw305}* allele (see M&M).

The expression of genes acting downstream of Shh (*foxa3*, *gli1*) has been affected in the *kcnb1* mutant (Figure 5K). *Foxa3* acting in concert with two other members of this family (*Foxa1* and *Foxa2*)

to positively regulate axial structures, including the floor plate (Dal-Pra et al., 2011). Notably, the *foxa3* transcript level increased significantly in the 24-hpf-old *kcng4b^{waw304}* mutants (Figure 5L) unlike that in *kcnb1^{sq301}* homozygotes (Figure 5K) suggesting a correlation between the activity of Kv2.1 and expression of floor plate markers. In contrast, the transcript level of the related gene *foxa*, which is mainly expressed in the neural crest and gut during this period (Odenthal and Nüsslein-Volhard, 1998), increased in both mutants (Figures 5K,L).

Defects in RF development are intrinsically linked to RF disassembly in the ampulla terminalis at the posterior end of the neural tube (Figure 4C) (Meiniel et al., 2008; Yang et al., 2021). The level of total fluorescence in this region increased in all mutants compared to wild type controls (Figure 6E). At the same time, the signal spread relatively broadly in the *kcng4b^{waw305}* mutant (Figures 6C,H), in contrast to controls and *kcnb1^{sq301}*, where it seems to be more compact (Figures 6A,D,G). It seems that the *Kcnb1-Kcng4b* mutants affected not only the assembly of RF, but its disassembly also.

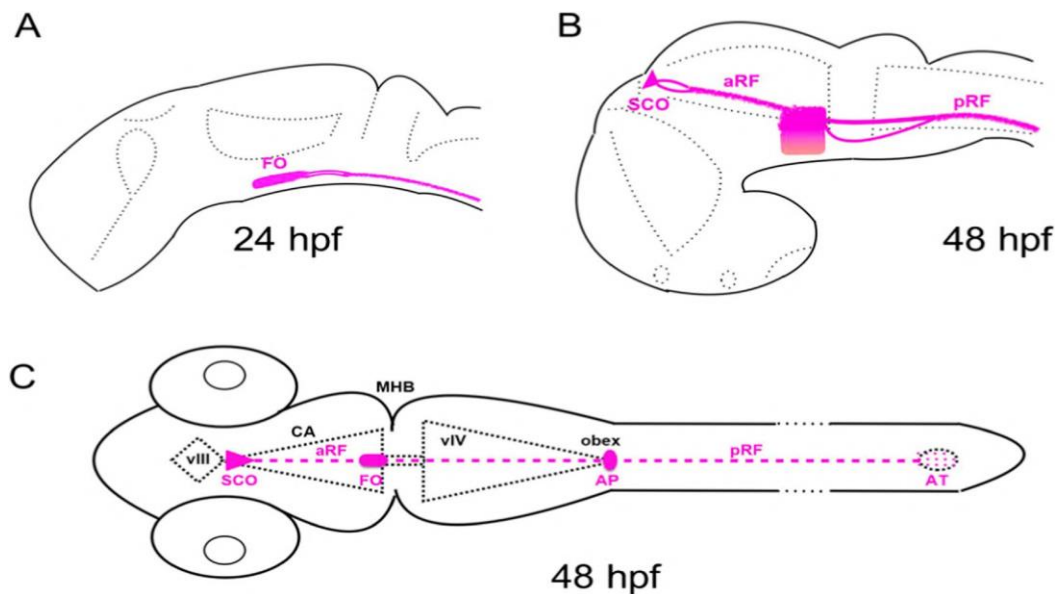


FIGURE 4

Schematic representation of the organs involved in formation of the RF. (A), The FO/floor plate-derived RF (pRF) forms on the apical surface of the floor plate. The AFRU + signal is duplicated posterior to the FO with the dorsal signal representing the posterior RF and the ventral one representing the AFRU + material on the apical surface of the ventrally bending floor plate. (B) The SCO-derived RF forms in the cerebral aqueduct (CA). (C) Both parts of the RF join at the FO. Abbreviations: aRF, anterior Reissner fiber; AT, ampulla terminalis; FO, flexural organ; MHB, midbrain-hindbrain boundary; pRF, posterior Reissner fiber; SCO, subcommissural organ; vIII, third ventricle; vIV, fourth ventricle; wt, wild-type.

Discussion

We conclude that mutations affecting the activity of Kv2.1 cause opposite changes in the expression of genes associated with RF formation, intracellular distribution of Sspo and its secretion, and the shape of the apical surface of the floor plate. Previous studies have demonstrated the effect of changing the curvature of the cell membrane on Kv2.1 activity (Schmidt et al., 2012). A picture emerging from analysis of the SCO apical membrane in *Kcnc1* and *Kcng4b* mutants suggested that changes in Kv2.1 activity modify the SCO apical surface topology (Figure 5). It is unclear whether this effect is direct. Whereas membrane tension regulates ion flow through the channel (Schmidt et al., 2012), the reverse process could be envisioned too. The membrane deformation could also be due to variations in the number and distribution of SCO-derived microfilaments that induce different tensions. In this case, initial deformation induced by microfilaments could trigger further changes in the activity of the mechanosensory mechanism (Porazinski et al., 2015; Takahashi et al., 2021; Voltes et al., 2019).

The effect of Kv2.1 seems to differ in the two sources of Sspo, i.e., midline floor plate and roof plate-derived SCO. It seems that Kv2.1 affects the formation of RFs at different levels, including expression of genes that encode RF components. Whether voltage-gated channels such as Kv2.1 are directly involved in regulation of transcription is unlikely. But as the principal component of ER-PM junctions involved in store-operated calcium entry, Kv2.1 may regulate activation of signal cascades, modification of

transcription factors and transcription (Johnson et al., 2018; Zarain-Herzberg et al., 2011).

The changes in Kv2.1 activity have been associated with developmental defects of the BVS (Shen et al., 2016), which as shown by studies of the RF development in *hyh* mice may impact RF formation (Pérez-Figares et al., 1998; Wagner et al., 2003). The results presented demonstrate that the defects of the BVS' spatial expansion affect RF development in zebrafish in a manner suggesting the Kv2.1 negative control over Sspo. This control may take place at different levels, including *sspo* expression and secretion, leading towards SCO and RF formation, and RF disassembly. Given that Kv2.1 is involved in the intracellular protein traffic (Jędrychowska and Korzh, 2019; Jensen et al., 2017) and insulin secretion (Jacobson et al., 2007), it is possible that Kv2.1 regulates Sspo secretion by cells producing RF proteins. The question is whether Kv2.1 effect is the same in these two different lineages, i.e., the midline floor plate and roof-plate-derived SCO cells representing the two main signaling centers of the neural tube.

The difference in the developmental expression profiles of *sspo* in the floor plate and SCO allows to discriminate to some extent the tissue-specific expression of this gene using the whole-body qRT-PCR. At 24 hpf only the floor plate expresses *sspo*, whereas later the floor plate expression is downregulated. In result, at 72 hpf the SCO is responsible for the bulk of *sspo* expression (Meinzel et al., 2008). Reduced Kv2.1 activity in *kcnb1* mutants (Jędrychowska et al., 2024) correlates with the decreased level of *sspo* transcript at 24 hpf, i.e., the time point *sspo* expression is confined to the floor plate

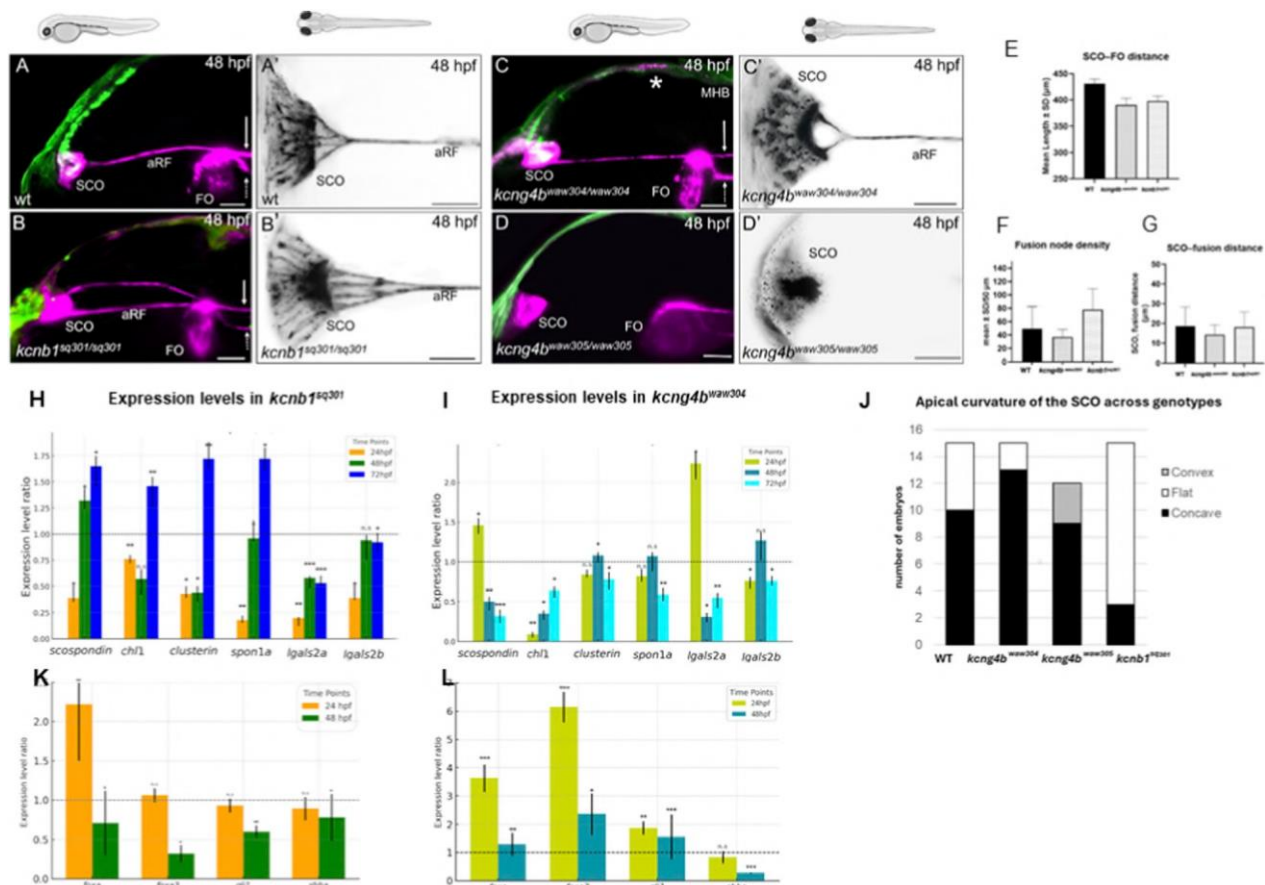


FIGURE 5

Mutations in the genes that encode the Kv channel subunits *Kcnb1* and *Kcng4b* affect RF development. (A) In the wild-type 48 hpf embryo ($n = 15$), the anterior RF connects the SCO and FO. A', Several SCO-derived filaments coalesce to form the anterior RF embryos ($n = 15$). The SCO apical surface was concave (10/15) or flat (5/15) (Table 1). (B) In the *kcnb1^{sq301/sq301}* 48 hpf embryo ($n = 15$), the anterior RF was abnormally enlarged (9/15) or duplicated (6/15). B', focus on the ventral anterior RF branch, where the number of SCO-derived filaments and fusion nodes increased compared to controls, and additional expression occurred in the roof plate anterior to the MHB. The SCO apical surface was concave (3/15) or flat (12/15). (C) The anterior RF was reduced in the *kcnb1^{sq301/sq301}* 48 hpf embryo ($n = 15$). C', The number of SCO-derived filaments was reduced to two with a single fusion node. The SCO apical surface was predominantly concave (13/15) or flat (2/15). (D) In the *kcnb1^{sq301/sq301}* 48 hpf embryo ($n = 15$), the anterior RF was significantly reduced (3/15) or absent (12/15). D', no SCO-derived filaments were detected. The SCO apical surface was concave (9/12) or convex (3/12) (Table 1). (A–D) Green - GFP expression in ET33-mi2a transgenics; magenta - anti-RF (AFRU) immunohistochemical staining detected by light sheet fluorescent microscopy (LSFM). A'–D', color signals of GFP in the ET33-mi2a embryos and AFRU staining converted to the black-and-white; (A–D) lateral view, A'–D', dorsal view. (E), Length of anterior RF was measured in Fiji using the segmented line tool, from the subcommissural organ (SCO) apical margin to the anterior tip of FO. Values are mean ± SD. $n = 6$ embryos per genotype were analyzed, selected by QC for clear lateral views with full SCO–FO span. (F) Fusion node density (junctions per 50 μm). (G) SCO–fusion distance measured on the same QC subset ($n = 6$ per genotype). The node density weakly correlated with SCO–fusion distance ($r = 0.22$), indicating that filament branching complexity and fusion point positioning represent distinct parameters of anterior RF assembly. (H,I) Developmental profiling of expression levels of the RF-associated genes detected by quantitative RT-PCR in the *kcnb1^{sq301/sq301}* (G) and *kcnb1^{sq301/sq301}* (H) mutants. (J) The shape of the apical surface of the SCO in the controls and mutants. (K,L) The level of transcripts associated with ventral midline signaling in the *kcnb1^{sq301/sq301}* (K) and *kcnb1^{sq301/sq301}* (L) mutants. Expression values were normalized to wild-type embryos at each time point; this approach avoids misinterpretation caused by stage-dependent shifts in *scspon* expression domains. Scale bar - 20 μm. Abbreviations: aRF, anterior Reissner fiber; FO, flexural organ; MHB, midbrain-hindbrain boundary; SCO, subcommissural organ.

(Figure 5H). In line with this result, increased Kv2.1 activity in *kcnb1* mutants correlates with an increase of the *scspon* transcript level (Figure 5I). In contrast, at 72 hpf, the opposite situation was detected (Figures 5H,I). Therefore, Kv2.1 likely may have an indirect positive effect on the *scspon* transcript level in the floor plate and an opposite effect on that in the SCO. This assumption is supported by the increase of the AFRU + signal associated with the anterior RF in *kcnb1* mutants and its reduction and/or absence in *kcnb1* mutants (Figures 5B–D). Thus, Kv2.1 subunits exert antagonistic effects on

BVS enlargement, as previously demonstrated (Shen et al., 2016). Within this process, Kv2.1 subunits have different effects on BVS signaling centers, such as the roof and floor plates.

Mutations in Kv2.1 subunits also affect the levels of several other transcripts implicated in RF development. Interestingly, changes of Kv2.1 activity correlate with expression level and function of another regulator of RF formation - *chl1a/camel* (Figures 5H,I). The gain of function of Kv2.1 and *Chl1a/Camel* causes the formation of ectopic RF, though in Kv2.1 mutants the defects seem localized

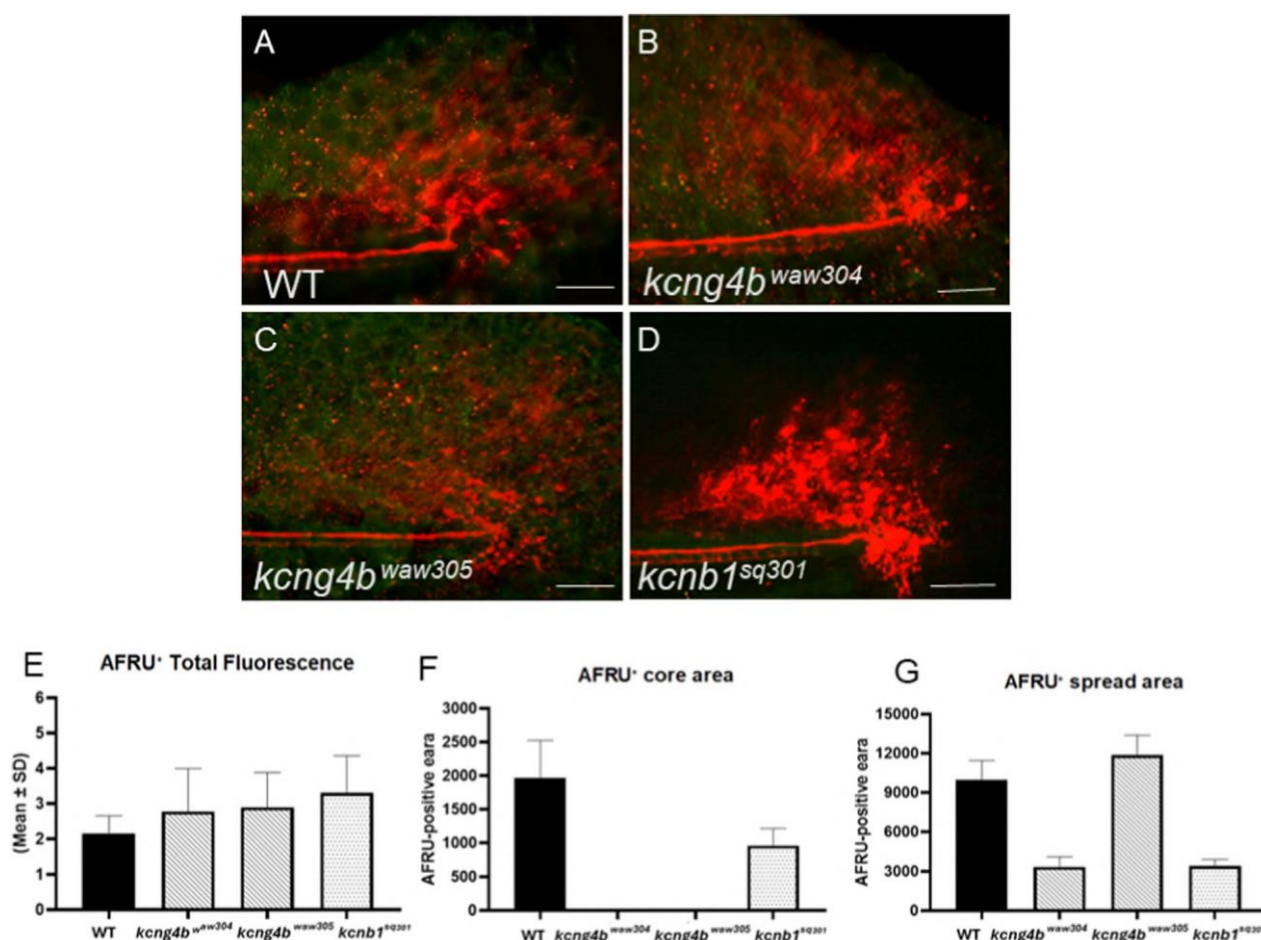


FIGURE 6

The mutations of Kv2.1 subunits affect RF disassembly in the ampulla terminalis (AT). The AFRU-stained AT in representative control (A), *kcnq4b waw304* (B), *kcnq4b waw305* (C), and *kcnb1 sq301* (D) embryos at 48 hpf. (A–D)–lateral view, anterior to the left-hand side. The AFRU + signal is red. The fluorescence of AFRU signal was quantified using ImageJ via thresholding. (E) The AFRU + total fluorescence; (F) The area of AFRU + intense fluorescence; (G) The total area of AFRU + spread (intense and diffuse regions). N = 6. Scale bar: 20 μ m.

to the third ventricle and cerebral aqueduct, unlike defects caused by manipulation of *Chl1a/Camel* levels, which spread into the hindbrain. Kv2.1 and *Chl1a/Camel* loss-of-function results in RF reduction or absence (Figures 5B–D) (Yang et al., 2021). Taken together, these results reveal the complex nature of Kv2.1's effects on RF formation. It could be intrinsically linked to the development of BVS as a whole (Pérez-Figares et al., 1998; Shen et al., 2016; Wagner et al., 2003). However, the effect of Kv2.1 on specific elements of BVS differs and likely extends beyond membrane ion flow. This may result from other Kv2.1 functions, such as intracellular protein transport, the formation of ER-plasma membrane junctions, the maintenance of the plasma membrane potential, and the formation of cholesterol-enriched lipid rafts (Deutsch et al., 2012; Johnson et al., 2019; Tamkun et al., 2007).

The RF forms in several steps. First, the midline floor plate, which is derived from the embryonic shield, transiently secretes Scospondin, forming the posterior RF along nearly the entire neural tube from the midbrain to the neural tube's most posterior tip (Halpern et al., 1997; Meinert et al., 2008; Shih and Fraser, 1996; Strähle et al., 2004). Thus, the posterior RF, which is transient

and derived from the midline floor plate, is an acellular structure generated by the cells originating from the embryonic organizer. As a transient embryonic structure, the posterior RF acts as a template organizing the SCO-derived anterior RF and extending in such a way the role of the embryonic organizer. Furthermore, the FO is not only an attachment point for the RF. It also connects the anterior and posterior parts of the RF. Thus, the early input from the embryonic organizer can be traced all the way to the RF, which coordinates information on body movements along the body axis (Driever, 2018).

Floor plate cells located anterior to the MHB give rise to FO (Figures 1A–C). Unlike the rest of the posterior floor plate, this circumventricular organ maintains contact with the RF. The second circumventricular organ, the SCO, then forms the anterior RF (Figures 1A–C). The anterior RF connects to the FO and, over time, replaces the posterior RF derived from the floor plate. Therefore, the posterior RF, derived from the embryonic shield, acts as a template for the definitive “anterior” RF.

The molecular mechanism responsible for the induction of *sspo* expression in roof plate-derived SCO likely relies on the

ventralization of the diencephalon. The process seems to depend on rapprochement of the ventral and dorsal signaling centers (roof plate and floor plate) at the mid-diencephalic boundary, which results in the expression of downstream Shh pathway components, such as Smo in the SCO and Isl1 in the pineal gland and nuclei of the posterior commissure (Baeuml et al., 2019; Korzh et al., 2007; Korzh et al., 1993; Traiffort et al., 1999).

Although the effect of Kv2.1 mutations on gene expression is likely indirect, its quantitative evaluation provided valuable insights, particularly in the context of morphological changes caused by mutations. RF development relies on the coordinated activity of many genes that encode proteins that form the RF (*sspo*, *lgals1*, *clu*, *chl1a/camel*, etc.) (Cantaut-Belarif et al., 2018; Muñoz et al., 2019; Yang et al., 2021). RF development also depends on BVS developmental regulators such as the α -SNAP deficient in *hyh* mutant mice (Pérez-Figares et al., 1998; Wagner et al., 2003). Interestingly, the BVS phenotype of zebrafish *kcng4b* mutants resembles that of *hyh* mice (Shen et al., 2016). Developmental changes in the BVS of zebrafish mutants deficient in the Kv2.1 subunits suggest that Kv2.1 activity negatively regulates RF formation and disassembly (see Figures 5, 6). This appears to be due to changes in the transcript levels of genes involved in RF formation, such as *sspo*, *lgals2a* and *chl1a/camel* (Figures 5H,I). The formation of RFs emerges as a chain of complex events, and Kv2.1 appears to be one of the factors that regulate this process.

This analysis raised some questions that could be answered by follow-up studies. The formation mechanism of the duplicate anterior RF in *Kcnc1*-deficient embryos remains unknown. This phenomenon could be linked to the premature closure of the cerebral aqueduct, which is caused by a reduction of the BVS, and the formation of the two separate channels for CSF circulation (Fame et al., 2016). However, there is a lack of clear evidence. Given the multitude of roles of *Kcnc1* discussed above, it would be interesting to determine which role—maintaining the membrane potential, trafficking secreted proteins, or regulating secretion at the plasma membrane or all of these—could be involved in the development of the anterior RF.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: GEO submission no. GSE194272.

Ethics statement

The animal study was approved by II Local Ethical Committee for Animal Experimentation in Warsaw. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RA: Data curation, Visualization, Investigation, Validation, Methodology, Writing – original draft, Writing – review and editing, Formal Analysis. RJ: Investigation, Writing – review and editing, Formal Analysis, Resources, Writing – original draft. JJ:

Visualization, Formal Analysis, Writing – review and editing, Conceptualization, Writing – original draft, Investigation. VK: Data curation, Validation, Formal Analysis, Writing – review and editing, Project administration, Supervision, Methodology, Writing – original draft, Conceptualization, Resources, Funding acquisition.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at:

<https://www.frontiersin.org/articles/10.3389/fcell.2025.1720752/full#supplementary-material>

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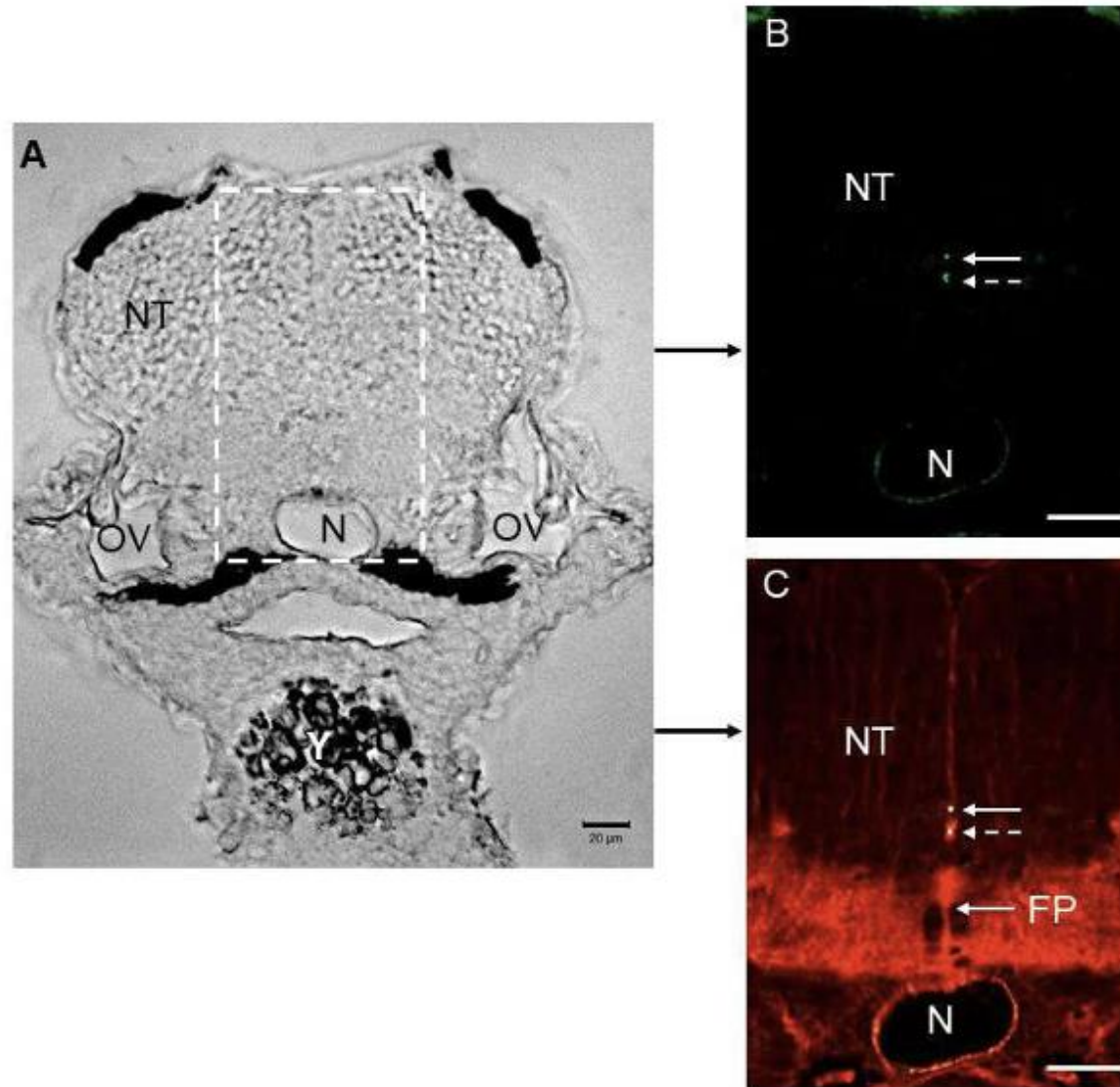
SUPPLEMENTARY FIGURE S1

The cross section of the 48 hpf wildtype embryo at mid-hindbrain (ear) level. A – bright field image; B, C – confocal images of the same specimen. Green, AFRU antibody, red, anti-F-actin (phalloidin) staining. FP – floor plate; N – notochord, NT – neural tube, OV – otic vesicle, Y – yolk. Scale bar – 20 µm.

SUPPLEMENTARY FIGURE S2

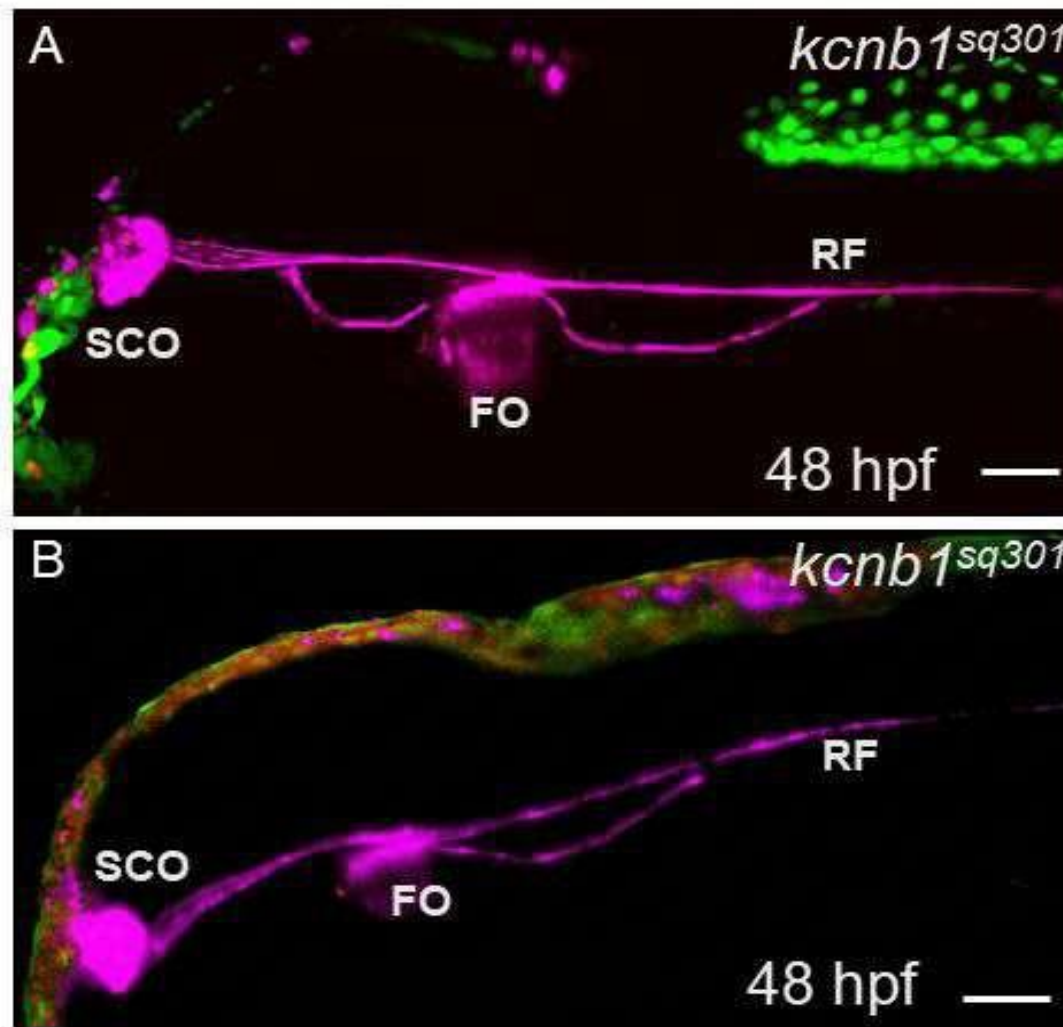
Some Kcnb1 mutants develop duplicated or enlarged anterior RF. A, duplicated anterior RF; B, enlarged anterior RF (lateral views). SCO, subcommissural organ, FO, flexural organ, RF, Reissner fiber. Green, GFP expression in the choroid plexus of the compound *kcnb1^{sq301}*/Tg (ET33-20) embryos. Scale bar – 20 µm.

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SUPPLEMENTARY FIGURE S1

The cross section of the 48 hpf wildtype embryo at mid-hindbrain (ear) level. A – bright field image; B, C – confocal images of the same specimen. Green, AFRU antibody, red, anti-F-actin (phalloidin) staining. FP – floor plate; N – notochord, NT – neural tube, OV – otic vesicle, Y – yolk. Scale bar – 20 μm.



SUPPLEMENTARY FIGURE S2

Some *Kcnb1* mutants develop duplicated or enlarged anterior RF. A, duplicated anterior RF; B, enlarged anterior RF (lateral views). SCO, subcommissural organ, FO, flexural organ, RF, Reissner fiber. Green, GFP expression in the choroid plexus of the compound *kcnb1sq301/Tg* (ET33-20) embryos. Scale bar – 20 μm.

2.2 Manuscript 2

R. Rosa Amini, Ruchi P. Jain, Vladimir Korzh (2026)

Body orientation affects the development of the brain ventricular system and mechanosensory gene expression in zebrafish embryos. Currently submitted to *npj Microgravity*



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I hereby declare that I, Razieh Amini, made a substantive contribution to the development of this publication. My contribution consisted of:

- conducting experiments,
- data analysis,
- writing sections of the manuscript

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Body orientation affects brain ventricular system development and mechanosensory gene expression in zebrafish embryos

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Key words: microgravity, body orientation, cerebrospinal fluid, zebrafish development, Reissner fiber.

Abstract

Most vertebrates maintain a horizontal body orientation, whereas humans and great apes exhibit postures varying from horizontal to vertical. This exposes the body and its fluid-filled organs to changing gravitational vectors. This study examines how body position influences brain ventricular system development under normogravity. The zebrafish embryos were maintained in distinct orientations for 24 hours. The development of the Reissner fiber, an essential element of the brain ventricular system and proprioception, has been analyzed along with expression of genes associated with mechanotransduction and formation of Reissner fiber. Embryos held in vertical positions exhibited disrupted Reissner fiber, body axis deformities, and altered expression of gravity-responsive genes from the Hippo pathway, particularly *yap1a*. Among the genes regulating the development of Reissner fiber, the transcript level of *chl1a/camel* increased dramatically. These findings suggest that body orientation modulates cerebrospinal fluid flow and mechanosensory pathways during development. Understanding these effects provides insight into the mechanotransduction processes underlying proprioception and may inform studies of gravity's impact on vertebrate neurodevelopment.

Introduction

Most vertebrates maintain a predominantly horizontal body orientation, whereas humans and great apes spend much of the day in a vertical posture, shifting to horizontal during sleep. This relatively recent evolutionary adaptation coincided with the transition to bipedalism. Nevertheless, humans frequently change body position throughout daily activities such as working, exercising, walking, and sitting, resulting in continuous exposure of cellular cytoplasm and bodily fluids to varying directions of gravitational force. The ability to tolerate brief periods in atypical positions, such as being upside down, varies depending on individual health and training. Notably, reports from incidents such as prolonged immobilization on roller coasters indicate that most individuals can withstand inverted positions for at least 1.5 hours. Therefore, body orientation relative to the gravitational vector is a critical factor, as gravity is a constant force influencing life on Earth. Living organisms detect gravity through specialized proprioceptive organs containing mechanosensory or gravisensory proteins, which respond to changes in gravitational force and transmit this information via signaling pathways (Aventaggiato et al., 2020).

Astronauts exposed to prolonged microgravity on the International Space Station experience redistribution of body fluids, including cerebrospinal fluid (CSF), increased brain and CSF volumes, altered aqueductal CSF hydrodynamics, impaired balance, and anemia (Kramer et al., 2020; Trudel et al., 2024; Van Ombergen et al., 2019). However, the effects of microgravity on animal development, both in space and under simulated conditions such as clinostat-induced microgravity, remain poorly understood (Harrison et al., 2020). A key challenge is to identify the molecular and physiological responses underlying gravity's impact on cell and tissue structure (Davis et al., 2024). Studies have shown that mechanotransduction relies on specific sensors (gravisensors), such as Yap1, which are affected by changes in gravity (Aventaggiato et al., 2020; Porazinski et al., 2015; Takahashi et al., 2021). In developing zebrafish, yap1 is expressed in regions of active cell

proliferation, including the ventricular zone of the neural tube and otic vesicles (Loh et al., 2014), and is associated with the development of the retinal pigmented epithelium, posterior body, and hematopoietic stem cells (Kimelman et al., 2017; Lundin et al., 2020; Miesfeld et al., 2015; Miesfeld and Link, 2014). Yap1 regulates several genes, such as *lats2* and *nf2a* (Yi et al., 2018) and acts upstream of *prox1a* to modulate Wnt signaling during development of the otic placode and the lateral line, both involved in proprioception (Loh et al., 2014; Lunsford et al., 2019; Skandalis et al., 2021).

To systematically mimic the space-flight conditions during controlled clinical studies, healthy adult volunteers have been subjected to the 60 head-down tilt (HDT) in bed (Pavy-Le Traon et al., 2007). These studies have shown that HDT can reasonably replicate some physiological responses observed in microgravity (Watenpaugh, 2016), although actual spaceflight conditions are likely to be more extreme. Research on snakes has highlighted the significance of hydrostatic pressure gradients in vertical blood columns (Lillywhite and Lillywhite, 2017), and similar gradients are likely present in the CSF column. These gradients may vary depending on the body's orientation relative to the gravitational vector (Klarica et al., 2014; Sagirov et al., 2023; Trimmel et al., 2022).

The development of the brain ventricular system (BVS) relies on the activity of the heart and motile cilia, both of which are relatively inefficient in 24 hpf zebrafish embryos due to the lack of a mature cardiac conduction system, heart valves, and established CSF flow in the brain (Fame et al., 2016; Olstad et al., 2019; Poon et al., 2016, 2010). The Reissner fiber (RF), a hallmark of the BVS in animals with horizontal body posture such as zebrafish, serves as a marker for BVS development (Rodríguez et al., 1984; Pérez-Fígares et al., 1998; Wagner et al., 2003; Lehmann and Naumann, 2005; Meiniel et al., 2008; Fernandez-Llebrez et al., 2001). The posterior (primordial) RF is formed by the floor plate and its derivative, the flexural organ (FO), while the anterior (definitive) RF is produced by the subcommissural organ (SCO) beginning at 30 hpf and is complete by 48 hpf (Amini et al., 2026). This process depends on CSF flow generated by motile cilia (Bearce et al.,

2025; Cantaut-Belarif et al., 2018). The RF has been proposed to act as a proprioceptive organ (Aboitiz and Montiel, 2021; Kolmer, 1921; Nicholls, 1917). Its development is regulated by genes expressed in the roof and floor plates and their derivatives, SCO and FO, respectively. Among these, *chl1a/camel*, encoding a divergent member of the L1-CAM superfamily of cell adhesion molecules, is notable; its deficiency in zebrafish leads to hydrocephalus similar to that observed in *kcng4b* mutants (Yang et al., 2021; Shen et al., 2016). In humans CHL1 deficiency has been associated with mental impairment and schizophrenia (Angeloni et al., 1999; Chu and Liu, 2010; Frints et al., 2003; Sakurai et al., 2002), and Chl1 is required for the development and maintenance of inhibitory neuronal subpopulations (Schmalbach et al., 2015).

We hypothesized that prolonged changes in body position could alter the distribution of bodily fluids during development. To test this, we examined the development of the BVS and RF in zebrafish embryos maintained in various fixed orientations. Our results show that maintaining 24 hpf embryos in specific orientations for 24 hours leads to defects in RF development. Such defects are reflected at the level of transcription as shown for several genes associated with RF development and mechanosensory responses to gravity. These were affected by vertical body postures, with *chl1a/camel* transcript levels showing the most significant changes. These findings suggest that *chl1a/camel* transcript levels may serve as a BVS-specific indicator of CSF flow.

Results

The abnormal distribution of bodily fluids and in particular the CSF is one of the immediate effects of microgravity. Such changes have been associated with the developmental defects of the BVS mechanism (Pérez-Fígares et al., 1998; Shen et al., 2016; Wagner et al., 2003). RF is a constitutive element of BVS. The development of the anterior RF is sensitive to changing CSF flow (Rose et al., 2020; Troutwine et al., 2020). Thus, we decided to study the effect of the body position on the RF development, which was studied in detail (Amini et al., 2026). With this aim, the 24 hpf embryos were mounted in the low melting point

agarose in transparent tubing and left to develop for 24 hpf in different horizontal and vertical positions (Fig. 1). Such an experimental setup fixed the body axis in desired position and restrained body movements. At 24 hpf the head remains tilted more than 90° in respect to the body axis. The head-to-tail angle (HTA) has been used as one of markers for the staging of developing embryos (Kimmel et al., 1995). HTA decreases from 120° at 24 hpf to 45° at 48 hpf due to straightening of the body axis that changes its position in respect of the vector of gravity (Fig. 1).

RF was detected by immunohistochemistry (Fig. 2A) or *in vivo* in transgenics (Fig. 2B, C). The embryos held in the horizontal “head down” position formed the anterior RF in the majority of embryos as detected at 48 hpf (Fig. 2A, D, H; n = 17/25) or 72 hpf (Fig. 2C, F, H; n = 23/25). When the embryos were placed in the “head up” “horizontal” position, the outcome was similar. The anterior RF was present in 20 (absent in 5) out of 25 embryos at 48 hpf (Fig. 2G, I). At 72 hpf the anterior RF was present in 14 (absent in 11) out 25 embryos (Fig. 2H, I).

When the embryos developed in the “vertical” (erect) position, small deformations to the body axis were noted at 48 hpf (Fig. 3A-C). The Reissner fiber failed to develop normally in the majority of embryos (N = 48/55; Fig. 3B', B'', C', C'', G). The RF became defasciculated or displaced (Fig. 3B', B''). Upon release from agarose for “compensation” only 23 out of 30 embryos had RF (Fig. 3A, C, E). An even more dramatic effect on the anterior RF development was detected in the embryos that developed in the “reversed vertical” (reversed erect) position. Here almost all embryos developed curled-down the body axis and cardiac edema (N = 52/55; Fig. 3D-F, G) and failed to develop the anterior RF (Fig. 3D', D'', E', E'', F', F'').

The studies in mammals suggested that the developmental window of the SCO-derived (anterior) RF formation could be relatively short compared to that in birds. When an initiation of the anterior RF is disrupted, it fails to develop during later period (Lang et al., 2006; Picketts, 2006). This posed a question whether similar developmental window for

the anterior RF formation exists in zebrafish. To check whether anterior RF could be restored in embryos after its cultivation in “vertical” positions the 48 hpf *scospondin-GFP^{ut24}* embryos after incubation in fixed position were released from agarose to free swim in a Petri dish until 72 hpf (“compensation” experiment, Fig. 4). Some embryos held in “vertical” position, where “no” anterior RF was found at 48 hpf, after being “free” in Petri dish from 48 hpf to 72 hpf, formed the anterior RF (Fig. 4A, B). In contrast, all embryos held in the “reversed vertical” position, where at 48 hpf no anterior RF was found, failed to form the anterior RF by 72 hpf after 24 hpf period in a Petri dish (Fig. 5-2). Thus, not only the number of embryos held in the reverse vertical position that fail to form the anterior RF was higher compared to embryos in “vertical” position. After “reversed vertical” position embryos were unable to restore anterior RF unlike some embryos held in “vertical” position. Upon release from agarose for “compensation” only 13 out of 30 embryos had RF (Fig. 4B, D, E). In fact, in both vertical positions the number of embryos having RF was slightly reduced between 48 hpf and 72 hpf (Fig. 4E).

Previously, several genes were linked with the development of the anterior RF (Amini et al., 2026; Muñoz et al., 2019; Troutwine et al., 2020; Yang et al., 2021). In the embryos kept in the “vertical” position, the transcript level of *sspo*, *lgals2a*, *lgals2b* was reduced whereas *chlla*, *spon1a*, and *chlla/camel* increased with the latter transcript level increasing most dramatically (Fig. 4). In the “reversed vertical” group of embryos, the level of *chlla* transcript increased to a value much higher than that in embryos kept in the “vertical” position. In addition, the levels of *clu*, *spon1a*, *lgals2a* transcripts also increased, whereas those of *sspo* and *lgals2b* decreased more significantly compared to controls or embryos kept in “vertical” position (Fig. 5; Supplemental Table 1).

We hypothesized that the changes of body position may affect the mechanical properties of cells involved in the RF’s development. Previously, several genes of the Hippo pathway such as *yap1* have been linked to mechano-elastic properties of cells in medaka, and their expression was affected by changes in gravity (Porazinski et al., 2015; Takahashi et al.,

2021). The developmental defects of the RF were reminiscent of the *kcnbl1*^{-/-} mutant where the activity of the voltage-gated potassium channel Kv2.1 was affected (Amini et al., 2026). Thus, the candidate mechanosensory genes were cross-referenced against the results of the whole-body RNAseq of Kv2.1 gain-of-function zebrafish embryos (Jędrychowska et al., 2024). The genes with highest level of changes of expression level were selected for analysis of the embryos of “vertical” groups by qRT-PCR. Here the levels of at least two transcripts equally increased in both “vertical” positions – *yap1* and *actn3b* (Fig. 6; Supplemental Table 2). The level of these transcripts seems to be “very sensitive” to changes in body position. In the embryos held in the “reversed vertical” position much more significant increase of the level of *lats1*, *itgb1b.1*, *ctnna2* transcripts was detected compared to the “vertical” group of embryos (Fig. 6; Supplemental Table 2). The levels of these transcripts may represent the “sensitive” category, whereas *itgb2*, *itga3b*, *nf2a*, *mob1*, which levels increased much less formed the “less sensitive” category. Thus, the genes associated with Yap1 signaling are very sensitive to changes in body position.

The development of anterior RF was easily affected by both vertical orientations of zebrafish embryos during the second day of development. Such changes correlate well not only with changes in the level of transcripts linked to development of RF like *chlla/camel*, but also with changes in the level of transcripts (*yap1*, *actn3b*, etc.) associated with mechano-elastic properties of cells. This suggests that the relatively simple developmental tests *in vivo* at normal gravity using small vertebrates such as zebrafish could be efficiently applied to model *in vivo* the developmental processes depending on gravity. Here we restricted our analysis to the ventricular system and Reissner fiber. Yet the cardiac edema observed in embryos that developed in vertical positions suggested that the other systems of organs (cardiovascular, excretory, etc.), which development depends on fluid flow could be studied using this approach too.

Discussion

The development of anterior RF has been affected by changes in orientation of zebrafish embryos at normogravity. The experimental setup to mimic the effect of microgravity is based on a notion that changing body position in adult animals and humans will induce an abnormal CSF flow (Klarica et al., 2014; Lillywhite and Lillywhite, 2017; Pavy-Le Traon et al., 2007; Sagirov et al., 2023; Trimmel et al., 2022; Watenpaugh, 2016). Here we applied the same concept to study the effect of the changing body position on Reissner fiber development, the proprioceptive element of animals with the horizontal body posture (Aboitiz and Montiel, 2021; Kolmer, 1921; Nicholls, 1917).

Such changes result in the deformation of the anterior RF and affect the levels of transcripts associated with the mechano-elastic properties of the RF-associated BVS elements connected to the RF (SCO, FO) or interacting with it (ependyma, CSF-cNs,). Given the suggested role of RF as the component of proprioceptive system, its deficiency may contribute to changes in space orientation and potentially influence structural changes in the neural tube. For example, the midbrain floor plate (or FO) acts as a source of many cell lineages in the brain, including CSF-cNs, dopaminergic and serotonergic neurons, *etc.* (Wyart et al., 2023), whereas SCO has been implicated in the development of posterior commissure (Stanic et al., 2010). The RF defects may affect these CVOs and related cell lineages.

It seems that some embryos held in horizontal position were affected (Fig. 2). In such positions at 24 hpf the anterior neural tube which forms the BVS is oriented almost vertically and only the central canal in the spinal cord is horizontal (Fig. 1). This may cause some disturbance in CSF distribution. As development progresses and the head-to-tail angle decreases by 48 hpf from 120° to 45° (Kimmel et al., 1995), the effect of CSF redistribution in horizontal position will likely decrease.

In contrast, the straightening of the body axis in vertical positions most likely will progressively affect the CSF flow. No wonder that the cultivation of embryos in both

“vertical” orientations which are way more severe comparing to the 6⁰ HDT in bed (Pavy-Le Traon et al., 2007), affects the development of the body axis and not only causes the defect or failure of the RF, but scoliosis and cardiac edema also (Fig. 3D, F). This is in line with previous studies of the developmental role of the RF (Amini et al., 2026; Cantaut-Belarif et al., 2018; Rose et al., 2020; Wyart et al., 2023; Yang et al., 2021). The change of the body axis of developing zebrafish impacts genes expression linked to the mechano-elastic properties of cells (Fig. 6; Supplemental Table 2). The transfer of mechanical cues depends on conformational changes of the mechanosensory molecules or graviceptors able to connect to mechanically stable cellular elements like cytoskeleton and modify its conformation depending on mechanical input (Aventaggiato et al., 2020; Lim et al., 2018).

Within the group of mechanosensory genes, *yap1* seems to be the most sensitive. Despite more severe morphological changes in embryos cultivated in the reversed vertical position, its expression has changed equally in both vertical positions indicating that the detected values may represent the maximal level of expression possible under experimental conditions during this developmental period (Fig. 6; Supplemental Table 2). Given the role of Yap1 in carcinogenesis such increase associated with significant morphological changes in the BVS-associated structures may be a worrying sign (Low et al., 2014; Thomas et al., 2026). Similar trend was demonstrated by *actn3b* (Fig. 6; Supplemental Table 2). Notably an increase in expression of *ACTN3* in humans has been associated with acute myeloid leukemia (Yang et al., 2019).

chlla was the most affected among the genes linked to development of RF (Fig. 5; Supplemental Table 1). Furthermore, its transcription level reacted to the changing body position much more significantly compared to *yap1*, which is well established as a gravisensor. Previously, we reported the regulatory role of *chlla* in the RF’s development. Its deficiency caused the deficiency of the RF and scoliosis (Yang et al., 2021). As the transmembrane protein interacting with various signaling pathways and cytoskeleton, Chlla may act as the developmental regulator of the mechano-elastic properties of BVS.

In mammals Chl1 is expressed in small to medium size sensory neurons and involved in development of the ventral midbrain (VM) dopaminergic (DA) pathways, a process critical for motor and cognitive function (Alsanie et al., 2017; Zhang et al., 2000). Chl1 interacts with extracellular matrix and potassium channels of the plasma membrane (Kung, 2005). The mammalian Chl1 was linked to regulation of axonogenesis, and its mutations in humans cause neurodegenerative diseases (Alsanie et al., 2017; Angeloni et al., 1999; Frints et al., 2003; Schlatter et al., 2008; Senchenko et al., 2011), and has been linked to scoliosis in different species (Sharma et al., 2011; Yang et al., 2021). Since the lipid bilayer of plasma membrane behaves as the mechanoreceptor (Butler and Chien, 2009; Mukherjee et al., 2026), perhaps, *chl1a/camel* functions as a sensitive gravisensor. This suggestion should be validated under microgravity conditions.

Materials and Methods:

Animals

Zebrafish (*Danio rerio*) were maintained according to established protocols (Westerfield, 2007) in the Zebrafish Core Facility at the International Institute of Molecular and Cell Biology in Warsaw. This facility is licensed for breeding and research (PL14656251, registry of the District Veterinary Inspectorate in Warsaw; 064 and 051: registry of the Ministry of Science and Higher Education). The experiments involving zebrafish embryos, larvae, and adults were conducted in accordance with the European Communities Council Directive (63/2010/EEC). The developmental stages in hours post-fertilization (hpf) are based on (Kimmel et al., 1995). The *scospondin-GFP^{ut24}* transgenic line is a knock-in allele, where the C-terminal portion of the *scospondin* gene was precisely tagged with the GFP coding sequence (Troutwine et al., 2020).

Embryo mounting in capillaries under defined body orientations

Zebrafish embryos were mounted in fluorinated ethylene propylene (FEP) capillaries

(BOLA, Germany) using a low-melting agarose-based multilayer mounting approach adapted from a published protocol (Kaufmann et al., 2012). FEP tubing was cleaned by sequential washes with NaOH, double-distilled water (ddH₂O), and 70% ethanol with ultrasonic treatment, cut into ~3 cm segments, and stored in ddH₂O until use. Low-melting agarose was prepared in E3 medium, melted, and maintained at 38 °C; tricaine (0.04%) and PTU (0.003%) were added immediately before mounting to anesthetize embryos and suppress pigmentation. At 24 hpf, embryos were manually dechorionated, transferred individually into molten agarose, and aspirated into FEP capillaries using a syringe-based loading system. Embryos were positioned within the capillary in predefined body orientations (horizontal or vertical), with orientation verified visually prior to agarose solidification. The horizontal positions “head-down” and “head-up” do not recapitulate the prone and supine positions of adult human body. This is due to curvature of the body, where the head-to-tail angle (HTA) at 24 hpf is 120°. It decreases to 45° by 48 hpf due to straightening of the body axis (Kimmel et al., 1995). For the same reason, the “vertical” and “reversed vertical” positions are not equivalent to “erect” and “upside-down” positions of adult human body, but mimic those as close as possible under experimental conditions. Capillaries were sealed by insertion into a solid agarose layer and maintained in E3 medium at 28.5 °C. Embryos were kept in capillaries from 24 hpf to 48 or 72 hpf and subsequently processed for live imaging, fixed for antibody staining, collected for gene expression analysis, or recovered from agarose and returned to E3 medium for further development.

Microscopy

Live imaging: Zebrafish embryos were raised in E3 medium (2.5 mM NaCl, 0.1 mM KCl, 0.16 mM CaCl₂, and 0.43 mM MgCl₂) with the addition of 0.2 mM 1-phenyl-2-thiourea (PTU, Merck, Germany) to block pigmentation. At selected developmental stages, the embryos were manually dechorionated and anesthetized with 0.02% tricaine (Sigma-Aldrich, USA). Then, they were oriented after embedding in 2 % methylcellulose (Merck, Germany) on the glass slides. A research stereomicroscope SMZ25 (Nikon, Japan) was

used for imaging.

Light-sheet fluorescent microscopy: *In vivo* imaging and imaging of fixed specimens were performed as previously described (Jedrychowska et al., 2021). For imaging of fixed specimen, 0.8 % low-melting agarose in phosphate-buffered saline (PBS) was used instead of the E3 0.02 % tricaine medium. Zeiss Lightsheet Z.1 microscope with W Plan-Apochromat objectives (20x/1.0 UV-VIS (for *in vivo* imaging and 40x/1.0 UV-VIS or 63x/1.0 UV-VIS for fixed embryos) were used. Transmitted LED light was used to obtain high-resolution bright-field images. The data were saved in the LSM or CZI format and processed using ZEN (Zeiss) or ImageJ 1.51n (Fiji) software. Maximum intensity or sum slice projections were generated for each z-stack. Brightness and contrast adjustments, as well as resizing were performed using FastStone viewer 7.4 (FastStone Soft).

Immunohistochemistry: Embryos were stained using two-color immunohistochemistry for RF with the polyclonal rabbit AFRU antibody (1:1000), a gift of Drs. J. Grondona [Malaga, Spain], E. Rodriguez, and M. Guerra [Valdivia, Chile]), and secondary Alexa Fluor 594 tagged donkey anti-rabbit antibody (1:1000; Invitrogen, USA) according to the described protocol (Korzh et al., 1998). The detected GFP was expressed in transgenic embryos.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR analysis was performed to assess the expression of selected genes at 48 hpf in embryos cultured under vertical and reverse-vertical conditions (Supplemental Table 3). Total RNA was extracted from pooled zebrafish embryos using the TRIzol–chloroform method (Sigma-Aldrich, USA), quantified by NanoDrop™ 2000 (Thermo Scientific, USA), and reverse-transcribed from 1 µg RNA using the iScript™ Reverse Transcription kit (Bio-Rad, USA). qPCR reactions were carried out using SsoAdvanced™ Universal SYBR Green Supermix on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA). Gene-specific primers were designed based on ZFIN annotations, and *eef1a111* was used as the reference (housekeeping) gene. Threshold cycle (Ct) values were obtained using

Bio-Rad CFX Maestro software. Relative gene expression was calculated using the $\Delta\Delta C_t$ method, with wild-type embryos serving as the reference condition. Data are presented as $\log_2$ fold change relative to wild-type, with the dotted line indicating no change in expression ($\log_2 = 0$). Error bars represent the standard deviation (SD) of biological replicates. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test, with comparisons made relative to wild-type controls (** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; n.s., not significant). Primer efficiency (90–110%) and amplification specificity were confirmed by standard curve analysis, melting-curve profiling, and agarose gel electrophoresis.

Figure Legends:

Fig. 1. Experimental setup. The 24 hpf embryos were mounted in low-melting point agarose in capillaries in four horizontal positions (head-down, A; head-up, B; lateral left hand-side down, not shown; lateral right hand-side down, not shown) and two vertical positions (vertical upright, C; reversed vertical, D). During development from 24 hpf to 48 hpf the body axis straightens. The different regions of the hollow neural tube (line divided into three sections) are differentially exposed to the vector of gravity (g, arrow).

Fig. 2. The formation of the anterior RF in zebrafish embryos. A-C, Representative images of embryos demonstrate the anterior RF formed in embryos mounted in horizontal position and detected by AFRU antibody (magenta, A) or seen in *sospondin-GFP^{ut24}* transgenics (B, C). The small whole-body schematics illustrate orientation of the embryos cultivated from 24 hpf to 48 hpf (A, B) or to 72 hpf (C). The position of the images is shown by the green box on the scheme of the brain at corresponding stage. Most embryos developing in horizontal positions form the anterior RF by 72 hpf. The embryos reliably form the anterior RF when developed in the horizontal head-down position, except for the “head-up” position, where the anterior RF was not observed in some embryos at 48 hpf, but was detected at 72 hpf. Scale bar = 20 μ m.

Fig. 3. Most embryos fail to form the anterior RF when developing in the vertical position.

A – horizontal position, control. B – C, embryos cultivated in upright vertical position. The mild group (B, B', B'') developed the defasciculated anterior RF and FO staining was weak. The severe group (C, C', C'') developed mild body deformation and cardiac edema. In a minority of embryos, the anterior RF formed (N = 9/55; B', B''). The anterior RF failed to connect to the weakly stained FO (N = 46/55; C', C'').

When developed in reversed vertical position (D-F), most embryos failed to develop the anterior RF (N = 52/55; D', E', F', D'', F'') and the AFRU+ material was found accumulated ectopically in the ventral part of the III ventricle (F'). whereas in the minority of embryos (N = 3/55; E'') the much reduced anterior RF formed. Anterior RF was visualized either by AFRU immunostaining in fixed embryos or by live imaging of scospondin-GFP^{ut24} transgenic embryos, as indicated in the corresponding panels. Quantification of anterior RF formation under different body orientations (G).

Bars represent the fraction of embryos with or without anterior RF. The number of embryos was shown on the bars. Scale bar, A-F = 100 μ m; B'-F', B''-F'' = 20 μ m.

Fig. 4. The development of embryos in vertical positions reduced recovery of Reissner fiber. Most embryos in vertical position failed to form detectable RF at 48 hpf (25/30) (A, G; only very thin microfilaments were detected). Some recovered relatively normally during 48-72 hpf period in Petri dish after release (B) and some did not (C). In this group the SCO retained the shape like that at 48 hpf. The anterior RF defect is even more pronounced in embryos in reversed vertical position, where 27/30 embryos (D, G) failed to form detectable RF at 48 hpf. Some embryos recovered during 48-72 hpf period in Petri dish after release from agarose (E) with others developing the deformed RF (F) or none (not shown). Quantification of anterior RF recovery after release from vertical and reversed vertical positions (G; same graph for both conditions). Scale bar = 20 μ m.

Fig. 5. The transcript levels of the genes associated with development of the RF in vertical and reversed vertical group at 48 hpf. Data are shown as log²-transformed fold change relative to WT. Error bars represent propagated SD.

Fig. 6. The transcript levels of the genes linked to mechano-elastic properties of cells changed depending on body position. These changes were more dramatic in embryos held in the reversed vertical position compared to the vertical position. Data are shown as log²-transformed fold change relative to WT. Error bars represent propagated SD.

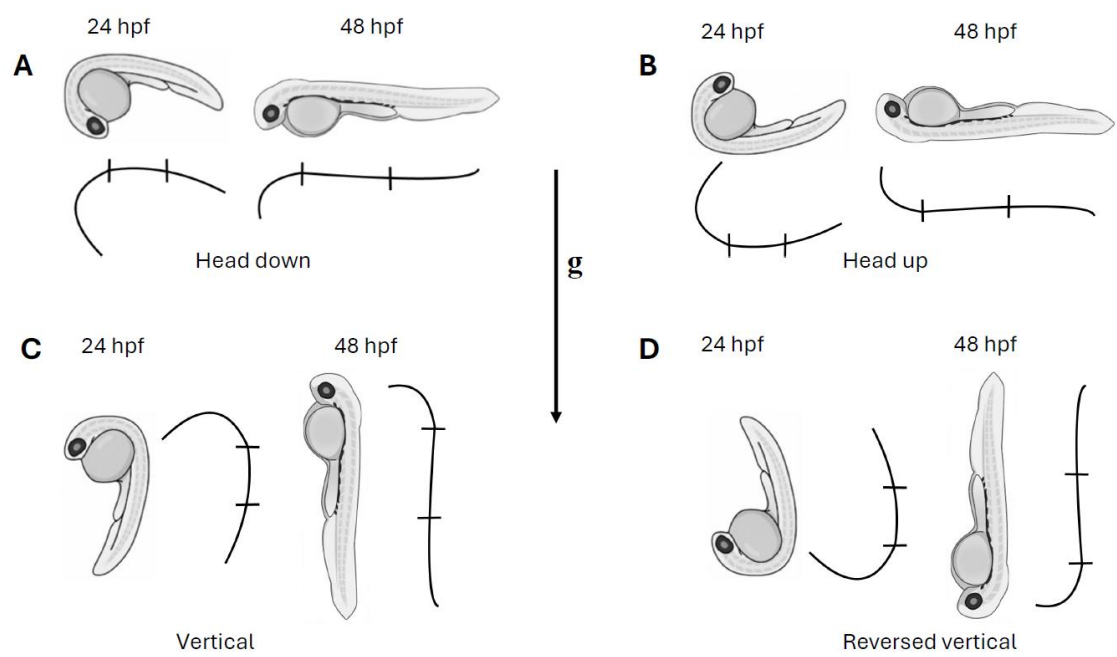


Figure 1

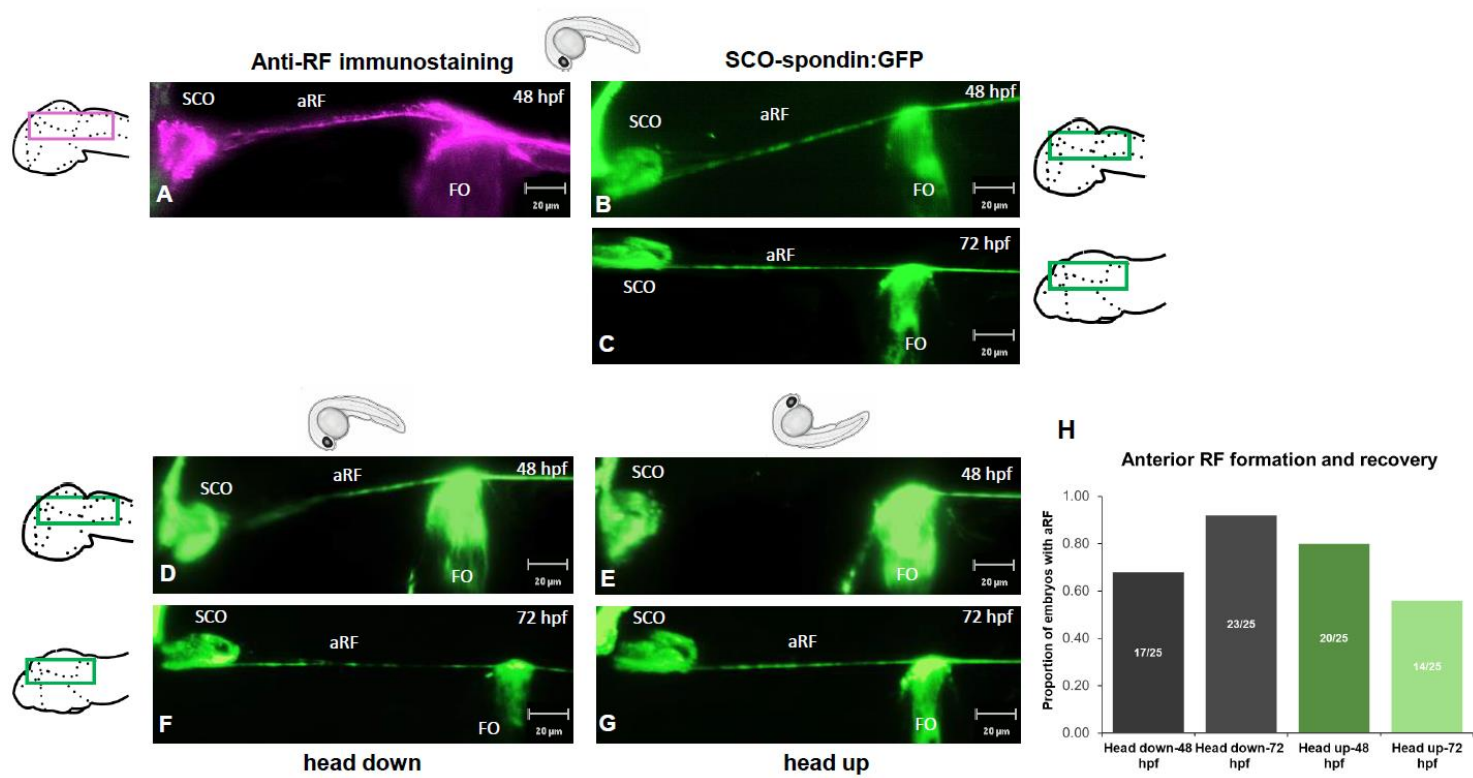


Figure 2

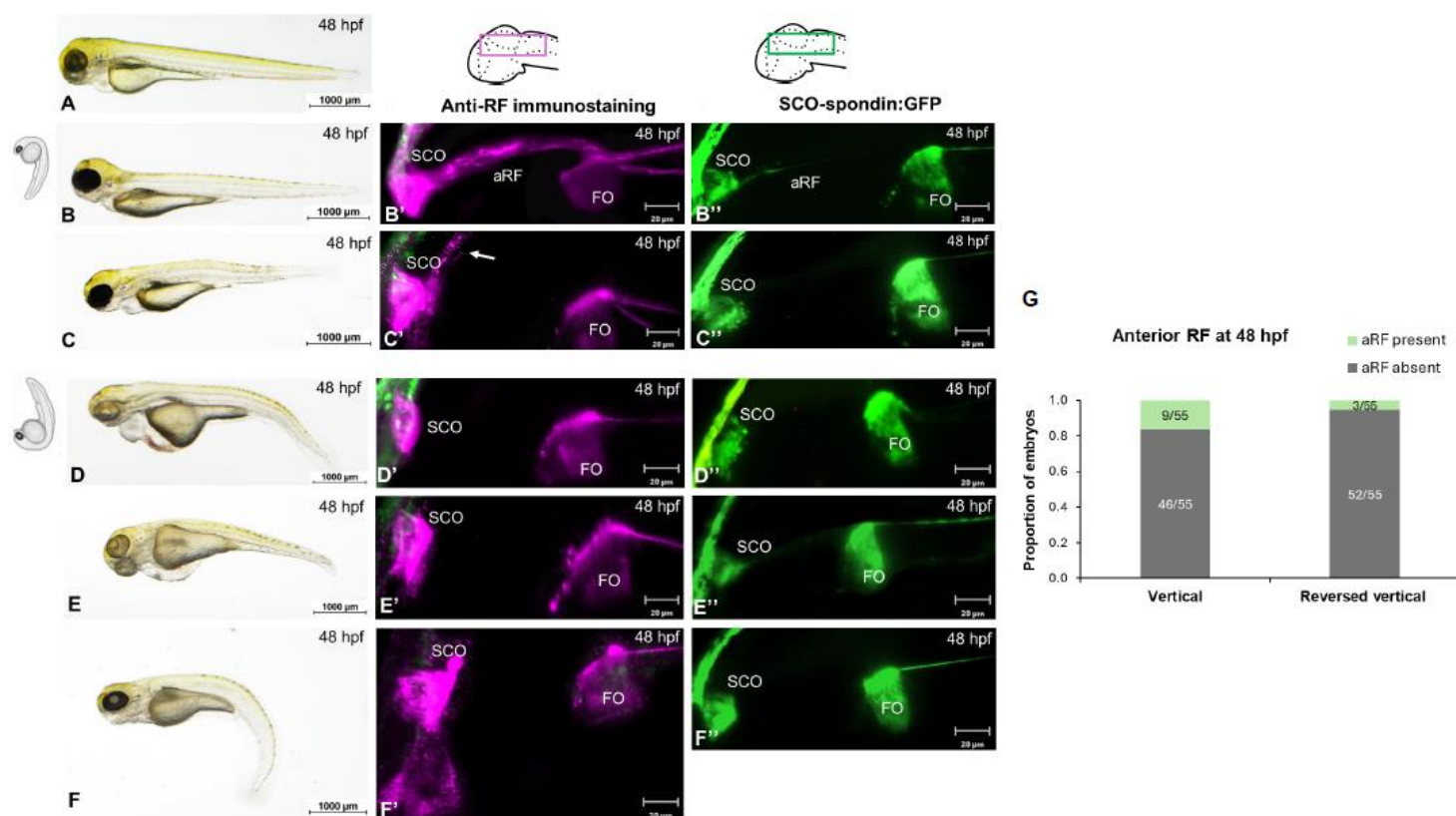


Figure 3

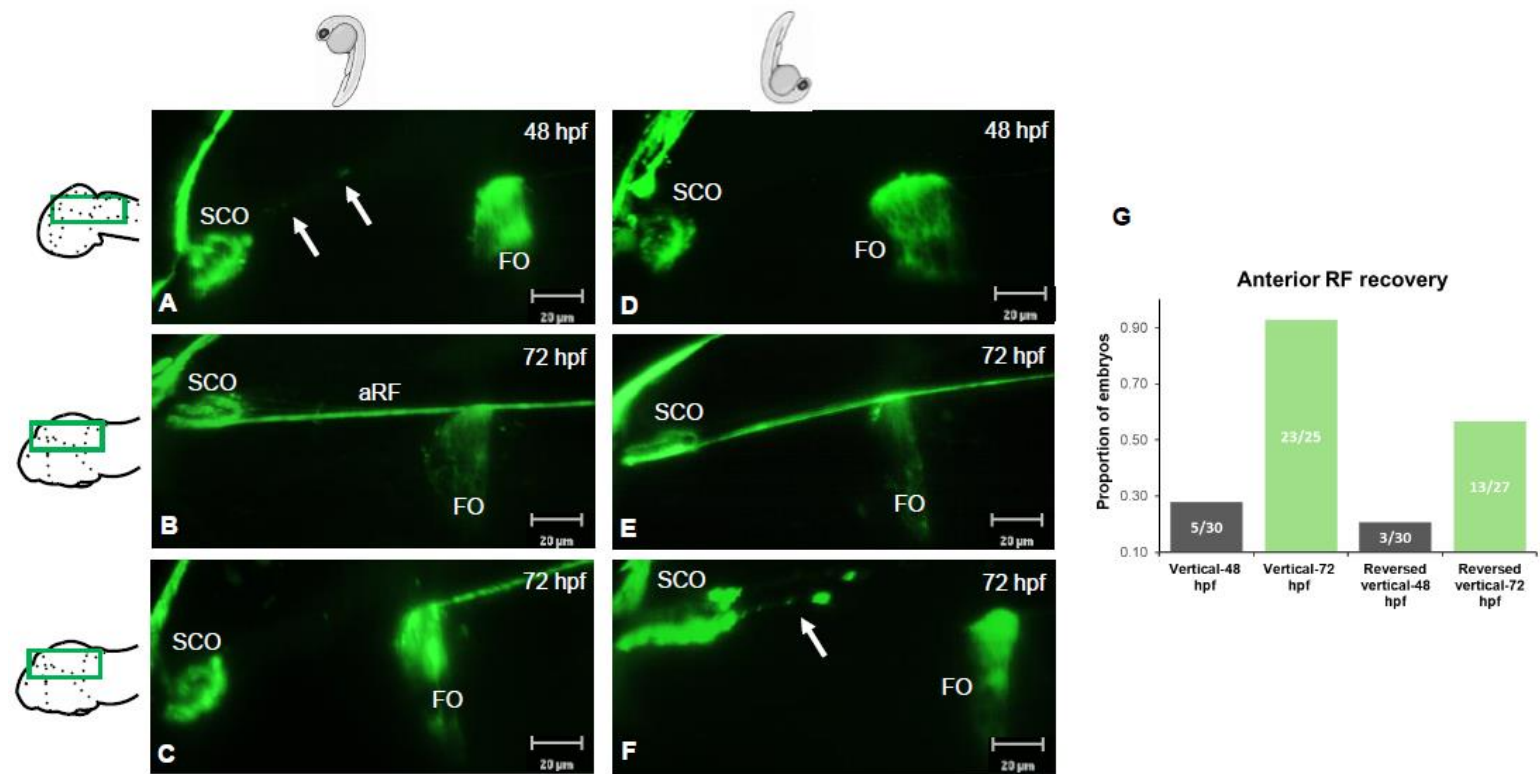


Figure 4

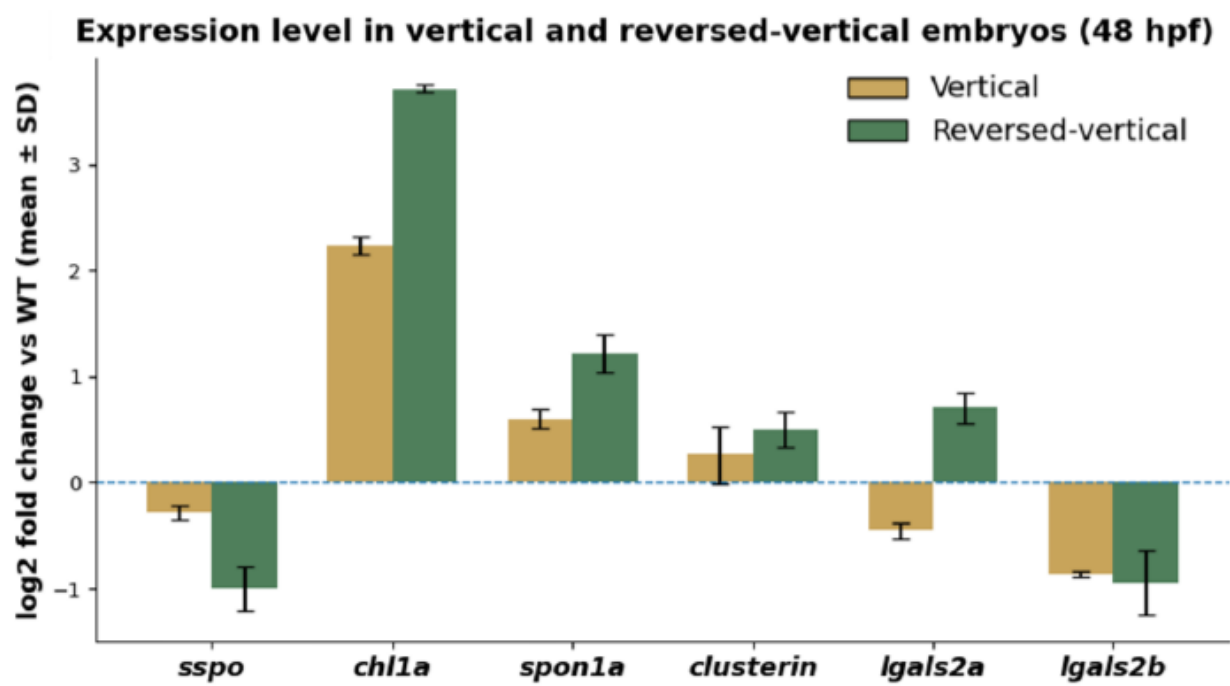


Figure 5

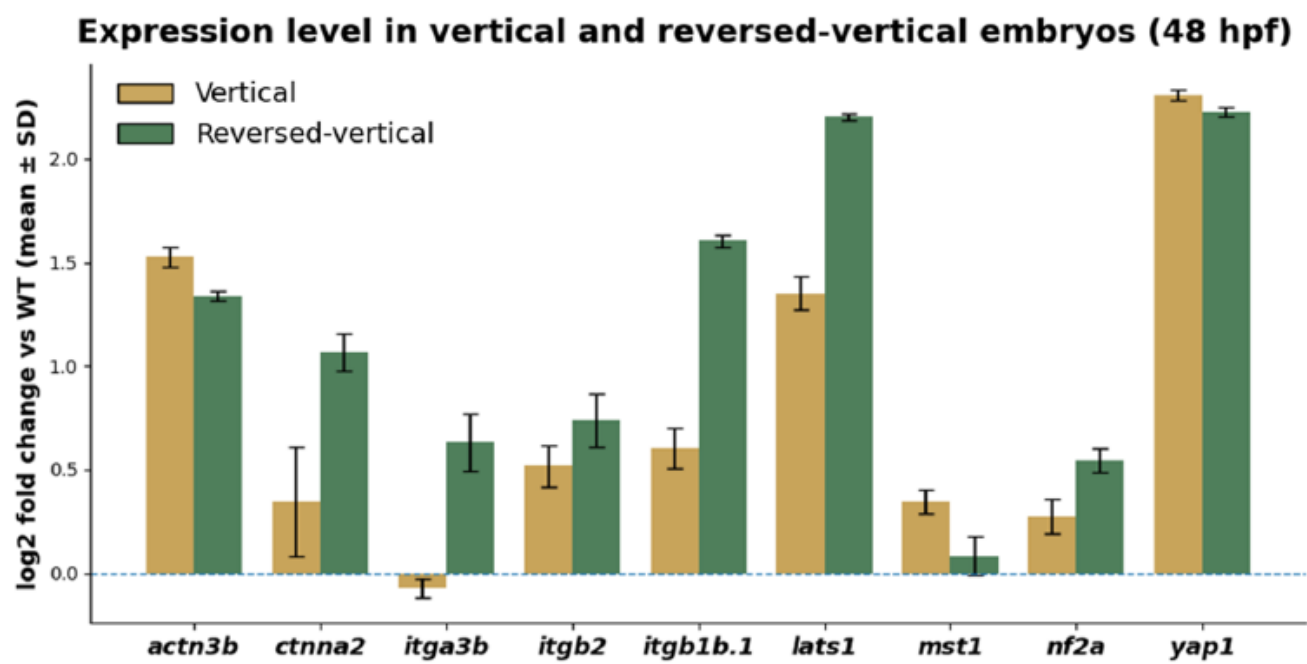


Figure 6

Supplementary materials:

Supplementary Table 1: The level of RF associated transcripts in different body positions.

N	Genes	Expression level ratio, vertical position/ wt, SD 48 hpf	p value	Expression level ratio, reversed vertical position/ wt, SD 48 hpf	p value
1	<i>chl1a</i>	4.71±0.27	0.001	13.11±0.3	0.0001
2	<i>clusterin</i>	1.2±0.22	n.s	1.42±0.16	0.01
3	<i>lgals2a</i>	0.73±0.04	0.001	1.63±0.16	n.s
4	<i>lgals2b</i>	0.55±0.01	0.0001	0.52±0.21	0.05
5	<i>spon1a</i>	1.52±0.09	n.s	2.33±0.29	n.s
6	<i>sspo</i>	0.82±0.14	n.s	0.5±0.24	0.01

Supplementary Table 2: The levels of transcripts linked to mechano-elastic properties of cells in different body positions.

N	Genes	Expression level ratio, vertical position/ wt, SD 48 hpf	p value	Expression level ratio, reversed vertical position/ wt, SD 48 hpf	p value
1	<i>actn3b</i>	2.88±0.1	0.01	2.53±0.04	0.01
2	<i>ctnna2</i>	1.27±0.23	n.s	2.09±0.13	0.01
3	<i>itga3b</i>	0.95±0.10	0.05	1.55±0.15	n.s
4	<i>itgb2</i>	1.43±0.10	0.01	1.67±0.15	0.01
5	<i>itgb1b.1</i>	1.52±0.1	0.01	3.04±0.06	0.001
6	<i>lats1</i>	2.55±0.14	0.01	4.6±0.05	0.001
7	<i>mst1</i>	1.27±0.15	0.05	1.06±0.07	n.s
8	<i>nf2a</i>	1.21±0.07	0.05	1.46±0.06	0.01
9	<i>yap1</i>	4.95±0.08	0.001	4.68±0.07	0.001

Supplementary Table 3: The list of primers

	Genes	qRT-PCR primers, 5' -> 3'
1	<i>actn3b</i>	ACACAGACCAGACCTCATC CTCCATCAGCCGTCATTC
2	<i>ctnna2</i>	GCCTCTCGCCTCGTCTAC TAAGTTGGTCATCTTCTGTCTGG
3	<i>chl1</i>	TGCAGCATTCTGTGCTCAACGT AACAGCCGATGAGGACAAGCA
4	<i>clusterin</i>	TCGCAAGTTGGTGAGAAATAC GGAGGCTGTGAAACAAGGAGT
5	<i>itga3b</i>	GGCAGTAACAGACCTCAACAATG CTCCGACCGCAGCAATGG
6	<i>itgb2</i>	TGGCTGGAGTGGCATTG AGTGGTTGTGGCATTCTGG
7	<i>itgb1b.1</i>	GCTGGTTATGCCTTACATTCTG TCTGCTTGCTGACTTCTTGG
8	<i>lgals2a</i>	GGAGCACAGAGACAACAAC ATCCTGACTTCGCCTTCG
9	<i>lgals2b</i>	CAACAACCTCAGCAAGATGG TGAAGTGAAGAGCGATGG
10	<i>lats1</i>	TCGGAGGAAGAGAAGAAGC TGAGGCGGATGTAGTTGG
11	<i>mst1</i>	GAGGTCCAGGTTAAGAATC CCATCATAGCAGTGTATCC
12	<i>nf2a</i>	TGGAGGCTGAGATGATTG TGCTGAAGTTGAAGTTAGG
13	<i>spon1a</i>	GTGACCAAGAGGAGGATTATGCT TGTCATGAATTGCGTGTCTTCCT
14	<i>sspo</i>	CAGTGTGGATTGTGATGTG CAGAGCAGAAGCGATACC
15	<i>yap1</i>	CTACTACATCAATCACAAGAAC CCACTCATCACTCCACTC

2.3 Manuscript 3

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Candidate's Contribution Statement

Article's title: Kv2.1-Kv6.4 subunit deficiencies impair inhibitory signaling and visual circuit dynamics in zebrafish

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Journal: BioRxiv

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I hereby declare that I, Razieh Amini, made a substantive contribution to the development of this publication. My contribution consisted of:

- genotyping of the mutant zebrafish line
- conducting the RT-qPCR
- data analysis



Candidate's Signature



Signature of the Corresponding Author

Kv2.1-Kv6.4 subunits deficiency impairs inhibitory signaling and visual circuit dynamics in zebrafish

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<https://doi.org/10.1101/2025.10.30.685505>

ABSTRACT

Voltage-gated potassium channels (Kv) play a crucial role in maintaining the cell's resting potential. Mutations in the Kv2.1 voltage-gated potassium channel are associated with developmental epileptic encephalopathy. Previous analysis of the loss-of-function zebrafish mutants *kcnb1sq301* and *kcng4bwaw304*, which affect genes that encode Kv2.1 subunits (the α subunit *Kcnb1* and the modulatory subunit *Kcng4b*), has shown that they play an antagonistic role in the development of hollow organs, such as the brain and ear. In this study, we investigated the behavioral effects of these mutations. Under normal light conditions, both mutants exhibited reduced activity at 5 days post-fertilization. However, exposure to a low concentration of 5 mM of the chemoconvulsant pentylenetetrazole increased their locomotor activity and induced seizures. Quantitative RT-PCR (qRT-PCR) analysis of the mutants revealed an increase in the transcript levels of *c-fos* and *gad2*, and a decrease in the transcript level of *gabra1*. This suggests that mutations cause a disruption to inhibitory neurotransmission. Local field potential recordings from the optic tectum of the mutants under baseline conditions showed an increase in spontaneous electrical activity. The *kcnb1* mutant is photosensitive and experiences freezing episodes under high-intensity light. Together, these findings suggest that defects in Kv2.1 subunits impact both locomotor behavior and light-evoked responses.

Keywords: *kcnb1*, *kcng4b*, DEE, zebrafish, light-evoked response, inhibitory neurotransmission

INTRODUCTION

Voltage-gated potassium channels facilitate the re-polarization of the action potential (1–3). Consequently, genetic mutations in these channels are a frequent cause of severe neurological disorders (4–7). Among these, mutation in the electrically active subunit Kv2.1 gene, encoded by KCNB1, has been linked to a spectrum of conditions, including developmental epileptic encephalopathy (DEE) (8–11). More than 50 de novo pathogenic variants in the KCNB1 gene have been identified in DEE patients. The patients suffer from unprovoked seizures, intellectual disability, autism spectrum disorder (ASD), and often severe movement disorders (12–18). Kv2.1 is predominantly localized to the somatodendritic region of the central neurons, where it generates a major component of the delayed rectifier K⁺ current that is critical for shaping the action potential duration and regulating overall excitability (19, 20). Therefore, these mutations alter the channel's gating properties or its membrane localization due to the gain-of-function (GOF), loss-of-function (LOF), and dominant negative effects, i.e. the changes that disturb the intricate balance of excitation and inhibition within the neural circuits (21–23). However, despite As the number of cases of KCNB1-related DEE increases, the mechanism by which Kv2.1 dysfunction influences circuit function and sensory-driven behavior during early brain development remains poorly understood.

Kv2.1 often forms heterotetrameric complexes with electrically silent/modulatory subunits KvS, such as Kv5, Kv6, Kv8, and Kv9, which do not form functional channels independently, but instead co-assemble with Kv2.1 to modulate its biophysical and functional properties (24–26). One such silent subunit is Kv6.4 (encoded by KCNG4), a key partner that imposes distinct regulatory effects. Kv6.4's co-assembly with the mammalian central nervous system drastically hyperpolarizes the inactivation kinetics of Kv2.1 by approximately -40 mV (27,28). In developing zebrafish, *kcnb1* and *kcng4b* show overlapping expression patterns in the brain,

suggesting that the Kv2.1 function is conserved in evolution. These genes play a role in the development of hollow organs like the brain ventricles and inner ear, and an assembly of Reissner Fiber. This highlights the developmental aspect of Kv2.1/Kv6.4 interactions, extending beyond their canonical role as characterized by electrophysiological studies (29–32). Conversely, in the mammalian photoreceptors, Kv2.1 forms heterotetrameric channels with Kv8.2, encoded by KCNV2. This assembly is important as the presence of Kv8.2 shifts the voltage-dependence of Kv2.1, generating a continuous ‘window current’ that is vital for regulating the photoreceptor’s dark current and subsequent signal transmission (33,34). Despite this clear link between KCNB1 and severe DEE, the precise mechanisms by which Kv2.1 loss leads to complex phenotypes remain poorly understood. Specifically, there are two major gaps: First, while Kv2.1’s role in central neuronal hyperexcitability is accepted, the contribution of its modulatory subunit, Kv6.4/KCNG4, to the shared DEE pathophysiology has not been rigorously compared in a unified model. Second, a minority of KCNB1 patients exhibit photosensitive symptoms (such as myoclonic eyelid seizures) (35), yet the specific mechanism by which Kv2.1 regulates light-evoked behavior and whether this defect originates in the brain or retina remains unknown. To address these gaps, we used the direct, comparative assessment of the neural functions and behavior of the zebrafish LOF mutants *kcnb1*^{-/-} with defect in the electrically active α -subunit (*Kcnb1*), and *kcng4b*^{-/-} with defect in the silent modulatory subunit (*Kcng4b*). In this paper, we report two principal findings that define the neurological and sensory consequences of subunit deficiency. Firstly, we demonstrate that both *kcnb1*^{-/-} and *kcng4b*^{-/-} mutants share a pathophysiology of central neuronal hyperexcitability, characterized by increased spontaneous brain activity, elevated c-fos expression, and increased susceptibility to pentylenetetrazole-induced seizures. Furthermore, we reveal a compensatory mechanism involving the inhibitory neurotransmitter system (*gad2* and *gabra1*) that underlies this hyperexcitable state. Secondly, we identify a unique defect in *kcnb1*^{-/-} mutants - a profound and

acute increase in sensitivity to light and freezing behavior. We show that this behavioral phenotype is not caused by structural defects in the retina. Most likely, it is the result of a ‘two-hit’ mechanism where a subtle signaling defect originating in the photoreceptors is amplified by the central neural hyperexcitability. Collectively, our work establishes shared and distinct roles for the two Kv2.1 subunits, highlighting their essential contribution to both inhibitory circuit homeostasis and sensory processing.

MATERIAL AND METHODS

Animals

We used three groups of zebrafish (*Danio rerio*), including a wild type (AB) control (WT), the *kcnb1* null mutant *kcnb1sq301* (29), and the *kcng4b* mutant *kcng4bwaw304* (36). Only the homozygous mutants (*kcnb1*^{-/-} and *kcng4b*^{-/-}) were used. However, for the readability throughout the manuscript, we use the gene shorthand *kcnb1* and *kcng4b* to refer to these mutants, respectively. The fish were maintained following established protocols (37) at the Zebrafish Core Facility of the International Institute of Molecular and Cell Biology, Warsaw (License number PL14656251). Procedures involving embryonic and larval zebrafish were conducted per the guidelines set by the Polish Laboratory Animal Science Association and the Council Directive (63/2010/EEC) of the European Communities.

Behavioral experiments

All behavioral experiments were conducted on 5 days post fertilization (dpf) larvae using the Zebrafish system (Viewpoint, Life Sciences, France) and Ethovision XT 12 software (Noldus) for tracking and analysis. All experiments were repeated at least three times. The number of larvae used for each experiment were specified in the figure legends. Locomotor activity was quantified

by calculating the total distance travelled and mean velocity.

a. Pentylentetrazole (PTZ) induced seizures

4 dpf larvae were transferred to a 96-well plate with 100 µl of E3 medium acclimatized overnight at 28.5°C. The plate was then placed in the Zebrabox for a 30 min dark acclimatization period. Baseline behavior was recorded with 50% bottom light for 15 min. Subsequently, 100 µl of fresh PTZ solution (P6500, Sigma) at various concentrations (10, 20, and 30 mM) was added to the wells, resulting in final concentrations of 5, 10, and 15 mM. Control wells received 100 µl of E3 medium and were recorded for 30 min. Hyperactivity was determined by comparing total distance and mean velocity before and after PTZ treatment

b. Dual light pulse-induced seizures

4 dpf larvae were acclimatized overnight at 28.5°C in a 96-well plate with 200 µl of E3 medium. The next day, larvae were dark acclimatized for a 30 min period in the Zebrabox, followed by a 15 min baseline recording. To induce seizures, two light pulses (0.5 s each- 100% bottom light intensity) separated by 1 s of darkness were administered every 2 minutes for a total of 10 min (38). To assess photosensitivity, the total distance and mean velocity were calculated.

c. Light- dark preference

To assess light-dark preference, we used a 10 cm Petri dish. Half of the plate was designated as dark zone by covering its side with black tape and its top half with a photographic filter (Cokin P154 ND8, Rungis, France) to block the overhead light. The other half was designated as light zone. A single 5-dpf larva was placed in the dish containing 20 ml of E3 medium and transferred to the Zebrabox system with 70% top light intensity. The experiment started after 2 min setup period and locomotor activity was recorded for 15 min. The preference was quantified by

calculating the total time spent in each zone, the total distance traveled, mean velocity and frequency (39). Heat maps were obtained using the following Ethovision settings: merging method: mean; smoothing: 5; and map colors per heat map.

d. Open field test

Individual larvae were transferred to a 24-well plate and placed in the Zebrabox system. For dark conditions, the entire experiment was performed in complete darkness (light intensity = 0%), whereas for light conditions, the experiment was performed with 70% top light intensity. The locomotor activity was recorded for 15 mins, following a 2 min acclimatization. To determine the locomotor behavior under different light conditions, the total distance and mean velocity were calculated. Heat maps were generated using the same settings as described in the light-dark preference test.

e. Light intensity test

This protocol was developed to assess the larvae's tolerance to varying light intensity. Larvae were individually transferred to a 24-well plate and placed inside the Zebrabox system in 100% darkness for 2 min before the experiment. Locomotor activity was recorded for 12 min while top light intensity was gradually increased by 20% every 2 minutes, starting from 0 to 100% and returning to 0% at the 12th minute. Light intensity tolerance was determined by calculating the total distance, mean velocity, and freezing activity.

f. Behavioral data analysis and statistics

For all behavioral experiments, the raw data were processed using Ethovision XT 12 and exported as Microsoft Excel files. Statistical analysis was performed using GraphPad Prism (Version 9.5.1). Normality of data distribution was assessed using the Shapiro-Wilk test. Depending on the outcome, comparisons were done using appropriate parametric (unpaired Student's t-test, one-

way ANOVA, or two-way ANOVA followed by a post-hoc test) or non-parametric (Mann-Whitney U test or Kruskal-Wallis's test followed by a post-hoc test). A p-value of ≤ 0.05 was considered statistically significant.

quantitative Polymerase chain reaction (qPCR) analysis

qRT-PCR was performed to quantify gene expression related to epilepsy and photoreceptors. RNA was isolated from 50 whole 5 dpf larvae using the TRIzol-chloroform (T9424, Sigma) method according to the manufacturer's protocol. RNA concentration was quantified, and 1 μ g of RNA was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Biorad). qRT-PCR and subsequent data analysis were performed as previously described (31). A list of genes with their primer sequences are provided in the supplementary table 1

Immunohistochemistry

For staining sections, 5 dpf larvae were fixed in 4% PFA (P6148, Sigma)/PBS overnight. After PBS washes, they were fixed in 1.5% bactoagar (214010, BD Biosciences) mixed with 5% sucrose (772090110, Avantor)/PBS, followed by transfer to a 30% sucrose solution for overnight incubation at 4°C until they sank. The bactoagar-embedded larvae were mounted in OCT (361603E, VWR) and sectioned (14-18 μ m) using a Leica cryostat. Sections were allowed to dry overnight and processed for staining as per (40).

Primary antibodies used were rabbit polyclonal anti-gnat2 (zebrafish) (1:500) (#PM075, MBL) for cone photoreceptors, and mouse anti-glutamine synthetase (#610517, BD Biosciences) for Müller glia. Following PBSTx (1x PBS+ 0.1 % Triton-X 100) washes, embryos were incubated in secondary antibody Alexa Fluor anti-mouse 594 or anti-rabbit 594 (1:1000) for 2 hr. Hoechst (H3570, Invitrogen) was used for nuclear staining.

Retinotectal projection tracing via DiI labelling

Phenylthiourea (PTU, #L06690, ThermoFisher) treated 5 dpf larvae were fixed overnight in 4% PFA/PBS at 4°C, followed by three 1-hour PBS washes. Fixed larvae were embedded in 1.5% low-melting agarose (LMA). Anterograde labelling of retinal ganglion cell axons was performed by microinjecting 5mg/ml DiI (1,1'-Diocadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate, D282, Molecular Probes), diluted in dimethyl sulfoxide, into the anterior retina. Embryos were then left overnight at room temperature. Injected embryos were visualized under Zeiss Lightsheet Z1 microscope using a 40x lens. Local field potential recording Five-day-old larva was initially paralyzed using 300 µg of pancuronium bromide (P1918, Sigma) and then embedded in 1.5% low melting point agarose (LMA, #50080, Lonza) made in embryo medium containing pancuronium bromide, on the sample holder. The sample holder was then placed on the stage of a Zeiss Axio Examiner Z1 confocal microscope and filled with freshly prepared ACSF (artificial cerebrospinal fluid). A glass microelectrode (2-7 MΩ) was backloaded with ACSF and tactically inserted into the optic tectum. Spontaneous electrical activity was recorded for 15 min using a MultiClamp 700B amplifier (Axon Instruments, United States). The resulting voltage records were digitized at a sampling rate of 20 kHz using Digidata 1550B (Axon Instruments, United States), and band-pass filtered at 0.5 Hz (high-pass) and 0.3 kHz (low-pass).

Electrophysiology data analysis and statistics

The recordings were processed and analyzed using custom Python scripts (Version 3.13.5). Analysis included bandpass filtering, adaptive thresholding, and quantification of spontaneous transient events (STE) by amplitude and duration. The script is freely available upon request from the corresponding author. The resulting summary data were analyzed for statistical significance using GraphPad Prism (Version 9.5.1). Following the Shapiro-Wilk test for assessing

data normality, statistics were calculated using one-way ANOVA followed by Dunnett's multiple comparison test. A p-value of ≤ 0.05 was considered statistically significant.

RESULTS

Kv2.1-Kv6.4 subunit deficiency does not cause spontaneous seizure but alters baseline locomotion

To assess unprovoked seizure activity, a characteristic of KCNB1-related DEE (13), we recorded baseline locomotor behavior of larvae under standard light conditions (50% bottom light). Deficiency of Kv2.1 and Kv6.4 subunits did not result in spontaneous seizure activity. However, the mutants exhibited reduced baseline locomotor activity compared to the wild-type embryos (Fig. 1a and b).

Increased susceptibility to PTZ-induced seizures

We then evaluated the seizure susceptibility of the mutant larvae using PTZ, a non-competitive GABA antagonist that binds to the postsynaptic GABA-A receptor, resulting in reduced chloride ion conductance, thereby decreasing inhibitory response (41). Zebrafish have been a well-established model to study PTZ-induced seizure for the past two decades (42). We performed a dose-dependent behavioral study using PTZ concentrations of 5, 10, and 15 mM. Both WT and the mutant (*kcnb1* and *kcnng4b*) larvae exhibited a dose-dependent increase in locomotor activity, as measured by total distance travelled (Fig. 1c and d) and mean velocity (Fig. 1e and f) across the different concentrations. Critically, the magnitude of hyperactivity was significantly greater in both subunit mutants compared to the WT larvae. The seizure threshold was lower in the mutants, as 5 mM PTZ showed a significant increase in the locomotor activity of the mutants but not in the WT (Fig. 1g and h). Overall, these findings suggest that Kv2.1 and Kv6.4 subunit mutants are more susceptible to PTZ-induced generalized seizures.

Increased spontaneous brain activity

To understand the effect of Kv2.1-Kv6.4 deficiency on electrical activity, we recorded local field potential from the optic tectum of 5 dpf larval zebrafish (Fig. 2a). Analysis of the recordings showed that spontaneous brain activity was significantly increased in both the mutants compared to WT, as evidenced by increase in total event count (Fig. 2c) and event frequency/min (Fig. 2d). The median half-width was also significantly higher in *kcnbl* mutant (Fig. 2e). Collectively, these data demonstrate that the loss of subunits leads to a significant increase in spontaneous electrical activity within the optic tectum, suggesting an underlying state of neural hyperexcitability.

Kv2.1 deficiency altered locomotor activity triggered by dual light pulse

To determine if the Kv2.1 subunit mutants exhibit photosensitive seizures, a characteristic feature of Jeavons syndrome (43), we subjected the larvae to a dual light pulse protocol (100% - bottom light, two 0.5 s flashes separated by 1 s of darkness). Under baseline dark conditions, the overall locomotor activity of the mutant larvae was comparable to that of wild type (Fig. 3a and b). However, during the dual light pulse test, the locomotor activity of *kcnbl*^{-/-} larvae was significantly decreased, evidenced by reduced total distance and mean velocity (Fig. 3c and d). In contrast, the *kcng4b* mutant larvae behaved similarly to wild-type controls, except for a short increase in activity observed after the initial dual pulse (Fig. 3e and f). Although neither mutant displayed overt photosensitive seizures, the significant reduction in locomotor activity during the dual light pulse test indicates an altered photomotor response in the *kcnbl* mutant.

***Kcnbl* LOF mutants exhibit photosensitivity to high-intensity light**

To further evaluate the light-sensitive behavior of *kcnbl* mutants, a light/dark preference test was

performed in a Petri dish with 70% top light; half of the dish was covered using a photographic film to make the dark zone (Fig. 4a). Both the wild-type and *kcnbl* mutant larvae spent more time and covered larger distances in the light zone than in the dark, while the *kcng4b* mutant showed no significant preference for either zone (Fig. 4b and c). However, the mean velocity difference between the wildtype and mutant larvae remained unaltered (Supplementary Fig. 1a), whereas the zone transition frequency increased in the light zone for WT and *kcnbl* mutant, but not in the *kcng4b* mutant (Supplementary Fig. 1b). On the other hand, the heatmaps of 14/32 of the *kcnbl* mutant larvae exhibited freezing bouts (Fig. 4d) after being exposed to high-intensity light, while only 2/32 wild-type and 5/30 *kcng4b* mutant larvae froze in the same conditions. This experiment provided evidence that the *kcnbl* mutant was sensitive to high-intensity light. Based on the results of the light/dark preference test, we performed two additional behavioral tests to check the response of the *kcnbl* mutant to high-intensity light. The first one was an open field test in both dark and high-intensity light (70% top light). The total distance travelled in the dark decreased in the mutants, while the mean velocity of both groups, wild-type and *kcnbl*^{-/-}, was similar (Fig. 5A -a and b). However, under high-intensity light, the total distance travelled, and the mean velocity were significantly reduced in the *kcnbl* mutants (Fig. 5A- c and d). Moreover, normal behavior was restored in mutants as soon as the lights turned off at the end of the experiment. The second behavioral test checked the photosensitivity threshold by gradually increasing the top light intensity from 0 to 100% every two minutes (Fig. 5-Ba). Both groups showed a decrease in overall locomotor activity at 20% intensity, with a steady decline as the intensity increased, but the decrease was more robust in the mutant (Fig. 5B-b). However, the total distance travelled by the *kcnbl* mutant larvae significantly decreased (Fig. 5B-c). Together, these results show increased photosensitivity in the *kcnbl* mutant, evidenced by light-triggered decreases in mobility and freezing.

Mutations in the Kv2.1 and Kv6.4 subunits disrupt inhibitory neurotransmission

Quantitative PCR (qRT-PCR) was performed to analyze the transcriptional levels of several genes implicated in epilepsy. The results showed an increase in the expression levels of fosab (c-fos) in both the mutants, indicating increased brain activity or hyperexcitability (Fig. 6a). Moreover, in the mutants, there was a significant increase in the levels of glutamate decarboxylase 2 (gad2), an enzyme responsible for synthesizing the inhibitory neurotransmitter GABA (Fig. 6b). However, there was a significant reduction in the transcript of the key subunit of the GABA-A receptor-gamma-aminobutyric acid type A receptor subunit alpha-1 (gabra1) (Fig. 6c). No changes in the transcript level of bdnf (brain-derived neurotrophic factor) was observed (Supplementary table 2). These results suggest a disruption in the gene expression related to the inhibitory network, characterized by an upregulation in gad2 and a downregulation in gabra1, alongside elevated c-fos levels. Furthermore, transcript level of genes involved in visual perception, cry2 (cryptochrome circadian regulator 2), and gnat2 (guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2), were upregulated in the kcnb1 but not in the kcng4b. Cry1a (cryptochrome circadian regulator 1a), a gene associated with the circadian clock, was significantly downregulated in the kcnb1, whereas its expression was significantly upregulated in the kcng4b (Supplementary Table 2). The changes in the transcript level involved in visual perception suggest that Kv2.1 subunits may play a role in vision.

Kv2.1 deficiency does not affect the morphology of the retina or the retinotectal projections

Since both qRT-PCR and behavioral tests showed changes in the light-evoked response of the kcnb1 larvae, we next performed antibody staining to assess the retinal morphology. Nuclear staining did not show any morphological defects in the retinal cell layers in the kcnb1 mutant

(Supplementary Fig. 2A -a and d). The labelling of cone photoreceptors and Müller glia appeared normal in both genotypes (Supplementary Fig. 2A- b to f). Because there were no visible morphological defects in the retinal layers, we performed DiI staining to trace the retinotectal projections at 5 dpf. The anterograde tracing of retinotectal projections showed normal projections in the *kcnb1* mutant (Supplementary Fig. 2B- g and h). These results together show that neither the morphology of the retina, nor the integrity of retinotectal projections were compromised in the *kcnb1* mutants.

DISCUSSION

In this study, we characterized the effects of LOF of *kcnb1* and *kcng4b* on zebrafish behavior and circuit function. Our primary finding is a convergence of neural phenotypes: both the mutants exhibited increased susceptibility to PTZ-induced seizures, upregulation of the immediate early gene *c-fos*, and altered inhibitory neurotransmission. Unexpectedly, we also identified an interesting phenomenon, i.e., *kcnb1* loss led to an abnormal light-evoked behavioral response, manifested by reduced locomotor activity and freezing under high-intensity light, despite intact retinal and retinotectal morphology. Together, these findings reveal shared roles of *kcnb1* and *kcng4b* in the inhibitory network and a unique role for *kcnb1* in sensory processing. Previous study on the zebrafish *kcnb1* LOF mutant has shown hyperexcitability and GABA dysregulation, characterizing features of KCNB1-related encephalopathies in humans (44). Our study extends these findings by analyzing both α subunit (*kcnb1*) and modulatory subunit (*kcng4b*) mutants in parallel and by identifying a sensory component associated with *kcnb1* loss. Even though the developmental studies demonstrated the antagonistic effect of the Kv2.1 subunits during development of hollow organs (29–32), our data show that their neural functions converge at the level of circuit excitability and stability. These results highlight a context-dependent link between Kv2.1-Kv6.4 subunits across development and neural processes. Even though initially the mutants are hypoactive, the upregulated *c-fos* levels, spontaneous brain excitability, and

susceptibility to lower concentrations of PTZ-induced seizures collectively reveal an underlying state of neuronal hyperexcitability. This evident physiological hyperexcitability, but lack of spontaneous behavioral manifestations, suggests that these mutants can still serve as a valuable model for screening compounds that correct the fundamental hyperexcitability, even if it does not fully mimic the human DEE seizure phenotype. Furthermore, the data show a disrupted inhibitory system shared by both mutants. Kv2.1 channels are known to regulate neuronal excitability not only through delayed-rectifier potassium currents but also by clustering and interaction with other ion channel subunits in an activity-dependent manner (45,46). Therefore, losing either *kcnbl* or *kcng4b* likely alters the way these ion channel complexes work, leading to weakened inhibitory control across the neural circuit. This state of central hyperexcitability is further supported by the contradictory changes observed in the transcription levels of inhibitory neurotransmission markers. We found a significant increase in GABA-synthesizing enzyme *gad2*, which was also observed by other authors (44), which we interpret as a compensatory mechanism, where the central nervous system attempts to suppress excitability by increasing the supply of the inhibitory neurotransmitter GABA. However, these efforts appear ineffective due to a simultaneous downregulation of the GABA-A receptor subunit *gabral*. The depletion of postsynaptic GABA-A receptors represents a critical constraint, suggesting that despite an elevated level of GABA, the postsynaptic inhibitory infrastructure is severely compromised. This leads to a net reduction in inhibitory neurotransmission, thereby maintaining the hyperexcitable state confirmed by the *c-fos* and electrophysiology results. This interpretation aligns with the findings in the *gabral*^{-/-} zebrafish model, where loss of this subunit leads to a drastic reduction in inhibitory synapses and network complexity (47). In addition to the observed hyperexcitability, *kcnbl* loss uniquely leads to decreased locomotor responses and freezing under bright illumination, suggesting an abnormal photic response rather than photosensitive seizures. In absence of retinal or retinotectal defects the impairment may arise not from neuroanatomical deficiency, but rather from altered

signaling properties within the visual pathway. The severe behavioral freezing suggests a fundamental failure in the transduction or transmission of the light-evoked signal at the photoreceptor level. Although *kcng4b* has shown overlapping expression with *kcnbl1* in the eye (29), its loss does not recapitulate the acute light-evoked deficit. Prior mouse models have elegantly shown that Kv2.1 and Kv8.2 loss disrupts the retinal dark current and phototransduction kinetics (33,34,48), yet our findings provide the first evidence linking *Kcnbl1* to a specific, quantifiable, light-evoked behavior in a whole, developing vertebrate organism. This acute behavioral signature effectively validates the known functional disruption of the Kv2.1/Kv8.2 channel in a whole-animal context. The functional nature of this light-evoked response is further highlighted by the observation that the *kcnbl1* larvae locomotor activity normalizes as soon as the light is switched off. This rapid recovery confirms that the defect is a consequence of acute, dysregulated electrical signaling in the visual pathway, rather than a long-lasting or permanent structural or developmental defect. This faulty signal originating from the retina could be amplified by the pre-existing state of hyperexcitability in the central nervous system, confirmed by elevated *c-fos* transcript levels and the LFP recordings showing increased spontaneous activity. Crucially, this central hyperexcitability acts as an amplifier, converting the subtle retinal signaling error into the severe, dramatic behavioral freezing we observe under high-intensity light, which represents the basis for the ‘two-hit’ model. Furthermore, whereas WT and *kcnbl1* larvae display a natural preference for the light zone, the *kcng4b* mutant exhibits a complete lack of preference (Fig. 4b-c), suggesting a distinct, non-acute disruption of the light/dark processing pathway. Finally, the transcriptional changes observed in genes related to light signaling, specifically the upregulation of *cry2* and *gnat2* and the downregulation of *cry1a* in the *kcnbl1* mutant, provide molecular evidence for *kcnbl1*’s role in the visual circuit. Notably, the *kcng4b* mutant showed significant upregulation of the *cry1a* transcript level, highlighting a transcriptional divergence in the sensory/circadian network. While these changes may reflect

long-term compensatory responses to altered light input or an impact on the circadian network, their specific dysregulation in the *kcnbl* mutant (but not in *kcng4b*) reinforces the hypothesis that *kcnbl* plays a unique, *kcng4b*-independent role in modulating the photic response pathway. Together, these findings suggest that Kv2.1 contributes to the stability of visual circuits by regulating both essential photoreceptor signaling and central inhibitory tone. Overall, our findings highlight the essential contribution of Kv2.1 subunits to inhibitory homeostasis and sensory processing. The parallel phenotypes of *kcnbl* and *kcng4b* mutants reveal shared pathways that may underlie the variability observed in human *KCNBL*-related disorders. The zebrafish model provides a flexible system to dissect these mechanisms further, including subunit-specific interactions, developmental timing of the Kv2.1 function, and circuit-level consequences of the channel imbalance. Future studies combining genetic, electrophysiological, and pharmacological approaches will be crucial to clarify how Kv2.1 channel diversity modulates excitability across neural systems

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Zebrafish (*Danio rerio*) were maintained according to established protocols (Westerfield, 2007) in the Zebrafish Core Facility at the International Institute of Molecular and Cell Biology in Warsaw (licensed breeding and research facility, PL14656251, registry of the District Veterinary Inspectorate in Warsaw; 064 and 051: registry of the Ministry of Science and Higher Education).

All the experiments with zebrafish embryos, larvae and adults were performed in accordance with the European Communities Council Directive (63/2010/EEC).

Data availability declaration: the materials and reagents described in the paper are available from authors upon request.

Author contribution declaration:

Ruchi P. Jain: Conceptualization, Methodology, Investigation, Writing – original draft, Project administration, Data curation. R. Rosa Amini: Methodology, Investigation. Lukasz Majewski: Methodology, Investigation. Vladimir Korzh: Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition.

Declaration of generative AI and AI-assisted technologies in the writing process.

During the preparation of this work the author(s) used Grammarly and Quillbot to improve language and readability. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

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Competing Interest declaration.

Authors declare no competing interests.

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Figure Legends:

Fig. 1: Kv2.1 and Kv6.4 subunit mutants are more susceptible to PTZ-induced generalized seizures.

All experiments were performed at 5 dpf with 50% bottom light intensity in Zebrabox. **a-b) Baseline motor activity:** Baseline recording for 15 min shows decreased total distance (a) and mean velocity (b) in the *kcnbl*^{-/-} and *kcng4b*^{-/-} larvae compared to WT. Statistics calculated using non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. (N=3; n WT=93, n *kcnbl*^{-/-}=93, n *kcng4b*^{-/-}=89); **c-f) Concentration-dependent hyperactivity:** The total distance traveled and mean velocity are significantly increased in the *kcnbl* mutant larvae (c and e) and *kcng4b* mutant larvae (d and f) after treatment with different concentrations (5, 10 and 15 mM) of pentylenetetrazole (PTZ) for 30 min. Statistics were calculated using two-way ANOVA followed by Šídák's multiple comparison test. (N=3; n=22 for each concentration); **g-h) Seizure susceptibility at low concentration:** A significant increase in the total distance travelled by both *kcnbl*^{-/-} and *kcng4b*^{-/-} larvae was observed at the lowest used PTZ concentration (5 mM). Statistics were calculated using non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. (N=3; n=22 for each concentration). All data are represented as mean ± SEM with each dot representing individual larvae. A p-value of ≤0.05 was considered statistically significant.

Fig. 2: Increased spontaneous brain activity in *kcnbl*^{-/-} and *kcng4b*^{-/-} larva.

Local field potential (LFP) was recorded from the optic tectum of 5 dpf larvae. a) Schematic representation of LFP recording site in the larval zebrafish brain. b) Representative LFP traces from a WT, *kcnbl*^{-/-} and *kcng4b*^{-/-} larva, showing spontaneous brain activity over a 15-minute period. **c-e) Quantitative analysis of spontaneous events:** The number of total events (c), frequency of events (d), and median half width (e) were significantly increased in the *kcnbl* mutant. The *kcng4b* mutants also showed significant increase in total events and event frequency compared to wildtype.

The data are represented as box plots. The central line of box plot represents median while the whiskers extend to the minimum and maximum value of distribution. Statistics were calculated using one-way ANOVA followed by Dunnett's multiple comparison test. Number of larvae: WT=12; *kcnbl*^{-/-}=12; *kcng4b*^{-/-}=5. A p-value of ≤ 0.05 was considered statistically significant.

Fig. 3: The *kcnbl*^{-/-} larvae are sensitive to light.

The dual light pulse test was performed at 5 dpf with 100% bottom light. Red arrows mark the time when dual light pulse was flashed. **a-b) Baseline locomotor activity:** Baseline recording for 15 min in the dark showed no changes in the locomotor behaviour of *kcnbl*^{-/-} and *kcng4b*^{-/-} larvae as assessed by total distance (a) and mean velocity (b). Statistics were calculated using non-parametric Kruskal-Wallis followed by Dunn's multiple comparison test. (N=3; n WT=92, n *kcnbl*^{-/-}=85, n *kcng4b*^{-/-}=88); **c-f) Dual light pulse response:** The overall distance traveled (c) and the mean velocity (d) are significantly decreased in the *kcnbl* mutant larvae during dual light pulse experiment suggesting light sensitivity; Conversely, no significant changes were observed in the locomotor activity of *kcng4b* mutants (e-f) under same conditions. Statistics were calculated via non-parametric Kruskal-Wallis followed by Dunn's multiple comparison test. (N=3; n WT=68, n *kcnbl*^{-/-}=85, n *kcng4b*^{-/-}=88). All data are represented as mean $\pm$ SEM with each dot representing individual larvae. A p-value of ≤ 0.05 was considered statistically significant.

Fig. 4: High intensity light induces freezing behavior in the *kcnbl*^{-/-} larvae.

The light/dark preference test was performed at 5 dpf with 70% top light over a 15-minute period. **a) Schematic representation of the light-dark setup. b) Light zone preference:** The total time spent in light zone is higher in the WT and *kcnbl* mutant larvae, indicating a light preference. In contrast, the *kcng4b* mutant shows no preference, spending similar time in both zones. **c) Locomotor**

activity in light zone: The total distance travelled in the light zone is larger in the WT and *kcnbl* mutant groups. Statistics were calculated using two-way ANOVA followed by Šídák's multiple comparison test. (N=4; n WT=32, n *kcnbl*^{-/-}=32, n *kcnng4b*^{-/-}=30); **d) Freezing behavior:** The heatmaps obtained from Ethovision XT represent the individual trajectory and total distance covered across the arena. Note the freezing behavior of the *kcnbl* mutant larvae upon high-intensity light exposure. All data are represented as mean ± SEM. A p-value of ≤0.05 was considered statistically significant.

Fig. 5: The *kcnbl*^{-/-} larvae displayed increased photosensitivity to high-intensity light

A. Open field test under different conditions: a-b) Locomotor activity in dark:

Under dark conditions, the total distance travelled (a) was reduced in the *kcnbl* mutant compared to WT. However, mean velocity (b) showed no changes. Statistics for (a) were calculated using unpaired Student's t-test with Welch's correction while (b) used a non-parametric Mann-Whitney test. (N=3; n=54); **c-d) Locomotor activity in light:** The locomotor activity of the *kcnbl*^{-/-} larvae was significantly decreased (c-d) under high-intensity light (70% top light), suggesting sensitivity. Statistics were calculated using a non-parametric Mann-Whitney test. (N=3; n=54); **e) Freezing behavior:** Heatmaps illustrate the total distance travelled by the larva under light and dark conditions. Note the freezing behavior of the *kcnbl* mutant larvae upon high-intensity light exposure with the red arrow indicating the freezing location.

B. Light intensity tolerance test: a) Schematic representation of the experimental protocol involving a gradual increase in light intensity. b) The total distance covered by the *kcnbl* mutant larvae gradually decreased as the light intensity increased. Statistics were calculated using a mixed effect model followed by Šídák's multiple comparison test. (N=3; n=42); c) The overall distance traveled by the *kcnbl* mutant larvae is significantly decreased. Statistics were calculated using a non-parametric Mann-Whitney test. (N=3; n=42). All data are represented as mean ± SEM with

each dot represents an individual larva. A p-value of ≤ 0.05 was considered statistically significant.

Fig. 6: Alteration in transcript level of genes involved in inhibitory neurotransmission

qRT-PCR was performed to analyse the transcript levels of genes involved in epilepsy and inhibitory neurotransmission. **a) Neuronal activity marker:** Increased expression of *c-fos* (immediate early gene) transcript in both *kcnb1*^{-/-} and *kcng4b*^{-/-}. **b-c) Inhibitory neurotransmission markers:** The gene expression level of *gad2* (glutamate decarboxylase 2), GABA synthesis enzyme (b) is significantly increased in the mutants. The transcript level of *gabral* (GABA A receptor, subunit alpha-1) was significantly downregulated in both the mutants. Statistics were calculated using one-way ANOVA. All data are represented as mean $\pm$ SD with the p-value adjusted to ≤ 0.05 .

Supplementary Fig. 1: High intensity light induces freezing behavior in the *kcnb1*^{-/-} larvae.

The light/dark preference test was performed at 5 dpf with 70% top light over a 15-minute period. **a)** Mean velocity between the WT and mutants (*kcnb1*^{-/-} and *kcng4b*^{-/-}) larvae remains unaltered; **b)** Zone transition frequency for WT and *kcnb1*^{-/-} is higher in light than dark, but *kcng4b*^{-/-} shows no changes. Statistics were calculated using two-way ANOVA followed by Šídák's multiple comparison test. (N=4; n WT=32, n *kcnb1*^{-/-}=32, n *kcng4b*^{-/-}=30). All data are represented as mean $\pm$ SEM. A p-value of ≤ 0.05 was considered statistically significant.

Supplementary Fig 2: No morphological defects in the retina or retinotectal projections.

A. Retinal cross-section analysis: Transverse retinal section from 5 dpf WT (a-c) and *kcnb1*^{-/-} (d-f) larvae show no morphological defects. Nuclear staining (Hoechst, blue) highlights the retinal cell layers (a,d). Cone photoreceptors (red) are labelled by anti-gnat2 (b,e), and Müller glia (green) are labelled by anti-glutamine synthetase (c,f). All markers and layers appear normal between the

genotypes. MG- Müller glia.

B. Retinotectal projection Integrity: Anterograde labelling of the retina was performed using DiI (1,1'-Diocetadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate) in 5 dpf WT (g) and *kcnb1*^{-/-} (h) larvae. The retinotectal projections in the optic tectum appear normal, with no defects in *kcnb1* mutant compared to the wildtype. OT- optic tectum, E-eye

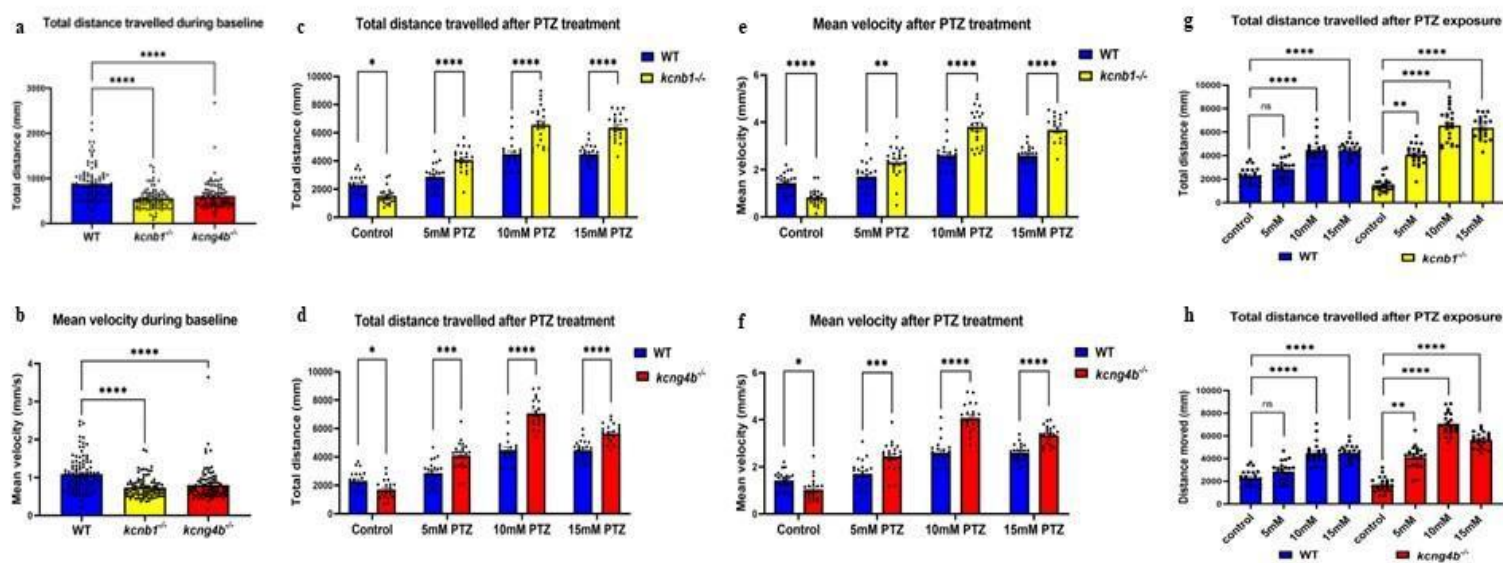


Fig. 1

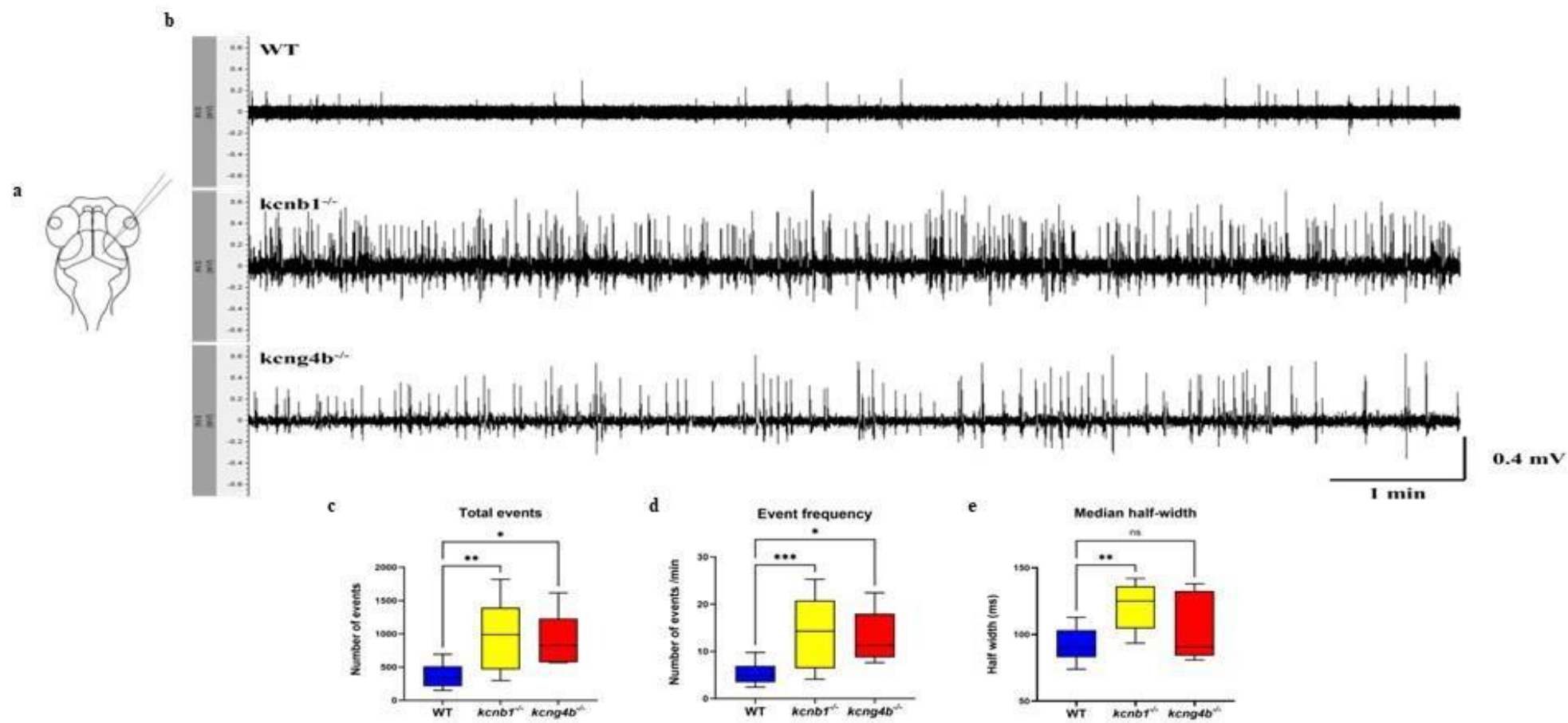


Fig. 2

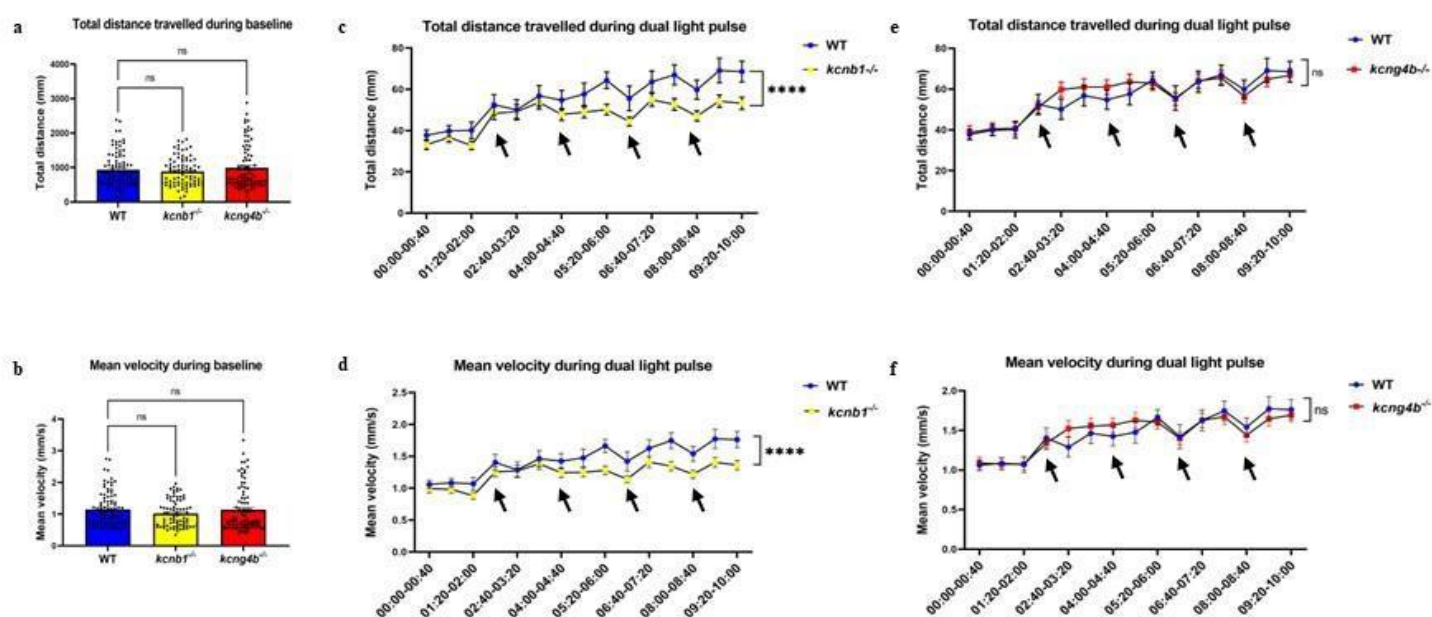


Fig. 3

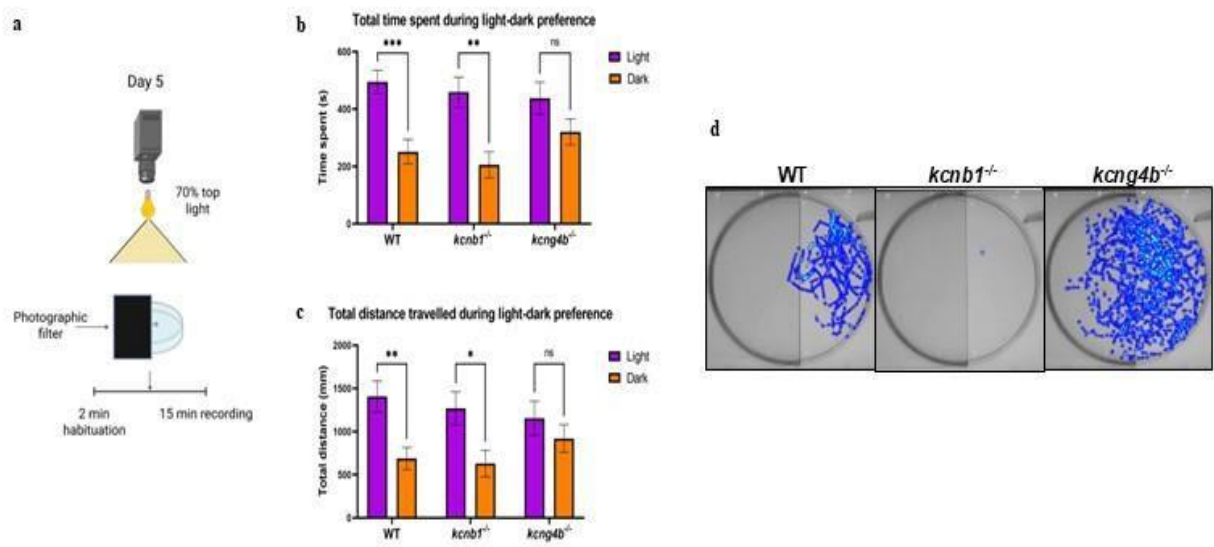


Fig. 4

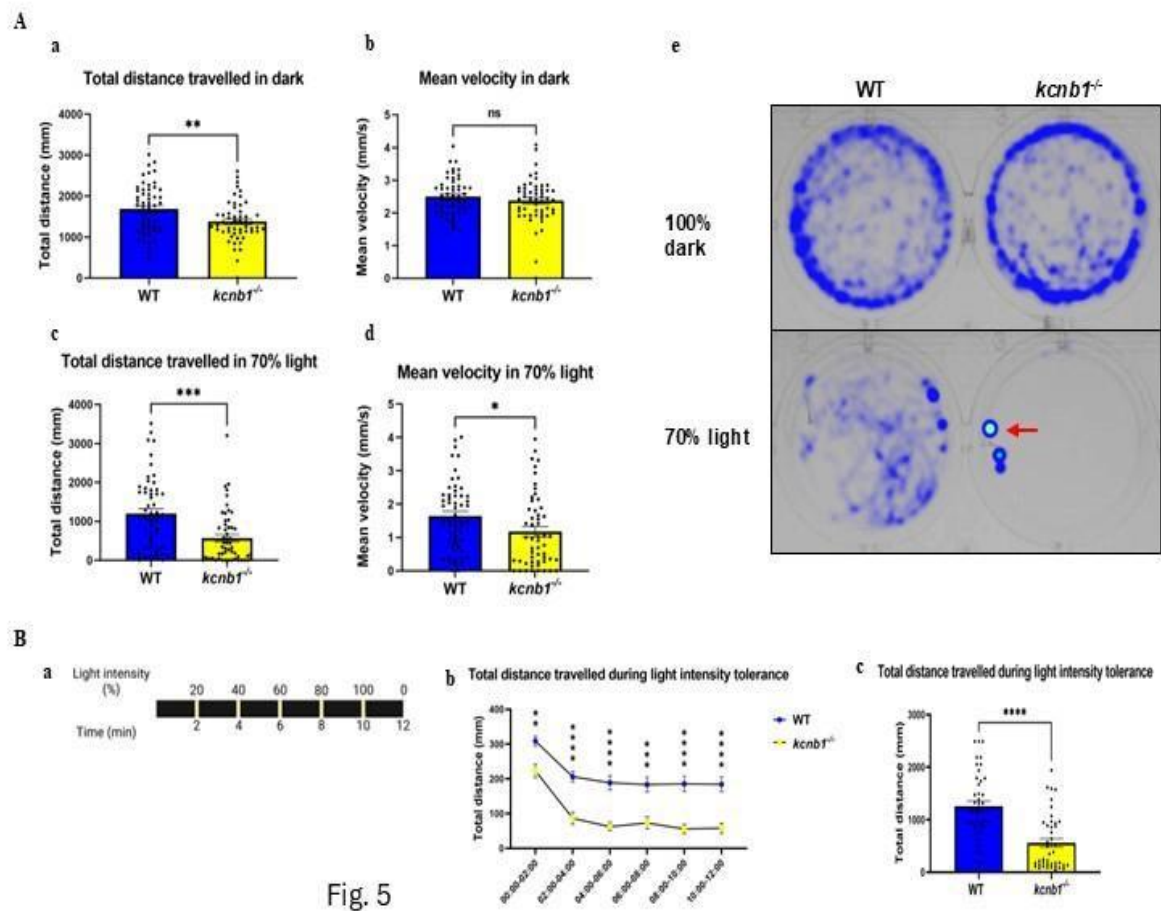


Fig. 5

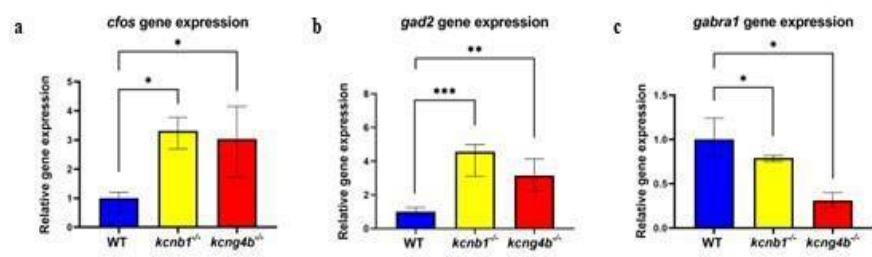


Fig. 6

Transcription level of genes involved in neuronal excitation, inhibitory transcription and visual perception at 5 dpf.

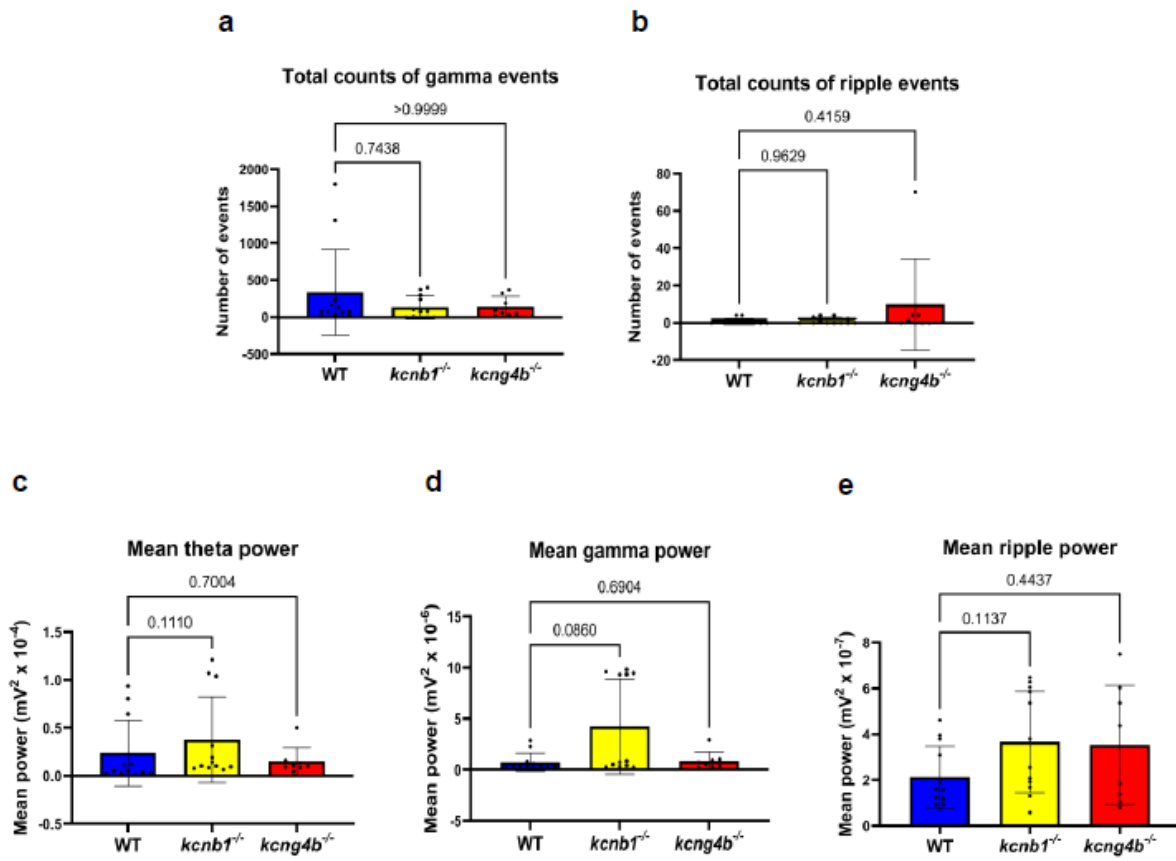
Genes	Expression level ratio, <i>kcnb1</i> / WT, SD 5dpf	p-value	Expression level ratio, <i>kcnb4b</i> / WT, SD 5dpf	p-value
<i>bdnf</i>	0.97±0.15	ns	0.74±0.16	ns
<i>fosab</i>	3.31±0.15	0.05	3.03±0.26	0.05
<i>gad2</i>	4.56±0.25	0.001	3.15±0.1	0.01
<i>gabra1</i>	0.79±0.05	0.05	0.31±0.08	0.05
<i>cry1a</i>	0.23±0.02	0.0063	6.35±0.28	0.001
<i>cry2</i>	1.84±0.43	0.046	0.85±0.14	ns
<i>gnat2</i>	2.31 ±0.27	0.018	0.46±0.11	ns

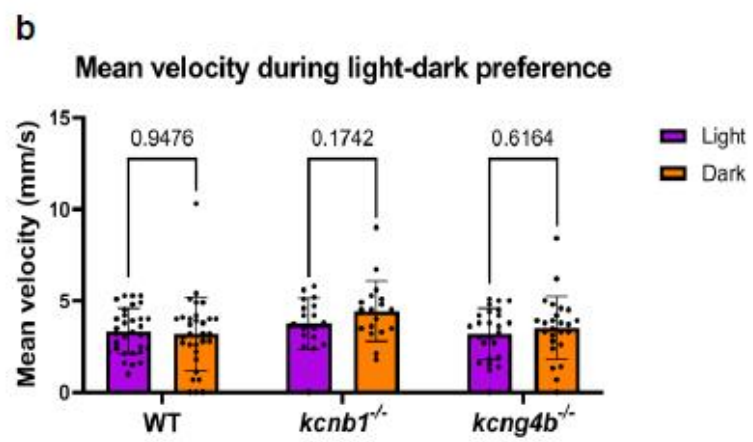
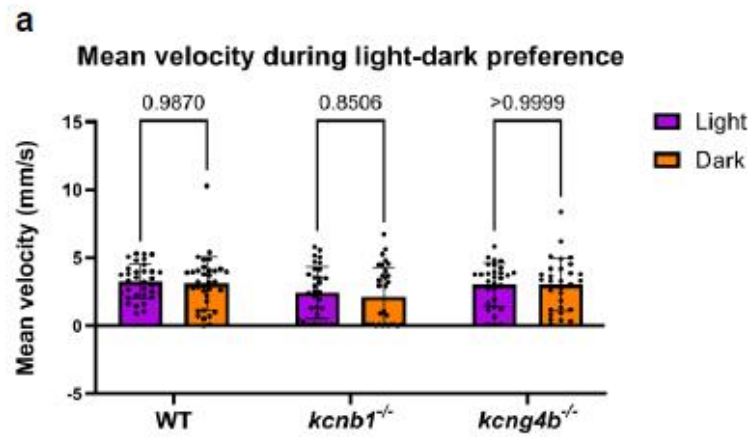
Transcription level of genes involved in eye development

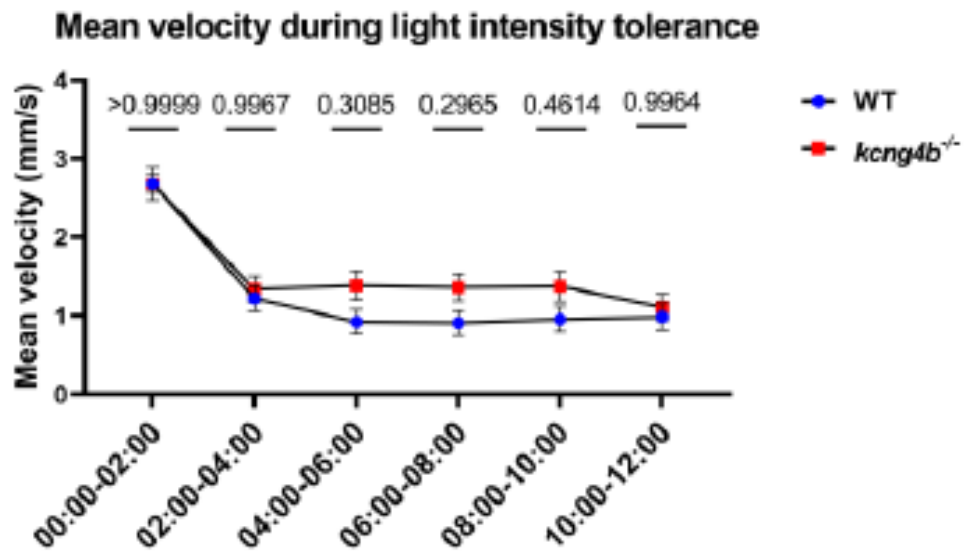
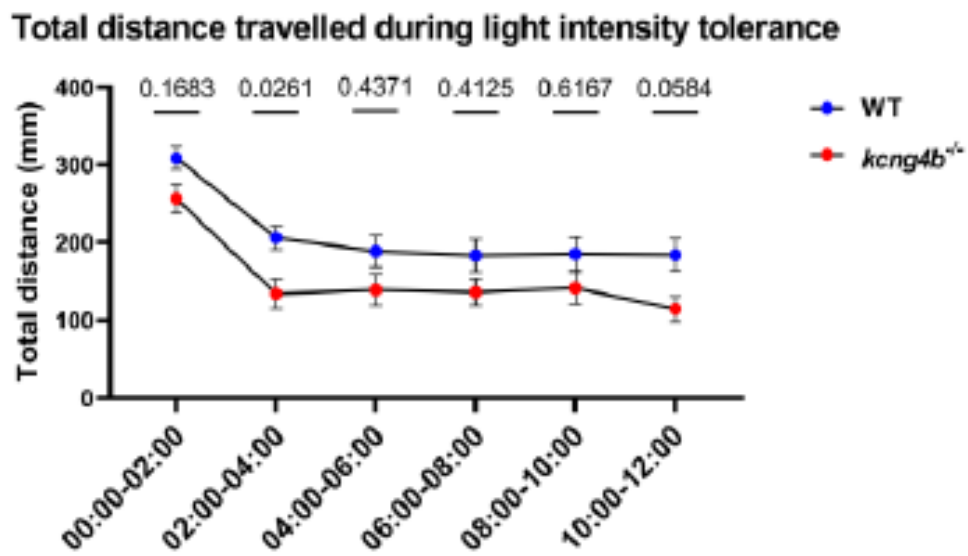
Genes	Expression level, <i>kcnb1</i> /WT, SD 1 dpf	P value	Expression level, <i>kcnb1</i> /WT, SD 2 dpf	P value	Expression level, <i>kcnb1</i> /WT, SD 3 dpf	P value
<i>foxe3</i>	0.101 ±0.03	0.0046	0.489 ±0.05	0.0057	0.625±0.34	ns
<i>pitx2</i>	0.297 ±0.23	0.0269	0.596 ±0.15	0.04	5.656 ±0.59	0.0014

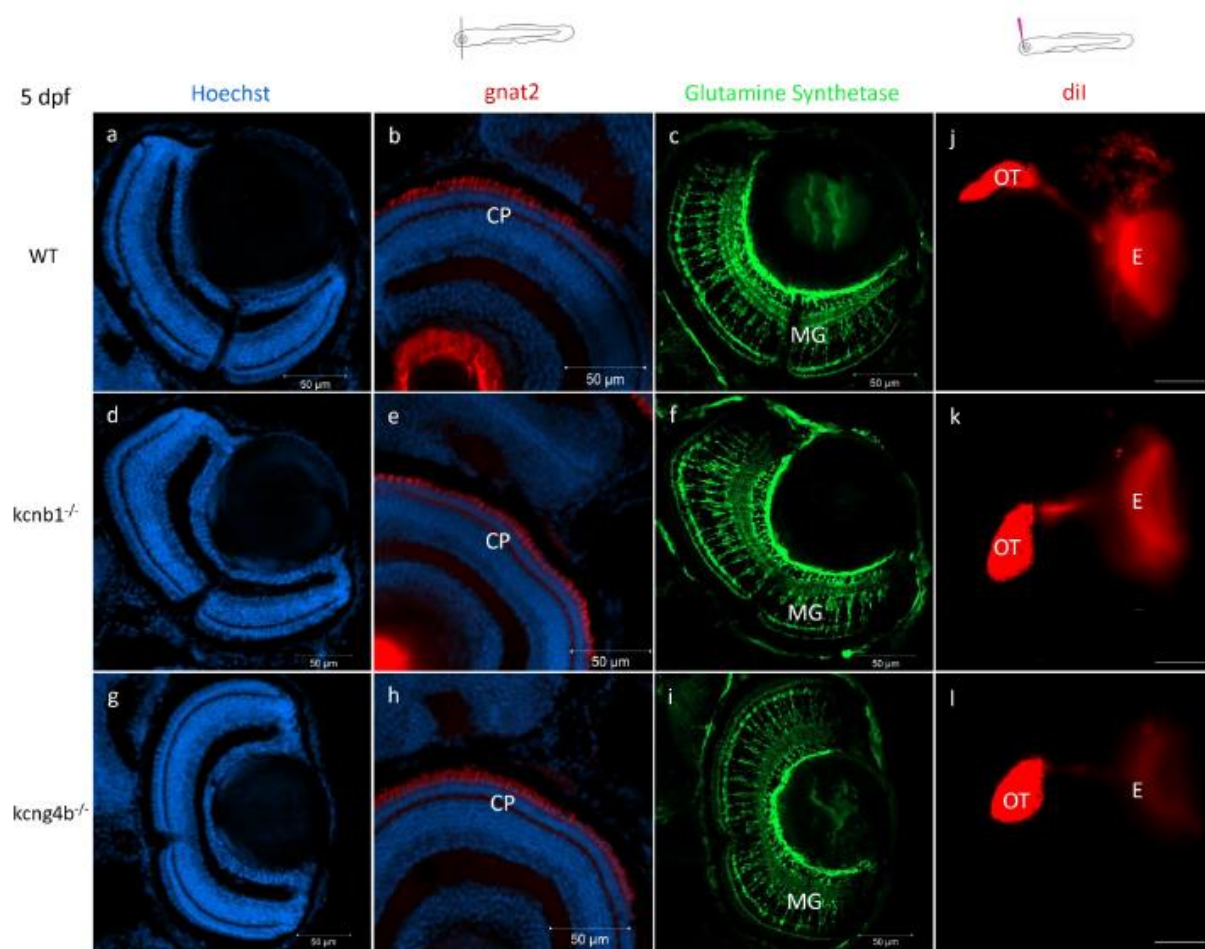
List of primer

Genes	qRT-PCR primers, 5'-> 3'
<i>bdnf</i>	TGACAGTATTAGCCAGTG CATTGCGAGTTATAGTGC
<i>fosab/c-fos</i>	GATGGCTGCTGCGAAATGC CGGCGAGGATGAACTCTAACC
<i>gad2</i>	CATTGAAGCCAAGCAGAAG GAACCAGAAGAGCAGAGC
<i>gabra1</i>	TGTGTGGAGTTTGTGTGAAAGC TCATTTGCGTAGCGAGGAGG
<i>cry1a</i>	TTCTTCCAGCAGTTCTTCC ATCGCCGTATGTAGTCTCC
<i>cry2</i>	CTGTTGGATGCGGACTGG CTCGTAGATGTAGCGGTTAGG
<i>gnat2</i>	CCACTTGTCCACCTCCAC CTTCCTTATCGGCATCTTCC
<i>pitx2</i>	AACGCTTGCCGATACTTC GTGGTGATTGTGATGATGG
<i>foxe3</i>	CACAGACAGAGAGGAAGTTG GGTAAGGTTATGCCGTATGG





a**b**



2.4 Manuscript 4

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Article's title: Mutant analysis of Kcng4b reveals how the different functional states of the voltage-gated potassium channel regulate ear development

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I hereby declare that I, Razieh Amini, made a substantive contribution to the development of this publication. My contribution consisted of:

- genotyping of the mutant zebrafish line
- conducting the RT-qPCR and data analysis

Candidate's Signature

Signature of the Corresponding Author



Mutant analysis of Kcng4b reveals how the different functional states of the voltage-gated potassium channel regulate ear development

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ABSTRACT

The voltage gated (Kv) slow-inactivating delayed rectifier channel regulates the development of hollow organs of the zebrafish. The functional channel consists of the tetramer of electrically active Kcnb1 (Kv2.1) subunits and Kcng4b (Kv6.4) modulatory or electrically silent subunits. The two mutations in zebrafish *kcn4b* gene – *kcn4b-C1* and *kcn4b-C2* (Gasnov et al., 2021) – have been studied during ear development using electrophysiology, developmental biology and in silico structural modelling. *kcn4b-C1* mutation causes a C-terminal truncation characterized by mild Kcng4b loss-of-function (LOF) manifested by failure of kinocilia to extend and formation of ectopic otoliths. In contrast, the *kcn4b-C2* mutation causes the C-terminal domain to elongate and the ectopic seventh transmembrane (TM) domain to form, converting the intracellular C-terminus to an extracellular one. Kcng4b-C2 acts as a Kcng4b gain-of-function (GOF) allele. Otoliths fail to develop and kinocilia are reduced in *kcn4b-C2*. These results show that different mutations of the silent subunit Kcng4 can affect the activity of the Kv channel and cause a wide range of developmental defects.

1. Introduction

Development is regulated by biochemical gradients, transcriptional networks, and bioelectrical signals generated by ion channels and pumps (Levin, 2014). Voltage-gated potassium (K_v) channels play an important role in the maintenance of the membrane potential. Their activity is tightly regulated. This is critical for the development and function of various cell lineages (Jędrychowska and Korzh, 2019; Shen et al., 2016; Vacher et al., 2008). In humans, mutations in K_v channels-encoding genes cause various diseases (Felix, 2000; Simons et al., 2015). For example, Kv2.1 (KCNB1) dysfunction has been associated with epilepsy (Bar et al., 2020; de Kovel et al., 2017; Kang et al., 2019; Latypova et al., 2017; Marini et al., 2017; Torkamani et al., 2014). This disease is likely caused by developmental abnormalities (Hawkins et al., 2021; Jędrychowska and Korzh, 2019; Speca et al., 2014).

In some human tissues including brain K_v 2 channels consist of the electrically active (KCNB1) and modulatory (or silent, KCNG4) α -sub-units. Subunit monomers assemble functional channel heterotetramers. The stoichiometry of the subunits in any given tetramer is variable, which adds to the functional complexity of K_v channel (Bocksteins, 2016; Möller et al., 2020). The genes encoding the subunits are expressed in the cell lineage- and tissue-specific pattern. Antagonizing activities of different subunits of K_v channels establish a regulated balance *in vitro* (Bocksteins and Snyders, 2012; Ottschytch et al., 2005) and *in vivo* (Jędrychowska et al., 2021; Jędrychowska and Korzh, 2019; Shen et al., 2016).

Most human KCNB1 mutants are heterozygotes carrying a single amino acid substitution. They develop early-induced epileptic encephalopathy (EIEE26), which is characterized by severe cognitive impairment (de Kovel et al., 2017; Jędrychowska and Korzh, 2019; Niday and

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Tzingounis, 2018; Saitsu et al., 2015; Srivastava et al., 2014; Thiffault et al., 2015; Torkamani et al., 2014). In comparison, the functional and pathological role of the mammalian *Kcng4* is much less clear. The GWAS study suggests that defects of *KCNG4* may be associated with migraine, multiple sclerosis and modulation of the uterine nociceptors excitability during labor (Lafrenière and Rouleau, 2012; Lee et al., 2020; Vilar-in-o-Güell et al., 2019), while targeted deletion of *Kcng4* in mice results in male sterility (Regnier et al., 2017) and hydrocephalus (<https://www.mousephenotype.org/data/genes/MGI:1913983#phenotypesTab>).

Some *KCNB1* heterozygotic human patients have developmental delay and speech defects (Bar et al., 2020; Latypova et al., 2017; Marini et al., 2017), which may be due to *KCNB1* gain-of-function (GOF) (Veale et al., 2022). Speech develops with the contribution of many inputs, including the hearing of sound (Anne et al., 2017). The orientation of aquatic and terrestrial animals in three-dimensional (3D) space depends on the inner ear, which coordinates the hearing of sound and the sense of balance. The latter relies on the vestibular system - three semicircular canals, where mechanosensory cells detect changes of fluid dynamics depending on the body position in 3D space and gravity. Sensation depends on the interaction of sensory cells with aggregates of biominerals such as otoconia in mammals or otoliths (utricle, saccule, and lagena) in non-mammalian vertebrates. Otoliths are composed of calcium carbonate and specialized proteins [Otopetrin (Otop1), Starmaker-like 1 (Sml-1), etc.] (Bezares-Calderón et al., 2020; Go et al., 2010; Hudspeth and Logothetis, 2000; Liedtke et al., 2000; Rozycka et al., 2014). Otolith formation depends on motile cilia that move the K^+ -enriched endolymph and tether cilia to which the otoliths attach (Colantonio et al., 2009; Riley et al., 1997; Yu et al., 2011; Wu et al., 2011).

Genetic analysis of ear development in various vertebrates, such as zebrafish, medaka, and mice, has identified a few genes that regulate ear and otolith development. Deficiency of these genes causes reduction, fusion, displacement, or absence of otoliths, resulting in hearing and balance defects (Chatterjee and Lufkin, 2011; Dror and Avraham, 2009; Malicki et al., 1996; Soñlner et al., 2004; Steel and Brown, 1994; Whitfield et al., 1996). For example, the proton-selective ion channel Otopetrin1 (*otop1*) is required for the formation of otoconia/otoliths (Hughes et al., 2004; Soñlner et al., 2004; Whitfield, 2020). The ciliary cytoskeleton and intraflagellar trafficking are essential for cilia extension (Choksi et al., 2014; Pathak and Drummond, 2009). Failure of cilia extension has been associated with language defects (Stawicki et al., 2016).

We have shown that the zebrafish *kcnb1* starts to be expressed at 19 hpf and its transcripts were detected in the brain ventricular system (BVS), eye, otic vesicle and somites. The zebrafish *kcnb1* mutant develops microcephaly, reduced otocyst and otoliths, and deformed kinocilia (Jedrychowska et al., 2021; Shen et al., 2016). In contrast, the homozygotic N-terminal truncated zebrafish *kcnq4b* mutant develops hydrocephalus (Shen et al., 2016).

kcnq4b is a developmental gene first expressed at 8–9 hpf and after 20 hpf in the brain ventricular system (BVS), otic vesicle, eyes and inter-segmental vessels (Shen et al., 2016). Its role in the ear development remains not fully understood. To address this issue, we generated two experimental *kcnq4b* mutants with deficient C-terminal (Gasanov et al., 2021). Unexpectedly, these mutants have opposite developmental ear defects.

2. Materials and methods

2.1. Animals

The wild-type (AB) and mutant zebrafish (*Danio rerio*) lines were maintained in the Zebrafish Facility of the International Institute of Molecular and Cell Biology in Warsaw (license No. PL14656251) according to the standard procedures and ethical practices (Westerfield, 2007). All experiments with zebrafish embryos/larvae were performed in accordance with the rules of the Polish Laboratory Animal Science

Association and European Communities Council Directive (63/2010/EEC). Developmental stages (in hours, hpf, and days post fertilization, dpf) were described according to (Kimmel et al., 1995). The transgenic line, *Tg(Brn3c:GAP43-GFP)^{z356}* (Xiao et al., 2005), which expresses cytosolic EGFP in inner ear and lateral line mechanosensory hair cells, was used in this study. The generation of two *kcnq4b* mutations, C1 and C2, by CRISPR-Cas9 has been described previously (Gasanov et al., 2021).

Xenopus laevis frogs (Nasco, Chicago, IL) were maintained in the animal facility of the Institute of Molecular Biology NAS RA (permission number 13-1F322) according to the guidelines of the local animal welfare authorities. All the quantified data are presented as mean $\pm$ SD.

2.2. Heterologous expression of *K_v* channels in *X. laevis* oocytes and electrophysiological measurements

Human $K_v2.1$ channel was co-expressed with zebrafish wild-type and mutant *Kcng4b* in the *X. laevis* oocytes. *Kcng4b-wt*, *Kcng4b-C1*, *Kcng4b-C2*, and *Kcng4b-trunc* constructs were subcloned from pTnT vector into the oocyte pGEM-He-juell oocyte vector using *Bam*HI and *Hind*III sites, between the *X. laevis* β -globin 5' and 3'-untranslated sequences to increase the efficiency of expression. The *in vitro* transcription was made using the mMessage mMachine T7 Kit (Ambion, USA). Quality of synthesized RNA was checked in agarose gel-electrophoresis. The synthesized mRNAs were quantified with RNA 6000 Nano kit (Agilent Technologies, USA).

X. laevis oocytes were surgically removed and defolliculated using 2 mg/ml collagenase (Sigma-Aldrich, USA) in Ca^{2+} -free OR₂ solution: 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.5. Stage V and VI oocytes were selected for mRNA (12 pg/oocyte) injection using manual nano-injector (Drummond Scientific, USA). Injected oocytes were incubated in OR₂ solution supplemented with 2 mM CaCl₂, 5 mM sodium-pyruvate and 50 μ g/ml gentamycin for 1–7 days prior to electrophysiological recording. The molecular ratio of $K_v2.1$ /*Kcng4b* mRNA injected in oocytes during co-expression experiments was 1:1.

Currents mediated through homomeric $K_v2.1$ and heteromeric $K_v2.1$ /*Kcng4* channels were analyzed by two-electrode voltage clamp (TEVC) method at room temperature. TURBO TEC-03X (NPI Electronic GmbH, Germany) amplifiers linked to Pulse software (HEKA Electronics, Germany) through ITC-16 digitizer were used for data acquisition and monitoring. Pipettes were pulled from borosilicate glass (WPI, Germany) using the PB/7 puller (Narishige, Japan). Tips of the recording electrodes were pre-filled with 1% agar-3M KCl and backfilled with 3M KCl solutions to prevent KCl leakage into the cells. Recordings were performed in modified ND96 solution containing: 75 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.5. Membrane voltage was continuously monitored during the recordings.

Conductance-voltage relationships for wild-type and mutant K_v channels were determined from tail currents. The Pulfitt (HEKA), Igor Pro 6.0 (WaveMetrics, Lake Oswego, OR, USA) and Kaleidagraph 4.0 (Synergy Software, Reading, PA, USA) software were used for data analysis. Nonspecific leak current was estimated by averaging the currents of 4–7 water-injected oocytes in parallel batches of each experiment. The average unspecific current was subtracted for GV and steady-state inactivation analysis. Normalized data points were fitted with Boltzmann function of the form $G/G_{max} = 1/(1 + e^{((V_{1/2}-V)/K)*F/RT})$ for GVs and $I_{test}/I_{cont} = ((1 - offset)/(1 + e^{((V_{1/2}-V)/K)*F/RT})) + offset$ for steady-state inactivation, $V_{1/2}$ is the midpoint voltage of activation and inactivation, respectively, K is the slope factor, F , R and T have their usual meanings.

2.3. Computer modelling of *Kcng4b*/*K_v6.4b* mutant proteins

The isolated S7 helix model was generated by Tasser web server (Yang et al., 2014) using a sequence of 34 amino acids predicted to form a TM helix. The structure of the $K_v1.2$ – $K_v2.1$ paddle chimera channel (PDB

id: 2R9R) was used as a template for the tetramer model after discarding beta-2 chains. Two opposing monomers served as the basis for modelling Kcng4b, whereas the other two were left unchanged. When constructing the Kcng4b monomer structures, the S7 helix was added based on the isolated helix model with constraints on the distances between the ends of S7 and S1 helices. The Modeller software (Webb and Sali, 2016) was used for these operations. Both models were embedded in membranes using MembraneBuilder from the CHARMM-GUI web server (Wu et al., 2014) and simulated using Gromacs 5.1.4 software (Abraham et al., 2015) with the CHARMM36m force field at physiological ionic strength (0.15 M KCl). Each system was minimized using the standard MembraneBuilder protocol and simulations were performed under isothermal-isobaric (NPT) conditions with semi-isotropic pressure coupling, temperature maintained at 310 K, and using the standard particle mesh Ewald (PME) cutoff of 1.2 nm. The single helix was simulated for 1.3 μ s in the liquid-ordered (Lo) phase – 50 dipalmitoyl-phosphatidylcholine (DPPC) and 20 cholesterol molecules per leaflet – and 1.5 μ s in liquid-disordered (Ld) phase – 50 phosphatidylcholine (POPC) molecules per leaflet. The tetramer was first simulated with positional restraint on Ca for 250 ns to ensure its stability, then with root-mean-square deviation (RMSD) restraint: RMSD of every 3rd Ca of helical parts of the TM domain was restrained at 0.15, in reference to the Modeller model. The restraint force constant was kept at 100,000 for 200 ns, 25,000 for 400 ns and 5000 for 450 ns. Finally, the model of Kv1.2–2.1/Kcng4b-C2 tetramer with S7 helix joined to the structure via loops based on the C2 mutant sequence was simulated without restraints for 1.2 μ s. We used Gromacs for position restraints and plumed for RMSD restraints (Tribello et al., 2014).

Mean square displacement (MSD) and angle calculations were performed using the MDTraj library for Python (McGibbon et al., 2015). Only backbone atoms were used for both analyses.

Minimum distances between S7 helix and the remainder of the channel have been computed in the following way: i) amino acid residues in the channel within 0.5 nm of the S7 helix in the first frame of the trajectory have been selected for analysis, ii) a minimum distance to aforementioned channel amino acid residues has been computed for each of the S7 residues. To predict the structure of the tetramer with S7 helices, sequences of the C2 mutant of Kcng4b and Kv1.2–2.1 have been used as an input to the AlphaFold Multimer v3 as implemented by Colabfold (Mirdita et al., 2022). The model was run for 30 recycles and relaxed with Amber.

2.4. Microscopy

2.4.1. Live imaging

Zebrafish embryos were raised in E3 medium (2.5 mM NaCl, 0.1 mM KCl, 0.16 mM CaCl₂, and 0.43 mM MgCl₂) with the addition of 0.2 mM 1-phenyl-2-thiourea (PTU, Merck, Germany) to block pigmentation. At selected developmental stages, embryos were manually dechorionated, anesthetized with 0.02% tricaine (Sigma-Aldrich, USA) and oriented by embedding in 2 % methylcellulose (Merck, Germany) on the glass slides. A research stereomicroscope SMZ25 (Nikon, Japan) was used to image acquisition.

Vital dye staining of lateral line hair cells of the 4 dpf embryos was performed using DASPEI (2-(4-(dimethylamino) styryl)-N-ethylpyridinium iodide) (Sigma-Aldrich, USA, D0815), 0.8 μ g/ml, and Calcium Crimson, AM ester (Thermo Fisher, USA, C3018), 6.8 μ g/ml in E3 medium, 28 °C, 15 min 3 mM FM 1-43FX was injected into an ear of 5 dpf larvae as described in (Smith et al., 2020).

2.4.2. Light-sheet and confocal fluorescence microscopy imaging

In vivo imaging and imaging of fixed specimens were performed as previously described (Garcia-Lecea et al., 2017; Jedrychowska et al., 2021). For imaging of fixed material, 0.8 % low-melting agarose in phosphate buffer saline (PBS) was used instead of E3 0.02 % tricaine medium. Zeiss Lightsheet Z. 1 with W Plan-Apochromat 20x/1.0 UV-VIS

(for *in vivo* imaging), 40x/1.0 UV-VIS or 63x/1.0 UV-VIS (for fixed embryos) objectives were used, transmitted LED light was also used to obtain the high-resolution bright-field images of zebrafish ear. Confocal imaging was performed using the Zeiss LSM 800 inverted microscope with Airyscan (Carl Zeiss, Germany). 405, 493 and 575 nm lasers were used to excite fluorescence with emission detected using emission filters (425–430, 514–530 and 592–625 nm BP), respectively. Data were saved in the LSM or CZI format and processed using ZEN (Zeiss) or ImageJ

1.51n (Fiji) software. Maximum intensity or sum slices projections were generated for each z-stack. Brightness/contrast adjustments and resizing were performed using FastStone viewer 7.4 (FastStone Soft).

2.5. Immunohistochemistry and *in situ* hybridization

Whole-mount immunohistochemistry was performed according to a previously established protocol (Korzh et al., 1998). A 1:100 dilution of mouse monoclonal anti-acetylated tubulin antibody (Sigma-Aldrich, USA, T7451) was used as the primary antibody and 1:2000 dilution of donkey anti-mouse Alexa Fluor 488 (Thermo Fisher, USA, R37114) was used as the secondary antibody. Alexa Fluor 568 Phalloidin (Thermo Fisher, USA, A12380) staining (1:200) was performed simultaneously

with the secondary antibody incubation (Tanimoto et al., 2011). DAPI (4',6'-Diamidine-2'-phenylindole dihydrochloride, Abcam, USA) was added to some samples along with secondary antibodies to final concentration 10 μ M. RNA probes for *in situ* hybridization were transcribed

in vitro from the pTnT vector (Promega, Madison, WI, USA) with sequences of *kcnb1*, *kcnq4b*, *otop1*, *slc12a2* and *atp2b1a* cloned in 3'-5' direction cDNA using Digoxigenin-labelled uridine triphosphate (DIG-UTP). The probe against *kcnb1* was synthesized from a previously described plasmid (Shen et al., 2016). The synthesis of the *otop1* and *atp2b1a* probes is described previously (Jedrychowska et al., 2021), while the *kcnq4b* and *slc12a2* probes were generated from pKcng4b-probe/pTnT and slc12a2-probe/pTnT vectors. These vectors were prepared by PCR using the following primers: Kcng4b-p-fwd

(5'-ATATATGAATTCATGCCATCATCAGCAATGCTAACC) and Kcng4b-p-rev (5'-ATATATGAATTCCTGAAGCCCTTGTCCTGTGCAAGAGG), Slc12a2-fwd (5'-ATATATGAATTCATGTCGGCGTCACCTCCAATCTCCG) and Slc12a2-rev (5'-ATATATGAATTCCTGATCCATCCGAACCTTCACCACTCC), as well as zebrafish cDNA as a template. The PCR product was cloned into a pTnT vector using the *EcoRI* restriction site (underlined in the primer sequences) in both orientations to get the sense and antisense probes.

2.6. Transcriptome analysis by RNAseq and bioinformatics

150 embryos for each sample were collected at 24 hpf [wild type control and Kcng4b-deficient crispants and morphants (Shen et al., 2016)] and RNA preparation was performed as previously published (Shen et al., 2017; Winata et al., 2013). mRNA levels were quantified using a standard pipeline based on TopHat-cufflinks59 with the *Danio rerio* gene annotation file (assembly GRCz10) from the Ensembl data-base. For differential expression analysis, gene-level FPKM values were converted into log ratios (KO/WT, base 2) and the threshold for differential expression to meet 1% FDR was determined using a mixture model-based approach called EBprot60. The transcripts with largest FPKM values were below 25 percentiles of all FPKM values across the sample were removed from further analysis as unreliable. For genes whose expression changed in crispants and morphants compared to WT, a gene was considered differentially expressed if the quantified FPKM value (Trapnell et al., 2010) was above the 25 percentiles of the whole transcriptome in the given sample. Functional enrichment test (Gene Ontology) was performed using an in-house program that calculates the significance of enrichment by hypergeometric test, using the GO annotation of genes from ZFIN database (<http://zfin.org>). The RNAseq data have been deposited online (GEO submission no. GSE194272).

2.7. qRT-PCR

The SsoAdvanced Universal SYBR Green Supermix and CFX Connect real-time PCR system (Bio-Rad, USA) was used for the Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) according to the manufacturer's instructions. The TRIzol-chloroform RNA extraction protocol (Sigma-Aldrich, USA) was used to extract total RNA from 20 to 50 zebrafish embryos at 28, 48, and 72 hpf. RNA concentration was assessed using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, USA), and cDNA was synthesized from 1 µg of RNA using the iScript™ Reverse Transcription kit (Bio-Rad, USA). qRT-PCR was performed using gene-specific primers for *eef1a1l1*, the reference reaction for the housekeeping gene, and other primers (Suppl. Table 1) were used to amplify mRNAs of interest selected based on results of the RNAseq analysis described above. The threshold cycle of each target and reference gene amplification in control and mutant embryos was determined automatically by LightCycler® 96 Software (Roche Diagnostics, USA). Fold change in mutants versus controls was calculated with delta-delta-C(t) method and Student's two tailed *t*-test with respect to the mismatch control was used to determine statistical significance. Statistical analysis including standard deviation calculation, was performed using Micro-soft Excel (Microsoft, USA) and GraphPad Prism 5 (GraphPad, USA) software. Melting curve analysis and agarose gel electrophoresis were performed as product specificity controls, while the reaction efficiency

(E) was calculated separately for each gene analyzed.

2.8. Balance assessment via needle touch

Individual larvae were placed in a Petri dish filled with E3 medium and stimulated by needle touch at 4 dpf. Their response and swimming behavior were observed and recorded. Normal larvae react to each touch by a startle reaction and swimming along a straight line. Mutant larvae exhibit vestibular defects manifested by an irregular swimming pattern (e.g., circling).

2.9. Statistical analysis

Mutant's analysis: The mutant phenotypes of the ear size, otoliths and kinocilia numbers were detected and counted in at least 20 samples. Data are expressed as mean ± SEM, unpaired Student's *t*-test. A representative subset of mutant embryos and larvae (N = 6) was shown. For behavioral response, each larva was tested in triplicate, and larvae with at least one response were considered responsive.

gRT-PCR: All RNA samples used as the templates were extracted independently from three or four different groups of embryos, and each was analyzed by qRT-PCR at least twice after cDNA synthesis. Data are expressed as mean ± SEM, unpaired Student's *t*-test. The value above 1.0 corresponds to an increase of mRNA level, the value below 1.0

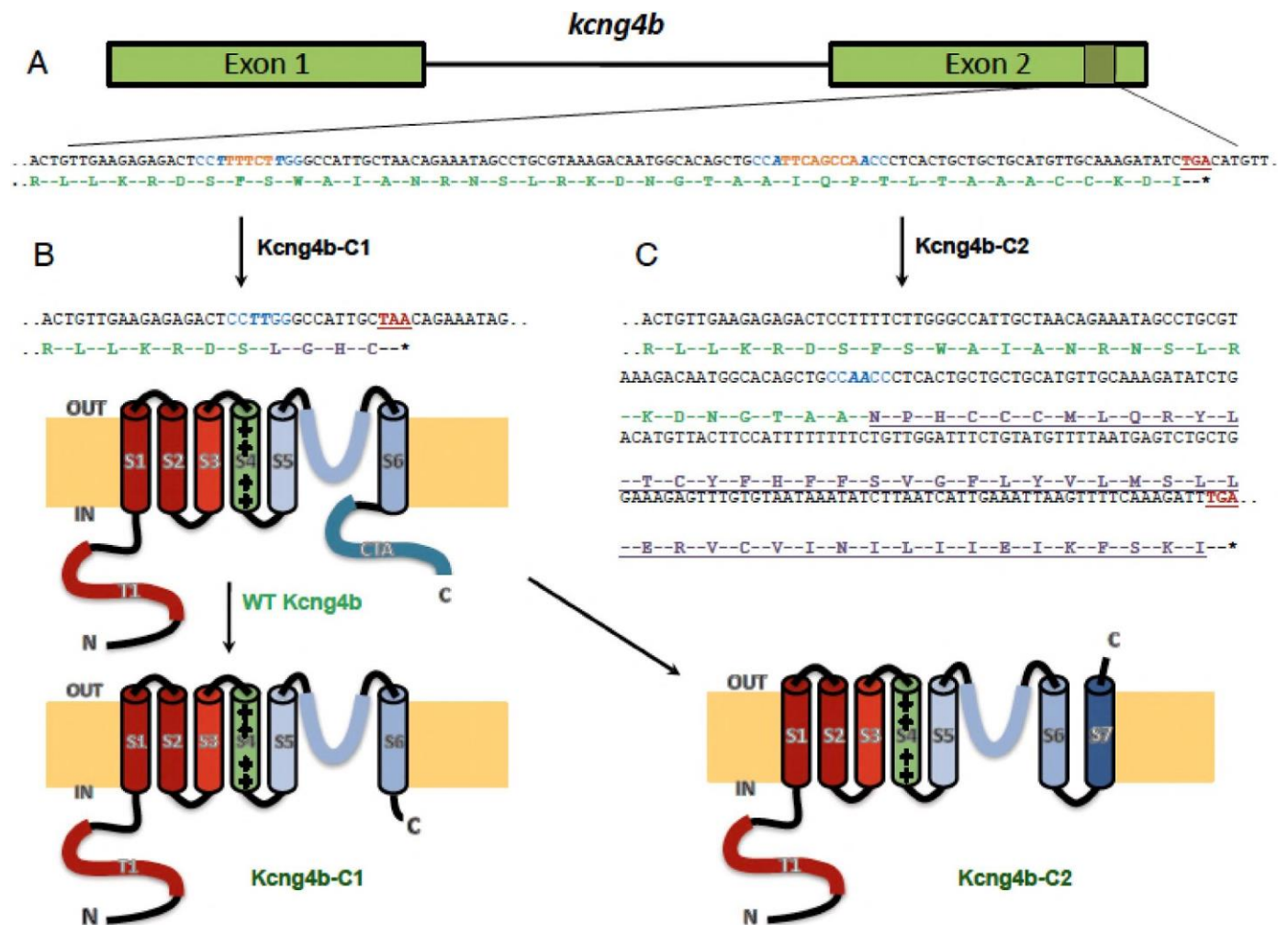


Fig. 1. *Kcng4b-C1* and *Kcng4b-C2* mutations differently affect *Kcng4b*'s C-terminal. A - *Kcng4b* nucleotide sequence is in black, stop-codons are in red, gRNAs' PAM-sequences are in blue, and deleted nucleotides are in orange. The *Kcng4b* amino-acid sequence is in green, the modified one in lilac. B - mutation *Kcng4b-C1* caused *Kcng4b* C-terminal truncation. C - mutation *Kcng4b-C2* caused an extension of *Kcng4b* polypeptide and formation of an additional C-terminal TM domain (S7, underlined). T1 and CTA - N- and C-terminal cytoplasmic domains, S1-S7 - TM domains. IN, OUT - intracellular and extracellular space.

corresponds to a decrease of mRNA level and “n.c.” - “not changed” is the same value as in the controls.

3. Results

Kcng4b plays the developmental role in the brain ventricular system (BVS) (Shen et al., 2016). Here we focused on the analysis of Kcng4b function during ear development, where the third (ectopic) otolith was previously detected in the insertional mutant *kcng4b-trunc* (*kcng4b^{sq301}*) (Suppl. Fig. 1). In absence of this mutant line, which has been lost, to study the developmental role of Kcng4b, we generated two novel Kcng4b mutant alleles (*kcng4b-C1*, *kcng4b-C2*) (Fig. 1) both of which were lethal as homozygotes.

3.1. Computational analysis of Kcng4b mutants

The deletion of five nucleotides in the second exon of *kcng4b* in the *kcng4b-C1* mutant caused the open reading frame (ORF) shift (Fig. 1A and B). The C-terminal of Kcng4b polypeptide was prematurely terminated with the loss of 29 C-terminal amino-acid residues (Gasanov et al., 2021) (Fig. 1B) resulting in a phenotype similar to that of *kcng4b-trunc* mutant (Suppl. Figs. 1 and 3). In the second exon of *kcng4b-C2* mutant, eight nucleotides were deleted, resulting in the ORF shift that replaced 14 C-terminal amino acids with 49 ectopic ones (Gasanov et al., 2021) (Fig. 1C). To verify the structure of mutant polypeptides, we analyzed the putative amino acid sequences in silico using TMHMM software (<https://services.healthtech.dtu.dk/service.php?TMHMM-2.0>) and

molecular modelling and predicted the C2 mutant structure with AlphaFold multimer v3. These predicted no significant changes in the structure of the *kcng4b-C1* mutant other than its C-terminal truncation (Fig. 2A–C; Suppl. Figs. 2A and B). In contrast, in the *kcng4b-C2* mutant, the ectopic seventh TM domain (S7) was predicted in the C-terminal of the putative Kcng4b-C2 polypeptide (Fig. 2B–D; Suppl. Fig. 2C; Suppl. Movie 1). Such a domain may flip the cytoplasmic C-terminal to an extracellular one, which will have a significant impact on the hetero-tetramer structure and function. Indeed, this S7 helix was predicted to be bound to the remainder channel by the AlphaFold model (Fig. 2C and D).

To explore this possibility molecular dynamics simulations of the S7 helix in model lipid membranes were performed (see M&M for details). Since the behavior of TM helices is phase-dependent, we used POPC lipids as a model for liquid-disordered (Ld) domains, and DPPC lipids with 40% cholesterol as a model of liquid-ordered (Lo) domains (Fig. 3A). The S7 behaved differently in these cases: in the thinner Ld

phase, the helix tilted by 44° to better accommodate its hydrophobic

thickness in the membrane hydrocarbon core, while in the much thicker Lo phase, it remained stably upright with average tilt of 11° (Fig. 3A). To explore whether the S7 helix forms a stable complex within the K_v2.1 (Kcnb1)-K_v6.4 (Kcng4) heterotetramer, we set out to simulate the entire TM portion of the channel. Since a structure of an entire K_v6.4 has not been solved, we used the K_v1.2-K_v2.1 paddle chimera channel (PDB ID: 2R9R) as a basis for the homology model in approach used previously (Matthies et al., 2018).

To show that the S7 helix remains stably bound to the channel, we

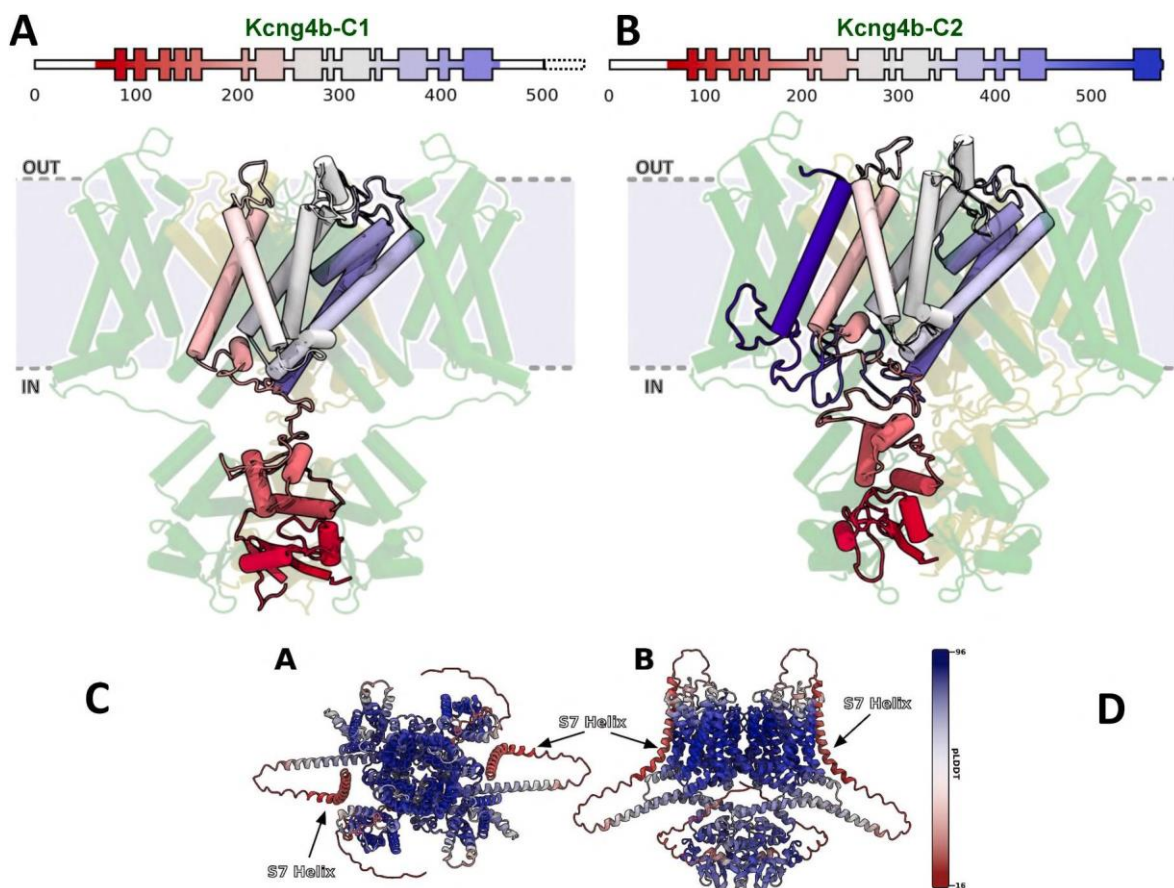


Fig. 2. Modelling of Kcng4b/Kv6.4 mutant monomers. Kcng4b-C1 (A), Kcng4b-C2 (B–D). IN, OUT – internal and external space. The deleted C-terminal region of Kcng4b-C1 is indicated by the dashed box. The additional transmembrane domain of Kcng4b-C2 (S7) is in dark blue. (C) Top and (D) side view of structure of Kv1.2–2.1 + Kcng4b tetramer as predicted by AlphaFold Multimer v3, colored by per-residue model confidence score (pLDDT). Two monomers of each protein were used to form the tetramer, analogous to the simulated model. AlphaFold clearly predicts the S7 helix in the membrane, bound to the core of the tetramer. Confidence score of the S7 helix prediction is low, which is expected from the terminus of the protein and a sequence that was not under evolutionary pressure.

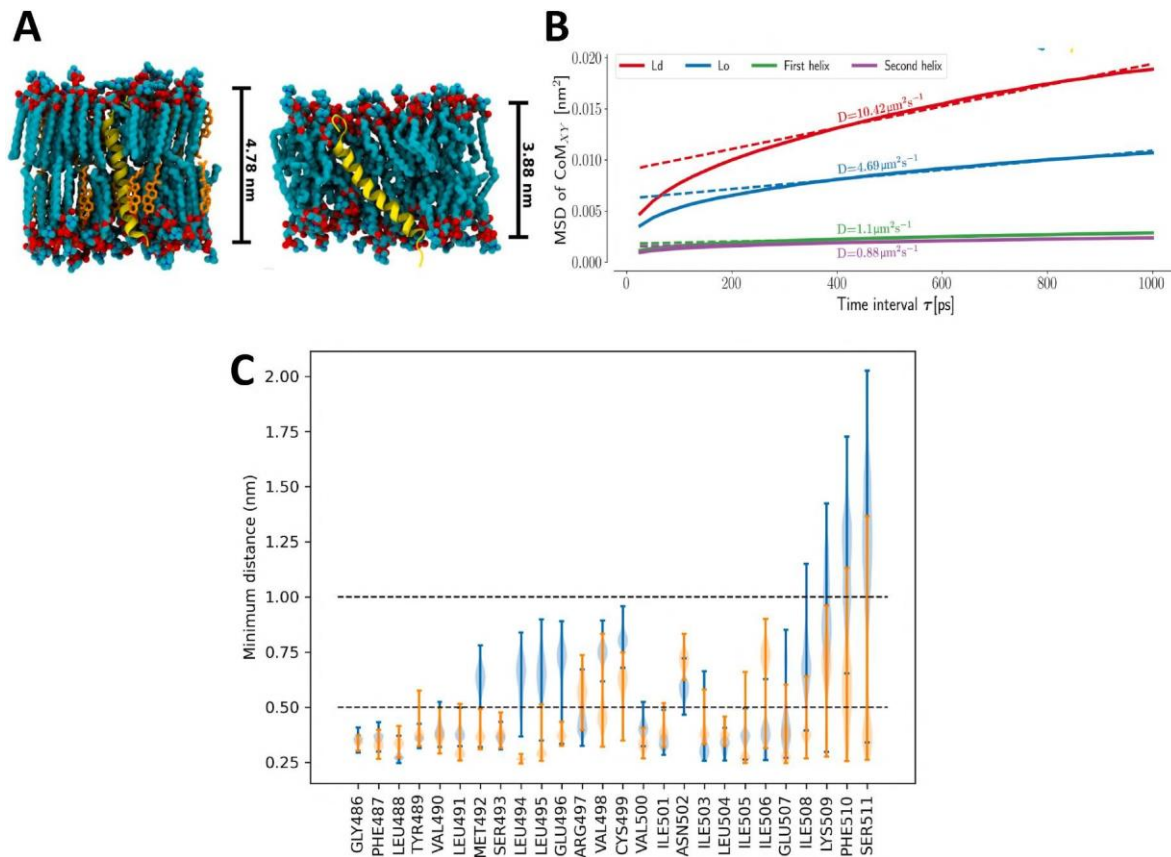


Fig. 3. Characterization of S7 domain in the Kcng4b-C2 mutants. A - Representations of isolated helix systems of Kcng4b-C2; S7 in a thicker, more stiff liquid ordered (Lo) phase (left), S7 in a thinner, looser liquid disordered (Ld) phase (right). B - Plots of mean square displacement (MSD) against time interval τ . Helices described as first and second refer to the S7s from two different Kcng4b/Kv6.4 monomers in the tetramer. Here, MSD measures displacement of the center of mass of the helix in membrane plane within the time interval τ . The slope of the MSD at long times is related to the diffusion coefficient D , equivalent to one obtained by standard experimental methods, e.g., single-molecule tracking. C - Distributions of minimum distances between the residues of S7 helices and the remaining membrane-bound part of the channel. Distributions for each of the helices are shown separately in blue and orange.

compared the diffusion coefficients of the S7 helix either in pure lipid bilayers or in the context of the heterotetramer model by plotting mean squared displacement (MSD) against the time interval τ (Fig. 3B). The helices in the heterotetramer model are much less mobile than in the pure bilayer models, indicating that the S7 helix remains bound to the rest of the TM domains. To confirm that even further, we have computed the minimum distances distributions between S7 residues and the remainder of the channel, in heterotetramer simulations (Fig. 3C). This analysis shows that both helices remain stably bound and do not attempt to dissociate. Although we were unable to predict its exact rotational orientation with respect to the tetramer, the fact that its protein-protein interface has not been evolutionarily optimized strongly suggests that there is no single high-affinity binding site, and that the location of the additional helix is largely determined by an entropic tendency to displace lipids from the lateral surface of the protein where they undergo conformational ordering (Fig. 3A).

While the 35 amino acids of Kcng4b-C2 mutant discussed above clearly form a helix, this region is preceded by the 14 amino acids sequence whose secondary structure remains unknown. However, since this sequence results from the same ORF shift, in the absence of strong predictions, we found it reasonable to assume that this region is un-structured. Indeed, in our simulations, this region remained unstructured for a total of 2.25 μ s, forming tangled loops with no clearly defined secondary structure (Fig. 3A).

Overall, both models confirm that S7 has the properties of a TM helix. The computer modelling revealed a possibility of formation of the Kcnb1-Kcng4 heterotetramer with the participation of Kcng4b-C2 and

without obvious effect on channel structure (Fig. 3A; Suppl. Movie 1) other than the flip of the C-terminal of Kcng4b-C2, making it an extra-cellular one. The *kcnng4b-C2* mutation likely affects an interaction of the Kcng4b (Kv6.4) C-terminal and Kcnb1 (Kv2.1) N-terminal (Bocksteins et al., 2014).

3.2. Kcng4b mutations differentially affect electrophysiological properties of Kv2.1 channels

To assess the functional effect of the *kcnng4b-C1* and *kcnng4b-C2* mutations on the expression and biophysical properties of Kv2.1 channel, we co-expressed these *kcnng4b* mutant variants along with the human Kv2.1 in *Xenopus* oocytes and compared the current densities and biophysical properties of the resultant channels. The co-expression of *kcnng4b-wt* and Kv2.1 resulted in a reduction of the potassium current density and shifted the steady-state inactivation curve of Kv2.1 toward the hyperpolarized potentials (Fig. 4; Table 1). These findings were consistent with the previous results obtained by co-expression of *KCNGB1* with *KCNGB4* in *Xenopus* oocytes (Müller et al., 2020) as well as in mammalian cells (Shen et al., 2016). The co-expression of Kv2.1 and *kcnng4b-C1* had a very similar effect on the biophysical properties of the channel as did the *kcnng4b-wt* (Fig. 4; Table 1). However, we observed slightly less effective modulation of Kv2.1 activity by Kcng4b-C1 compared to Kcng4b-wt (Table 1) as the analysis of peak currents at +60 mV indicated. The almost identical biophysical properties of Kv2.1/Kcng4b-C1 compared to Kv2.1/Kcng4b-wt suggest that Kcng4b-C1 forms typical heterotetramers with Kv2.1 (Fig. 4).

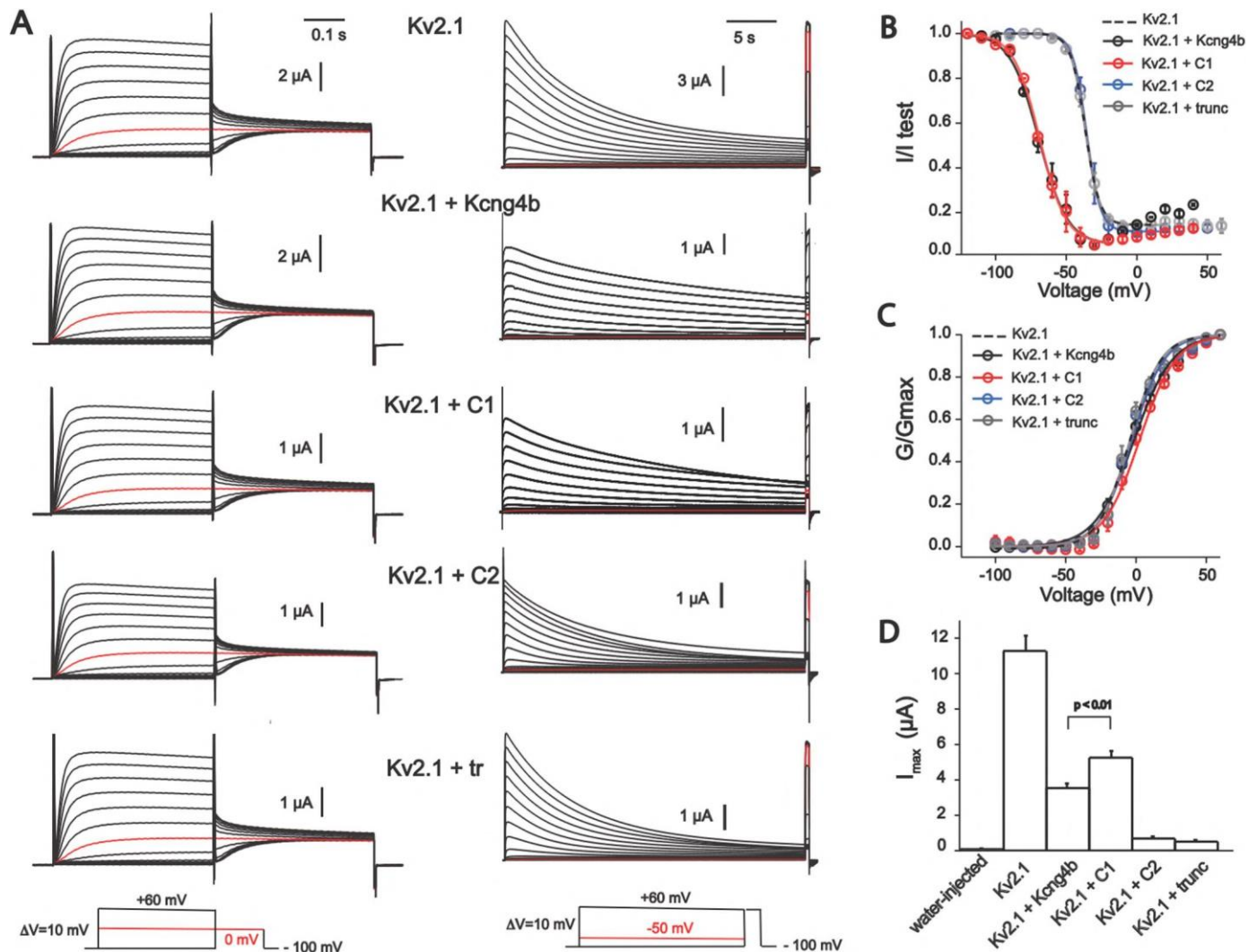


Fig. 4. Biophysical properties of homomeric Kv2.1 (KCNB1) and heteromeric Kv2.1/Kv6.4. (Kcng4b) channels studied in *X. laevis* oocytes. A - Whole-oocyte current traces of Kv2.1 homomeric and Kv2.1/Kcng4b heteromeric channels elicited by step-depolarization of the oocyte membrane from -100 mV to +60 mV with 10 mV increments (left) for estimation of steady-state activation. Current traces for estimation of the steady-state inactivation of channels is shown to the right. Current was induced by step-depolarization of membrane from -100 mV with 10 mV increments followed by short test pulse to +60 mV for GV curves. The holding potential of oocytes expressing Kv2.1+kcng4b-wt and Kv2.1+kcng4b-C1 was -120 mV in inactivation protocol. The final depolarization step is +40 mV for these channels. Current traces shown in red color correspond to 0 mV for IV protocol and -50 mV for estimation of Steady-state inactivation. B - Steady-state inactivation curves of channels. Solid curves correspond to the fit of the Boltzmann function with offset to the data points. C - Conductance-voltage (GV) relation-ship of channels shown in A. Solid curves correspond to the fit of the Boltzmann function to data. D - Current density of channels measured as peak current + 60 mV. Due to very low expression of Kv2.1 + C2 and Kv2.1 + Kcng4b-trunc channels, current traces shown in panel A and data points in B and C correspond to measurements 72–96 h post injection period whereas current amplitudes shown in D reflect 48 h after injection. Error bars in the graphs represent $\pm$ SEM. $P > 0.01$ – Student's unpaired T-test was used to compare the groups; $n \geq 7$ for all experiments. Abbreviations: C1 - kcng4b-C1; C2 - kcng4b-C2; Kcng4b-trunc - N-terminal truncated Kcng4b (Shen et al., 2016).

In contrast, a dramatic reduction of current density was observed upon co-expression of both Kcng4b-C2 and Kcng4b-trunc (Shen et al., 2016) variants with Kv2.1 (Fig. 4D). The biophysical properties of channels formed by co-injection of KCNBI mRNA with these Kcng4b variants were almost identical to those of Kv2.1 homomers (Fig. 4; Table 1), indicating that the measured currents were likely to be mediated by Kv2.1 homomers. Since the current amplitudes of KCNBI/Kcng4b-C2 and KCNBI/Kcng4b-trunc combinations are approximately 1/16 of those mediated by corresponding homomeric channels (Fig. 4D), it becomes obvious that these Kcng4b mutants exert a dominant-negative effect on Kv2.1. A similar dominant-negative effect, but to a lesser extent, was previously shown by co-expression of Kcng4b-trunc with Kv2.1 in HEK 293 cells (Shen et al., 2016). The stronger effect observed in our experiments may reflect the more precise control of injected mRNA quantity and therefore the ratio of expressed

proteins in oocytes. The simplest explanation of the substantial current reduction by co-expression of Kcng4b-C2 Kcng4b-trunc variants with Kv2.1 is that the heterotetrameric channels containing Kcng4b mutant variants are non-functional. In the case of Kcng4b-trunc, this could be explained by truncation of protein domains forming an ion permeation pathway, but retention of tetramerization domain (Fig. 3).

The explanation of the dominant-negative effect of Kcng4b-C2 on Kv2.1 is more complex and could be associated with presence of S7 transmembrane domain (Figs. 1 and 2). This extra domain may induce more global structural changes that result in nonfunctional channels. Altogether, our experiments indicate that Kcng4b-C1 likely acts as a weaker Kv2.1 modulator with partial LOF, resulting in the Kv channel GOF in zebrafish, whereas Kcng4b-C2 strongly inhibits Kv2.1, causing its LOF in zebrafish.

Table 1 Electrophysiological properties of K_v2.1 homomeric channels as well as channels resulting from co-injection of K_v2.1 with different forms of *kcng4b* protein.

Channel	Activation			Inactivation			
	V _{1/2}	K	n	V _{1/2}	K	offset	n
K _v 2.1	−3.70 ± 1.80	12.5 ± 0.42	18	−35.3 ± 2.71	5.19 ± 0.17	0.07 ± 0.02	11
K _v 2.1 + <i>kcng4b</i> - wt	−0.57 ± 1.72	13.2 ± 0.43	7	−72.6 ± 4.60	8.67 ± 0.42***	0.09 ± 0.04	7
K _v 2.1 + <i>kcng4b</i> - C1	1.31 ± 1.72	12.8 ± 0.33	12	−69.8 ± 3.75	9.01 ± 0.31***	0.07 ± 0.03	11
K _v 2.1 + <i>kcng4b</i> - C2	−2.37 ± 1.63	11.6 ± 0.37	11	−36.2 ± 0.89***	4.81 ± 0.27***	0.11 ± 0.02	9
K _v 2.1 + <i>kcng4b</i> - trunc	−4.81 ± 2.10	11.4 ± 0.42	7	−37.1 ± 1.16***	4.70 ± 0.14***	0.14 ± 0.03	7

Values are given as mean ± SEM, n indicates the number of cells analyzed for each protocol. V_{1/2} –midpoint of activation and inactivation (mV), K is the slope factor. Parameters are obtained from the Boltzmann fit of the steady-state activation and inactivation data. ***- P < 0.001, Student’s unpaired T-test.

3.3. *kcng4b* mutants develop abnormal ears

kcng4b is expressed in the developing ears at 24 and 48 hpf (Fig. 5A and B). The *kcng4b-C1*^{−/−} embryos exhibited cell delamination in the third ventricle and mild hydrocephaly at 28 hpf (Suppl. Figs. 3A–D) along with abnormality of the heart, peripheral vasculature (Suppl. Figs. 3, E and F) and ear, which is like that of the *kcng4b-trunc* mutant (Shen et al., 2016). The ear development was affected in the *kcng4b-trunc* mutant, where the ear size and number of otoliths increased (Suppl. Fig. 1). In contrast, in the *kcnb1* mutant the ear size and number of otoliths were reduced (Jedrychowska et al., 2021). In the

kcng4b-C1^{−/−} mutants, the ear was enlarged: at 28 hpf [7.3 ± 5.7 %, p = 0.01 (20 embryos, left + right ear)]; at 72 hpf [19.8 ± 7.9 %, p = 0.0001 (20 embryos, left + right ear)]. The additional third otolith was detected in abnormally high proportion during the first two days of development (Fig. 5C–F; Suppl. Figs. 4A and B) like that in *kcng4b-trunc* (Suppl. Fig. 1), but at 72 hpf it disappeared in some larvae, likely due to a fusion with the saccular otolith (Fig. 5F; Suppl. Figs. 5A and B). Unlike *kcng4b-C1*^{−/−} mutants, *kcng4b-C2*^{−/−} mutants develop relatively normally (Fig. 6A–D) except for the ears, where the opposite “no otolith” phenotype was observed for 8 days (Fig. 6E–H; Suppl. Figs. 4C and D; 6A, B).

3.4. *Kcng4b* mutations affect kinocilia of sensory cells

The abnormal development of the ears may include defects of patterning and/or differentiation of sensory cells (Jedrychowska et al., 2021). In Tg(Brn3c:GAP43-GFP)^{s356t} embryos, EGFP is expressed in the ciliated sensory cells of the ear and lateral line (Xiao et al., 2005). Analysis of *kcng4b-C1*^{−/−}/Tg(Brn3c:GAP43-GFP)^{s356t} and *kcng4b-C2*^{−/−}/Tg(Brn3c:GAP43-GFP)^{s356t} composite embryos and larvae (28 hpf and 72 hpf) did not reveal any obvious defect in the number or morphology of ear sensory cells (Suppl. Figs. 7 and 8). However, the kinocilia of the sensory cells were affected. In 28 hpf *kcng4b-C1*^{−/−} mutants, kinocilia morphology was mildly affected, and their number decreased (Fig. 7A, B, D), whereas in the 28 hpf *kcng4b-C2*^{−/−} mutants, the kinocilia size and number were severely reduced (Fig. 7A–C, D).

3.5. *Kcng4b* mutations affect larvae balance

The wild-type embryos hatched on day 3, while the *kcng4b-C1*^{−/−} and *kcng4b-C2*^{−/−} embryos fail to hatch on day 4 and were chemically dechorionated with pronase (1 mg/ml). Most of mutants were touch-responsive (Suppl. Table 1). Upon touching the wild-type embryos moved along the straight line for 2–3 s at a significant distance (Suppl.

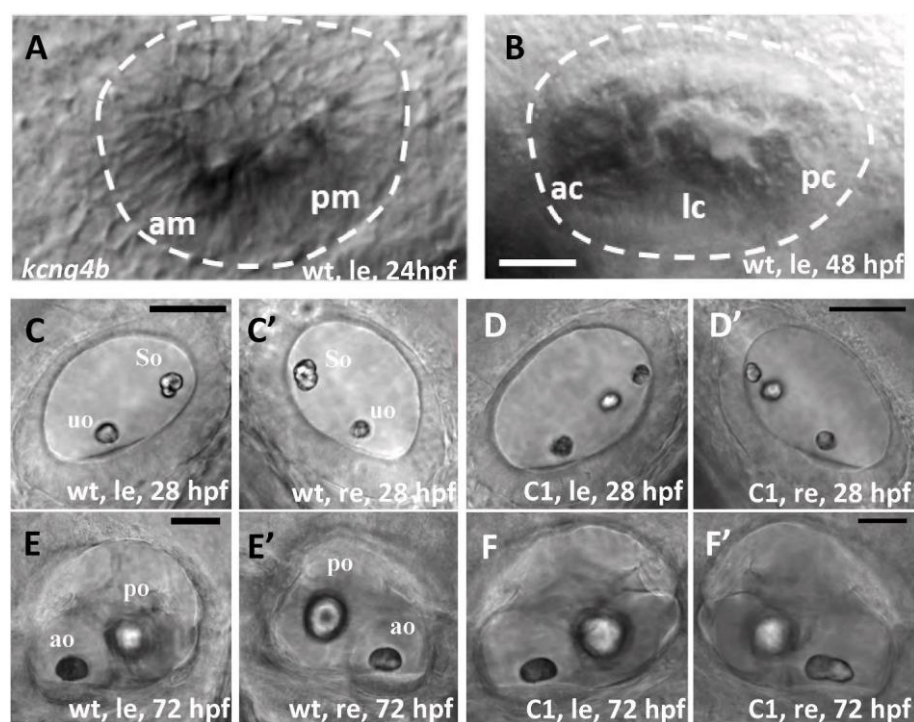


Fig. 5. *Kcng4b* plays a role in ear development. All images are lateral views (anterior towards the left). A, B – whole mount *in situ* hybridization, *kcng4b*, left ear, A – 24 h; B – 48 hpf. C–F – DIC microscopy. C–E – ears at 28 hpf; and D, F – 72 hpf, le and re – left and right ears, uo and so – utricular and saccular otoliths, correspondingly; A, B, C, E – wildtype, D, F – *kcng4b-C1* mutant. Abbreviations: C1 – *kcng4b-C1*; hpf – hours post fertilization; le – left-hand side ear; re – right-hand side ear; wt – wildtype. Scale bar = 50 μ m.

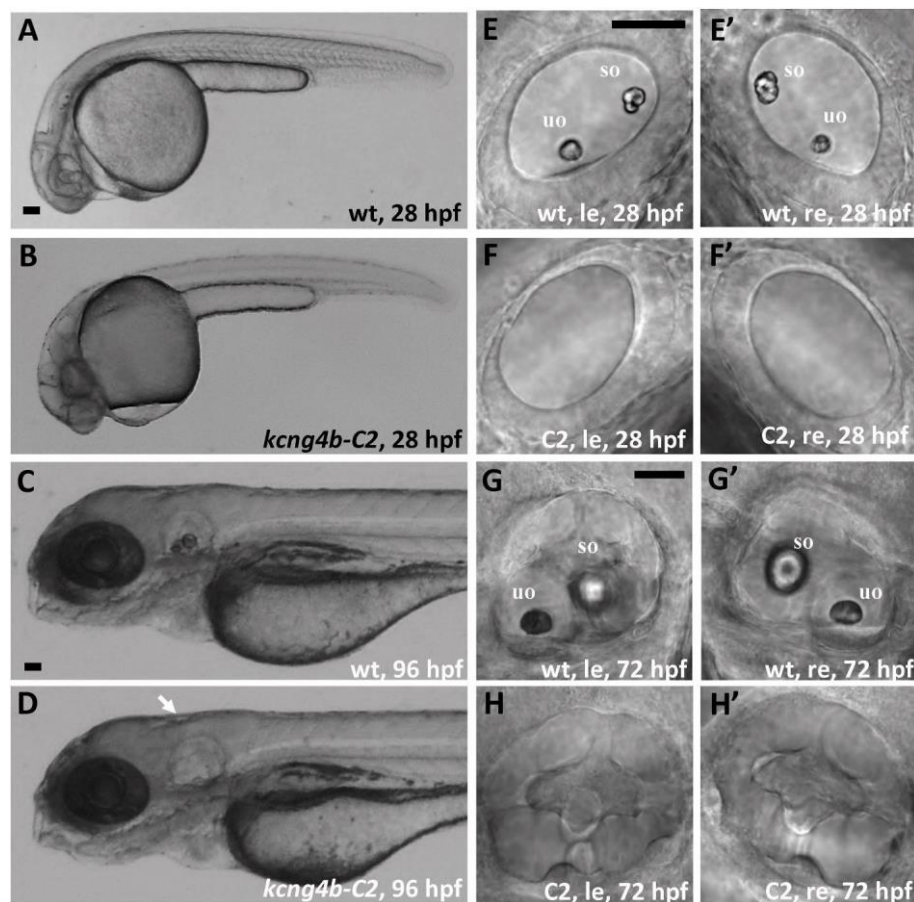


Fig. 6. *kcnng4b-C2* mutation causes an absence of otoliths. A, B – 28 hpf, arrow – ear; C, D – 96 hpf; E–H – ears at 28 (E, F) and 72 hpf (G, H), le and re – left and right ears, uo and so – utricular and saccular otoliths, correspondingly. All images are in lateral view. A, C, E, G – wild type, B, D, F, H – *kcnng4b-C2*. Abbreviations: C2 – *kcnng4b-C2*; hpf – hours post fertilization; le – left-hand side ear; re – right-hand side ear; wt – wildtype. Scale bar = 50 μ m.

Movie X). The mutant embryos moved in short burst along curved line (Suppl. Movie Y, Z).

3.6. *Kcnng4b* mutations affect expression of multiple genes that regulate ear development

We have previously shown that the *Kcnb1* LOF causes changes in gene expression (Jedrychowska et al., 2021). Here, we asked whether it is also true for mutations affecting the modulatory “silent” subunit of Kv2.1, *Kcnng4b*. The two LOF mutants *kcnng4b-trunc* and *kcnng4b-C1* show rather similar phenotypes: enlarged ear and increased number of oto-liths. In contrast, the *kcnng4b-C2* mutants show the small ears and “no otoliths” phenotype. This phenomenon could be faithfully reproduced using the morpholino- or CRISPR-mediated knockdown (Shen et al., 2016). Aiming to address a cause for developmental changes in these mutants, we started with the comparative RNAseq analysis of tran-scriptomes of the *Kcnng4b*-deficient embryos (crispants and morphants) by RNAseq (Suppl. Fig. 9). This analysis revealed changes in transcrip-tion of several genes known to play a role during development of the ear and otoliths.

These results were used as a guide to select the candidate genes for qRT-PCR analysis of the *kcnng4b-C1* and *-C2* mutants at 24, 48 and 72 hpf (Suppl. Table 2). We focused on genes expressed in the mechanosensory hair cells, sensory patches, and otic vesicles. First, genes whose expression was affected in the same way at all three developmental stages (marked in bold black font in the Suppl. Tables 3–5). These included *atp1b2b*, *atp2b1a*, *cldn7b*, *dachb*, *myo6b*, *sox9a*, i.e., the genes expressed in the mechanosensory hair cells and otic vesicle. Second, the

genes, whose expression varied from stage to stage, suggesting feedback autoregulation (marked in bold magenta in the Suppl. Tables 3–5) – *igfbp3*, *kcnng4b*, *mif*, *otofa*, *otogl*, *otop1*, *prom1a*, *sparc*, *wwc1*. Most of these genes were associated with otoliths formation. Third, *cldn7b*, *fgf3*, *fgf8a*, *gas8*, *gsdmeb*, *hapln1a*, *jag1* were expressed in two mutants in opposite ways during at least one developmental stage, which seems to be a common theme for changes in expression of all genes.

Previously, it was shown that in *Kcnb1* mutants, *otop1* expression increased in parallel with reduction in otolith number (Jedrychowska et al., 2021). According to the qRT-PCR results, *otop1* expression was significantly affected in the *Kcnng4b* mutants. The whole mount *in situ* hybridization analysis of *otop1* expression supported these results and showed that the level of *otop1* expression changed dramatically. It was decreased in the *kcnng4b-C1* mutants (Fig. 8A and B) and increased in the *kcnng4b-C2* mutant (Fig. 8A–C), like that in the *Kcnb1* LOF mutant.

4. Discussion

The plasma membrane (PM) potential (V_m) plays a role in cell sur-vival, proliferation and regulation of transcription (Levin, 2014). Mod-ulation of the electrically active KCNB1 subunit by the electrically silent KCNG4 subunit of the Kv channel controls channel activity (Bocksteins, 2016). The interaction of *Kcnb1* and *Kcnng4b* regulates cell survival, proliferation and development of zebrafish BVS, where *Kcnng4b* LOF or *Kcnb1* GOF increases and *Kcnng4b* GOF or *Kcnb1* LOF decreases Kv channel activity (Shen et al., 2016). Whereas the two genes are co-expressed in the BVS, eyes, and ear, their expression pattern differs in the somites, where only *kcnb1* is expressed and intersegmental vessels,

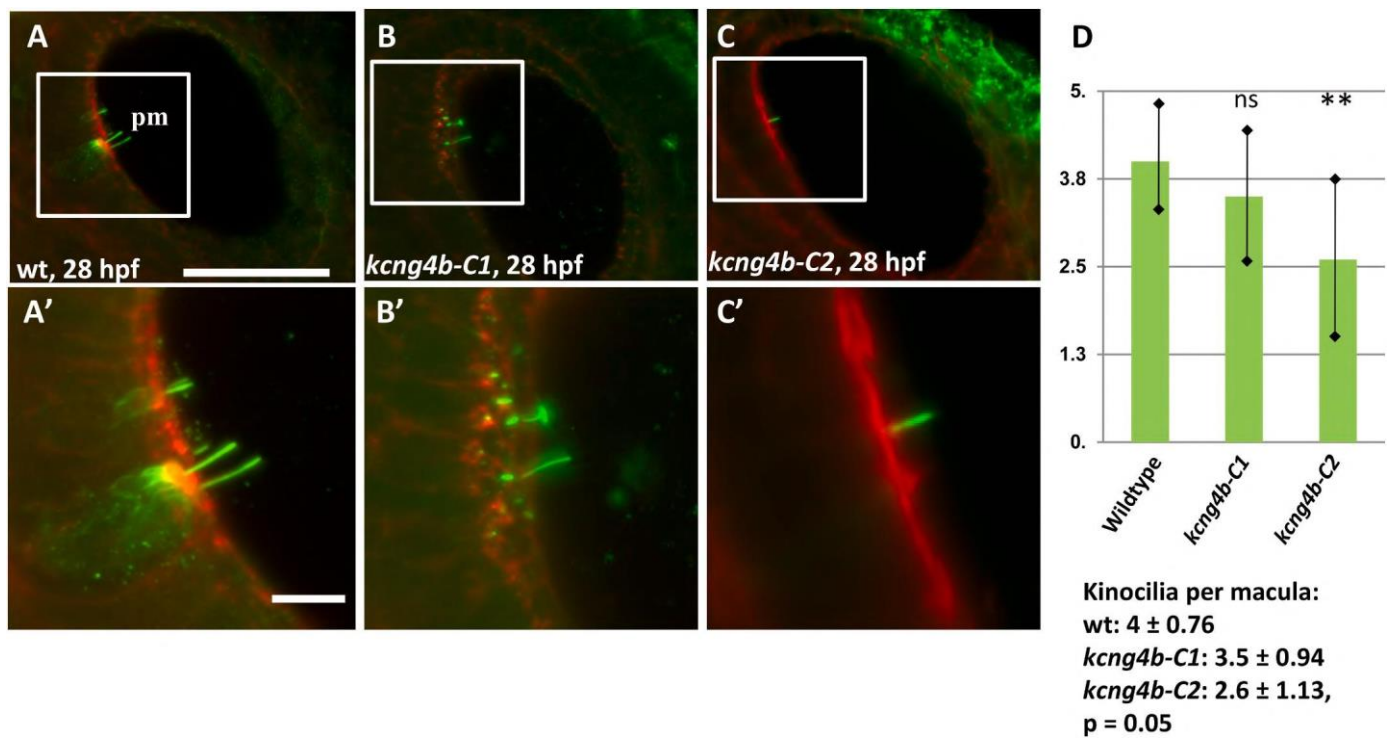


Fig. 7. The development of inner ear sensory cells is affected in *kcng4b-C1* and *kcng4b-C2* mutants. Immunohistochemical staining using the anti-acetylated tubulin antibody (green)/phalloidin (red), 28 hpf, right-hand side ear. A – wild type, B – *kcng4b-C1*, C – *kcng4b-C2*, D – number of kinocilia per macula.

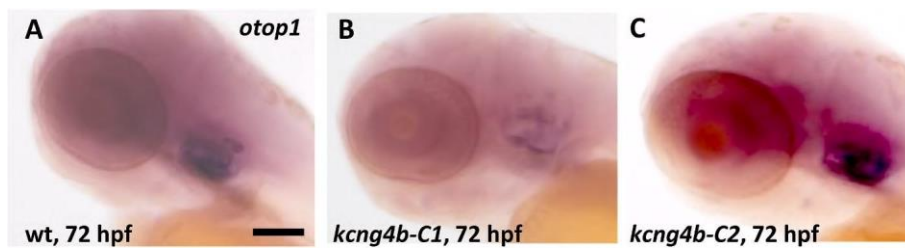


Fig. 8. The expression of *otop1* was affected in *kcng4b-C1* and *kcng4b-C2* mutants as detected by whole mount *in situ* hybridization at 72 hpf. A – wildtype, B – *kcng4b-C1*, C – *kcng4b-C2*, Scale bar, 100 μm.

where only *kcng4b* is expressed. The expression patterns of the two genes also differ temporarily, when only *kcng4b* expression has been detected so far at 8–9 hpf (Shen et al., 2016). This suggests that while these subunits may interact in the BVS, eyes and ears, a different mechanism of Kcnb1 (or another electrically active subunit) modulation may be in place in somites (and intersegmental vessels).

As far as the ear development is concerned the Kcnb1 and Kcng4b seem to act by antagonizing each other activity like that shown previously for the developing BVS (Shen et al., 2016). Noteworthy is the significant difference in the otoliths development caused by the two novel mutations presented here, which, unlike the *kcng4b-trunc* mutation affecting the N-terminal, affect the Kcng4b C-terminal.

The difference may be due to the structural difference of the putative mutant proteins. Kcng4b-C1 represents a milder version of the *kcng4b-trunc* mutation causing Kcng4b LOF, which according to electrophysiological data (Table 1) is less efficient in modulating the channel compared to the wildtype Kcng4b (Table 1), likely resulting in the mild channel GOF. It looks that a small increase in channel activity detected *in vitro* triggers a rather distinct developmental effect (Fig. 5). It may be less surprising since the mild LOF effect detected *in vitro* likely is further enhanced by the progressive reduction of Kcng4b-C1 transcript *in vivo* (Suppl. Tables 3–5). Thus, the developmental effect may represent the

sum of the mild LOF of a putative polypeptide and *kcng4b* transcript reduction.

In contrast, the ectopic TM domain of Kcng4b-C2 causes a dominant negative effect on Kcnb1 *in vitro* (Table 1), which alone can strongly inhibit Kv channel activity. Of interest is a difference in the functional effects of the two potentially inhibitory forms of Kcng4b – Kcng4b-trunc and Kcng4b-C2 (Table 1; Fig. 6; Suppl. Fig. 1). This could be due to the differential stability of these two mutant mRNAs *in vivo*. The electro-physiological experiments were performed in *Xenopus* oocytes (Fig. 4), where all heterologous mRNAs are stable unlike the short *kcng4b-trunc* mRNA (Shen et al., 2016). Its net effect *in vivo* is the Kv channel GOF. The mRNA of Kcng4b-C2 is longer than that of the wild type Kcng4b and relatively stable *in vivo*. While its level in the mutant at 24–48 hpf is moderately downregulated (Suppl. Tables 3–5, entry 12), it increases rather significantly by 72 hpf (Suppl. Table 5, entry 12). Thus, it may be translated into the dominant-negative protein, which causes Kv channel LOF.

The electrophysiology data (Fig. 4, Table 1) suggest that Kcng4b-C2 suppresses the current amplitude of Kv2.1 like Kcng4b-trunc. Thus, despite structural changes caused by the mutation in the C-terminal by the appearance of ectopic 7TM domain, at least some interaction may take place between Kcnb1 and Kcng4b-C2. The remaining current

kinetics are almost identical to those of Kcnb1 homomers, suggesting that interaction with Kcng4b-C2 renders the channel non-functional. This contrasts with Kcng4b-C1, which has similar but functionally weaker characteristics compared to Kcng4b-wt.

These data suggested that a fraction of Kcnb1 homomers remains active in the *kcnb1*-C2 mutant. It may explain the lack of phenotype in the brain (Fig. 5), where the activity of the Kcnb1 homomer fraction may be just sufficient to avoid cell delamination. In contrast, in the ear, which is known to have a much higher concentration of K ions in the endolymph, this activity may not be sufficient to maintain otoliths development.

The spectrum of KCNB1 human heterozygotic mutant phenotypes includes a number of defects ranging from epileptic encephalopathy to developmental delay and speech defects (Bar et al., 2020; Latypova et al., 2017; Marini et al., 2017; Veale et al., 2022). Previously, our study demonstrated a reduction in otic vesicle size and number of otoliths associated with hearing and balance defects in zebrafish *kcnb1* LOF mutants (Jedrychowska et al., 2021). In the absence of otoliths in Kcng4b-C2, the developing zebrafish is likely to lack proper balance, as it was shown for embryos deficient in otoliths/otoconia (Hughes et al., 2004; Jedrychowska et al., 2021; Lundberg et al., 2015; Sołłner et al., 2004). This illustrates that the role of Kcnb1-Kcng4b axis in the inner ear development is conserved in evolution between teleosts and mammals. The changes of membrane potential and ion flows play a role in regulation of developmental signaling pathways (Levin, 2014). The

changed membrane potentials and levels of K⁺ and other ions were shown to trigger changes in gene expression (Adams et al., 2016; Jedrychowska et al., 2021; Paine et al., 1981; Shen et al., 2016). The expression profiles of several developmental genes in different mutant alleles of the Kv silent subunit largely correlate with the observed developmental changes (Figs. 5, 6 and 8; Suppl. Tables 3–5).

Language development is closely linked to hearing (Anne et al., 2017). In some human heterozygotic patients carrying a KCNB1/Kv2.1 mutation, speech development is impaired (Latypova et al., 2017; Marini et al., 2017; Bar et al., 2020). The current study of the two *kcnb1* mutants shows that different mutations affecting the silent α -subunit of the Kv channel can cause both LOF and GOF of the silent subunit, like what has been shown for the human electrically active subunit KCNB1. Given the antagonistic interaction between the two types of subunits (Shen et al., 2016), the LOF and GOF mutations of Kcng4 may respectively result in the GOF and LOF effects on the functional hetero-tetramers of Kv.

CRediT authorship contribution statement

Justyna Jedrychowska: Writing – original draft, Methodology, Investigation. **Vitya Vardanyan:** Writing – original draft, Methodology, Investigation. **Milosz Wieczor:** Writing – original draft, Methodology, Investigation, Conceptualization. **Antoni Marciniak:** Writing – original draft, Methodology, Investigation. **Jacek Czub:** Writing – original draft, Supervision, Conceptualization. **Razieh Amini:** Methodology, Investigation. **Ruchi Jain:** Methodology, Investigation. **Hongyuan Shen:** Methodology, Investigation. **Hyungwon Choi:** Validation, Formal analysis, Data curation. **Jacek Kuznicki:** Writing – review & editing, Supervision, Resources. **Vladimir Korzh:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used DeepL Write in order to improve language and readability. After using this tool/ service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ydbio.2024.03.002>.

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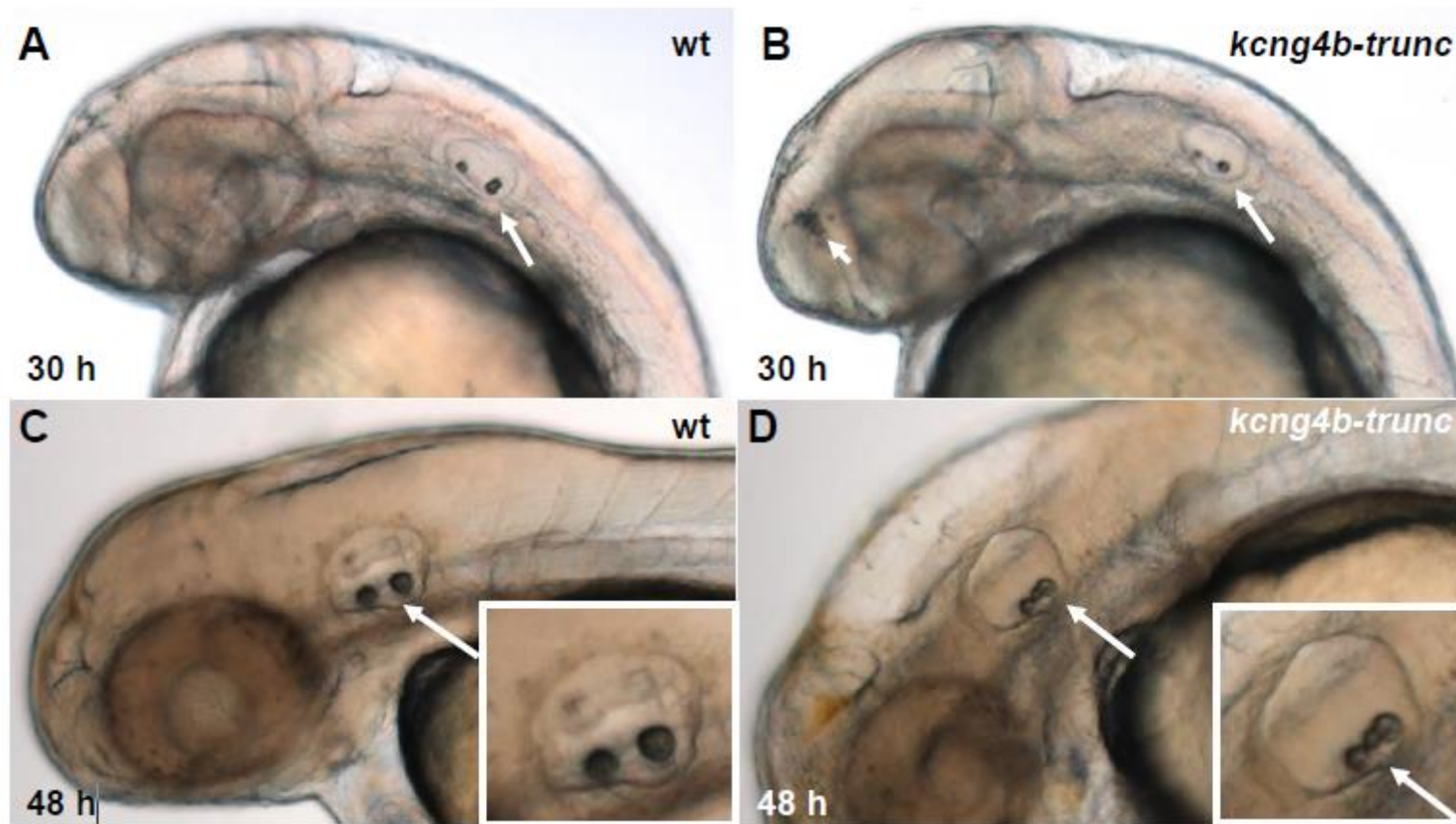


Fig. S1

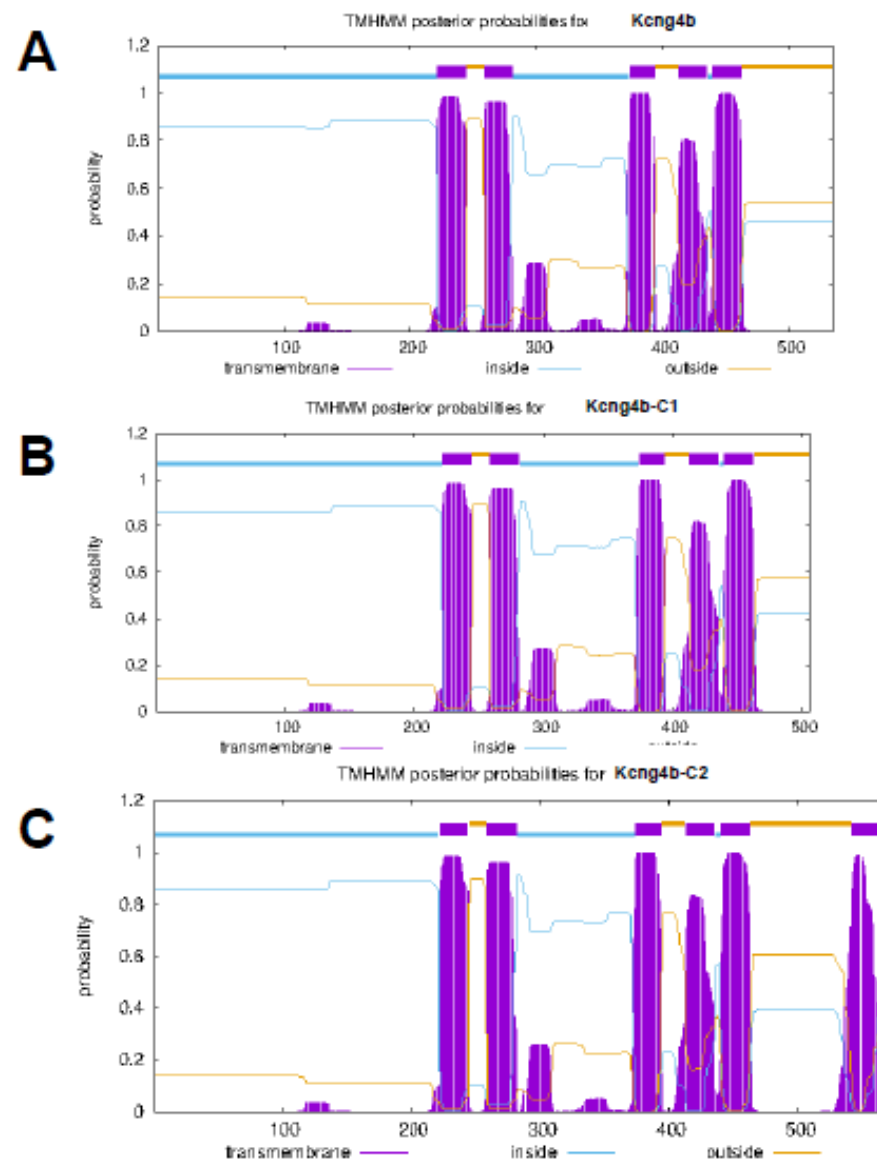


Fig. S2

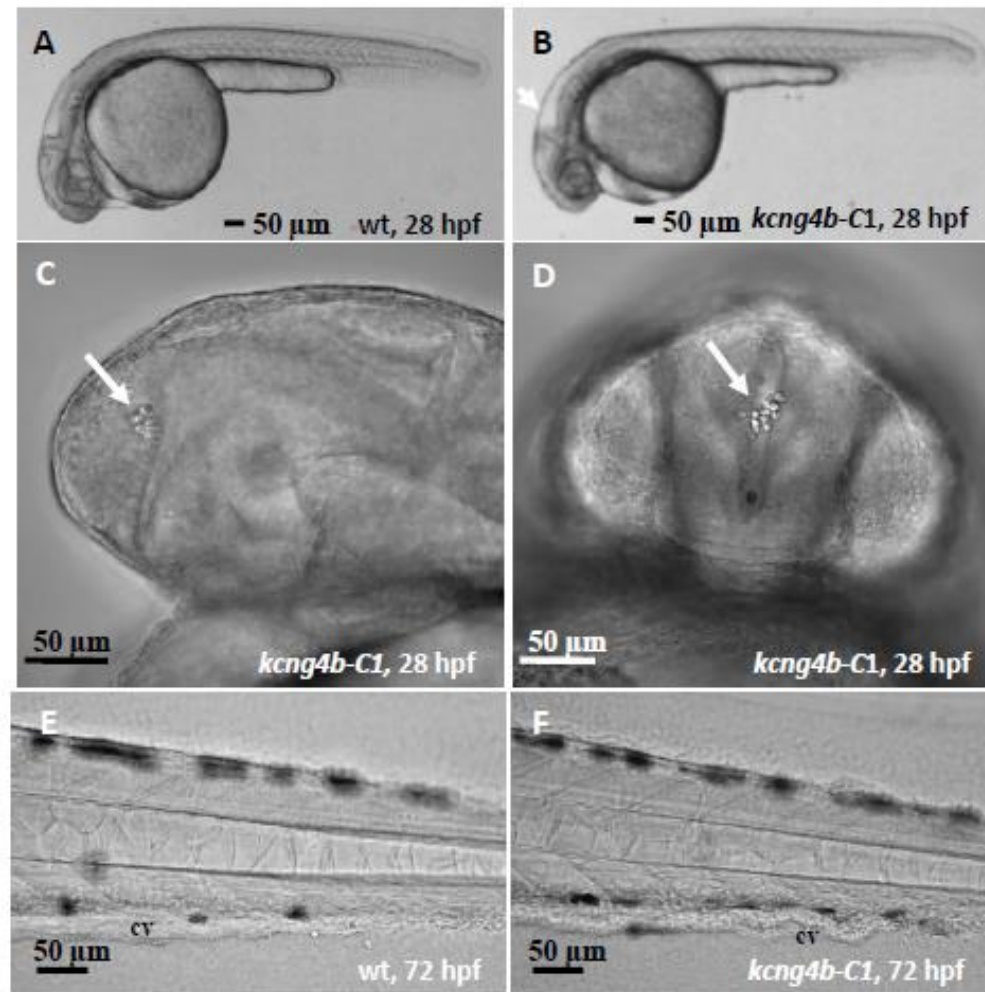
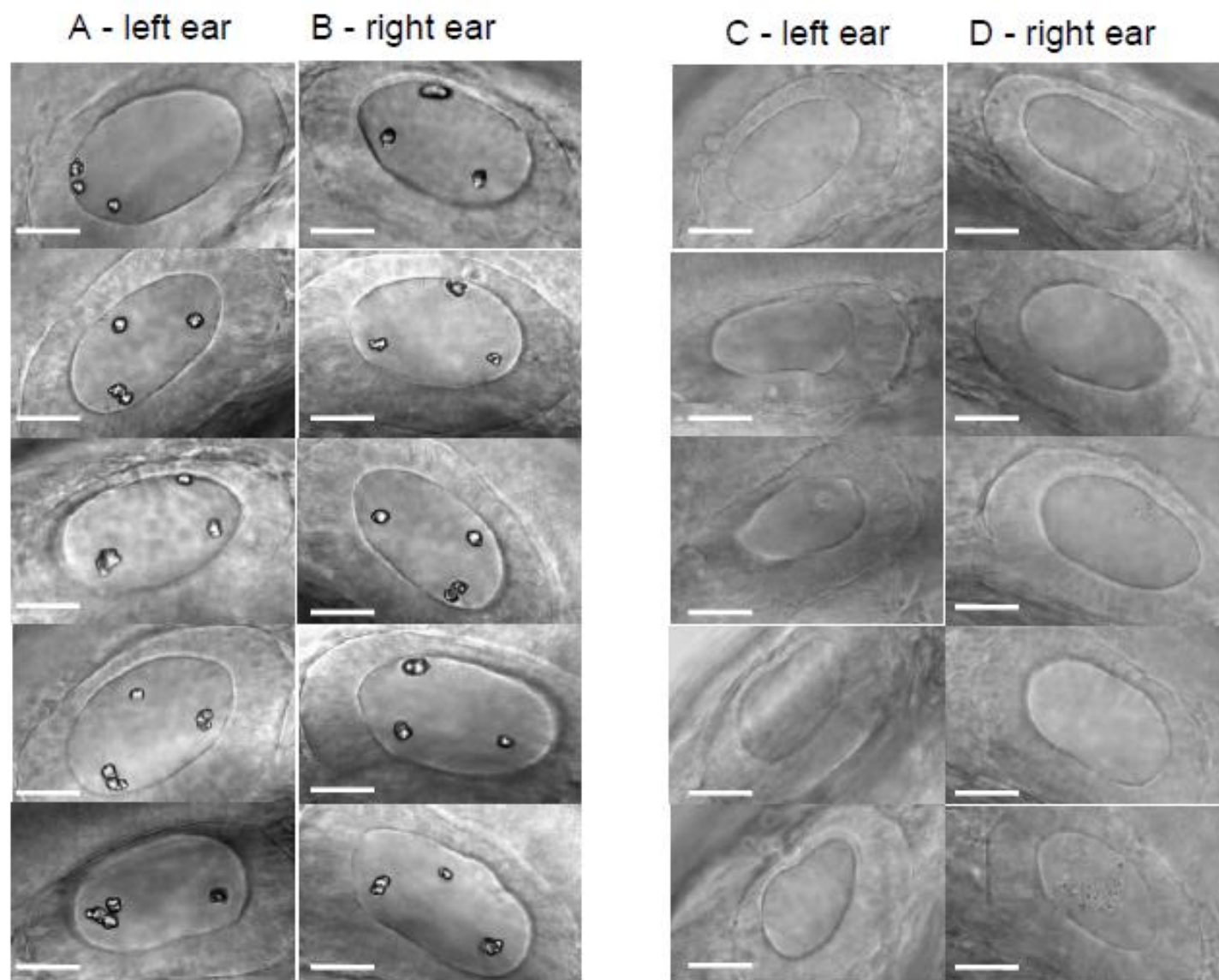


Fig. S3



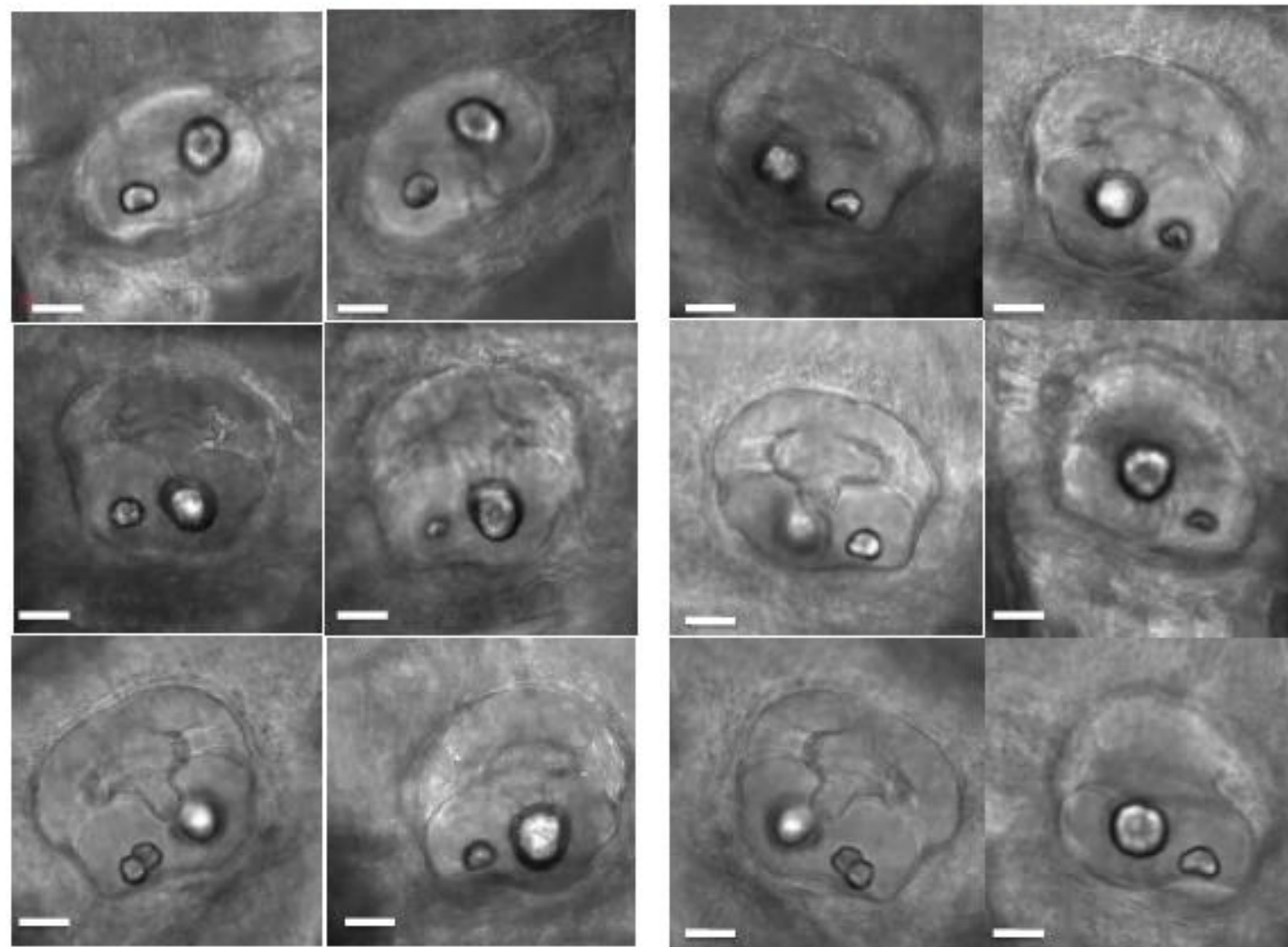
kcng4b-c1^{-/-} 28 hpf, n=5

Fig. S4

kcng4b-c2^{-/-} 28 hpf, n=5

A - left ear

B - right ear

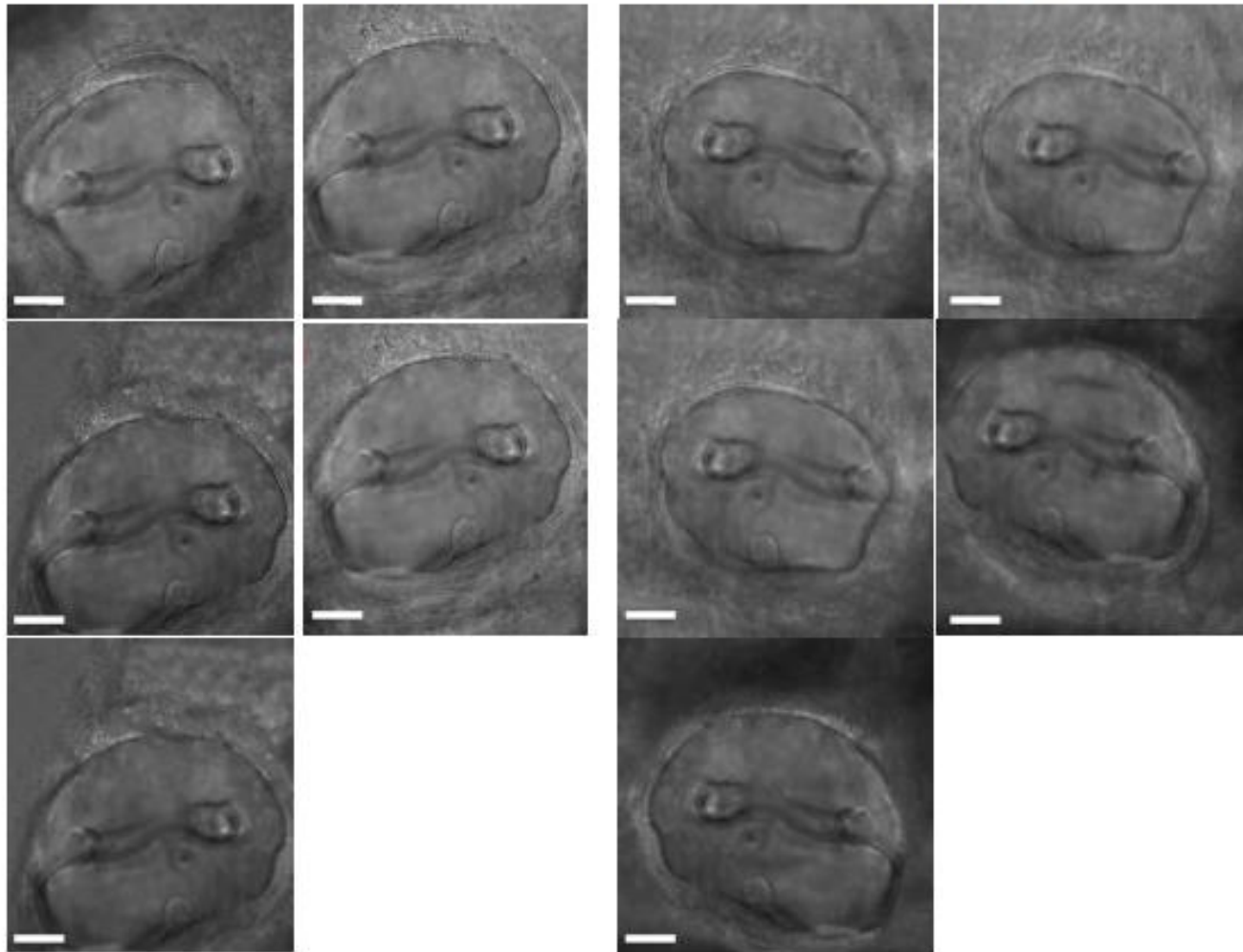


kcng4b-c1^{-/-} 72 hpf, n=6

Fig. S5

A - left ear

B - right ear



kcng4b-c2^{-/-} 72 hpf, n=5

Fig. S6

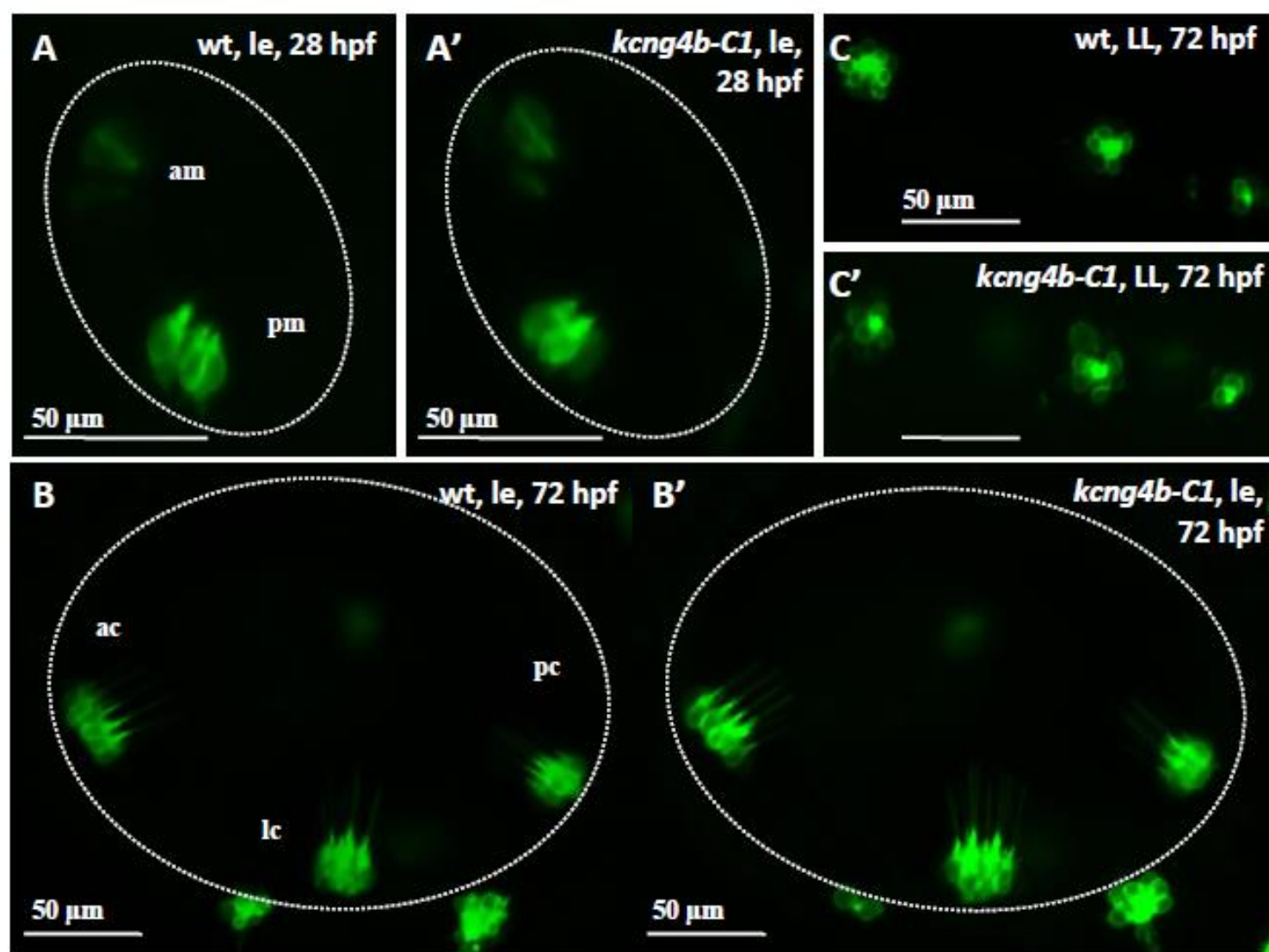


Fig. S7

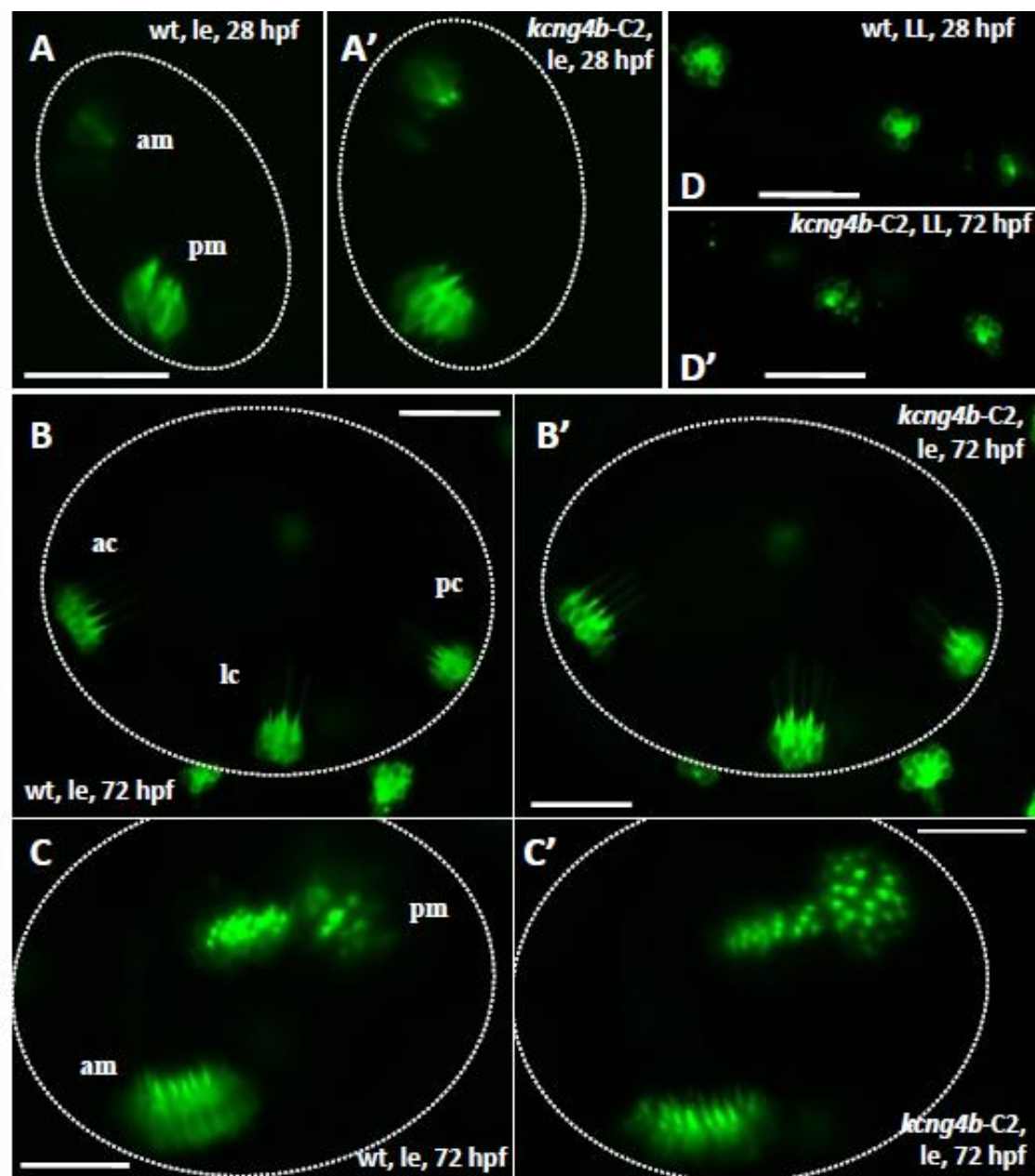


Fig. S8

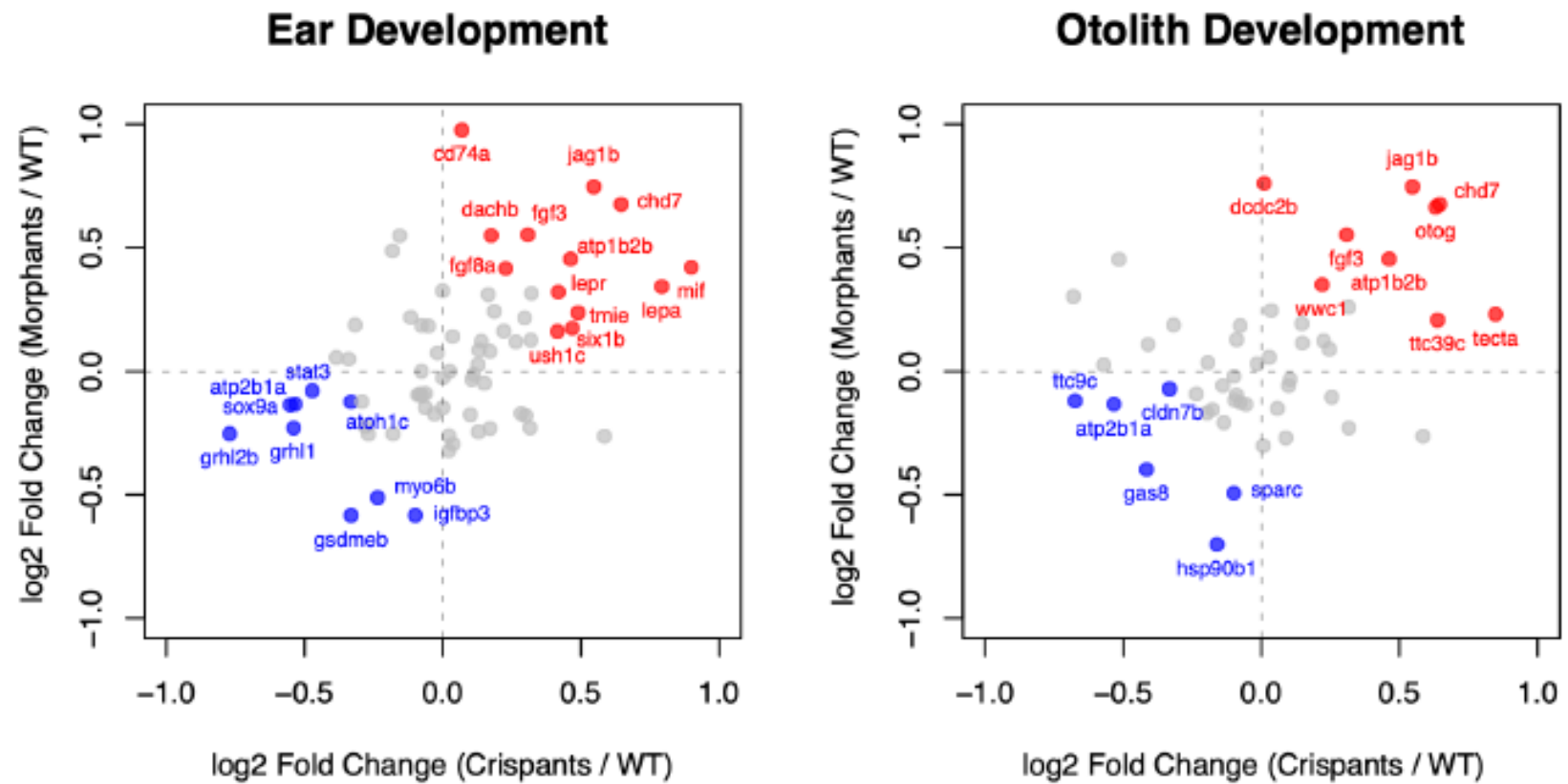


Fig. S9

Supplemental Table 1

	Wild-type	kcng4b-C1 -/-	kcng4b-C2 -/-
Total sample	70 (N=3)	70 (N=3)	21(N=2)
Responsive	68	68	19
Movement	Movement towards Petri dish periphery	Short burst	Short burst

N	Genes	qRT-PCR primers, 5' - > 3'
1	<i>atp1b2b</i>	GCGGCGGTTTGAAGAACTTT TCAGTCCACTCATCTTTCCCA
2	<i>atp2b1a</i>	CAGATCCTCTGGTTTAGAGG TCCGAGTCCTCTATCCGG
3	<i>cldn7b</i>	GCCTTTGGATGTCGTGCGCT GCCACACCCAGTCCAGCTACTG
4	<i>dachb</i>	CAGCGAGAGAATAAGGAAA TGGAATGTTGTGAGGATG
5	<i>fgf3</i>	CCCAAGGGCGCCTTGTGCCAGGG CCGTGTTTTAAAGCCCCTCCTGG
6	<i>fgf8a</i>	GCCGTAGACTAATCCGGACC TTGTTGGCCAGAACTTGCAC
7	<i>gas8</i>	ACGAGTTTGCAAGGCCCATAA TGGCCAACACTCTGTCCATT
8	<i>gsdmeb</i>	TATGAACTGTGTCTGCTG ATCTGCTGGAATAACGAG
9	<i>hapln1a</i>	GGTGTGAGTTCATCGATGGG CAAACGAGGGGAGTATGGGA
10	<i>igfbp3</i>	ATCTCACGGACACGACAAGC GATGGACCGTCTCAGCTTCC
11	<i>jag1b</i>	GTGTGGATGGTGAAAACCTGGT GGGAGAAGACTGACACTCATTTATATT
12	<i>kcng4b</i>	ATTGCAGTGACAGTTATCAGC AGCGCAACAAAACTCCATGG
13	<i>mif</i>	GCGCAGAATAAACAATACTCC TCCAAAGGTGCTGTTGTTCC
14	<i>myo6b</i>	TTTGAATGAGGCCACTCTCC CAAAGAGCGTCCCTGATAGC
15	<i>otofa</i>	CACTGAAGTACACATGGAGC GGTGACTTCAAAGCTGATGG
16	<i>otogl</i>	CAAAATGACCGAAGCGCACA GCCCAGCTGTTCCCAAATTC

17	<i>otop1</i>	ATCTCTCAGGTACGGTACG CGTCTCCTGTGGTTGTGC
18	<i>prom1a</i>	CGCTGCTGTGATAACTGTGG CTCAGGTTCTGATTGGCTGC
19	<i>sox9a</i>	AGCACATCAGCTACGGTTCCTTCA TCTGACCTCCAGCATGGGTGTAAT
20	<i>sparc</i>	TTGAGGTCGTGGAGGATGTT GTATTTGCAGGGTCCGATGT
21	<i>stat3</i>	TGCCACCAACATCCTAGTGT GCTTGTTTGCACTTTGTACTGA
22	<i>tecta</i>	CCTGGTATTGAGGGAGAGGAC CGATCCACACCGTGTCTTG
23	<i>tmie</i>	GGTGCAAAGAAGGTAAAGAAGAGG GCTTCTTTCTTTGGTGCTCCT
24	<i>ttc39c</i>	GGGAGAATAGATGAAGCC GGAAGGTGAGGAATGGTA
25	<i>wwc1</i>	CGTTCTTCACTGTCTTCC TTGTTTGTGTCCTGGTAG
House- keeper	<i>eef1a111</i>	AAAATCGGTGGTGCTGGCAA GGAACGGTGTGATTGAGGGA

Supplemental Table 3.

Comparison of expression of selected genes in the 24 hpf *kcng4b* mutants.

N	Genes	Expression level ratio, <i>kcng4b</i> -C1 / wt, SD	p value	Expression level ratio, <i>kcng4b</i> -C2 / wt, SD	Comment
1	<i>atp1b2b</i>	0.34± 0.27	0.01	2.41±0.05	hair cells, semicircular canal
2	<i>atp2b1a</i>	0.05±0.90	0.05	7.04±0.008	hair cells
3	<i>cldn7b</i>	1.12±0.17	0.05	2.35±0.009	otic vesicle, etc
4	<i>dachb</i>	0.57±0.64	n. s	4.62±0.36	otic vesicle, etc
5	<i>fgf3</i>	2.97±0.59	0.05	1.87±0.005	otic vesicle, etc
6	<i>fgf8a</i>	2.80±0.63	0.05	1.65±0.02	otic vesicle, etc
7	<i>gas8</i>	2.76±2.58	n. s	4.43±0.002	otic vesicle, not specific
8	<i>gsdmeb</i>	1.79±0.18	0.01	1.28±0.05	otic vesicle, not specific
9	<i>hapln1a</i>	1.31±0.47	n. s	6.01±0.1	semicircular canals, etc
10	<i>igfbp3</i>	0.26±2.20	0.05	1.61±0.02	otic vesicle, macula
11	<i>jag1b</i>	0.17±0.60	0.001	1.72±0.008	otic vesicle, etc
12	<i>kcng4b</i>	3.43±0.79	0.05	0.63±0.02	ependyma
13	<i>mif</i>	1.47±0.02	0.0001	0.37±0.04	otic vesicle, etc
14	<i>myo6b</i>	0.2±1.64	0.05	5.4±0.006	hair cells
15	<i>otofa</i>	0.19±0.27	0.05	1.71±0.07	hair cells
16	<i>otogl</i>	0.33±0.72	0.05	1.71±0.08	hair cells, otolith

17	<i>otop1</i>	0.15±0.04	0.0001	3.17 ±0.1	otic vesicle, etc
18	<i>prom1a</i>	1.76±1.40	0.01	0.76±0.12	GFP-tagged in ET33-mi2a
19	<i>sox9a</i>	0.38±0.82	0.05	5.27±0.09	otic vesicle, etc
20	<i>sparc</i>	0.64±0.35	0.05	1.88±0.12	otic vesicle, etc
21	<i>stat3</i>	0.30±0.91	0.05	0.55±0.009	otic vesicle, etc.
22	<i>tecta</i>	0.46±0.92	0.05	0.65±0.07	hair cells
23	<i>tmie</i>	1.17±0.06	0.001	1.55±0.09	otic vesicle, etc
24	<i>ttc39c</i>	1.18±1.08	n. s	7.48±0.07	brain
25	<i>wwc1</i>	1.44 ±0.25	0.05	0.69±0.06	not known

Supplemental Table 4.
Comparison of expression of selected genes in the 48 hpf *kcng4b* mutants.

N	Genes	Expression level ratio, <i>kcng4b</i> -C1/ wt, SD	p value	Expression level ratio, <i>kcng4b</i> -C2 / wt, SD	Comment
1	<i>atp1b2b</i>	0.70±0.089	0.05	3.31±0.03	hair cells, semicircular canal
2	<i>atp2b1a</i>	0.57±0.32	0.05	3.07±0.07	hair cells
3	<i>cldn7b</i>	0.28±1.76	n. s	2.85±0.008	otic vesicle, etc
4	<i>dachb</i>	0.55±1.98	n. s	1.58±0.03	otic vesicle, etc
5	<i>fgf3</i>	1.21±1.52	n. s	0.82±0.005	otic vesicle, etc
6	<i>fgf8a</i>	0.83±0.15	n. s	0.88±0.06	otic vesicle, etc
7	<i>gas8</i>	2.64±1.12	0.05	2.06±0.01	otic vesicle, etc
8	<i>gsdmeb</i>	0.53±0.02	0.05	9.16±0.1	not specific
9	<i>hapln1a</i>	0.45±1.42	0.05	1.90±0.03	semicircular canals, etc
10	<i>igfbp3</i>	0.19±0.74	0.01	0.27±0.16	otic vesicle, macula
11	<i>jag1b</i>	0.17±0.60	0.01	0.27±0.08	otic vesicle, etc
12	<i>kcng4b</i>	0.60±0.28	0.05	0.68±0.03	ependyma
13	<i>mif</i>	0.51±0.21	0.05	5.21±0.18	otic vesicle, etc
14	<i>myo6b</i>	0.2±0.26	0.001	2.16±0.02	hair cells
15	<i>otofa</i>	2.46±0.72	0.05	0.47±0.05	hair cells
16	<i>otogl</i>	3.75±1.62	0.01	0.34±0.05	hair cells, otolith
17	<i>otopl</i>	0.04±0.001	0.05	2.57 ±0.25	otic vesicle, etc

18	<i>prom1a</i>	0.62±0.31	n. s	0.61±0.14	GFP-tagged in ET33-mi2a
19	<i>sox9a</i>	0.33±0.55	0.05	7.45±0.22	otic vesicle, etc
20	<i>sparc</i>	1.50±0.21	0.05	3.56±0.01	otic vesicle, etc
21	<i>stat3</i>	0.44±0.4	0.05	0.60±0.001	otic vesicle, etc.
22	<i>tecta</i>	0.38±0.58	0.05	0.29±0.09	hair cells
23	<i>tmie</i>	2.07±0.12	0.05	0.48±0.07	otic vesicle, etc
24	<i>ttc39c</i>	5.00±0.50	0.05	0.63±0.04	brain
25	<i>wwc1</i>	0.6±1.62	n. s	2.21±0.16	otic vesicle, etc

Supplemental Table 5.
Comparison of expression of selected genes in the 72 hpf *kcng4b* mutants.

N	Genes	Expression level ratio, <i>kcng4b</i> -C1/ wt, SD	p value	Expression level ratio, <i>kcng4b</i> -C2 / wt, SD	Comment
1	<i>atp1b2b</i>	0.13± 1.54	0.01	9.57±0.003	hair cells, semicircular canal
2	<i>atp2b1a</i>	0.15±0.01	0.01	2.11±0.01	hair cells
3	<i>cldn7b</i>	0.75±0.48	0.05	1.54±0.001	otic vesicle, etc
4	<i>dachb</i>	0.07±0.83	0.01	1.62±0.01	otic vesicle, etc
5	<i>fgf3</i>	3.15±0.49	0.05	0.46±0.01	otic vesicle, etc
6	<i>fgf8a</i>	1.75±0.18	0.01	0.8±0.04	otic vesicle, etc
7	<i>gas8</i>	0.62±0.11	0.05	1.90±.026	otic vesicle, etc
8	<i>gsdmeb</i>	1.42±0.25	0.05	0.02±0.04	not specific
9	<i>hapln1a</i>	0.91±1.04	n. s	0.48±0.06	semicircular canals, etc
10	<i>igfbp3</i>	2.98±0.17	0.05	0.56±0.04	otic vesicle, macula
11	<i>jag1b</i>	0.25±0.4	0.01	0.54±0.07	otic vesicle, etc
12	<i>kcng4b</i>	0.28±0.22	0.01	7.05±0.01	ependyma
13	<i>mif</i>	0.76±0.17	0.01	0.01±0.02	otic vesicle, etc
14	<i>myo6b</i>	0.41±0.37	0.01	2.24±0.05	hair cells
15	<i>otofa</i>	0.37±0.43	0.01	1.11±0.03	hair cells
16	<i>otogl</i>	0.40±0.58	0.01	0.77±0.04	hair cells, otolith
17	<i>otopl</i>	0.06±0.006	0.001	1.44 ±0.2	otic vesicle, etc

18	<i>prom1a</i>	0.66±0.76	n. s		2.19±0.01	GFP-tagged in ET33-mi2a
19	<i>sox9a</i>	0.73±0.80	n. s		1.50±0.1	otic vesicle, etc
20	<i>sparc</i>	0.34±1.51	0.05		1.02±0.04	otic vesicle, etc
21	<i>stat3</i>	0.47±0.30	0.05		0.34±0.04	otic vesicle, etc.
22	<i>tecta</i>	0.45±0.41	0.01		0.66±0.06	hair cells
23	<i>tmie</i>	0.76±0.20	0.01		1.21±0.07	otic vesicle, etc
24	<i>ttc39c</i>	0.26±1.54	0.05		2.52±0.04	brain
25	<i>wwc1</i>	0.76±1.32	n. s		0.59±0.22	otic vesicle, etc

3. DISCUSSION

3.1 Role of Kv2.1/Kv6.4 axis in Reissner fiber development (Manuscript 1-Major contribution)

Our findings support the view that Kv2.1 influences RF assembly. Because RF is an acellular structure formed through the secretion and polymerization of Sspo, the effects of Kv2.1 are most plausibly mediated through regulation of secretory processes in the SCO and floor plate (Rodríguez et al., 1998; Meiniel et al., 2008; Muñoz et al., 2019). Although membrane excitability and membrane potential may contribute to secretion, the present data do not distinguish between conductance-dependent and non-conductance-dependent roles of Kv2.1 in this context (Tamkun et al., 2007; Deutsch et al., 2012; Johnson et al., 2019).

Mutations affecting Kv2.1 subunits produced strong and opposing effects on anterior RF formation. Reduced Kv2.1 activity was associated with expansion or duplication of the anterior RF, while increased Kv2.1 activity led to reduction or loss of the anterior RF. These phenotypes were accompanied by changes in RF-associated transcript levels, altered Sspo distribution, and differences in SCO apical surface topology and SCO-derived microfilament organisation. Together, these observations place Kv2.1 upstream of the anterior RF assembly, exerting a net negative influence on the early steps required for RF formation (Shen et al., 2016; Amini et al., 2026). In this framework, changes in SCO morphology are more likely to be downstream effects of altered RF-dependent ventricular conditions than primary defects. This interpretation aligns with the idea that SCO architecture is sensitive to the mechanical and fluid environment of the ventricular system and may participate in feedback interactions involving membrane tension and local cytoskeletal forces (Schmidt et al., 2012; Porazinski et al., 2015; Voltes et al., 2019; Takahashi et al., 2021).

Available expression data further suggest that Kv2.1 influences RF-associated transcription indirectly. While *sspo* expression is confined to the SCO and floor plate, Kv2.1 subunits seem to be expressed more broadly in the CSF-facing ventricular lining. This distribution supports a hierarchical organization where broadly expressed regulators affect more spatially restricted effectors. In line with this, whole-embryo qRT-PCR data show that Kv2.1 modulates *sspo* expression in a stage-dependent and context-dependent manner, aligning with the previously noted antagonistic roles of Kv2.1 subunits during BVS development (Shen et al., 2016; Jędrychowska et al., 2024). Since a direct transcriptional role for voltage-gated potassium channels is unlikely, these effects are more plausibly mediated indirectly through signaling and membrane-associated processes (Johnson et al., 2018; Zarain-Herzberg et al., 2011).

Across different biological systems, Kv2.1 has been linked to regulated secretion, supporting the view that secretion is a central functional axis affected by Kv2.1 activity (Jacobson et al., 2007; Jędrychowska & Korzh, 2019; Jensen et al., 2017). At the same time, the effect is unlikely to be limited to Sspo alone, because additional RF-associated factors, including *Chl1a/Camel* and galectins, are also altered in Kv2.1-subunit mutants (Muñoz et al., 2019; Yang et al., 2021). The relationship between Kv2.1 and Chl1a/Camel appears to be partially convergent rather than strictly linear, implying that Kv2.1 acts through additional mechanisms, likely at the level of ventricular architecture and the cellular organisation required for Sspo secretion (Yang et al., 2021; Amini et al., 2026).

RF formation is a staged developmental process involving transient floor plate-derived and later SCO-derived components, which may help explain why perturbations affecting these tissues produce distinct phenotypes (Halpern et al., 1997; Shih & Fraser, 1996; Meiniel et al., 2008; Strähle et al., 2004; Lehmann & Naumann, 2005). In addition, induction of *sspo* expression in the roof plate-derived SCO depends on coordinated dorsal-ventral signalling, including Shh-dependent mechanisms (Korzh et al., 1993; Traiffort et al., 1999; Korzh et al., 2007; Baeuml et al., 2019). Kv2.1-driven changes in membrane organisation and intracellular trafficking may therefore influence how SCO cells integrate morphogenetic signals, thereby affecting RF-related developmental programmes without requiring a direct transcriptional role for the channel itself (Johnson et al., 2018; Jędrychowska & Korzh, 2019).

Taken together, these findings support a model in which Kv2.1 negatively regulates anterior RF formation and stability by coordinating secretory activity, membrane organisation, ventricular architecture, and local CSF routing and flow dynamics. Kv2.1 likely integrates several cellular processes that determine when and where Sspo is secreted and how efficiently RF is assembled (Deutsch et al., 2012; Johnson et al., 2019; Tamkun et al., 2007; Amini et al., 2026).

The duplicated anterior RF phenotype observed in *kcnbl* mutants may reflect altered ventricular geometry. Specifically, reduction of the cerebral aqueduct within a diminished BVS may alter CSF routing and promote the premature emergence of parallel dorsal and ventral flow paths, thereby supporting abnormal anterior RF assembly (Fame et al., 2016; Amini et al., 2026) (Fig. 2).

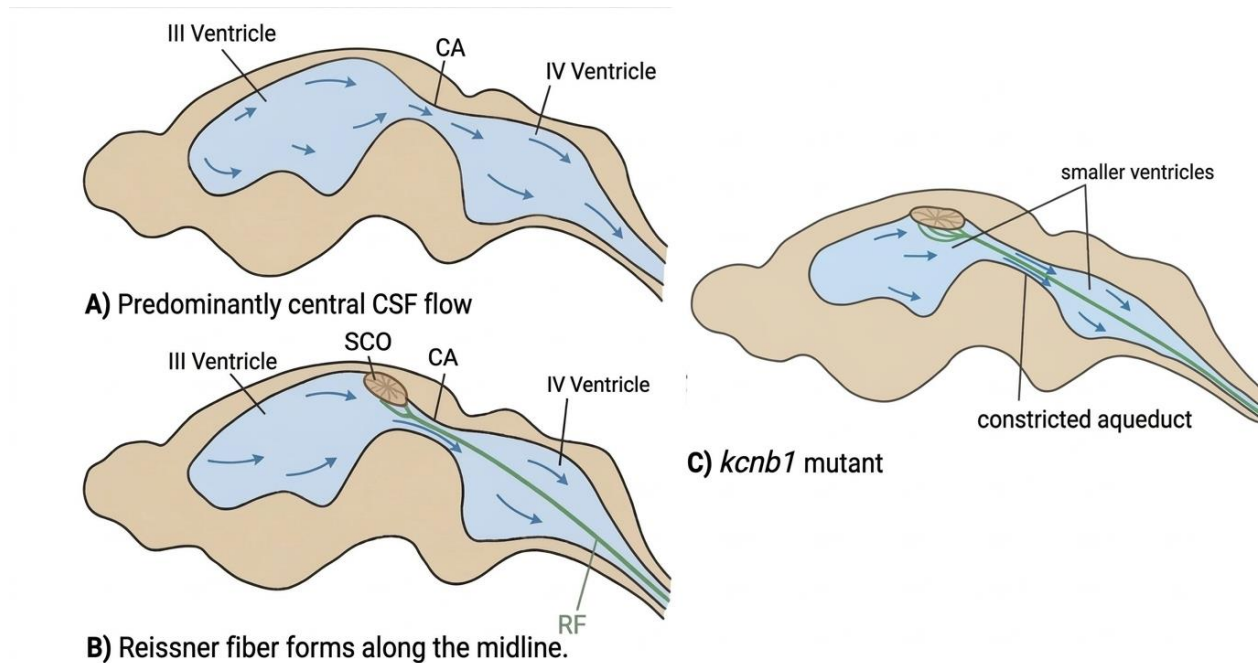


Fig. 2. Model of CSF flow and RF assembly in the zebrafish brain

(A) In wild-type embryos, CSF flow is regionally variable but overall directed toward the cerebral aqueduct, with no continuous RF present.

(B) In wild-type, a continuous RF extends along the midline from the SCO through the aqueduct, supported by directional CSF flow.

(C) In *kcnb1* mutants, reduced ventricular size and aqueduct constriction alter CSF routing, leading to more confined and partially aligned flow that may facilitate RF assembly.

3.2 Effects of Body Orientation on Brain Ventricular System Development Under Normogravity (Manuscript 2- Major contribution)

In this study, I showed that changing the orientation of zebrafish embryos relative to gravity disrupts anterior RF formation and alters the organization of the BVS. These changes are orientation-dependent and are accompanied by body axis abnormalities and shifts in gene expression associated with RF formation and mechanosensitivity. Overall, these findings identify body orientation as an important physical factor during early development, capable of modifying the mechanical environment of ventricular tissues during a critical developmental period (Amini et al., submitted).

The influence of body position on fluid distribution is well established in adult physiology, where changes in posture or gravity can affect CSF dynamics and, in turn, brain structure and function (Pavy-Le Traon et al., 2007; Watenpugh, 2016; Klarica et al., 2014; Trimmel et al., 2022; Sagirov et al., 2023). The present results suggest that related principles also operate during development, when the ventricular system is still forming, and compensatory mechanisms may be limited. In this context, sustained changes in body orientation appear sufficient to disrupt the physical conditions required for proper anterior RF assembly.

The orientation-specific effects highlight the importance of developmental geometry. Embryos in a horizontal position formed anterior RF, whereas vertical and reversed-vertical orientations produced progressively stronger defects. This indicates that deviations from the normal body axis geometry between 24 and 48 hpf interfere with RF formation, consistent with previous observations that the embryonic axis aligns with gravity during this period (Kimmel et al., 1995). Increased hydrostatic asymmetry and altered CSF distribution are likely to contribute to these effects. The observation that only some embryos recover RF formation after release from constraint further suggests that anterior RF initiation depends on a restricted permissive period (Lang et al., 2006; Picketts, 2006).

At the molecular level, RF disruption was accompanied by altered expression of genes associated with RF development and mechanosensitive responses. Among RF-related genes, *chlla* showed the strongest response to altered body orientation, consistent with previous work linking this gene to RF integrity and body axis maintenance (Yang et al., 2021). More broadly, altered expression of RF-associated genes indicates that disturbed body orientation affects not only morphology but also the transcriptional state of ventricular tissues involved in RF formation (Muñoz et al., 2019; Troutwine et al., 2020; Yang et al., 2021). A similar conclusion arises from analysis of genes linked

to cellular mechano-elastic properties, including *yap1* and *actn3b*, whose altered expression is consistent with activation of a conserved mechanosensitive response (Porazinski et al., 2015; Takahashi et al., 2021; Aventaggiato et al., 2020).

At the same time, the interpretation should remain cautious. Immobilisation in agarose and confinement within capillaries may impose additional physiological stress, and some phenotypes, particularly cardiac edema and body curvature in reversed-vertical embryos, may reflect broader systemic disturbance rather than CSF redistribution alone. Nevertheless, because horizontally immobilised embryos were exposed to the same general experimental constraints without showing defects of comparable severity, body orientation itself is likely to be the principal factor underlying the vertical-position phenotypes (Pavy-Le Traon et al., 2007; Klarica et al., 2014; Lillywhite & Lillywhite, 2017).

Overall, these findings demonstrate that sustained changes in embryo orientation reshape CSF distribution and mechanical forces in the developing BVS, activate mechanosensitive gene programs, and disrupt anterior RF formation. The strong response of *chlla* emphasizes its sensitivity to mechanical change, while increased Hippo/Yap signaling supports activation of conserved mechanotransduction pathways. More broadly, this work supports a central conclusion of the thesis: early ventricular tissues and RF assembly depend on precise coordination between molecular regulation and physical environment (Cantaut-Belarif et al., 2018; Rose et al., 2020; Wyart et al., 2023; Yang et al., 2021).

3.3 Mechanistic pathways linking Kv2.1 activity to RF morphogenesis (unpublished data)

3.3.1 Wnt/ β -catenin signaling provides a permissive framework for RF formation

Our data suggest that canonical Wnt/ β -catenin signaling is required for SCO to support RF assembly. In zebrafish, the roof plate-derived SCO expresses Wnt ligands such as Wnt3 and activators of Wnt signalling, such as *Rspo1*, and functions as a dorsal signaling center during development of the BVS (García-Lecea et al., 2017, Teh et al., 2015, Thisse et al., 2001, Veerapathiran et al., 2020). Consistent with this, Wnt3 and *Sspo* are co-expressed in the SCO and FO at the onset of anterior RF formation (Fig.1 A – C).

Pharmacological perturbation of the pathway supports this requirement. Inhibition of Wnt/ β -catenin signaling abolished RF formation, whereas pathway activation promoted an expansion of

the anterior RF. Activation of Wnt/ β -catenin signaling with LiCl or CHIR-99021 increased the density of the associated microfilament network such that it resembled the RF observed in *kcnbl* loss-of-function mutants, suggesting that elevated Wnt activity can increase Sspo secretion.

Transcriptomic data align with the same interpretation and point to Kv2.1 as a potential upstream modulator of Wnt responsiveness. Several genes encoding positive regulators of Wnt/ β -catenin signaling (including *rspo1*, *dvlla*, *gskip*, and *lrp5/6*) show altered expression in mutants of Kv2.1 subunits, with opposite trends in *kcnbl* and *kcng4b* mutants (Tables 1). By contrast, the expression of genes associated with Wnt ligand secretion was largely unchanged. This argues against the primary effect of the channel on Wnt secretion and instead suggests that Kv2.1 regulates the response of SCO cells to Wnt/ β -catenin signaling. In summary, Wnt/ β -catenin signaling appears to counteract the negative regulation of Kv2.1 during RF formation.

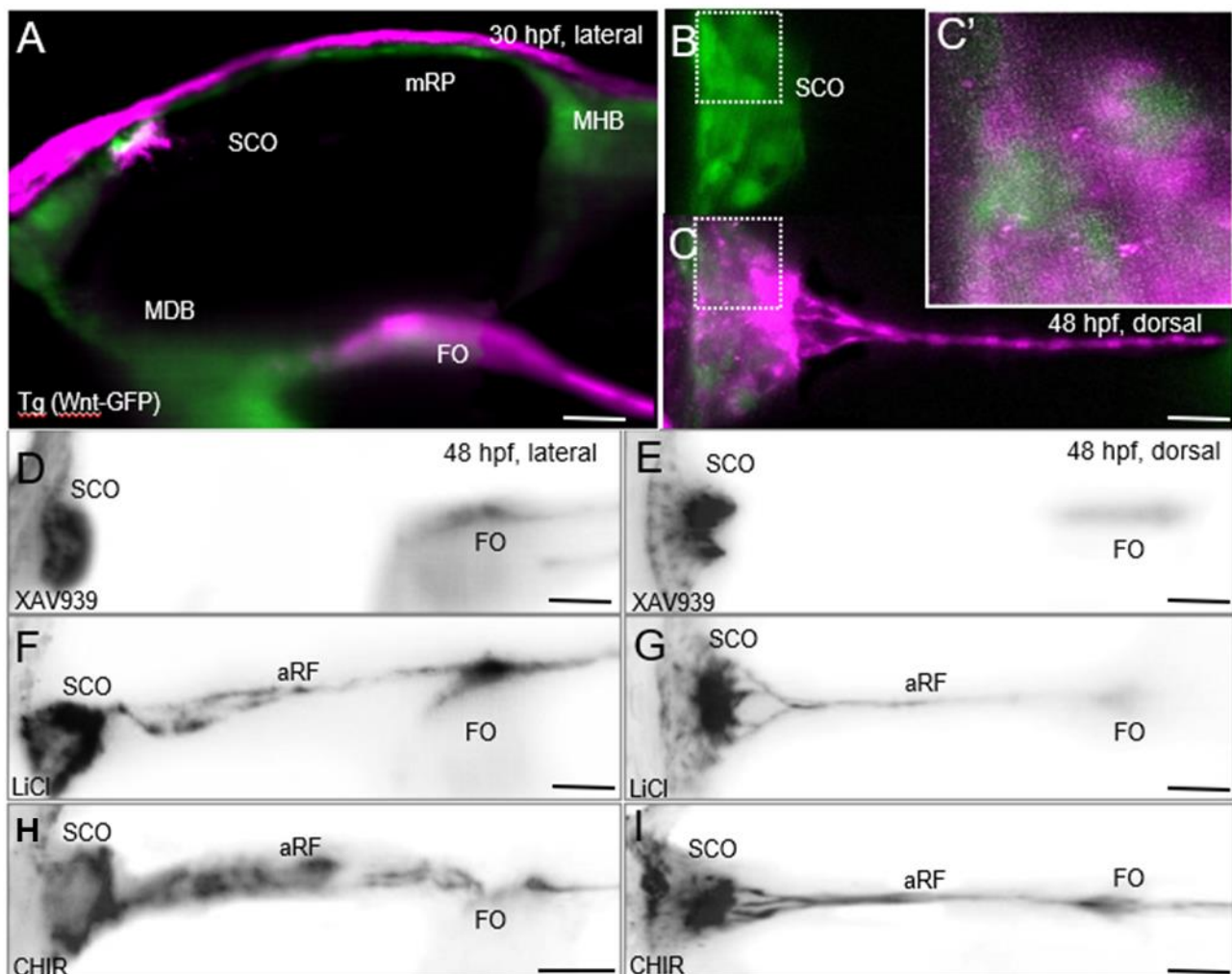


Fig. 1. Wnt/ β -catenin signaling regulates anterior RF formation. The SCO cells co-expressed Wnt3 and Sspo.

A) SCO of the 48 hpf Wnt3-GFP embryo.

B) The 30 hpf Wnt3-GFP transgenics (green) stained with the AFRU antibody (magenta).

C) SCO of the same 48 hpf Wnt3-GFP transgenics stained with AFRU antibody, C' - the 3x magnification of the boxed area (B, C). Wnt3-GFP and AFRU localize to the same cell; the GFP is mainly in the perinuclear space, and AFRU targets the peripheral cytoplasm.

D, E) The pan-Wnt inhibitor (XAV939) blocks anterior and posterior RF development without noticeably affecting Sspo level in the SCO. Body axis defects were observed in 25/45 embryos.

F, G, H) The Wnt/ β -catenin agonists [LiCl (F, G) or Laduviglusib (CHIR, H) (H, I)] increased the anterior RF-associated signal along the D-V axis.

A-C – Wnt3-GFP transgenics; D-I – ET33-mi2a transgenics. A, D, F, H - lateral view, B, C, E, G - dorsal view.

N = 45 in all samples. Scale bar = 20 μ m.

Abbreviations: aRF – anterior Reissner fibre; FO – flexural organ, MDB – mid-diencephalic boundary; MHB – midbrain-hindbrain boundary; MRP – midbrain roof plate; SCO – subcommissural organ.

Table 1. Mutations of genes encoding Kv2.1 subunits affect the level of transcripts associated with the Wnt/ β -cat signaling.

N	Genes	Expression level ratio, <i>kcnb1</i> / wt, SD 24hpf	p value	Expression level ratio, <i>kcnb1</i> / wt, SD 48hpf	p value
1	<i>dvl1a</i>	1.51±0.11	0.05	0.24±0.05	0.01
2	<i>fat1a</i>	0.99±0.08	0.05	0.45±0.27	0.05
3	<i>gskip</i>	1.46±0.18	0.05	0.66±0.22	0.05
4	<i>kcnma1a</i>	1.11±0.15	n.s	0.56±0.06	0.001
5	<i>lrp5</i>	1.14±0.51	n.s	0.39±0.18	0.05
6	<i>rspo1</i>	1.53±0.34	0.05	0.63±0.29	0.05
7	<i>wls</i>	1.14±0.1	0.05	0.94±0.08	0.01
8	<i>wnt4a</i>	1.48± 0.28	n.s	0.39±0.02	0.01

N	Genes	Expression level ratio, <i>kcng4b-C1</i> / wt, SD 24hpf	p value	Expression level ratio, <i>kcng4b-C1</i> / wt, SD 48hpf	p value
1	<i>dvl1a</i>	0.42±0.07	0.01	0.15±0.06	0.05
2	<i>fat1a</i>	0.77±0.3	n.s	0.23±0.08	0.01
3	<i>gskip</i>	0.91±0.22	n.s	0.76±0.11	0.05

4	<i>kcnma1a</i>	1.43±0.26	0.05	0.5±0.04	0.001
5	<i>lrp5</i>	3.07±0.4	0.001	1.9±0.27	0.001
6	<i>rspo1</i>	0.45±0.08	0.05	0.31±0.03	0.05
7	<i>wls</i>	1.24±0.11	0.05	0.88±0.02	0.01
8	<i>wnt4a</i>	1.02±0.07	0.05	0.97±0.08	n.s

3.3.2 Hedgehog signaling regulates ventral components of the RF

Shh is the axial mesoderm-derived morphogen that acts downstream of the Nodal signaling to ventralize the neural tube and develop the lateral floor plate (Krauss et al., 1993; Roelink et al., 1994; Sampath et al., 1998; Schier et al., 1997; Strähle et al., 2004). Previous studies have shown that Nodal deficiency affects *sspo* expression in the floor plate but not in the SCO, raising the question of how Hedgehog signaling contributes to RF formation (Lehmann and Naumann, 2005). The Hh inhibitor cyclopamine was used to efficiently block the Hh activity and an agonist of Hh, purmorphamine (PMA), was used to increase the Hh signaling in the stage-specific manner during 24-48 hpf period (Winata et al., 2009).

Inhibition of Hedgehog signaling selectively disrupted anterior RF formation and reduced Sspo secretion from the floor plate, while the SCO-associated signal was preserved or increased. The apical surface of the FO shrunk along the A-P axis and the duplicated signals in the hindbrain were reduced to such extent that the ventral signal was no longer detectable. In contrast, the intensity of SCO-associated signal increased (Fig. 2 A-D). The PMA treatment did not cause any major changes compared to the wild-type control, although the curvature of the hindbrain floor plate increased (Fig. 2 E, H). Thus, Hh signaling plays a critical role in RF formation by regulating floor plate- and SCO-related Sspo secretion in a manner that opposes that of Wnt/ β -catenin signaling.

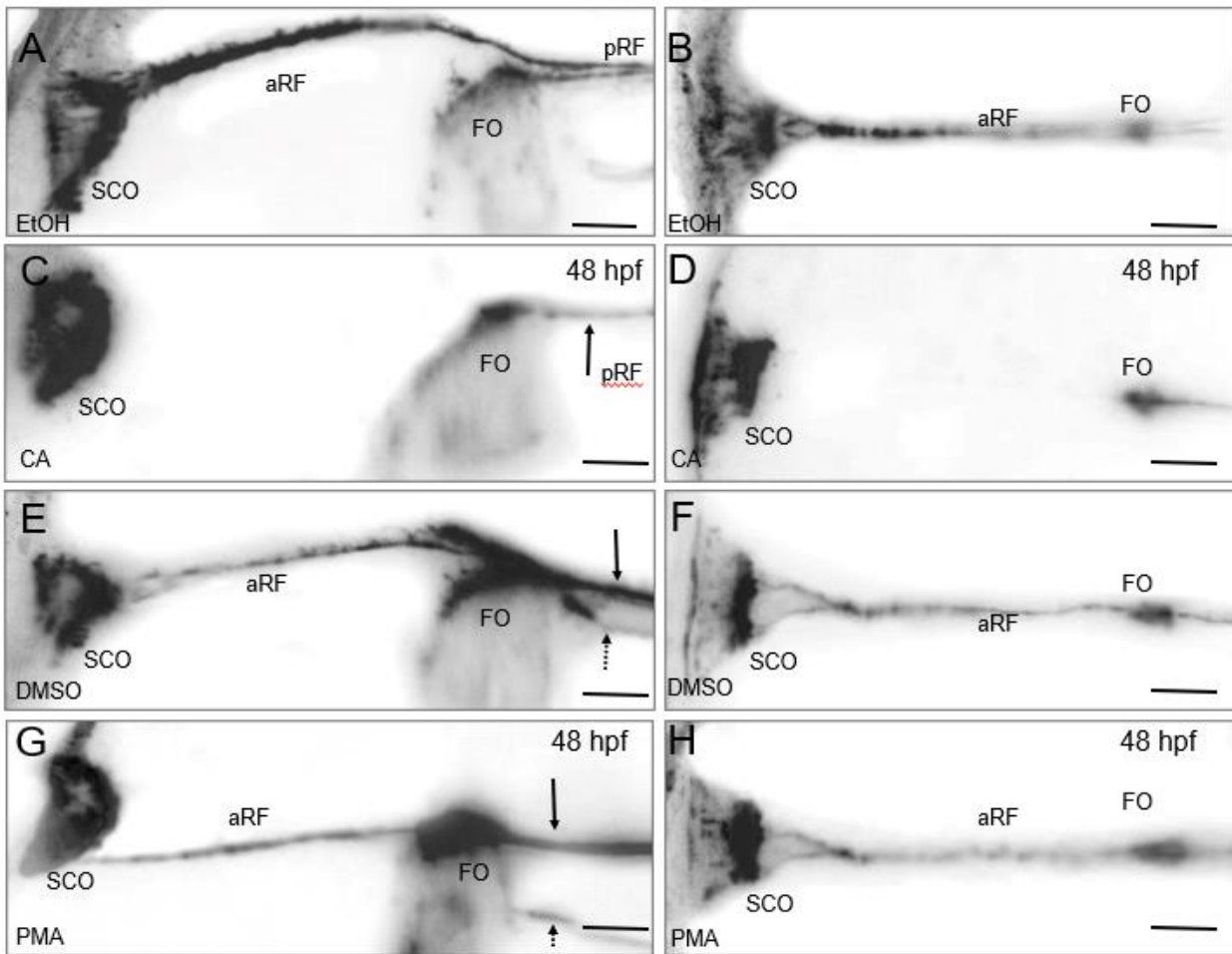


Fig. 2. Shh signaling regulates RF formation.

C, D) Cyclopamine (EtOH) treatment blocks anterior RF development (48 hpf; (scoliosis – 30/45).

A- lateral view, B- dorsal view.

G, H) Purmorphamine (DMSO) has no apparent effect on RF development.

N = 45 in all samples.

Scale bar = 20 μ m. Abbreviations: aRF – anterior Reissner fibre; FO – flexural organ, pRF – posterior Reissner fibre, SCO – subcommissural organ.

3.3.3 Cholesterol availability integrates membrane signaling and Kv2.1-dependent RF regulation

Previous work shows that loss-of-function mutations in *kcng4b* influences cholesterol metabolism, suggesting a functional connection between Kv2.1 channel activity and membrane lipid homeostasis (Jędrychowska et al., 2024). Given the established role of Wnt/ β -catenin signaling in anterior RF formation, we asked whether cholesterol availability is required for Sspo secretion and subsequent RF assembly. Cholesterol helps to organize signaling platforms in the plasma membrane. Consistent with this, previous studies have shown that acute cholesterol depletion using methyl- β -cyclodextrin (m β CD, 2.5 μ M) reduces the association of Wnt3 with cholesterol-dependent membrane domains in zebrafish embryos (Ng et al., 2016). Consistent with these observations, acute m β CD treatment in wild-type embryos disrupted anterior RF formation. It was well documented that statins inhibit cholesterol biosynthesis (Signore et al., 2016). The incubation of embryos in the presence of an inhibitor of cholesterol metabolism, Atorvastatin (10 μ M) abolished anterior RF formation, despite continued Sspo production by the SCO (Fig. 3). Therefore, these results suggest that cholesterol is not required for *sspo* expression *per se*, but is essential for efficient secretion and/or extracellular assembly of the coherent RF.

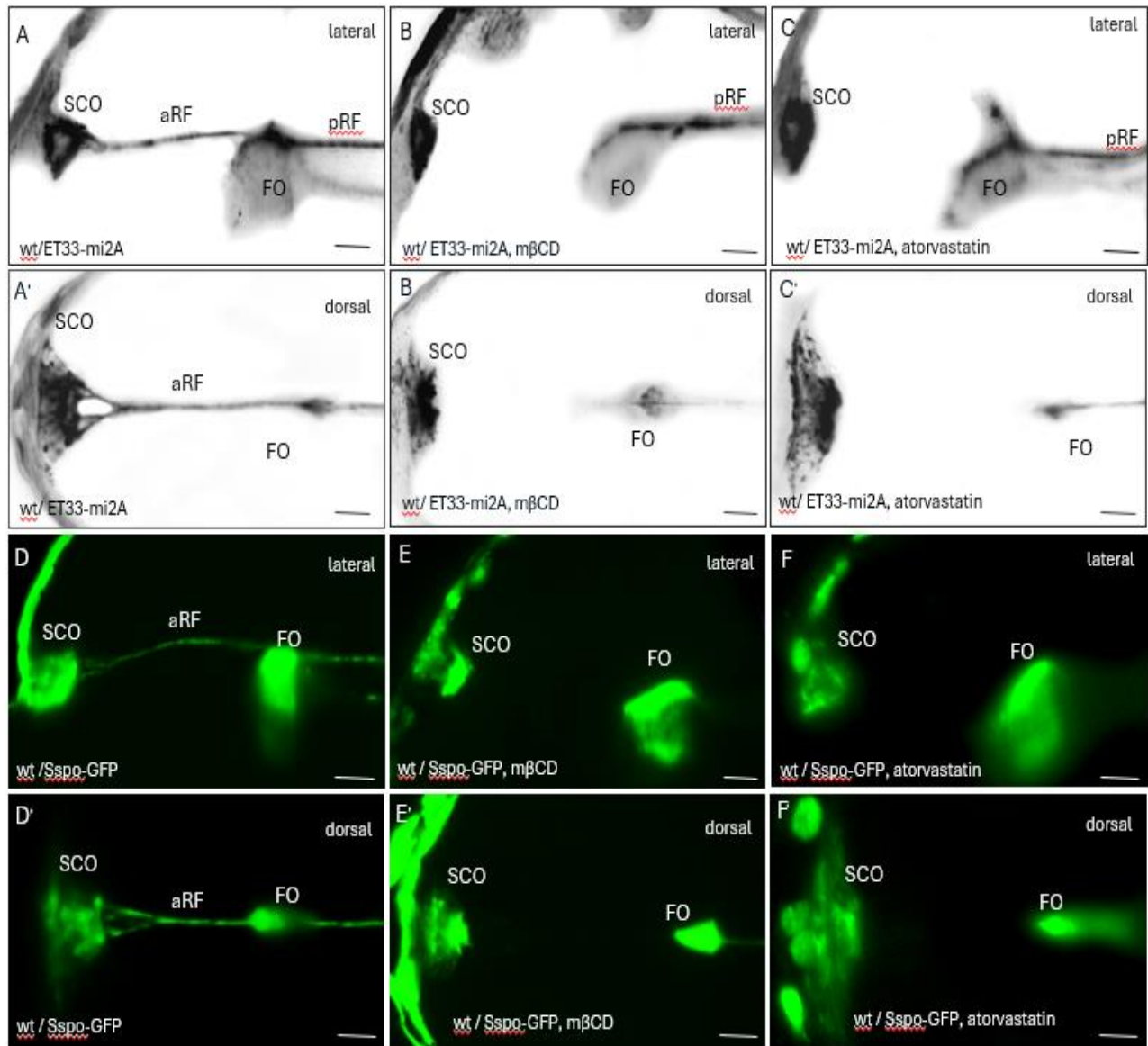


Fig. 3. Cholesterol depletion affects the formation of the anterior RF in wild-type embryos.

A, A', D, D') control.

B, B', E, E') 40 min treatment of 48 hpf embryos with 2.5 μ M m β CD abolished the anterior RF. (body axis defects – 15/30) - (body axis defects – 21/30).

C, C', F, F') 24→48 hpf treatment with Atorvastatin (10 μ M) abolished the anterior RF. (body axis defects – 12/30) - (body axis defects – 20/30).

A-F – lateral view; A'-F' – dorsal view; A-C, A'-C' – color signals of GFP in the ET33-mi2a embryos and AFRU staining converted to the false black-and-white signals; D-F, D'-F' – green (Sspo-GFP transgenics). N = 30 in all samples. Scale bar = 20 μ m.

Abbreviations: aRF – anterior Reissner fibre; FO – flexural organ, pRF – posterior Reissner fibre, SCO – subcommissural organ.

The relationship between cholesterol availability and Kv2.1 activity was particularly evident in *kcnbl* mutants. Given the elevated Sspo levels observed in *kcnbl^{sq301}* embryos, we asked whether this phenotype might be linked to altered cholesterol homeostasis and whether modulating cholesterol levels could influence RF formation in this genetic background. Acute cholesterol depletion with mβCD partially impaired anterior RF morphology in *kcnbl* mutants (Fig. 4B, C); however, the RF was preserved and maintained its orientation along the SCO–FO axis. In contrast, chronic inhibition of cholesterol synthesis by Atorvastatin resulted in a much more severe phenotype, with near-complete loss of anterior RF (Fig. 4D, E).

These observations suggest that reduced Kv2.1 activity in *kcnbl* mutants can partially buffer the effects of acute cholesterol depletion, allowing Sspo secretion and anterior RF formation to proceed. However, this buffering effect is lost when cholesterol biosynthesis is chronically blocked, indicating that a minimal level of cholesterol remains indispensable for this process.

In contrast to *kcnbl* mutants, the relatively mild RF phenotype of *kcng4b^{waw304}* embryos was largely insensitive to cholesterol depletion (Fig. 4G–J). This finding suggests that the moderate increase in Kv2.1 activity associated with the loss of the modulatory subunit Kcng4b may help protect against complete suppression of Sspo secretion under cholesterol-limiting conditions. In summary, these results support the view that cholesterol availability is a determinant of Kv2.1-dependent regulation of Sspo secretion.

This interpretation is further supported by transcriptional analyses of genes involved in cholesterol transport and metabolism. *npc1* encodes the cholesterol transporter (mutated in Niemann–Pick disease type C1). It was upregulated in *kcnbl* mutants and downregulated in *kcng4b* mutants, whereas *npc2* showed no such change. In addition, the transcript level of *apoeb*, a gene encoding the key mediator of cholesterol efflux, was reduced in *kcnbl* mutants but increased in *kcng4b* mutants (Fig. 4K–L). These opposing transcriptional profiles suggest that altered Kv2.1 activity is associated with differential regulations of cellular cholesterol handling, which could feed back onto membrane organisation and secretory capacity.

As a membrane-embedded protein complex, Kv2.1 can directly interact with cholesterol, a major constituent of the plasma membrane. Cholesterol depletion phenocopies key aspects of the severe *kcng4b* loss-of-function phenotype, characterized by increased Kv2.1 activity and pronounced RF defects (Jędrychowska et al., 2024). These effects are likely mediated by the disruption of multiple membrane-associated processes, including intracellular trafficking, ER–plasma membrane junctions, membrane potential homeostasis, and organisation of cholesterol-enriched lipid rafts (Deutsch et al., 2012; Fox et al., 2015; Johnson et al., 2019; Ottschytsch et al., 2002; Tamkun et

al., 2007). In this context, cholesterol may act as a regulatory factor for Kv2.1 function, in a manner conceptually similar to modulation by the Kcng4b subunit.

Interestingly, phenotypes associated with *kcng4b* loss show parallels to α -SNAP-deficient models, including hydrocephalus and RF abnormalities (Pérez-Fígares et al., 1998; Wagner et al., 2003). The α -SNAP-syntaxin complex has been implicated in cholesterol trafficking from the plasma membrane to mitochondria, which is essential for steroid biosynthesis (Deng et al., 2019; Rodríguez et al., 2011). Moreover, both Kcng4 mouse mutants and *kcng4b*^{waw305} zebrafish exhibit severe fertility defects (Regnier et al., 2017; Jędrychowska et al., 2024).

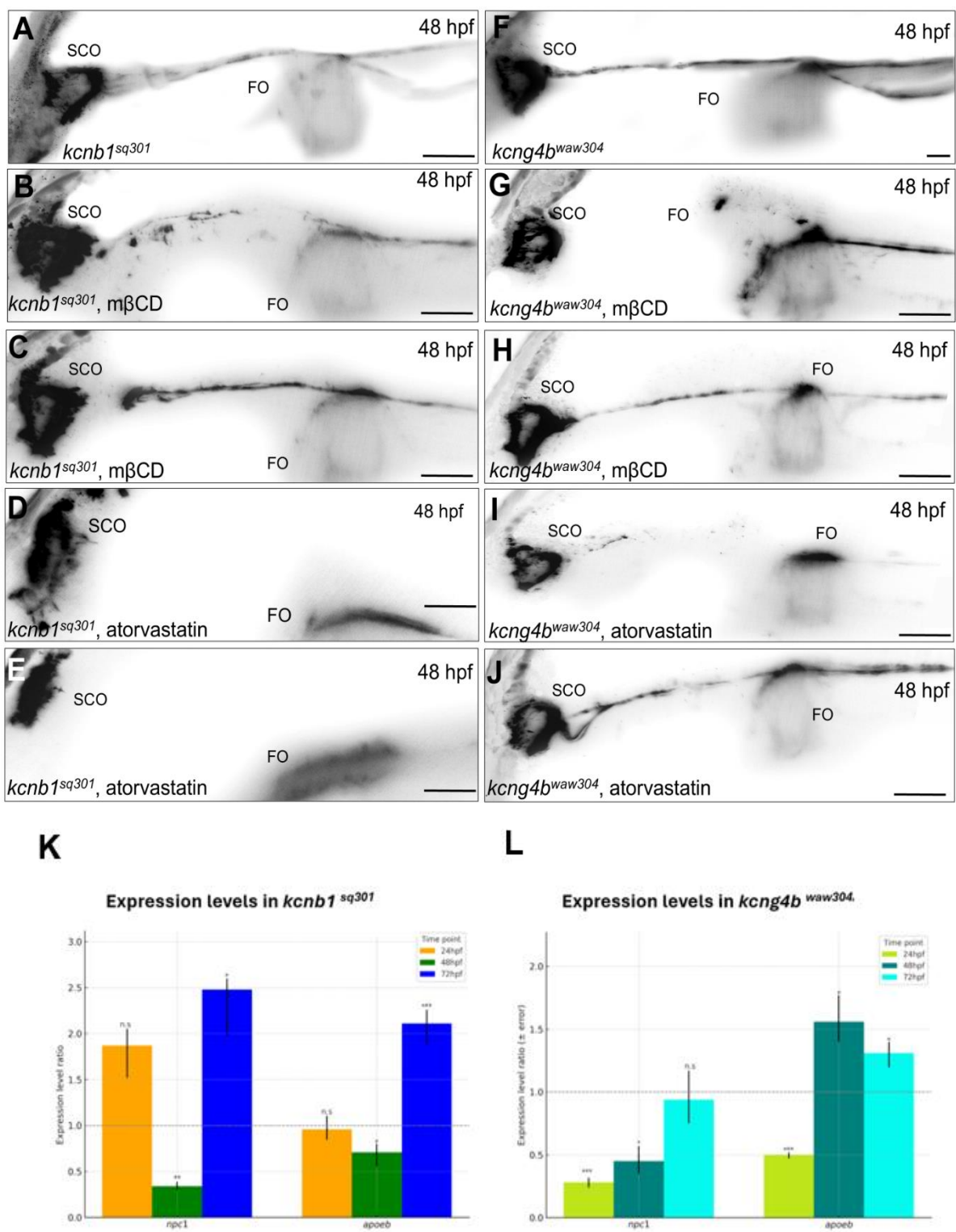


Fig. 4. Cholesterol depletion affects the anterior RF formation in mutants.

A) Control *kcnb1^{sq301/sq301}* mutant.

B-C) 40 min treatment of 48 hpf *kcnb1^{sq301}* with 2.5 μ M m β CD. (body axis defects – 12/40).

D, E) 24→48 hpf atorvastatin treatment (10 μ M) of *kcnb1^{sq301}*. (body axis defects – 3/20).

F) Control *kcng4^{waw304}*- mutant; G, H – 40 min treatment of 48 hpf *kcng4b^{waw304}* mutant with 2.5 μ M m β CD (body axis defects – 10/20).

I, J) 24→48 hpf atorvastatin treatment (10 μ M) of *kcng4b^{waw304}* mutant (body axis defects – 3/20).

All images are in lateral view. The color signals of GFP in the ET33-mi2a embryos and AFRU staining were converted to false black-and-white signals. Scale bar = 20 μ m.

3.3.4 RF assembly is highly sensitive to AFRU antibody and oxidative stress

Disruption of anterior RF formation following oxidative stress induction or direct interference with extracellular Sspo provides additional evidence that RF assembly is highly sensitive to conditions within the ventricular lumen. Notably, these manipulations impair RF integrity even as Sspo synthesis continues in the SCO. This suggests that defects are unlikely to result from reduced production of RF components. Instead, the findings indicate that RF formation depends on extracellular conditions within the ventricular lumen. These conditions must support polymerization and stabilization of Sspo-derived microfilaments.

Changes in redox conditions are one example of extracellular perturbations that can influence the physicochemical properties of secreted proteins. Oxidative modifications can alter protein–protein interactions. They may also compromise the stability of large extracellular assemblies across diverse biological contexts (Finkel, 2011; Schieber & Chandel, 2014). Within this framework, the disruption of RF integrity after oxidative stress supports the idea that RF is a dynamic extracellular structure. Its assembly and maintenance depend on a tightly regulated luminal environment rather than solely on intracellular synthesis of its constituent proteins (Fig. 5C, D).

Similarly, disruption of RF formation following AFRU antibody injection underscores the need for intact extracellular interactions between Sspo molecules during RF assembly (Fig. 5E, F). Antibody-mediated interference likely disrupts normal association and fusion of Sspo microfilaments. This prevents their incorporation into a continuous fiber. Together, these observations support the interpretation that RF morphogenesis relies on extracellular self-assembly mechanisms operating within the CSF. These mechanisms are sensitive to perturbations that affect protein stability and intermolecular interactions (Cantaut-Belarif et al., 2018; Thouvenin et al., 2020).

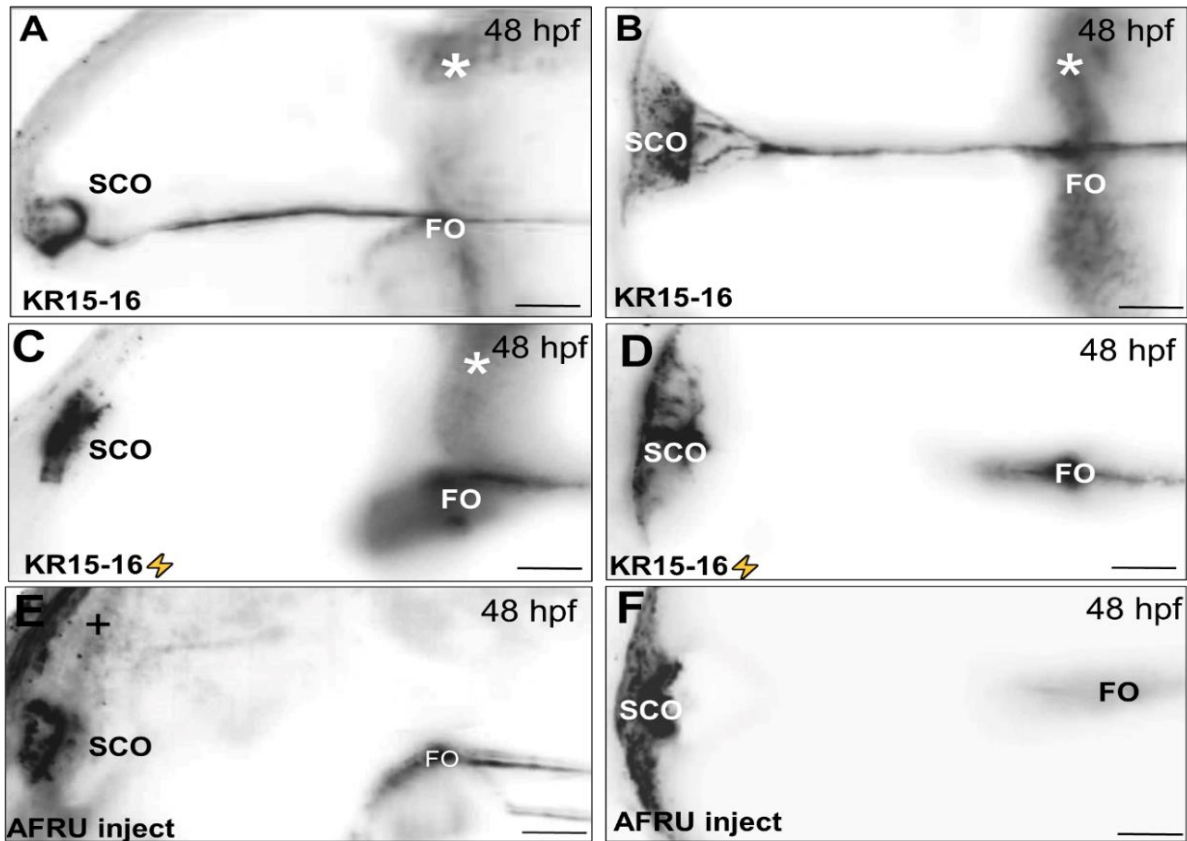


Fig 5. aRF development is blocked by ROS and AFRU antibody.

During development, KillerRed (KR) is expressed at the MHB (asterisk) of Tg (KR15-16) transgenics in the *En1b*-like manner

A, B) Irradiation with intense green light (24 hpf) triggered production of ROS, which blocked aRF formation (C, D).

A similar outcome has been caused in some embryos after injection of the AFRU antibody (E, F), which was previously shown to have neutralising activity in mammals (Rodriguez et al., 1990).

(+) - Increased background staining may represent the remnants of the injected AFRU antibody.

N = 20, Scale bar = 20 μ m. Abbreviations: FO – flexural organ, SCO – subcommissural organ.

3.3.5 The ciliated SCO microenvironment provides a permissive context for RF elongation

Previous studies have shown that disrupting motile cilia or cilia-driven cerebrospinal fluid flow impairs RF elongation and stability, highlighting the essential role of cilia in RF morphogenesis (Cantaut-Belarif et al., 2018; Olstad et al., 2019). In contrast, our analysis shows that Kv2.1 mutants exhibit pronounced RF defects despite preserved ciliary number, distribution, and overall morphology (Fig. 6). This distinction indicates that, while cilia-driven flow is necessary for RF formation, it is not sufficient. Instead, additional regulatory layers that act at the level of SCO secretion or extracellular assembly likely contribute to RF morphogenesis. Thus, the RF phenotypes observed in Kv2.1-related mutants are unlikely to arise from primary defects in ciliogenesis and instead point to cilia-independent mechanisms regulating SCO-derived secretion and extracellular fiber assembly.

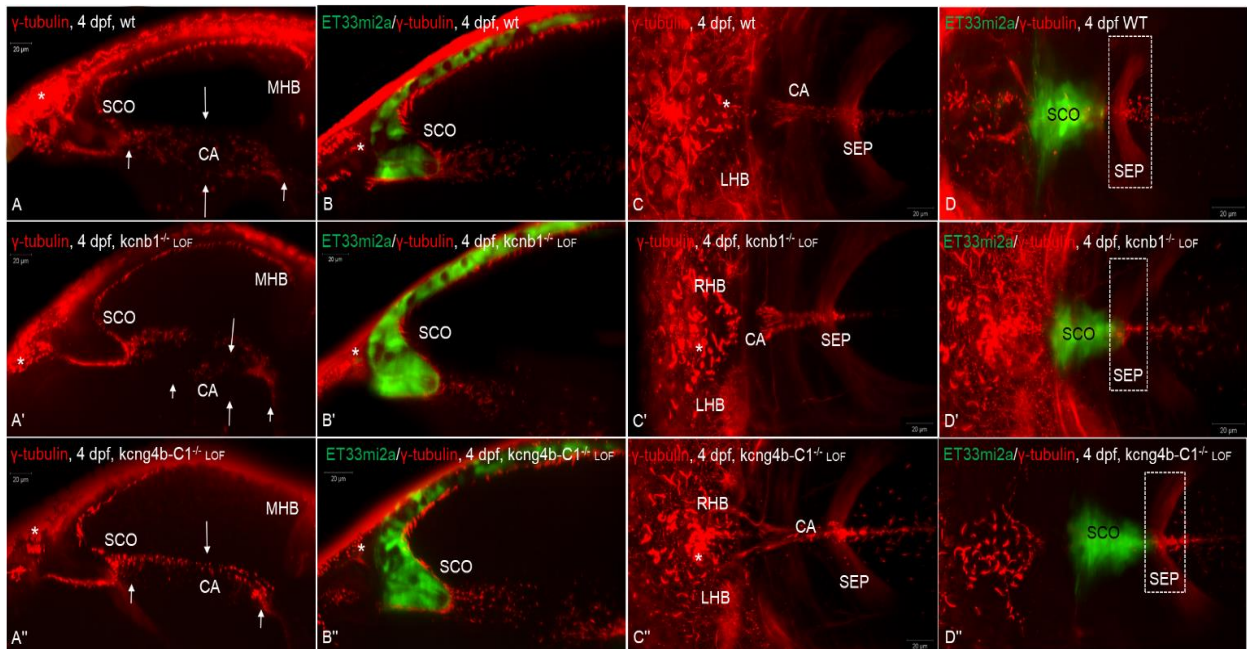


Fig. 6. SCO develops in close association with ciliated cells.

SCO develops in close association with ciliated cells.

A) The anti- γ -tubulin staining (red) of 4 dpf zebrafish reveals that ciliated cells outline the SCO and CA (lateral view). The CA narrows in the position of the SCO and FO (small arrows) and expands in between these organs (large arrows).

B) The GFP expression in SCO and midbrain RP (green) of 4 dpf ET33-mi2a zebrafish stained for anti- γ -tubulin (red) (lateral view).

C) The anti- γ -tubulin staining (red) of 4 dpf zebrafish (dorsal view) outlines the funnel-shaped CA and weakly stains the supra endymal plexus posterior to the SCO.

D) The GFP expression in SCO (green) of 4 dpf ET33-mi2a zebrafish stained for anti- γ -tubulin (red) (dorsal view).

N = 15, Scale bar = 20 μ m. Abbreviations: CA– Cerebral Aqueduct, MBH– Midbrain–Hindbrain Boundary, LHB – Left Hindbrain, RBH –Right Hindbrain, SCO – subcommissural organ, SEP – supra endymal plexus.

A key implication of the experiments in Sections 3.3.1–3.3.5 is that proper development of the BVS and RF formation depends on coordinated interactions between developmental signaling pathways and membrane properties in ventricular epithelia. Sonic Hedgehog and Wnt/ β -catenin signaling appear to require a cholesterol-dependent membrane environment to support normal secretory activity in roof- and floor plate cells. Accordingly, changes in membrane composition or Kv2.1 activity can alter ventricular morphology and disrupt RF assembly.

3.4 Loss of Kv2.1 and Kv6.4 subunits affects brain excitability and the visual circuit dynamics (Manuscript 3- Minor contribution)

The main aim of this part of the thesis was to investigate whether early developmental abnormalities associated with Kv2.1 dysfunction are linked to later changes in neuronal excitability and behavior. Since Kv2.1 is expressed in both ventricular epithelial cells and neurons, it can be regarded as a common regulator functioning at both developmental and physiological levels in the nervous system (Shen et al., 2016; Jędrychowska & Korzh, 2019).

Earlier chapters of this thesis showed that Kv2.1 activity is required for proper BVS development and correct RF assembly. Disruption of Kv2.1 affects ventricular integrity, secretory activity of the SCO, and extracellular RF formation within the CSF (Cantaut-Belarif et al., 2018; Thouvenin et al., 2020; Amini et al., 2026). In parallel, behavioural and electrophysiological analyses indicate that loss of either the electrically active *Kcnb1* subunit or the modulatory *Kcng4b* subunit increases neuronal excitability. Mutant larvae showed reduced baseline locomotor activity together with exaggerated responses to chemical or sensory stimulation, consistent with a lowered threshold for network activation rather than continuous spontaneous epileptic activity (Specia et al., 2014; Veale et al., 2022; Joshi et al., 2024; Kang et al., 2019; Bar et al., 2020).

This effect was particularly evident in *kcnb1* mutants, which showed altered visual responsiveness, freezing behaviour, and increased susceptibility to chemically induced seizures despite the absence of spontaneous seizures under baseline conditions and without obvious structural defects in the visual system. These observations suggest that the phenotype is more likely to reflect altered central sensory processing and impaired regulation of neuronal activity than a primary defect in sensory organ morphology (Specia et al., 2014; Kang et al., 2019).

One possible point of convergence between developmental and functional observations is that early abnormalities in ventricular architecture and RF formation may modify the environment in which neural circuits mature. RF is an acellular structure embedded in the CSF and linked to CSF-contacting neurons, body axis maintenance, and sensorimotor control (Cantaut-Belarif et al., 2018; Olstad et al., 2019; Wyart et al., 2023). In addition, ventricular morphology and CSF composition contribute to the developmental context in which neural circuits are established (Pérez-Fígares et al., 1998; Wagner et al., 2003; Fame & Lehtinen, 2020). At the same time, Kv2.1 may also affect circuit function more directly through its roles in progenitor-associated cells and in neuronal membrane excitability (Shen et al., 2016; Murakoshi & Trimmer, 1999; Vacher et al., 2008).

Collectively, these findings support the view that Kv2.1 links brain morphogenesis with neuronal excitability. The behavioural and electrophysiological abnormalities observed in *kcnb1* and *kcng4b* mutants are therefore best interpreted not as isolated neuronal defects, but as outcomes of a broader disturbance affecting both non-neuronal brain development and later neural circuit function. More broadly, this part of the thesis reinforces a central conclusion of the work: ventricular and CSF-associated structures contribute to the conditions necessary for neural circuit maturation and the emergence of behavior.

3.5 Role of Kcng4b in inner ear development (Manuscript 4 - Minor contribution)

As introduced earlier in this thesis, the Kv2.1 channel complex has well-established roles in zebrafish development, including effects on fluid-filled epithelial organs. Here, we focus specifically on the contribution of Kcng4b to inner ear morphogenesis, using two independently generated mutant alleles, *kcng4b-C1* (*kcng4b^{waw304}*) and *kcng4b-C2* (*kcng4b^{waw305}*) (Jędrychowska et al., 2021, Gasanov et al., 2021).

Inner ear morphogenesis is highly sensitive to the functional state of the Kv2.1/Kv6.4 channel complex. Although both alleles affect Kcng4b, they produce markedly different phenotypes, indicating that relatively small shifts in channel state can generate strongly divergent developmental outcomes. *Kcng4b-C1* is associated with enlarged inner ears and ectopic otoliths, whereas *kcng4b-C2* produces a more severe loss-of-function-like phenotype with reduced ear size and complete absence of otoliths (Jędrychowska et al., 2021; Jędrychowska et al., 2024). These opposing outcomes reinforce the idea that the balance of Kv2.1/Kv6.4 activity, rather than subunit

loss alone, is critical for normal morphogenesis of hollow epithelial structures (Shen et al., 2016; Jędrychowska et al., 2021; Jędrychowska et al., 2024).

The more severe *kcng4b*-C2 phenotype further suggests that abnormal channel architecture, rather than simple channel activity reduction, can disrupt Kv2.1/Kv6.4 function in a dominant-negative manner (Jędrychowska et al., 2024). More broadly, Kv2.1/Kv6.4 dysfunction appears to affect inner ear development by compromising luminal inflation, otolith formation, and organ morphology more than early sensory cell specification. Hair cell number was largely preserved, whereas kinocilia and balance defects were observed, indicating that altered channel activity impairs functional maturation of the inner ear more strongly than initial cell fate decisions. Supporting this interpretation, molecular analyses showed altered expression of otic and hair cell-associated genes, including upregulation of *otop1* in the absence of otoliths (Jędrychowska et al., 2021).

Overall, these findings indicate that *Kcng4b*-dependent modulation of Kv2.1 is crucial for inner ear development by supporting luminal homeostasis and structural maturation of the sensory organ. While this project is a minor component of the thesis, it supports the main conclusion that the Kv2.1/Kv6.4 axis sensitively regulates epithelial organization and luminal homeostasis in various developmental contexts.

3.6 General conclusion

This thesis consists of two parts. The first includes the studies in which I made the major contribution, and which directly addressed the main research questions of the thesis. The second includes collaborative studies that provide additional developmental and functional context for the roles of Kv2.1/Kv6.4.

General conclusion for the major contribution part

The primary chapters addressed three closely related questions about RF formation and BVS morphogenesis. Their main conclusions are summarized in a question-and-answer format for clarity.

Question 1. How does Kv2.1 affect RF formation?

Kv2.1, through the balanced activity of its *Kcnb1* and *Kcng4b* subunits, regulates early steps of anterior RF formation by controlling *Sspo* distribution, SCO organisation, and extracellular RF assembly. Thus, the Kv2.1/Kv6.4 axis acts as an upstream regulator of RF morphogenesis within the developing BVS.

Question 2. How do Wnt/Shh signalling pathways and membrane organisation regulate RF formation?

RF formation depends on the interaction between developmental signalling and cholesterol-dependent membrane organisation. In this framework, Kv2.1 helps establish the membrane conditions that permit localised secretion and proper RF assembly.

Question 3. How does body orientation and gravity-dependent CSF redistribution influence RF morphogenesis?

Anterior RF formation is highly sensitive to body orientation and gravity-dependent changes in CSF distribution within the developing BVS. These findings show that RF assembly emerges from integration of molecular regulation with physical and hydrodynamic cues.

Additional support from collaborative/minor contribution studies

The collaborative studies included in this thesis extend this model to later neural excitability and to inner ear morphogenesis, supporting the broader conclusion that Kv2.1/Kv6.4 contributes to epithelial organisation, luminal homeostasis, and functional maturation in fluid-filled systems

In summary, this thesis identifies Kv2.1/Kv6.4 as a developmental regulator linking membrane organisation, secretion, signalling, and physical environment during BVS and RF formation. More broadly, it shows that the development of CSF-associated structures depends on tight coordination between molecular and mechanical factors.

4 FUTURE PERSPECTIVES

The results presented in this thesis identify several unresolved questions that define clear directions for future work. Although the data support the view that RF formation depends on coordinated secretory activity, membrane organisation, and physical forces within the BVS, the relative contribution of each of these factors remains incompletely resolved. Based on this, the following future directions are proposed to clarify which mechanisms act as primary drivers of RF vulnerability and which represent downstream consequences of altered developmental conditions.

1. Defining the temporal window for anterior RF initiation

A major unresolved issue is whether anterior RF assembly depends on a restricted developmental window. The body-orientation experiments suggest that failure to initiate anterior RF, particularly under reversed-vertical conditions, is not rescued by later return to free swimming. This pattern is

consistent with the idea that SCO-derived RF assembly requires a permissive period during which BVS geometry, secretory competence, and CSF conditions are aligned (Cantaut-Belarif et al., 2018; Olstad et al., 2019; Thouvenin et al., 2020). Future work should therefore define this window more precisely by restricting altered orientations to shorter developmental intervals and by assessing RF recovery alongside readouts of SCO secretory state and ventricular morphology.

2. Direct measurement of CSF flow and BVS geometry

The present work supports a role for gravity-dependent CSF redistribution, but direct CSF movement has not yet been measured in this system. Previous studies in zebrafish and mammals show that body posture and gravity can alter CSF flow, pressure gradients, and ventricular expansion (Pavy-Le Traon et al., 2007; Klarica et al., 2014; Fame et al., 2016; Watenpugh, 2016; Fame & Lehtinen, 2020). A key next step will therefore be to combine tracer-based approaches with time-lapse imaging to visualise CSF transport, compartment mixing, and clearance under different body orientations, together with time-resolved quantification of BVS geometry. Such analyses will help distinguish alterations in fluid transport from those imposed by ventricular shape.

3. Separating local ventricular mechanisms from systemic stress effects

An important challenge is to distinguish local effects within the BVS from broader physiological stress responses. Under severe orientation conditions, cardiac edema and general developmental compromise may contribute to RF instability and ventricular phenotypes. Future work should determine whether anterior RF defects are already detectable before overt systemic abnormalities emerge and should compare conditions that produce general stress without reproducing the same CSF-facing mechanical environment. This will help clarify whether altered CSF redistribution is the primary trigger or one of several converging factors contributing to RF disruption.

4. Defining the molecular mechanisms linking membrane state, secretion, and RF assembly

The findings of this thesis indicate that RF formation depends on the interaction between membrane composition, developmental signalling, and secretion. Future studies should therefore determine whether RF defects arise primarily from altered membrane potential, disrupted lipid microdomains, impaired trafficking at ER–PM contact sites, or failures in extracellular RF assembly. These analyses should also clarify how mechanosensitive pathways such as Hippo/YAP, together with RF-associated factors including *chlla*, galectins, and *Clu*, act as causal regulators or downstream markers of RF vulnerability (Porazinski et al., 2015; Takahashi et al., 2021; Tamkun et al., 2007; Deutsch et al., 2012; Johnson et al., 2019; Jędrychowska & Korzh, 2019).

Overall perspective

The work presented here provides a framework for understanding RF formation as a process governed by the interaction of secretion, membrane biophysics, and CSF mechanics within the developing BVS. The next goal is to move from correlation to mechanism by defining when anterior RF initiation becomes vulnerable, directly measuring CSF redistribution together with BVS geometry, and distinguishing intracellular secretion defects from extracellular assembly constraints. Once these mechanisms are established under normogravity, the same framework can be extended to altered gravity conditions to test specific predictions about CSF transport, ventricular homeostasis, and anterior RF vulnerability.

5 MATERIALS AND METHODS

The methods described in this chapter are for the unpublished data in 3.3

5.1 Zebrafish Husbandry and Ethics

Wild-type and transgenic *Danio rerio* lines were maintained at 28.5 ± 0.5 °C under a 14 h light/10 h dark photoperiod in a recirculating Aquatic Habitats system at the Zebrafish Core Facility, IIMCB, Warsaw. Water quality was monitored and maintained at pH 7.0–7.5 and conductivity 300–500 $\mu\text{S}/\text{cm}$. Fish were fed Gemma Micro dry feed twice daily and live *Artemia nauplii* once daily.

All procedures involving embryos and larvae were conducted in accordance with institutional regulations and EU Directive 2010/63/EU, under license PL14656251. Standard zebrafish husbandry procedures were followed (Westerfield, 2007).

5.2 Transgenic Reporter Lines

Line	Symbol / Identifier	Function / Reporter Expression	Reference
sqet33mi2AEt	Tg[sqet33mi2AEt]	Enhancer-trap line labeling roof plate, SCO, and ventricular ependyma	Korzh et al., 1998
sq19Tg[Wnt3:GFP]	Tg[Wnt3:GFP]	Cytosolic GFP in roof plate/SCO	Amini et al., 2024

sqKR15ct16Tg	Tg[sqKR15ct16]	Membrane-bound KillerRed at midbrain–hindbrain boundary (MHB)	Amini et al., 2024
<i>scospondin-GFP</i>^{ut24}	sspo-GFP Knock-in	Endogenous Scospondin C- terminal GFP tag	Troutwine et al., 2020

5.3 Fixation and Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde (PFA), a tissue preservative, in phosphate-buffered saline (PBS, pH 7.4): 24–48 hours post-fertilization (hpf) embryos were fixed for 1–2 h at room temperature, while embryos ≥ 72 hpf were fixed overnight at 4 °C. After washing in PBS, samples were stored at 4 °C until further processing. Embryos older than 48 hpf were then permeabilized in 0.5% Triton X-100 (a detergent that makes membranes permeable) in PBS for 30 min.

Samples were blocked in 10% BSA (bovine serum albumin) in PBST (PBS + 0.1% Triton X-100) for 1 h at room temperature. Primary antibodies (which bind specifically to target proteins) were diluted in blocking solution and gently stirred overnight at 4 °C. After washing in PBST, Alexa Fluor–conjugated secondary antibodies (which recognize and bind to the primary antibodies and are labeled with fluorescent dye) were applied for 2 h at room temperature in the dark. Embryos were washed, embedded in agarose, and oriented for imaging.

Target	Antibody	Host	Dilution	Source
Reissner fiber	AFRU	Rabbit	1:1000	Rodríguez laboratory
Cilia	Acetylated α -tubulin	Mouse	1:1000	Sigma
GFP	Anti-GFP	Mouse	1:1000	Abcam

5.4 Quantitative Real-Time PCR

Total RNA was extracted from pools of 40–50 embryos using TRIzol reagent. RNA purity was evaluated using a NanoDrop spectrophotometer, and only samples that met standard purity criteria were selected for cDNA synthesis. Reverse transcription was performed with iScript Supermix (Bio-Rad). Quantitative PCR was conducted using SYBR Green chemistry on a CFX Connect system (Bio-Rad). Primer specificity and amplification efficiency were validated prior to analysis. Gene expression levels were normalized to *eef1a1ll* and calculated using the $2^{-\Delta\Delta C_t}$ method. For statistical analysis, Tukey's post hoc test and one-way ANOVA were employed.

5.5 Pharmacological and Chemical Treatments

General Procedures

All pharmacological treatments were performed on dechorionated embryos maintained in E3 medium at 28.5 ± 0.5 °C in 6-well plates (20 embryos per well, 5 mL medium). To begin, treatments were initiated at 24 hpf; unless otherwise stated, embryos were fixed at 48 hpf. Stock solutions for treatments were prepared in DMSO, ethanol, or distilled water, stored at -20 °C, and freshly diluted in E3 medium immediately before use. The final solvent concentration did not exceed 0.1% (v/v), and vehicle controls were included in all experiments. Following treatment, embryos were washed three times for five minutes each in fresh E3 prior to fixation or live analysis. Each experiment utilized three independent clutches.

Wnt/ β -Catenin Pathway Modulation

Canonical Wnt signaling was modulated pharmacologically between 24 and 48 hpf using the compounds listed below. Embryos were gently agitated on a rocking platform during exposure to ensure uniform diffusion.

Compound	Function	Working concentration	Solvent	Treatment window
XAV939 (Sigma-Aldrich #X3004)	Tankyrase inhibitor, β -catenin degradation (pathway inhibition)	5 μ M	DMSO	24–48 hpf
LiCl (Sigma-Aldrich #62476)	GSK-3 β inhibitor, β -catenin stabilization (activation)	50 mM	H ₂ O	24–48 hpf
CHIR-99021 (Tocris #4423)	Potent GSK-3 β inhibitor (activation)	10 μ M	DMSO	24–48 hpf

Hedgehog (Hh) Pathway Modulation

Hedgehog signaling was modulated between 24 and 48 hpf using the following compounds. Equivalent vehicle-treated controls were included for all conditions.

Compound	Function	Working concentration	Solvent	Treatment window
Cyclopamine (Sigma-Aldrich #C4116)	Smoothened antagonist (Hh inhibition)	100 μ M	EtOH	24–48 hpf
Purmorphamine (Tocris #4551)	Smoothened agonist (Hh activation)	10 μ M	DMSO	24–48 hpf

Cholesterol Depletion and Inhibition of Cholesterol Biosynthesis

Cholesterol availability was perturbed using two complementary approaches adapted from Ng et al. (2016) and Signore et al. (2016): acute membrane cholesterol extraction and chronic inhibition of cholesterol biosynthesis.

Acute membrane cholesterol depletion

At 48 hpf, embryos were incubated in 2.5 μ M methyl- β -cyclodextrin (m β CD; Sigma-Aldrich #C4555) for 40 min at 28.5 °C with gentle rocking. After 3 $\times$ 5 min of cold PBS washes, the embryos were quickly fixed in 4% PFA in PBS. This acute treatment reduced anterior RF integrity as assessed by AFRU staining.

Chronic inhibition of cholesterol biosynthesis

To inhibit *de novo* cholesterol synthesis, embryos were treated with 10 μ M atorvastatin (Sigma-Aldrich #A1456) from 24 to 48 hpf. After treatment, embryos were washed in E3 and fixed in parallel with m β CD-treated samples. RF outcomes were assessed by AFRU immunostaining and compared with untreated and vehicle controls.

Reactive Oxygen Species (ROS) Induction via KillerRed

Optogenetic ROS induction was performed using the Tg(sqKR15-16) line expressing membrane-bound KillerRed under an En1b-like promoter. At 24 hpf, embryos were mounted in 1% low-melting-point agarose in E3 containing tricaine and exposed to intense green light (~540–580 nm, 68 mW/cm²) for 90 min. After illumination, embryos were released from agarose and reared in E3 until 48 hpf, then fixed in 4% PFA.

5.7 Chronic inhibition of cholesterol biosynthesis

To inhibit *de novo* cholesterol synthesis, embryos were treated with 10 μ M atorvastatin (Sigma-Aldrich #A1456) from 24 to 48 hpf. After treatment, embryos were washed in E3 and fixed in parallel with m β CD-treated samples. RF outcomes were assessed by AFRU immunostaining and compared with untreated and vehicle controls.

5.8 Experimental Design and Replication

At least three biological replicates were performed for each pharmacological or chemical modification, with at least 15 embryos per experimental group. Embryos showing nonspecific developmental arrest, necrosis, or gross malformations unrelated to treatment were excluded prior to analysis. Phenotypes were classified based on SCO morphology, continuity of the anterior RF, and AFRU immunostaining patterns.

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