POLARITY-SELECTIVE AFFERENT INNERVATION OF THE ZEBRAFISH LATERAL LINE

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SUMMARY

The lateral line is a mechanosensory system present in fish and amphibians that allows them to detect changes in water flow in the surrounding environment. The lateral line is formed by individual organs called neuromasts where the hair cells in charge of the mechanoreceptive function are located. In every neuromast of the lateral line, we can find two subpopulations of hair cells oriented in opposite directions that are selectively innervated by two different types of afferent neurons. In this work, I have elucidated how the expression of the transcription factor Emx2 is responsible for the mechanism that governs selective innervation not only during development, but also during regeneration after damage of any of the components of the system. I describe a mechanistic algorithm that explains previous phenomenological observations. My work sheds light into the neuronal tuning mechanism that allows the correct transmission of different directional information in separate pathways to the central nervous system and its maintenance throughout the fish life even despite the exposure to frequent receptor cell turnover and neuronal peripheral damages.

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1. INTRODUCTION

The detection of external cues is essential for animals to properly react to environmental cues. Exterosensory systems are the interphase between the external world and the brain. These systems contain three elements: peripheral receptors in charge of information detection, transmission pathways, and neural representation of that input (Pujol-Martí and López-Schier, 2013). To ensure correct functioning of the system and accurate representation of the environment, it is essential that peripheral receptors are connected to the right processing centers and that the input from different sensory modalities is properly integrated. Different sensory modalities are organized in different ways, but some key elements are highly conserved across species. One example is the retinotopic map for vision or the tonotopic map for hearing in vertebrates (Pujol-Martí and López-Schier, 2013).

Mechanosensation is the sensory modality that allows organisms to detect mechanical cues. Mechanosensation is involved in the senses of touch, hearing and balance, and in the feeling of pain. It constitutes the basis of some forms of decision-making, directing behaviors that include spatial navigation and the detection and escape from predators. Posture during locomotion and rheotaxis are other examples of highly conserved processes that rely on mechanoreceptive hair cells (Flock and Wersäll, 1962; Hudspeth, 1989; Ghysen and Dambly-Chaudière, 2007; Bleckmann, 2008; Pujol-Martí and López-Schier, 2013; Basaldella et al., 2015).

During my thesis work I have investigated synaptic partner recognition between hair cells and ascending neurons. I have used the mechanosensory lateral line in larval zebrafish as experimental model (Dambly-Chaudière et al., 2003; Ghysen and Dambly-Chaudière, 2005).

The zebrafish is an optimal experimental model for studies of development, neuronal function and behavior. Three main features of the zebrafish stand out: 1) optical

transparency, 2) ease to genetic manipulation, and 3) fast development (Friedrich et al., 2010). Transparency is a great advantage for visualization of internal structures under the microscope. The genetic manipulation of the zebrafish allows us to obtain transgenic fish with directed expression of fluorescent proteins, which is useful for imaging, and it also can be used as a tool for disrupting/enhancing the function of selected gene products and study its effect on the organism. The fast development is an advantage because it allows the creation of stable transgenic and mutant strains in short periods.

1.1 LATERAL LINE

The lateral line is a mechanosensory system present in fishes and amphibians that mediates a sense of "distant touch". It detects water perturbations within short distances around the animal (Dijkgraaf, 1963; Bleckmann, 2008). It also allows the animal to detect the presence of a submerged animate or inanimate object and determine its movement direction through perception of the strength and direction of the water flow, and integrating the information received from different parts of the body. The organs of the lateral line, called neuromasts, show structural and physiological similarities to the auditory and vestibular systems of mammals. In both cases we find the same fundamental elements: mechanosensory hair cells at the peripheral level, afferent neurons transmitting the information centrally, and central processors in the brain (Suli et al., 2012; Pujol-Martí and López-Schier, 2013).

The lateral line plays a role in functions like escape responses, prey detection, predator avoidance, schooling, courtship and rheotaxis (the detection of the direction of water flow) (Ghyssen and Dambly-Chaudière, 2007; Bleckmann, 2008; Nagiel et al., 2008; Suli et al., 2012; Pujol-Martí and López-Schier, 2013). Neuromasts are formed by three cell types: sensory hair cells, non-sensory supporting cells and mantle cells (Bleckmann, 2008). Superficial neuromasts are sensitive to water velocity, whereas canal neuromasts are sensitive to water acceleration (Kalmijn, 1988). Larval zebrafish only has superficial neuromasts.

The lateral-line system is divided in two main branches, one corresponding to the head (Anterior Lateral Line or ALL) and another one located on the trunk and tail fin of the fish (Posterior Lateral Line or PLL) (Dambly-Chaudière et al., 2003; Ghysen and Dambly-Chaudière, 2007). The lateral line receives its name from the arrangement of the PLL neuromasts, which flank both sides of the animal organized in a line from the caudal part of the head to the tip of the tail (Ghysen and Dambly-Chaudière, 2004). Different fish species present variations in the number, location, shape, orientation and distribution of neuromasts (Bleckmann, 2008). Neuromasts receive both afferent and efferent innervation. Processing of lateral-line transmitted mechanical information involves several areas of the brain, including the medulla and the telencephalon (Bleckmann, 2008). The central projections of the afferent neurons in the hindbrain form a somatotopic map that represents the spatial distribution of the neuromasts, which may be relevant for the fish to understand directional features of hydrodynamic stimuli (Pujol-Martí et al., 2010).

1.1.1 POSTERIOR LATERAL LINE DEVELOPMENT

The posterior lateral line develops from a migrating primordium (Metcalfe, 1985). The sequence of development has been described by several authors (Figure 1, Ledent, 2002; López-Schier et al., 2004; Ghyssen and Dambly-Chaudière, 2005; Sapède et al., 2005 and Ghyssen; Dambly-Chaudière, 2007), and it consists of the following sequence:

1. A placode originates at 18-20 hours post-fertilization (hpf) posterior to the otic region. This placode divides giving origin to a rostral group of around 20 cells and a caudal group of around 100 cells.
2. The rostral group of 20 cells differentiates and generates the PLL ganglion, which comprises the afferent neurons that innervate the PLL neuromasts.
3. The caudal group forms the primordium (primI), which will start migrating at 20 hpf along the body following the horizontal myoseptum and in a caudal direction, reaching the tip of the tail at 40 hpf.

4. During this migration, also called first wave, the primordium deposits the groups of cells called proneuromasts that will generate the L1-5 and terminal neuromasts after differentiation. At the same time, neuronal axons extend from the PLL ganglion following the primordium and glial cells migrate following the axons.
5. While primI migration is occurring, at approximately 24 hpf, a second placode originates close to where the first one did.
6. The second placode gives rise to a group of cells that will further divide in three subgroups
    a. D1 neuromast
    b. Second primordium (primII): migrates following the same path as primI and originates a second wave of neuromasts: L2i, L2ii…
    c. Dorsal primordium (primD): migrates originating the dorsal lateral line.
7. The PLL, comprised by the primI and primII, migrates towards a more ventral position as the fish grows.
8. During the following weeks, the dorsal lateral line also moves dorsally and two new sets of neuromasts appear where the firs lines did.
9. The last step of development consists of the formation of neuromast clusters called “stitches”, generated by successive bisections and ventral migration of differentiated neuromasts.

Although the first placode that originates primI is also involved in the development of the lateralis afferent neurons, the formation of the neuromast occurs independently from the innervation. The neurons are determined by the expression of *neurogenin-1* and the formation of neuromasts is determined by *atonal-1a* (Ghysen and Dambly-Chaudière, 2004). The choice of cell fate between neurons or primordium is regulated by Notch signaling, favoring the primordium fate when is activated and neuron fate when is inhibited (Mizoguchi et al., 2011). The migration of the primordium is regulated by CXCR4 (receptor) and SDF1 (ligand). SDF1 is expressed along the horizontal myoseptum, from the first somite to the tip of the tail, which is the pathway that the primordium and, later, the neurons will follow. SDF1 serves as cue that guides the

primordium by interacting with the CXCR4 receptor (Ghysen and Dambly-Chaudière, 2004; Sapède et al., 2005).

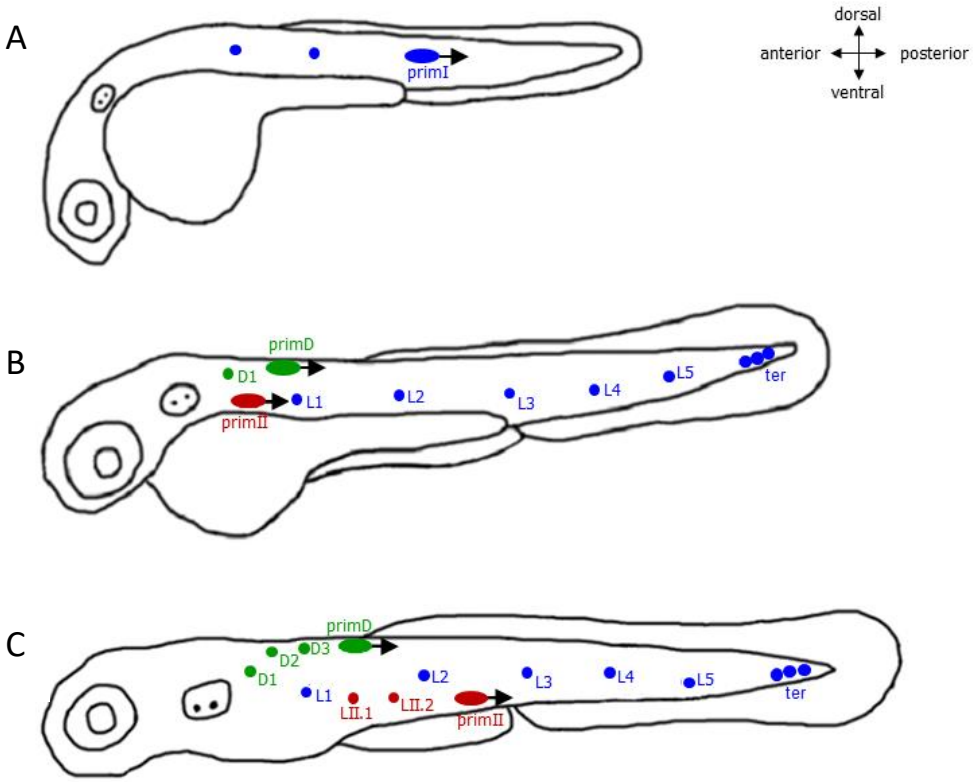

Figure 1. Development of the posterior lateral line. A: Representation of the zebrafish larva at 30 hpf. PrimI is migrating towards the tail and depositing the first wave of neuromasts. **B:** Representation of the zebrafish larva at 2 days post-fertilization (dpf). The neuromasts originated from primI are represented in blue. PrimII starts its migration and is represented in red. PrimD is shown in green. **C:** Representation of zebrafish larva at 3-4 dpf showing the first wave of neuromasts in blue, primII and neuromast originated in the second wave in red, and primD and dorsal neuromasts in green.

The posterior lateral line pattern at last stages of the embryonic development is highly conserved among teleosts, including 5 pairs of lateral neuromasts and 2-3 pairs of terminal neuromasts, and 1 pair of dorsal neuromasts. As the fish grows, the number of neuromasts and afferent neurons increases over time, but their complexity and also the

number of efferent neurons remains the same as in larval stages, so larval neuromasts have the same morphology and functionality as adult fish (Metcalfe, 1985; Ghysen and Dambly-Chaudière, 2007). The neuromasts deposited by the primordium are called primary and at a posterior stage, accessory neuromasts are originated from supporting cells of the primary neuromasts. Accessory neuromasts stay close to the primary neuromasts giving origin to ‘‘stitches‘‘, that are clusters of 10-20 neuromasts innervated by different branches of the same neurons. Unlike the embryonic pattern, the PLL pattern in adult animals is very diverse across species (Ghysen and Dambly-Chaudière, 2005; Ghysen and Dambly-Chaudière, 2007).

1.2 NEUROMASTS

Neuromasts are the functional unit of the lateral line. They are formed by clusters of mechanosensory hair cells and supporting cells that include sustentacular and mantle cells (Ghysen and Dambly-Chaudière, 2004). Each neuromast comprises around 80 cells, distributed approximately in 16-20 hair cells, 50 sustentacular cells and 10 mantle cells. The spatial distribution of the different cells consists of concentric rings, where hair cells and non-sensory sustentacular supporting cells are located in the central position and the group of mantle cells forms an exterior circumference around them (Pinto-Teixeira et al., 2015). This geometry originates radial symmetry. Larval neuromast have identical morphology as those found in the adults. (Metcalfe, 1985). Between two contiguous neuromasts we can find the connecting interneuromast cells (Pinto-Teixeira et al., 2015) (Figure 2A).

Hair cells are polarized in a coherent manner along the plane of the neuromast epithelium. This orientation generates two types of neuromasts in the PLL: horizontal neuromasts (anterior-posterior axis of the fish body) (Figure 2B) and vertical neuromasts (dorsal-ventral axis) (Figure 2C), which respectively originate from primI and primII (Pujol-Martí and López-Schier, 2013). The different orientation of the neuromasts is due to the direction of migration of their originating primordia. As primII advances towards

the tail, it deposits pro-neuromasts that rotate 90º in respect to the original primordium migration trajectory, giving origin to the vertical neuromasts (López-Schier et al., 2004; Ghysen and Dambly-Chaudière, 2007). The presence of both types of neuromasts endows the animal with the capacity to sense the direction of mechanical stimuli (López Schier et al., 2004).

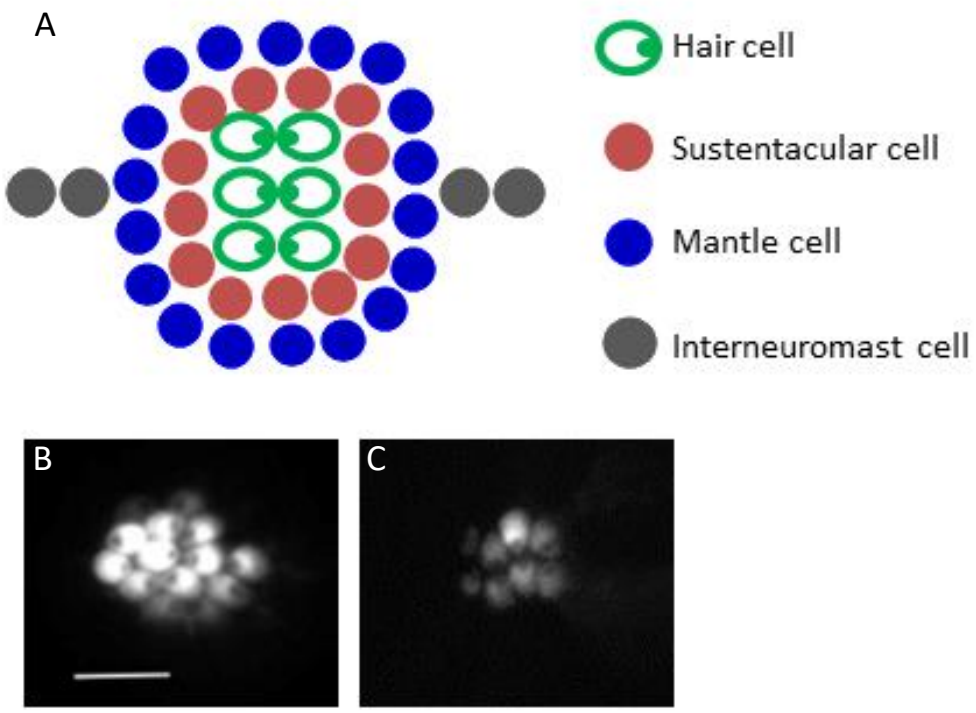

Figure 2. Neuromast anatomy. A: Schematic representation of the distribution of the different types of cells in the neuromast. Hair cells are shown in green and they occupy the central space of the neuromast. **B-C:** Hair cells of opposing polarities in horizontal (B) and vertical (C) neuromasts in the transgenic line Tg (myo6b:actb1-EGFP). Scale bar: 5 µm.

1.3 HAIR CELLS

Hair cells have a hair bundle formed by a set of stereocilia arranged in a staircase shape of increasing length and a kinocilium located next to the tallest one (Figure 3A). Stereocilia are interconnected at the tip by links that include mechanotransduction components. The hair bundles in neuromasts are included into a gelatinous cupula that

is in direct contact with the surrounding water and makes all hair bundles move together in response to a mechanical stimulus. The presence of the cupula may contribute to an increased system sensitivity because all hair bundles move at once, enhancing stimulus detection and transmission (Flock and Wersäll, 1962; Bleckmann and Zelick, 2009; Kindt et al., 2012; Mogdans and Bleckmann, 2012; Deans, 2013; Pujol-Martí and López-Schier, 2013).

The function of hair cells in the lateral line and in the inner ear is highly conserved across species (Hudspeth, 1989). Sound, head movements and changes in water currents are examples of stimuli that are captured by hair cells in the auditory and vestibular system of mammals and in the lateral line of fish and amphibians (Einhorn et al., 2012; Suli et al., 2012). The process of encoding external stimuli into electrical responses is known as sensory transduction (Trapani and Nicolson, 2010). Transduction channels in the hair cell mechanoreceptive bundle are controlled by mechanical forces, allowing for motion detection and constituting the basis for senses like hearing or equilibrium. Mechanical stimuli open or close transduction channels, altering the membrane potential and eliciting the release of neurotransmitters at the synapse. This excites the postsynaptic afferent neuron that transmits the information to the brain (Hudspeth, 1989; Mogdans and Bleckmann, 2012).

The location of the kinocilium provides the hair cell with planar polarization along the neuromast epithelium (Figure 3). In neuromasts, hair cells originate in pairs from pluripotent supporting cells giving rise to two populations of hair cells oriented 180º to each other, intermingled and in equal number (Figure 2). Sibling hair cells show opposing polarity (opposite position of the apical kinocilium) (López-Schier et al., 2004; Faucherre et al., 2009). This polarization pattern creates an axis of symmetry in the neuromast where half of the hair cells are polarized to one direction and the other half are polarized in the opposite direction. The axis of symmetry can be anterior-posterior (horizontal neuromasts) or dorsal-ventral (vertical neuromasts) (Figure 3C) and is evident only during the first hours of development or after regeneration, at later stages all cells are intermingled (Ghysen and Dambly-Chaudière, 2007; Faucherre et al., 2009; Pujol-Martí and López-Schier, 2013).

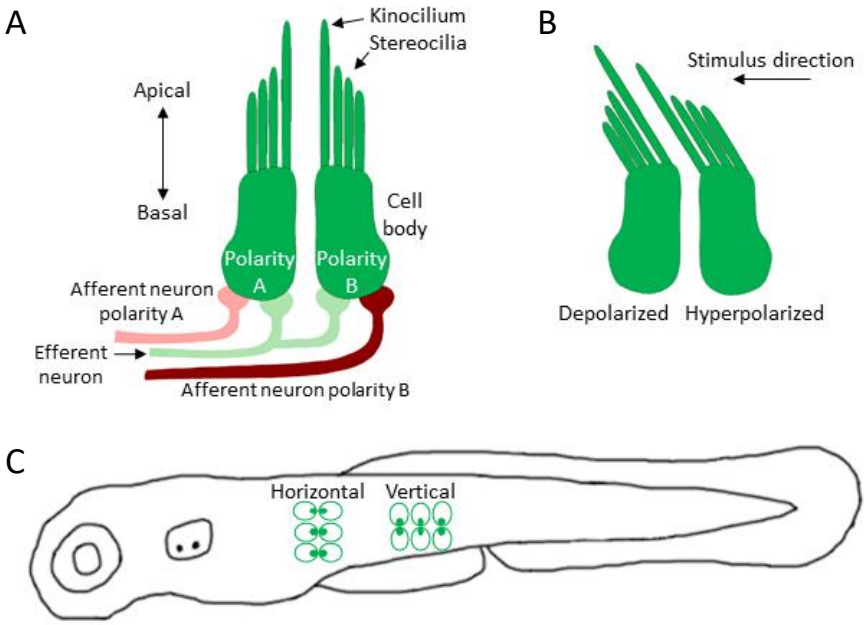

Figure 3. Hair cell anatomy and neuromast orientation. A: Representation of hair cell anatomy and innervation by afferent and efferent neurons. **B:** Representation of the mechanical simulation of the hair cells in the two possible directions of excitability. **C:** Horizontal neuromasts are oriented in the anterior-posterior axis and vertical neuromasts in the dorsal-ventral axis of the fish.

The polarization of the hair bundle defines the direction of excitability of the hair cell. Stimuli that push the hair bundle towards the kinocilium elicit depolarization of the hair cell via opening of mechanotransduction channels, whereas stimuli in the opposite direction result in cell hyperpolarization when the open probability of channel decreases (Figure 3B). Therefore, horizontal neuromasts are sensitive to anterior-posterior stimuli whereas vertical neuromasts detect stimuli in the dorsal-ventral axis. In case of perception of a diagonal stimuli direction, the response is intermediate and proportional to the vectorial projection on those orthogonal axes (Hudspeth, 1989; Denman-Johnson and Forge, 1999; Pujol-Martí and López-Schier, 2013). Following a mechanical stimulus along the anterior-posterior or dorsal-ventral axis, half of the hair cells will result depolarized whereas the other half will be hyperpolarized because of the presence of both polarities in each neuromast (Faucherre et al., 2009).

During development, the shape of the hair cells is dynamic, the soma extends projections that have been previously described as polarity-specific scaffolds for afferent innervation (Dow et al., 2015). Figure 4 shows how projections extend and retract over time in a neuromast of a 2-day post-fertilization (dpf) fish. Dynamic filopodia are not an exclusive feature of young hair cells, there is also evidence that during development, neurons' dendrites extend filopodia that aid them in the search for a synaptic input (Kishore and Fetcho, 2013).

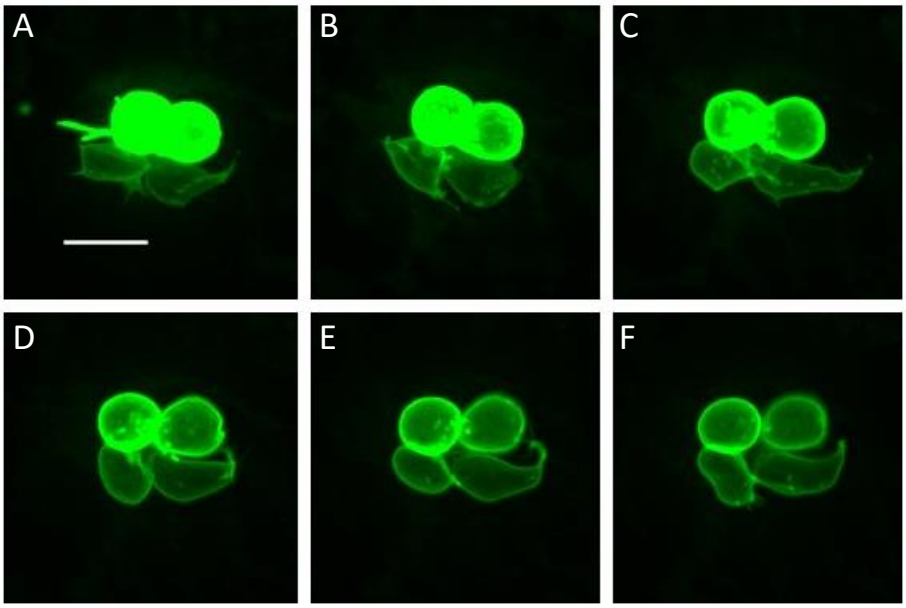

Figure 4. Hair cell projections. A-F: Images of a 2 dpf fish taken at 55 minutes intervals show the projections present in the hair cells of a developing neuromast. Tg (pou4f3:GAP-GFP) labels the membrane of the hair cells in green. Scale bar: 5 µm.

Hair cells in the inner ear and in the lateral-line neuromasts of fish and amphibians can very effectively regenerate after damage throughout life, in contrast to those in the mammalians inner ear, which have limited capacity of regeneration (Ghysen and Dambly-Chaudière, 2007; Nagiel et al., 2008; Suli et al., 2012; Steiner et al., 2014). This regenerative capacity is maintained through the whole life of the animal, regardless how many damage-regeneration cycles take place, and it is essential for keeping the function of the organ throughout the life of the animal, which is especially important in sensory

organs because of the risks associated to being exposed to the external environment (Cruz et al., 2015; Pinto-Texeira et al., 2015).

When lateral-line hair cells die, new hair cells regenerate in pairs giving rise to siblings with opposing planar polarities. Hair cells start reappearing 8-12 hours after ablation of existing cells. Neuromasts achieve complete recovery structure and function in 48-72 hours (López-Schier and Hudspeth, 2006; Nagiel et al., 2008; Pinto-Teixeira et al., 2015). The neuromast recovers the original distribution and symmetry axis, independently of the nature and characteristics of the damage.

1.4 PLANAR CELL POLARITY

Hair cells are coherently polarized relative to adjacent cells in the epithelium (Denman-Johnson and Forge, 1999). During hair-cell development, the kinocilium initially develops located at the center of the apical surface, it then moves to one side determining the polarity of the hair cell, and after that a group of staircase-like stereocilia forms next to it with the longest one being closest to the kinocilium (Figure 3A; Hudspeth, 1989; Ghysen and Dambly-Chaudière, 2007). Hair cell polarization is independent of innervation as demonstrated by experiments that block the formation of neurons by removing *neurogenin1* in zebrafish (López-Schier et al., 2004; Nagiel et al., 2008).

Planar polarity can be observed at three levels: 1) the subcellular level reflects the polarized organization of the stereocilia bundle and the location of the kinocilium; 2) Planar Cell Polarity (PCP), which refers to the overall organization at cellular level of hair bundles between neighboring cells, representing the coordinated organization of polarized hair cells in an epithelium in two dimensions; 3) the highest level refers to the organization across in organs or the whole organism and is important for stimuli integration (López-Schier et al., 2004; Deans, 2013).

The mammalian utricle and saccule are two organs located in the inner ear, in charge of detecting linear accelerations and involved in the control of balance and posture. Hair cells in the utricle and saccule are also polarized in opposite directions respective to the so-called line of polarity reversal (LPR), which is a figurative line that divides the organ in two hair cells compartments of opposite polarities similarly to a neuromast (Holley et al., 2010; Eatock and Songer, 2011; Deans, 2013).

The Planar Cell Polarity system is determined by the organization of the PCP proteins and regulates the establishment and maintenance of planar polarity in various organs, from the eye in *Drosophila* to the inner ear in mammals (Ghysen and Dambly-Chaudière, 2007; Deans 2013). The core Planar Cell Polarity proteins include Frizzled (Fz), Dishevelled, Van Gogh (Vangl2), Prickle, Diego and Flamingo (Hale and Strutt, 2015). The distribution of the planar cell polarity core proteins provides landmarks for polarization (Strutt, 2003; Deans et al., 2007; Axelrod, 2008; Deans, 2013; Shi et al., 2016). In *Drosophila*, planar cell polarity is stablished by a signaling pathway that includes accumulation of Frizzled (Fz), Dishevelled and Diego at the distal side of wing epidermal cells, opposite to Van Gogh and Prickle at the proximal part. In the mammalian vestibular epithelia, there is an analog asymmetric distribution of planar cell polarity proteins, where Frizzled is located opposite to Vangl2/Prickle (Deans et al., 2007; Deans, 2013).

Vangl2 is part of the core Planar Cell Polarity system in hair cells. It is required for the localization of the basal bodies that determine the position of the kinocilia and, consequently, the orientation of the hair bundles. A lack of Vangl2 affects the movement of the kinocilium away from the apical center resulting in a failure of orientation or randomization of the polarity, this means that hair cells remain polarized but in random directions (Figure 5). Vangl2 does not affect the division orientation of hair cell precursors (Ghysen and Dambly-Chaudière, 2007; López-Schier and Hudspeth, 2006; Copley et al., 2013; Jiang et al., 2017). In Vangl2 mutants as well as in Fz mutants, hair cells are incorrectly oriented but correctly polarized, (Deans, 2013). By contrast, an overexpression of the Vangl2 protein produces an anterior bias but it does not result in a randomized phenotype, suggesting therefore that proper polarization of the hair cells is achieved by an asymmetric distribution of Vangl2 (Mirkovic et al., 2012). In the case

of zebrafish, Vangl2 is localized caudally in horizontal neuromasts and ventrally in vertical neuromasts; this distribution is true for all the hair cells in the neuromast, regardless their polarity (Mirkovic et al., 2012).

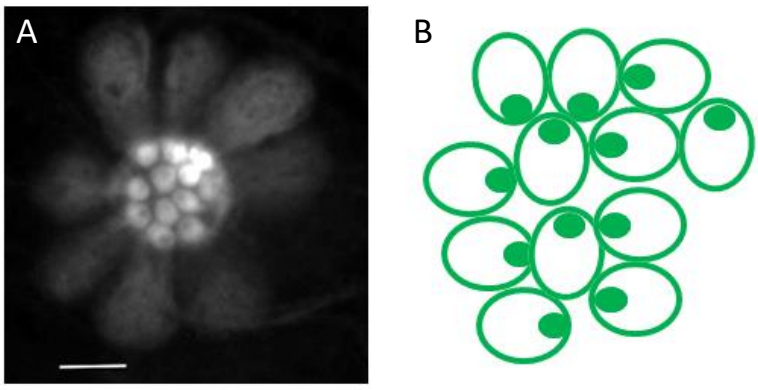

Figure 5. Vangl2 mutant phenotype. A: Apical view of a neuromast labelled with by fluorescent phalloidin. This staining shows random orientation of the hair cells in a *trilobite* mutant fish. **B:** Schematic representation of the *trilobite* mutant showed in A. Scale bar: 5 µm.

1.4.1 EMX2

The homeodomain protein Emx2 is a transcription factor that plays a critical role in the regulation of hair-cell development in the inner ear of mammals, in which the expression of Emx2 determines the pattern of hair cells in the organ of Corti (Holley et al., 2010).

Emx 2 expression determines the position of the hair cell kinocilium along a defined axis but it does not have an effect on the distribution of proteins of the core Planar Cell Polarity complex like Pk2 or Vangl2. In the case of the vestibular maculae, Emx2 is only expressed in the hair cells at one side of the line of polarity reversal. Observations of the hair cells in the saccular and utricular maculae show that the lack of Emx2 leads to the loss of dual polarization as all hair cells are found to be pointing in the same direction instead of divided in two of opposite orientations at both sides the line of polarity

reversal as observed in wild type animals (Figure 6; Holley et al., 2010; Deans, 2013; Jiang et al., 2017).

The organization of the lateral line hair cells resembles the arrangement of hair cells in the mammalian vestibular system. In neuromasts, Emx2 is responsible for the mirror of symmetry pattern observed at developmental stages because it mediates hair bundle polarity reversal in one half of the population of sensory cells. Hair cells are born in pairs giving rise to two sibling cells that display opposite polarities due to the asymmetric expression of Emx2 in only one of them. Emx2 expression is restricted to half of the population of hair cells, specifically in those oriented posteriorly in horizontal neuromasts and ventrally in the vertical neuromasts (Jiang et al., 2017).

Emx2 has been described as a global polarity cue of sensory hair cells that reverses the interpretation of the orientation indicated by the PCP proteins along a determined direction, resulting in opposite polarization respect to the sibling hair cell (Jiang et al., 2017).

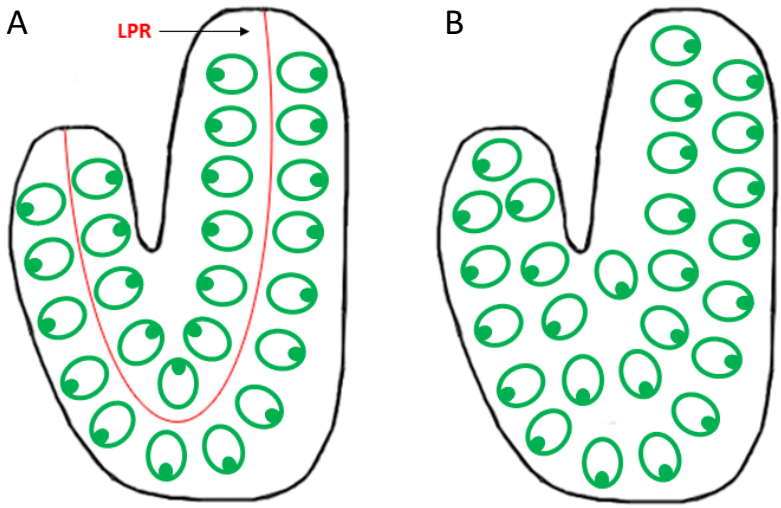

Figure 6. Saccula. A: Wild type hair cell orientation at both sides of the LPR (Line of Polarity Reversal). **B:** Emx2 mutant, hair cells are misoriented and the LPR disappears.

1.5 AFFERENT INNERVATION OF THE LATERAL LINE

Afferent neurons of the lateral line are in charge of transmitting the information detected by the hair cells to the brain. These neurons have their cell bodies located in a ganglion and extend peripheral axons to the neuromasts, and central axons to the hindbrain. The anterior lateral line ganglion is located anterior to the ear and the posterior lateral line ganglion posterior to it (Dambly-Chaudière et al., 2003; Pujol-Martí and López-Schier, 2013).

In the anterior lateral line there are two nerves, an anterior-dorsal nerve that innervates supraorbital, infraorbital and otic neuromasts, and an anterior-ventral nerve that innervates mandibular and opercular neuromasts. In the posterior lateral line, there can be distinguished also two nerves, the one that innervates the neuromasts in the body middle line, and the one innervating dorsal neuromasts (Raible and Kruse, 2000). In the posterior lateral line ganglion, we can find approximately 50 afferent neurons at 5 dpf, which is around four times the number of neuromasts (Liao, 2010). The ratio of afferent neurons to neuromasts in the anterior lateral line differs from that of the posterior lateral line, what suggest that may be differences in the sensitivity threshold or spatial resolution (Haehnel et al., 2012).

The central axons of the anterior and posterior lateral line extend ipsilaterally towards the hindbrain and bifurcate while forming two apposed but separated columns leaving the one corresponding to the posterior lateral line more dorsally located. The medial octavolateralis nucleus (MON) in the hindbrain receives afference from the posterior lateral line in the dorsal part, from the anterior lateral line in the medial part and from the inner ear in the ventral part. The torus semicircularis is a midbrain structure that receives indirect afferent input from the lateral line and inner ear and then conveys the information to higher centers, such as the thalamus and hypothalamus (Fame et al., 2006). The columns of central axons are organized in a somatotopic distribution that represents the position of the innervated neuromasts, allowing a topographic representation of the spatial distribution of neuromasts (Gompel et al., 2001; Nagiel et

al., 2008; Pujol-Martí et al., 2010; Pujol-Martí et al., 2012; Pujol-Martí and López-Schier, 2013).

Fish decode water flow direction through central integration of the information detected by neuromasts of different orientation across the left-right axis of the body (Oteiza et al., 2017) The components of a mechanical stimulus (location, direction and velocity) detected by the receptors need to be correctly transmitted to the brain for a correct neural representation of the information perceived to process environmental information and to react appropropiately. It is therefore crucial that peripheral sensory receptors are connected to processing centers in the central nervous system through coherent synapses with lateralis afferent (ascending) neurons (Coombs et al., 1998; Pujol-Martí and López-Schier, 2013).

1.6 SYNAPTIC SELECTIVITY

In larval zebrafish, each neuromast is usually innervated by four lateralis afferent neurons, and each neuron synapses with around half of the hair cells in individual neuromasts. Only exceptionally, in the cluster of terminal neuromasts located at the tip of the tail, each neuron innervates more than one individual organ. Because each afferent neuron contacts only hair cells of the same planar polarity, the neuronal group divides the neuromast into synaptic compartments of planar polarity. This indicates that the vectorial information transduced by hair cells is transmitted by separate ascending channels to the brain (Zimmerman, 1979; Bleckmann, 2008; Nagiel et al., 2008; Obholzer et al., 2008; Faucherre et al., 2009; Faucherre et al., 2010; Trapani and Nicolson, 2010; Pujol-Martí and López-Schier, 2013).

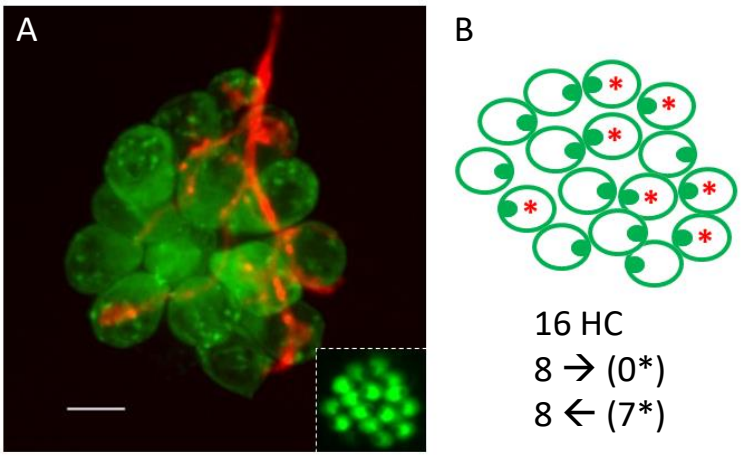

Figure 7. Afferent selectivity. A: Horizontal neuromast innervation in Tg(myo6b:actb1-EGFP) after Sill:mCherry DNA injection. Polarity of the hair cells (bottom right corner) is observed in a different plane. **B:** Schematic representation and quantification of the neuromast innervation. Scale bar: 5 µm. Adapted from Lozano-Ortega, Valera, et al., 2018, with permission.

In zebrafish, hair cells are able to regenerate after damage, recovering original neuromast structure and functionality. Afferent re-innervation after this damage-regeneration process maintains the original selectivity memory as neurons establish synapses again with only the original polarity they were innervating before the trauma, recovering thus the ability of the neuron to discriminate the vectorial component of a mechanical stimulus (Nagiel et al., 2008; Faucherre et al., 2009). Given the frequent turnover of sensory cells in the lateral line due to the exposure to the environment, understanding the underlying mechanism of this continuous refinement is a crucial matter of sensory biology.

Axons of the posterior lateral line are located in a very superficial position and, therefore, exposed to external damage. For this reason, the regenerative capacity of the lateral lines sensory nerves is crucial for the maintenance of its mechanosensory function. Unlike the central branch, in which regeneration is limited to only 5% of the cases, all or the majority of the peripheral afferent nerve of the zebrafish regenerates effectively in approximately 24 hours in the case of young fish or later in the case of adult fish (Xiao et al., 2015). After damage in the peripheral afferent axon, re-

innervation occurs maintaining the original selectivity, the system has polarity memory. Exceptionally, when all axons except one are removed, the remaining solitary neuron becomes promiscuous and takes over all the hair cells in the neuromast, regardless the polarity; the system recovers selectivity upon re-growth of neighboring axons (Pujol-Martí el al., 2014).

The separate transmission of the two polarities to the brain and the somatotopic organization of the neuromast in the central nervous system is an indicative of how the lateral line afferent neurons can precisely convey the information about the stimulus components.

The establishment of correct connections between afferent neurons and their partner neurons in the brain is essential for the proper assembly of functional neural circuits that convey sensory information correctly. The neuronal connectivity pattern is established during development, when growing axons start a connecting process that undergoes a refinement procedure that results in a fully functional system. The innervation process is dynamic, an afferent neurite reaches a neuromast and explores, establishing transient contacts with hair cells of any polarity and only stabilizing and forming synaptic connections with hair cells of the “right” polarity in which the neurite becomes bulged (Faucherre et al., 2009; Faucherre et al., 2010).

1.6.1 MECHANISM OF SELECTIVITY

The central aim of the work presented in this thesis is the elucidation of the mechanism that governs the selective synapse between hair cells of identical polarity and their partner afferent neurons. Using the lateral line of zebrafish, I tested several hypotheses using various experimental approaches.

1. **Is selectivity defined by afferent neurons?**

The hypothesis that states that the presence of afferent neurons may instruct hair cell polarity has been ruled out as a selectivity mechanism because hair cell polarity is established before and independently of afferent innervation (Faucherre et al., 2009).

Another possibility is that afferent neurons could be genetically predetermined to strictly innervate one polarity regardless what other afferent neurons synapse (Julien and Sagasti, 2014). Nevertheless, when an afferent neuron is left alone innervating a neuromast by removal of the others, it becomes promiscuous and innervates all hair cells in the neuromasts including the "wrong polarity". When the removed neurons regenerate, they reinnervate the hair cells of the same polarity that they connected before the removal and the promiscuous neuron retracts, recovering thus polarity-selectivity innervation (Pujol-Martí et al., 2014). This result evidences that selectivity is based not only in hair cell-neuron interaction but also amongst the different axons innervating a neuromast. Therefore, selectivity is not a predetermined characteristic of the neurons and it is influenced by the behavior of the rest of the neurons in the afferent nerve.

2. **Is selectivity defined by hair cell activity?**

It has also been hypothesized that neuronal exploration when looking for the right synaptic partner during development is based on evoked hair cell activity. Faucherre et al., 2009 suggested a two-step model in which afferent neurons would arrive to the neuromast and choose stochastically one of the polarities, then it would keep contacting hair cells in the neuromast establishing stable synaptic connections with cells of identical polarity as the first one. This idea emerged when comparing wild-type animals and *tmie* mutants, which are devoid of mechanorreceptive capability. The peripheral arbors of neurons in the mutants were more complex and less persistent and consistent than those in wild type animals (Faucherre et al., 2009). Accordingly, it was concluded that

neurons are able to read and detect the differences in evoked activity generated by the two subpopulations of hair cells and keep stable synapses only with those that are the right match for the neuron (Pujol-Martí et al., 2014).

3 **Is selectivity defined by hair-cell basal projections?**

During development, the hair cells' soma is very dynamic and shows long projections that extend and retract. These structures have been described to be relevant for the selectivity process based on a model in which new-born hair cells extend projections to contact axons of the correct directional tuning, pulling them towards the soma in order to stablish stable synaptic contacts (Dow et al., 2015). The arguments to support the model that hair-cell projections are specific scaffolds are based on the descriptive observation of these structures during development, but no perturbation experiments were performed to test whether basal projections are essential for synaptic selectivity. This hypothesis has been explored in this thesis.

4 **Is selectivity defined by hair-cell identity?**

The selective correlation between neurons and hair cells could be due to a molecular code. This model is based on chemoaffinity and it hypothesizes that hair cells of different polarities are molecularly asymmetric. Neurons would recognize the different markers and would innervate hair cells of the right polarity.

The challenge for testing this hypothesis relies on the difficulty of finding what is the distinctive element between the two subpopulations of hair cells and neurons to experimentally test their relevance during innervation.

5 **Is selectivity defined by efferent neurons?**

Neuromasts are innervated by both, afferent and efferent neurons. Efferent neurons in vertebrates receive input from ascending sensory neurons in the hindbrain and project peripherally to mechanoreceptive cells of the inner ear and lateral line in order to control the sensitivity of the mechanosensory organs (Flock and Russell, 1976; Metcalfe, 1985; Simmons, 2002; Tomchik and Lu, 2006). In birds and mammals, the formation of efferent synapses is one of the process that defines cochlear maturation and takes place prior to the onset of the auditory function but days after afferent-hair cells synapses occurred (Simmons, 2002). In mice, the efferent innervation is involved in the auditory hair cell spontaneous activity pattern during development, and this process is required in the correct formation of the tonotopic map. Al alteration in the efferent activity results in defective connections of the auditory pathways (Clause et al., 2014; Wedemeyer et al., 2018).

In the zebrafish, there are fewer efferent than afferent neurons and in the case of efferent nerves, the ones that innervate mid-body PLL also innervate dorsal and occipital neuromasts (Metcalfe, 1985). Efferent neurons differentiate and extend axons to the lateral line very early in development, which lead to the hypothesis that they can play a role in the growth or maintenance of peripheral structures, for example by aiding hair cells development or supporting hair cell-afferent synapses establishment and refinement (Bricaud et al., 2001; Simmons, 2002). Given the potential role of these neurons in development and refinement, I wanted to better understand their role in the lateral line and explore whether they are involved in the selectivity process.

It is unknown the role that efferent neurons play in afferent-synaptic stabilization in the lateral line. Afferent neurons develop earlier and in some cases they innervate hair cells before efferent neurons reach the neuromast (Figure 8). In other cases, the afferent innervation is closer in time to the efferent. One possibility is that afferent neurons start synaptic target exploration and efferent neurons are involved in the stabilization of the final synapses, but further experiments are required to confirm if they are essential for to the process of afferent synaptic selectivity.

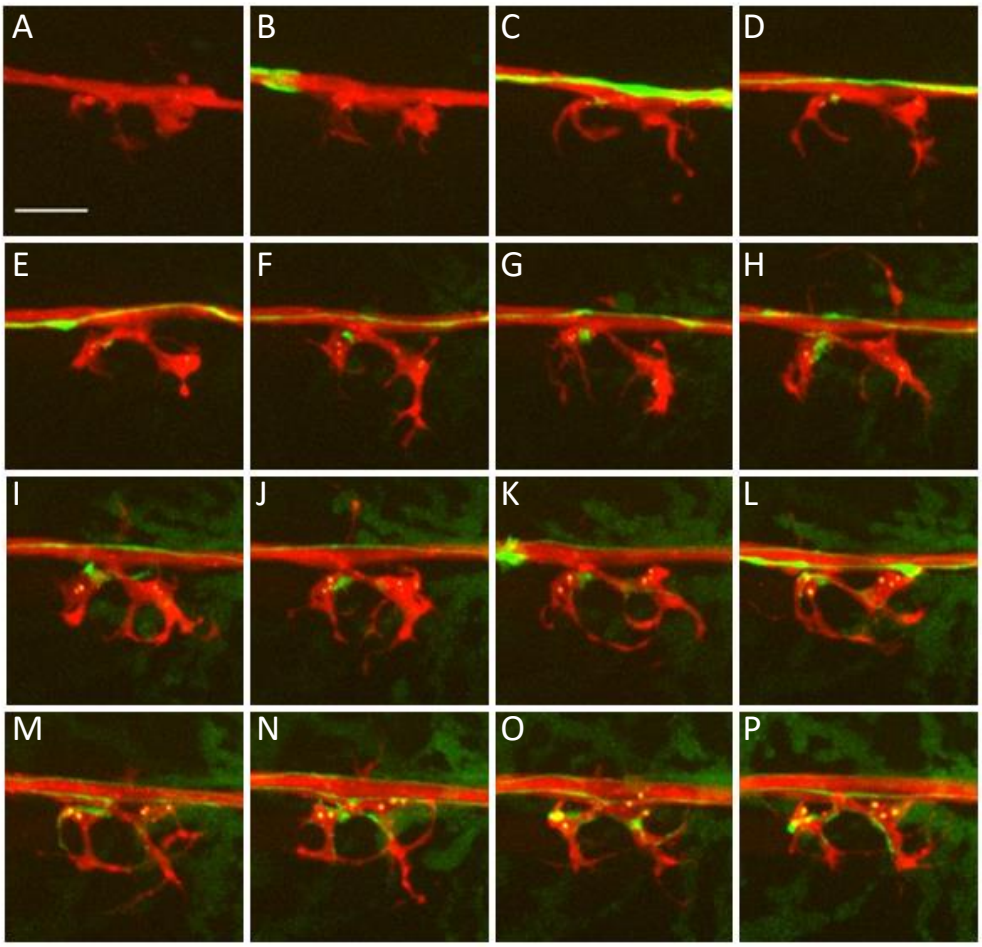

Figure 8. Afferent and efferent synaptogenesis in the posterior lateral line. A-P: Selected time points of a time-lapse imaging of triple transgenic Tg (Islet1:GFP; hsp701:mCherry-2.0cntnap2a; pou4f3:ctbp21-mKOFP) that shows afferent neurons in red, efferent neurons in green and Ribeye in orange. Scale bar: 5 µm.

Therefore, it should be reasonable to consider involvement of the efferent neurons in the establishment of the afferent selective innervation. However, this seems unlikely because efferent neurons contact all hair cells in the neuromast regardless the polarity, meaning that they are not selective (Faucherre et al., 2009).

6 **Is selectivity defined by axonal competition?**

The solitary neuron experiments (Pujol-Martí et al., 2014) suggest that selectivity depends on the interaction of the collective afferent axonal population. The fact that a solitary neuron becomes promiscuous means that selectivity requires the presence of at least two different neurons. This hypothesis nevertheless, does not explain recognition of synaptic partners. It establishes that there is a relationship between the different axons that allows to have two different information pathways corresponding to the two different excitability directions of the hair cells.

2. AIM OF THE THESIS

The aim of my thesis work is to elucidate the mechanism that underlies synaptic partnership between afferent neurons and hair cells of specific planar polarity, using the lateral line of the zebrafish.

Specifically, my thesis focuses on studying the following:

1. The role of hair-cell projections
2. The role of hair-cell identity

3. RESULTS

3.1 HAIR-CELL PROJECTIONS

At early stages of development, the hair-cells' somas are extremely dynamic, as they extend projections for several hours (up to 15hs) after they are born. These projections contain actin and microtubules and have been reported to serve as scaffolds that lead neurites towards the hair cell soma (Dow et al., 2015).

3.1.1 PROJECTIONS DURING DEVELOPMENT

I have studied these structures in order to confirm their function. For this, I have taken advantage of the transgenic line Tg(pou4f3:GAP-GFP), where hair cells' membrane was labeled and projections can be observed. The synapses between afferent neurons and hair cells are characterized by synaptic ribbons (in the presynapse, also called active zone, of the hair cells), which accumulate vesicles formed by the protein Ribeye, encoded by the gene ctbp2. Ribeye can be labelled with the transgenic line Tg(pou4f3:ctbp21-mKOFP). By using the combination of two transgenic lines: Tg(pou4f3:GAP-GFP; pou4f3:ctbp21-mKOFP), we can image hair cells and the active zone as the hair cells mature. Basal projections originating in hair cells normally occur at early stages of development, whereas we can observe that Ribeye starts to be visible in mature hair cells, which are less dynamic (Figure 9). The observation suggests poor temporal correlation between projections and synapses.

I next wanted to directly study the interaction between the afferent axons and hair cells, and test whether projections play any role in synaptic selectivity. For this purpose, I injected Sill:mCherry DNA in double transgenic embryos Tg(pou4f3:GAP-GFP; pou4f3: ctbp21-mKOFP) in order to sparsely label afferent neurons. Then, I screened 3-4 dpf larvae selecting those that had a single neuron per neuromast labelled and I observed the development process using time-lapse imaging (Figure 9).

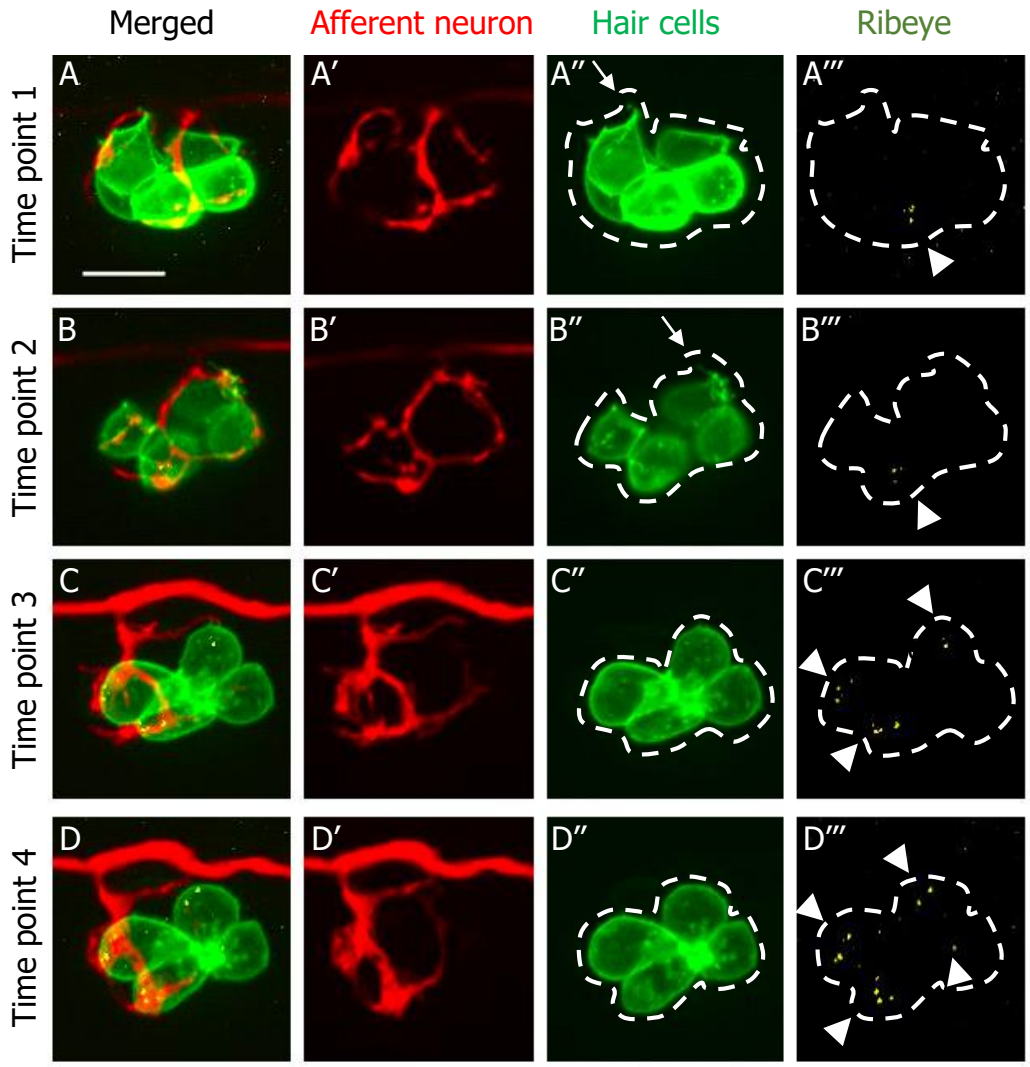

Figure 9. Hair cell projections during development. A-D‴: selected time points from live imaging innervation of hair cells by a single afferent neuron. Arrows point to hair cell projections and arrowheads indicate the presence of ribeye. **A-A‴:** in the first time point we can see how the upper left hair cell has a projection and ribeye is only present in the lower hair cell. **B-B‴:** in the second time point there is a transient contact between a projection of the upper right hair cell and the afferent neuron. **C-D‴:** in time points 3 and 4 the afferent neuron stabilizes the synapsis with the upper left hair cell and the contact is lost with the upper right hair cell, which starts expressing ribeye, proving that the presence of a hair cell projection-afferent neuron contact does not always lead to a stable synapsis. Scale bar: 5 µm. Adapted from Lozano-Ortega, Valera, et al., 2018, with permission.

The process of target exploration and hair-cell innervation is described in Figure 9. I show how the two upper hair cells extend projections that contact the neuron (arrows)

and then, the contact with the right cell is lost, whereas the contact with the left one is maintained and the neurite becomes bulged and stable. Ribeye accumulation can be observed (arrowhead) to co-localize with the marked neuron in the case of the left hair cell.

In early developmental stages, axons dynamically explore the possible synaptic partners in the neuromast. As this exploration process happens, axons may interact with hair cells of both polarities. This transient contact between the two synaptic partners is not always mediated by projections and is normally not permanent. Young hair cells randomly extend projections during development and afferent neurons randomly contact hair cells regardless the polarity. After a few hours, the searching process refines and axons selectively maintain contact with hair cells of only one polarity stabilizing the synapses.

The quantification of hair cell projections interactions with neurons also shows little correlation between the presence of these projections and innervation. Out of 40 hair cells that I monitored, only 3 had projections that contacted the neuron that would innervate that cell. In 13 cases there was a contact between projections and neurons but no final innervation and in the remaining 24 projections were not present or did not interact with the neuron.

3.1.2 HAIR-CELL PROJECTIONS DURING AFFERENT RE-INNERVATION

After neuronal or hair-cell injury, axons re-innervate hair cells of the original orientation. This is also true even in cases when neurons innervate a neuromast different than the original, indicating that the system has memory and keeps the innervation selective (Pujol-Martí et al., 2014).

In order to study if basal projections play any role in polarity-selective innervation, I have used the triple transgenic Tg(hsp70l:mCherry-2.0cntnap2a; pou4f3:GAP-GFP;

pou4f3:ctbp21-mKOFP), which allows the visualization of afferent neurons, hair-cell membrane and Ribeye. After severing the axons using an ultraviolet laser, I imaged the axonal regeneration process over time and I have observed how new connections between neurons and hair cells are established (Figure 10). Hair-cell re-innervation is completed within a few hours I started a time-lapse imaging of the fish approximately 2 hours after axon severing to see the entire re-innervation process.

Figure 10 shows how axons are capable of reengaging synaptic connections in absence of basal hair cell projections and, although this transgenic line does not allow the visualization of hair-cells polarity, it is known that axonal re-innervation after neuronal damage is always polarity selective, the system maintains its memory (Figure 11).

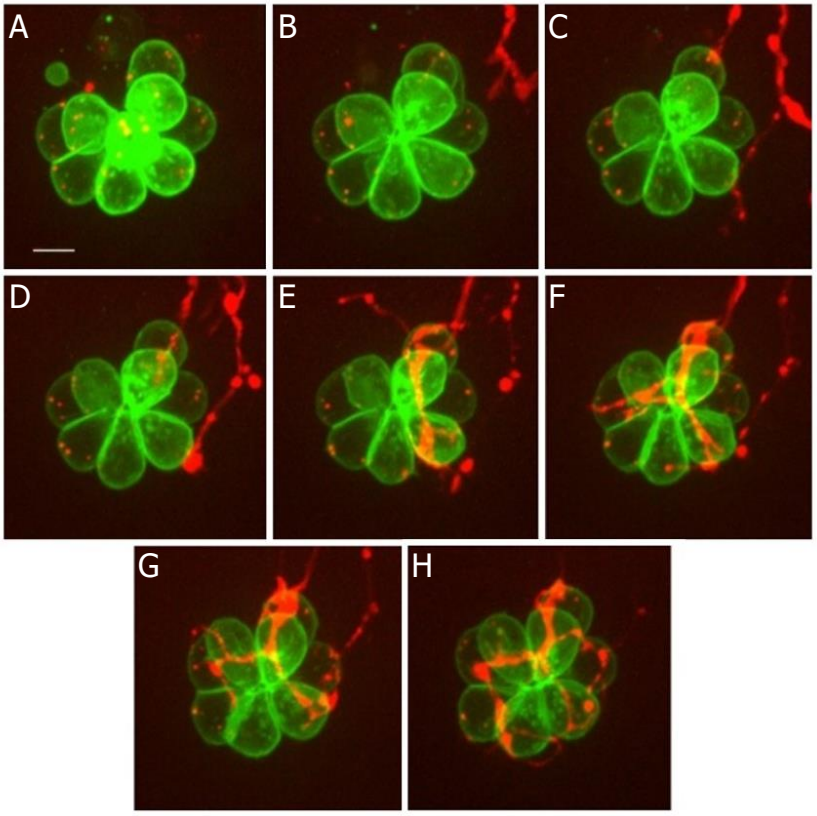

Figure 10. Hair cell projections during re-innervation. A-H: ca. 2.5 hours interval time lapse of afferent neuron regeneration after severing in a 5 dpf fish. Hair cells are labelled in green and neurons in red in the double transgenic Tg (pou4f3:GAP-GFP;

hsp70l:mCherry2.0cntnap2a). Innervation occurs in the absence of basal projections in the hair cells. Scale bar: 5 µm.

3.1.3 CONCLUSION

Hair-cell projections correlate in time with synaptogenesis but they are not associated with synaptic components. Looking together at the data during development and during regeneration, in neither case there is any correlation between projections and neuronal innervation. Projections are present during the first hours after hair cells are born and they do not reappear during re-innervation after neuronal damage, therefore, projections seem to be restricted to young hair cells.

These observations suggest that hair cell projections may have a facilitative role in synaptogenesis, expanding the surface of the soma and therefore increasing the chances of finding axon, but not necessarily in a polarity specific manner. Therefore, we can conclude that hair cell projections are not selective scaffolds of innervation and they do not direct the neurites to hair cells of the right polarity.

3.2 SELECTIVITY MEMORY

Unlike central branches, peripheral axons of the lateral line afferent neurons have a great regenerative capacity after injury, they re-grow and reestablish synapses with hair cells. This re-innervation can happen with the same neuromast they were innervating before or with different one, but in both cases, neurons always go back to the original polarity (Figure 11I).

In some cases, when peripheral axons are ablated closer to the ganglion, they re-grow towards a different target neuromast and, as a result, they innervate a different

neuromast than originally (Figure 11J left bar), but even in these circumstances, polarity selective innervation is always maintained (Figure 11I left bar).

Figure 11K-13N shows the results of an experiment that intends to further test the mechanism that underlies the selectivity recovery of regenerating axons after damaging both components, neurons and hair cells. The experiment consisted of a complete reset of the system by ablating the neurons and killing the hair cells in the Tg (myo6b:actb1-EGFP; nkhgn39d) with a single afferent neuron labelled by Sill:mCherry DNA injection. After ablation, fish were left to recover for a few hours and observed under the microscope, showing a fully recovered neuromast where the single labelled neuron was innervating the original polarity, recovering thus the selective innervation of the system.

Furthermore, there are special situations in which neurons change the type of neuromast that they innervate. In this case, a neuron originally innervating a horizontal neuromast re-grew and switched to innervate a vertical neuromast. Interestingly, there is a consistent correlation in which neurons originally innervating caudal neuromasts always switch to ventral neuromasts and neurons innervating rostral neuromasts always switch to dorsal neuromasts (Figure 11 O-S).

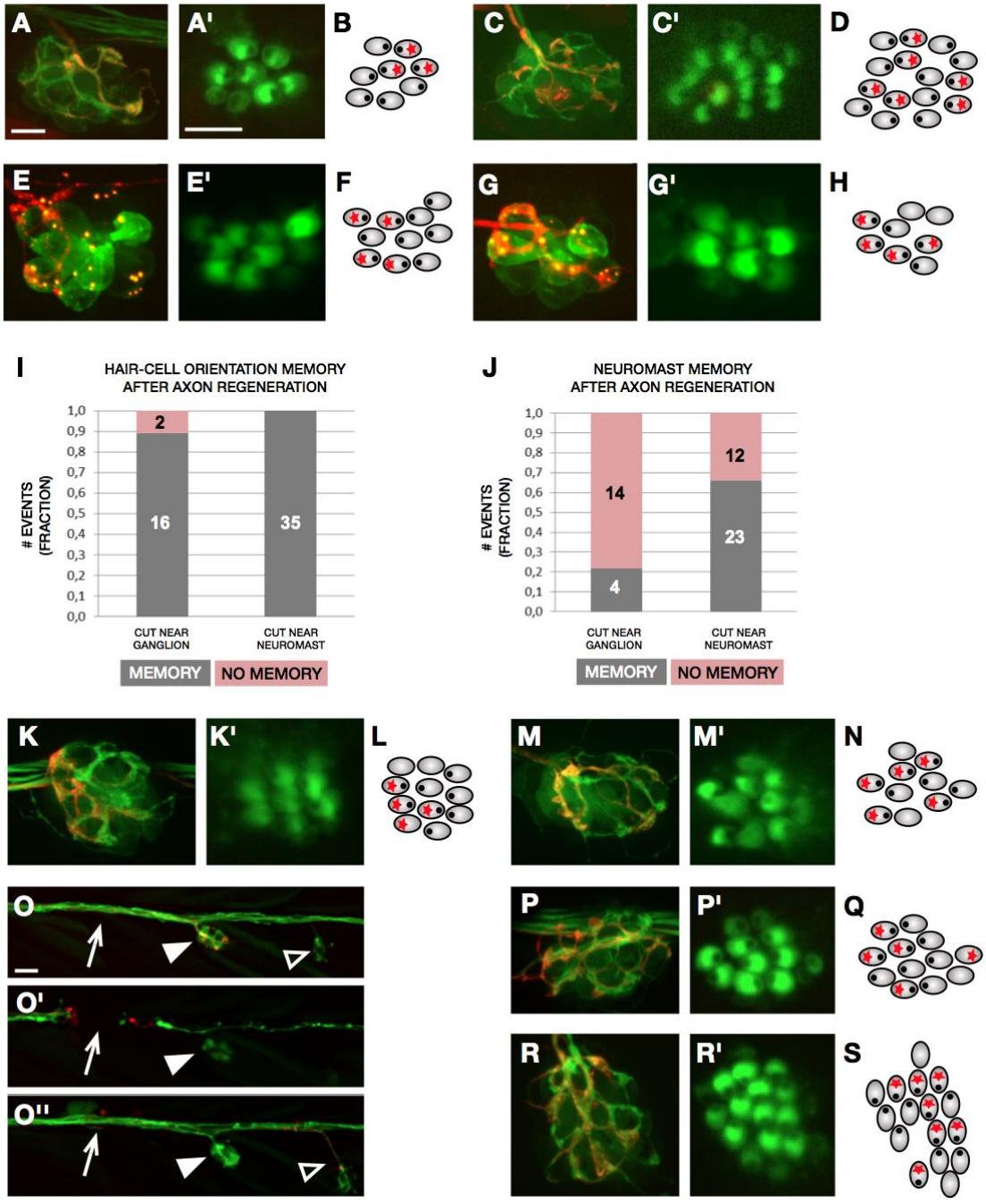

Figure 11. **Selectivity memory.** Transgenic Tg(myo6b:actb1-EGFP) labels hair cells and polarity in green, Tg (nkhgn39d) labels all afferent neurons in green but as they are severed with the laser, this green signal does not interfere with the hair cells, and one single neuron is labelled in red by injection of Sill:mCherry DNA. **A-C:** A neuromast of the transgenic line Tg(myo6b:actb1-EGFP; nkhgn39d) with mosaic expression of mCherry in a single afferent neuron. **A:** The individualized axon (red) synapses with crHCs oriented hair cells. **A':** Magnified view of the hair bundles of hair cells in panel A. **B:** Schematic representation of the example in panel A, in which hair cell orientation is indicated by an eccentric black dot and innervation by a red asterisk. **C:** After severing, the individualized regenerated axon (red) recapitulates synapses with crHCs in the same

neuromast. **C':** Magnified view of the hair bundles of hair cells in panel C. **D:** Schematic representation of the example in panel C, indicating hair cell orientation (eccentric black dot) and synapses (red asterisk). **E:** Neuromast double-transgenic Tg(myo6b:actb1-EGFP; Ribeye-Kusabira) showing a single LAN marked by mosaic expression of mCherry. The axon (red) synapses with rcHCs. **E':** Magnified view of the hair bundles of hair cells in panel E. **F:** Schematic representation of the example in panel E, in which hair cell orientation is indicated by an eccentric black dot and innervation by a red asterisk. **G:** After severing, the individualized axon (red) regenerated to recapitulate synapses with rcHCs. **G':** Magnified view of the hair bundles of hair cells in panel G. **H:** Schematic representation of the example in panel G, indicating hair cell orientation (eccentric black dot) and synapses (red asterisk). **I:** Quantification of hair cell innervation by regenerating singly marked axons of samples in which the axonal bundle was severed immediately below the neuromast (N = 35) (near neuromast) or furthest (N = 18) (near the posterior lateralis ganglion). All 35 axons cut near neuromast re-innervated hair cells of the original orientation, but 16 of the axons cut near the ganglion (0.89) re-innervated the original orientation after regeneration (memory in grey), and 2 (0.11) did not (no memory in pink). **J:** Quantification of neuromast innervation by regenerating singly marked axons of samples in which the lateralis nerves were severed immediately below the neuromast (near neuromast) (N = 35) or furthest (near the ganglion) (N = 18). A total of 23 (0.66) individualized axons cut near neuromast reinnervated the original neuromast (memory in grey), whereas 12 (0.34) re-innervated a different organ (no memory in pink). When axons were cut near the ganglion, 4 (0.22) re-innervated the original neuromast (memory in grey), and 14 (0.78) re-innervated a different organ (no memory in pink). **K-O'':** Neuromast of the transgenic line Tg(myo6b:actb1-EGFP; nkhgn39d) with mosaic expression of mCherry in a single LAN. **K-K':** Individualized axon (red) synapse with rcHCs. **K':** Magnified view of the hair bundles of hair cells in panel K. **L:** Schematic representation of the example in panel K. **M:** After axon severing and hair cell elimination, the individualized axon (red) regenerated to synapses with regenerated rcHCs in the same neuromast. **M':** Magnified view of the hair bundles of hair cells in panel M. **N:** Schematic representation of the example in panel L-M. **O-O'':** Selected time points from live confocal imaging of regenerative innervation of hair cells in the transgenic line Tg(myo6b:actb1-EGFP; nkhgn39d) (green) in an instance when a singly marked LAN (red) switched from a horizontal neuromast (solid arrowhead) to a vertical neuromast (empty arrowhead). Before laser-mediated severing (panel O), after severing (panel O'), and after regeneration (panel O''). The white arrow indicated the site of the cut. **P-P', R-R':** Neuromast of the transgenic line Tg(myo6b:actb1-EGFP; nkhgn39d) with mosaic expression of mCherry in a single LAN. **P:** The individualized axon (red) synapses with crHCs in a horizontal neuromast. **P':** Magnified view of the hair bundles of hair cells in panel P. **Q:** Schematic representation of the example in panel P. **R:** After severing, the individualized regenerated axon (red) switches to innervate a vertical neuromast, in which it synapses selectively with vdHCs. **R':** Magnified view of the hair bundles of hair cells in panel R. **S:** Schematic representation of the example in panel R. Scale bars are 10 µm in A and A' and 50 µm in O. crHC, caudorostral HC; HC, hair cell; LAN, lateral afferent neuron; rcHC, rostrocaudal HC; vdHC, ventrodorsal HC. The experiments described in this figure were performed by Gema Valera. Adapted from Lozano-Ortega, Valera, et al., 2018, with permission.

3.2.1 CONCLUSION

The presented results indicate that the consistently observed memory of polarity selectivity may not rely completely on the hair cells polarity. The fact that rostral neurons recognize dorsal hair cells and caudal neurons recognize ventral hair cells as appropriate synaptic partners suggest that selectivity is not linked to polarity, but to a different distinctive element. This means that afferents neurons would recognize hair cell identity rather than hair cell polarity.

During lateral line development, vertical neuromasts originate from primII when proneuromasts turn 90º. Considering this and the consistent selectivity observed even in those cases in which neurons switch from horizontal to vertical neuromasts, it seems reasonable to hypothesize that there must be a correlation between rostral-dorsal and caudal-ventral hair cells that plays a role in the process or neuronal recognition. These observations lead to the hypothesis that the distinctive landmark that neurons recognize might be the same in rostral-dorsal and caudal-ventral hair cells.

3.3 EMX2 EXPRESSION IN NEUROMAST HAIR CELLS

One of the possible mechanisms to explain how neurons selectively innervate hair cells of different polarities is the recognition of a molecular code that distinguishes the two orientations of hair cells. Emx2 is a transcription factor whose expression gives origin to two differently oriented populations of hair cells in the inner ear. Based on this observation, I investigated the role that Emx2 plays in the zebrafish neuromast as potential distinctive molecule to determine its involvement in hair cell orientation in the lateral line.

In order to investigate the Emx2 expression in the neuromasts, I did an immunostaining against Emx2 in a the Tg(myo6b:actb1-EGFP), in which it is possible to visualize the polarity of the hair cells (Figure 12). In both, horizontal and vertical neuromasts, Emx2

is expressed in only half the population of hair cells, specifically in the ones polarized in the caudal and ventral direction respectively.

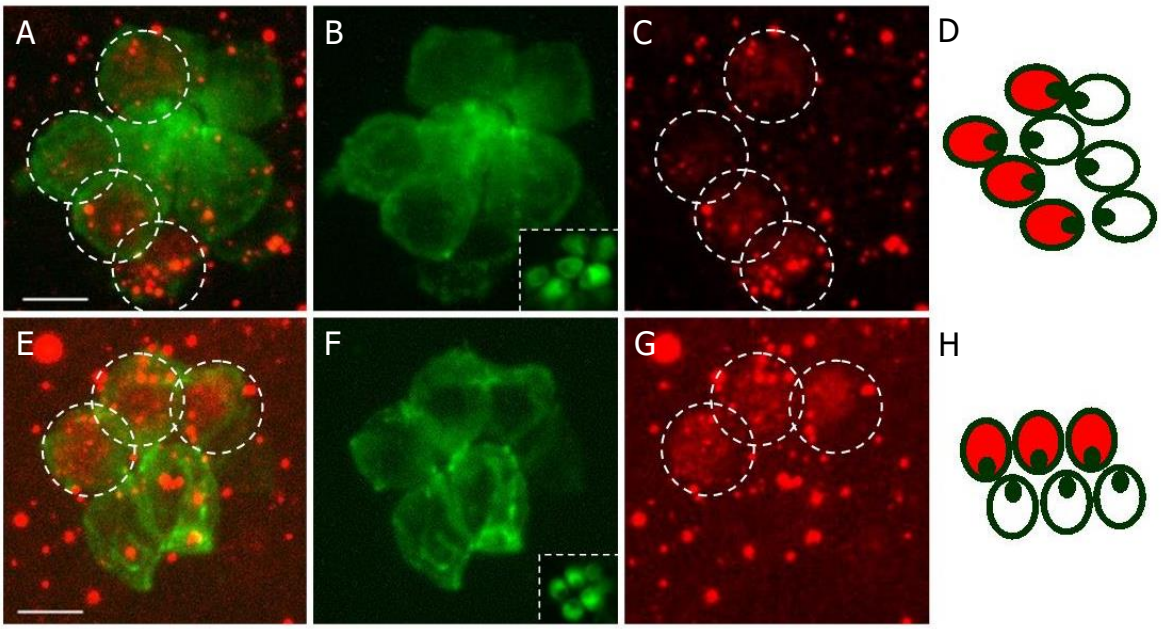

Figure 12. Emx2 immunostaining in wild type. A-D: Horizontal neuromast. **A:** Red and green channels merged showing how Emx2 expression in horizontal neuromasts is restricted to caudally oriented hair cells. **B:** Hair cells and polarity labelled by EGFP. **C:** Hair cells labelled by Emx2 antibody and red secondary antibody (circular dashed lines). **D:** Schematic representation of A; hair cells are represented based on their polarity and those filled in red are the Emx2 positive ones. **E-H:** Vertical neuromast. **E:** Red and green channels merged showing how Emx2 expression in vertical neuromasts is restricted to ventrally oriented hair cells. **F:** Hair cells and polarity labelled by EGFP. **G:** Hair cells labelled by Emx2 antibody and red secondary antibody (circular dashed lines). **H:** Schematic representation of E; hair cells are represented based on their polarity and those filled in red are the Emx2 positive ones. Scale bar: 5 µm. Adapted from Lozano-Ortega, Valera, et al., 2018, with permission.

This result confirms that hair cells of different polarities differ in terms of Emx2 expression and suggests that Emx2 may be the distinctive molecular cue that neurons recognize. Furthermore, the expression pattern observed in Figure 12 is consistent with

the correlation between innervation switch from rostral to dorsal and caudal to ventral orientations shown in Figure 11O-S.

3.3.1 EMX2 EXPRESSION AND AFFERENT INNERVATION

As a next step to further validate these observations, I observed Emx2 expression whilst also looking at the afferent innervation. For this, I did an immunostaining against Emx2 in fish of the transgenic line Tg(myo6b:actb1-EGFP) with a single afferent neuron labelled by Sill:mCherry DNA injection (Figure 13).

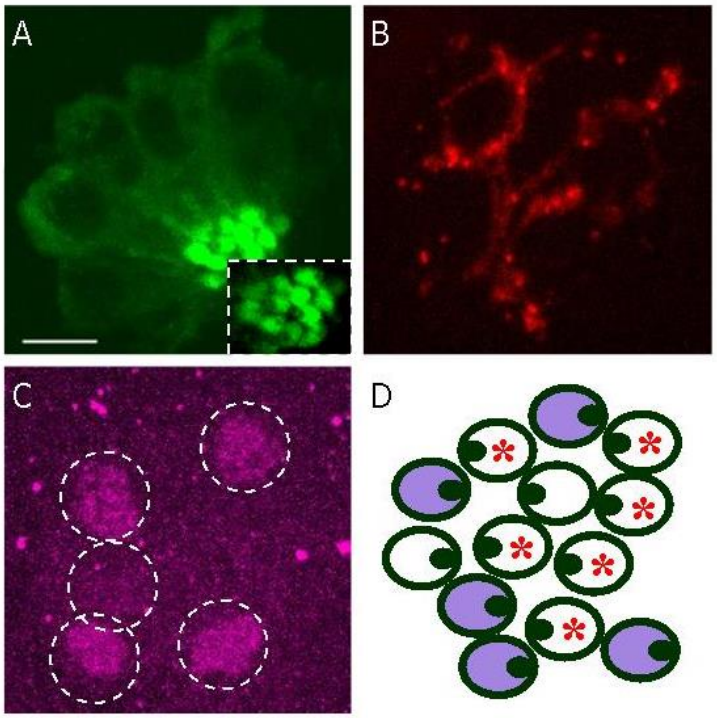

Figure 13. Emx2 expression and afferent innervation in wild type fish. A: Hair cells and polarity are shown in green, labelled by EGFP in Tg (myo6b:actb1-EGFP). **B:** An afferent single neuron is labelled by Sill:mCherry injection. **C:** Hair cells oriented caudally are labelled by Emx2 immunostaining. **D:** Schematic representation of the neuromast. Hair cells are represented based on their polarity and those filled in purple represent the Emx2 positive ones. The red stars in the hair cells indicate which hair cells are innervated by the single afferent neuron labelled. Scale bar: 5 µm. Adapted from Lozano-Ortega, Valera, et al., 2018, with permission.

The experiment shown in Figure 13 corroborates the correlation between Emx2 expression, polarity and innervation, supporting to the hypothesis that Emx2 expression status is the feature that a neuron recognizes to selectively choose its synaptic partner.

3.3.2 EMX2 EXPRESSION IN ABSENCE OF NEURONS

Next, I tested whether the presence of the afferent neurons influence in any way the expression of Emx2 or if it is intrinsic to the hair cells using of *neurogenin1* mutants as a model, which lack lateral line afferent neurons.

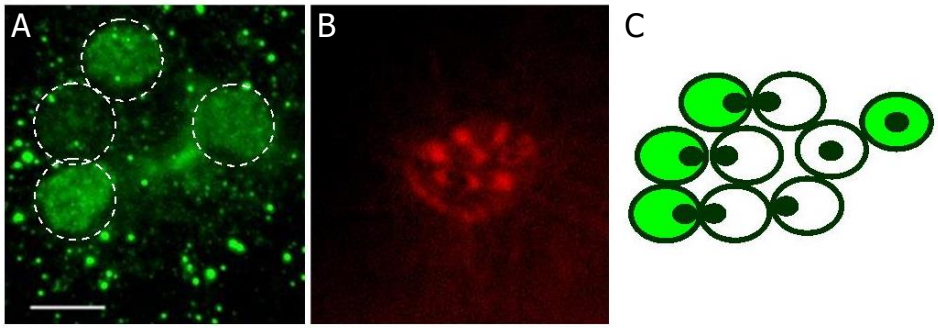

Figure 14. Emx2 expression in *neurogenin1* mutants. **A:** Staining using Emx2 primary antibody and Secondary antibody 488 labels hair oriented caudally. **B:** Red phalloidin staining labels hair cells polarity. **C:** Schematic representation of A and B shows how caudal hair cells are Emx2 positive. Scale bar: 5 µm.

Figure 14 shows how only caudal hair cells are labelled by the Emx2 antibody in *neurogenin1* mutants. This observation confirms that Emx2 expression is an intrinsic characteristic of the hair cells and does not depend on the presence of neurons.

3.3.3 CONCLUSION

These findings show that there is a specific molecular code for hair cell orientation based on asymmetric expression of Emx2 in half of the hair cell population in the neuromast and that this molecular pattern correlates with selective afferent innervation.

The following steps aim to confirm if innervation is instructed by the hair cell orientation of by the differences in Emx2 expression by looking at the innervation in fish that display randomized hair cell orientations in the neuromasts.

3.4 VANGL2 MUTANTS: AFFERENT INNERVATION AND EMX2 EXPRESSION

3.4.1 AFFERENT INNERVATION IN VANGL2 MUTANTS

Trilobite mutants harbor a loss-of-function mutation in the Vangl2 protein, which plays a role in the planar polarity of the hair cells. As a result, Vangl2 mutants show a complete randomization of the planar polarity and lose the axis of symmetry (Figure 5; Mirkovic et al., 2012).

Studying the pattern of afferent innervation in Vangl2 mutants can help understanding the link between synaptogenesis and polarity because it allows to test if a randomization in the polarity would lead to a random innervation or affect synaptic stability.

The experimental approach consisted in injecting Sill:mCherry DNA in *trilobite* fish. After pre-selecting those fish with the characteristic *trilobite* aspect and screening for red fluorescence in the afferent neurons, I did a phalloidin staining, which reveals the stereocilia of the hair cells and allows to visualize the polarity and confirm the mutation.

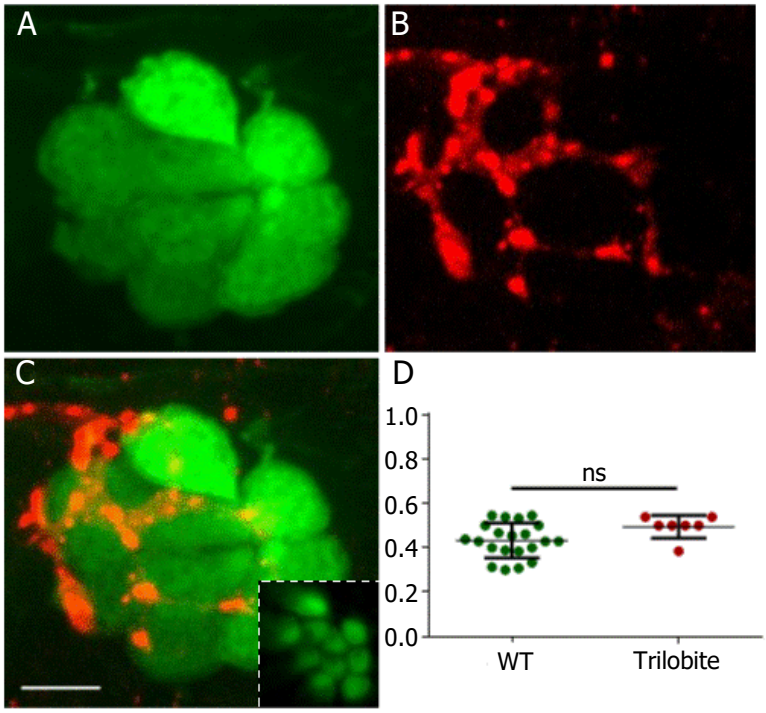

Figure 15. Afferent innervation in *trilobite* mutants. A: Hair-cell somas are labelled in green in the line Et(krt4:EGFP). **B**: A single afferent neuron is labelled in red by injection of Sill:mCherry. **C:** Merged of A and B, and visualization of the polarities labelled by the phalloidin staing in a different plane in the dashed line square. **D**: t-test statitical comparison of the fraction of hair cells that a single neuron innervates in wild type and *trilobite* mutants shows that in both cases a neuron innervates approximately half the population and the difference between these two groups is not significant. Scale bar: 5 µm.

Figure 15 shows that approximately half of the population of hair cells in the neuromast is innervated by a single afferent neuron, which is not different from a wild type case in normal conditions. Since the number of hair cells in a neuromast is not always exactly the same, I divided the number of cells innervated in a neuromast by the total number of hair cells, obtaining thus the fraction of innervated hair cells in that neuromast (Figure 15D). This result confirms that the proportion of hair cells innervated by an afferent neuron is also around 50%, even in the case of a system with a disruption in the polarization pattern.

These observations in Vangl2 mutants suggest that hair cell orientation is not the cue that instructs selective afferent innervation.

3.4.2 EMX2 EXPRESSION IN VANGL2 MUTANTS

Considering that the innervation ratio of a single neuron in *trilobite* is not different from a wild type animal, I decided to test the Emx2 expression in Vangl2 mutants to determine if the proportion of Emx2 positive and negative cells is also close to 50% as observed in wild type fish.

The Emx2 expression is revealed by immunostaining the *trilobite* larvae (Figure 16 A-C) and a phalloidin staining is required to visualize the polarization and confirm the mutant phenotype (not shown). I quantified the number of Emx2-positive hair cells in the *trilobite* mutant fish relative to the total number of hair cells in the neuromasts to overcome variability due to the size of the neuromast. I compared this parameter to wild type fish by doing a t-test (Figure 16D) and the result indicates a non-significant difference between wild type and *trilobite* fish, showing that in both cases approximately 50% of the hair cells in the neuromast express Emx2.

From the results observed in Figure 15 and Figure 16, I could conclude that the orientation of the hair cell does not affect neither the innervation pattern nor the Emx2 expression of the hair cells because the outcomes do not differ from a wild type situation.

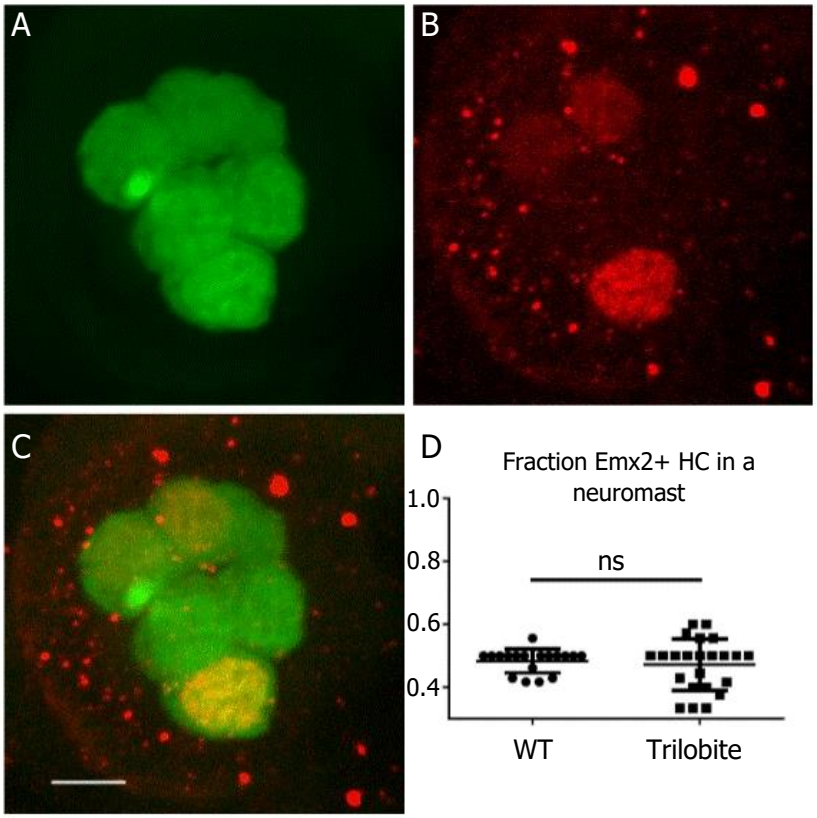

Figure 16. Emx2 expression in *trilobite* mutants. A: Et(krt4:EGFP)sqet4 line labels the hair cell somas in green. **B**: Antibody staining labels three somas out of a total of 6 hair cells. **C**: Merged A and B. **D**: Statistical comparison between the proportion of hair cells labelled by Emx2 Ab in a neuromast in wild type fish and *trilobite* mutants is in both cases around 0,5 (half the population of hair cells in the neuromast). Scale bar: 5 µm. Adapted from Lozano-Ortega, Valera, et al., 2018, with permission.

3.4.3 AFFERENT INNERVATION AND EMX2 EXPRESSION IN VANGL2 MUTANTS

Since the previous experiments suggest that hair cell orientation does not dictate selective innervation, I next wanted to investigate whether the expression of Emx2 is the responsible factor behind afferent selectivity by combining the previous experiments in one and looking at the innervation pattern and Emx2 expression together in *trilobite* fish.

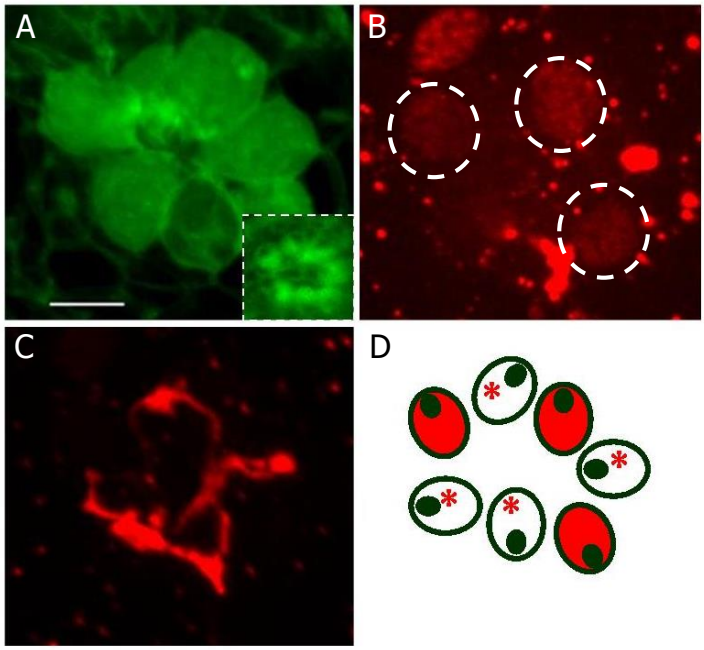

Figure 17. Emx2 expression and afferent innervation in *trilobite* mutants. A: Et(krt4:EGFP)sqet4 line labels the hair cell somas in green and the polarities are labelled by the Phalloidin staing in a different plane in the dashed line square. **B-C**: Antibody staining labels Emx2 positive hair cells (B) in red and the Sill:mCherry injection labels the afferent neuron (C) also in red. Both elements are distinguishable because of their anatomy and plane location. **D:** Schematic representation of the experiment: hair cells are represented by their polarities, those filled in red are the Emx2 positive ones and the white stars mark the afferent innervation. Scale bar: 5 µm. Adapted from Lozano-Ortega, Valera, et al., 2018, with permission.

I injected Sill:mCherry DNA in *trilobite* fish. After pre-selecting larvae with the typical *trilobite* body shape and screening for red fluorescence, I performed the Emx2 staining and the phalloidin staining to confirm the mutant phenotype (Figure 17).

Figure 17 shows that a single afferent neuron innervates hair cells which are equal in terms of Emx2 expression regardless the orientation of the hair bundle. This result confirms how Emx2 expression consistently correlates with synaptic partnership and hair cell orientation is separable from selectivity.

3.4.4 CONCLUSION

The experiments conducted in Vangl2 mutants confirm that afferent innervation is determined by Emx2 expression rather than by hair cell orientation as we can observe that the proportion of innervated hair cells is maintained the same as in wild type fish (50%) and correlates with the Emx2 expression status.

3.5 EMX2 LOSS OF FUNCTION MUTANTS

Having confirmed the role of Emx2 in innervation selectivity, I next investigated the system when Emx2 expression is affected to better understand the link between Emx2 and afferent innervation.

I induced an Emx2 loss-of-function mutation in fish of the transgenic line Tg(myo6b:actb1-EGFP), which reveals the polarity of the hair cells by looking at the fluorescence expression and to determine if the mutagenesis has induced changes in the phenotype. I used the CRISPant approach for somatic mutagenesis (Burger et al., 2016), which allows to generate CRISPR mutants directly in the larvae resulting from the injected eggs. The sgRNAs were designed with the tool crispr.cos.uni-heidelberg.de and injected in combination with Cas9 protein following the CRISPant protocol into 1-2 cell stage eggs.

As this method aims to obtain mutated fish in F0, larvae were screened for green fluorescence at 4-5 dpf and then examined under the microscope to select the mutated fish, in which all hair cells in a neuromast had identical orientation (Figure 18).

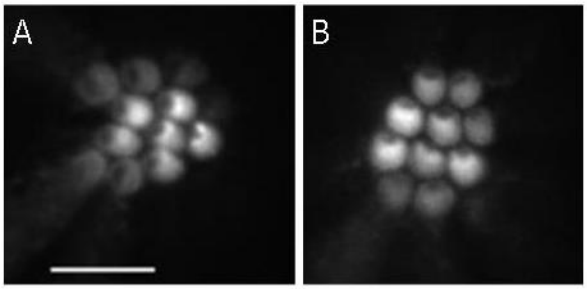

Figure 18. Emx2 mutants. Imaging of fish mutated by using the CRISPant technique. **A:** Example of mutated horizontal neuromast, all hair cells are oriented rostrally. **B:** Example of mutated vertical neuromast, all hair cells are oriented dorsally. Scale bar: 5 µm.

One limitation of this method is that the mutation was not "complete" and in the same fish it was possible to find wild type-looking neuromasts (50% of each polarity), and neuromasts with a high predominance of only one orientation but not reaching 100%.

Across all the samples under examination, I observed that the predominant orientation of the hair cells was always rostral in horizontal neuromasts and dorsal in vertical neuromasts (Figure 18). Provided that the Emx2 immunostainings always showed as positive the caudal and ventral directions, this result is a solid confirmation that the identity of the hair cells is determined by their Emx2 expression status rather than by their orientation.

3.5.1 FUNCTIONALITY OF THE EMX2 CRISPANT MUTANTS

Before conducting any further experiment on the Emx2 mutants, I first wanted to assess the functionality of the mutated neuromasts. For this, I used a DiASP (4-(4-(diethylamino)styryl)-N-methylpyridinium) staining. DiASP is a vital dye that enters in the hair cells through the mechanically gated transduction channels on the stereocilia when they open, therefore we can use it to test functionality of the channels and subsequently of the hair cells.

I incubated 4-5 dpf larvae in DiASP solution for 2min and then washed them with Danieau water. In all observed neuromasts, all hair cells were labelled by DiASP, revealing thus a perfect functioning of the mechanotransduction channels and, therefore, a complete functionality of the neuromast (Figure 19).

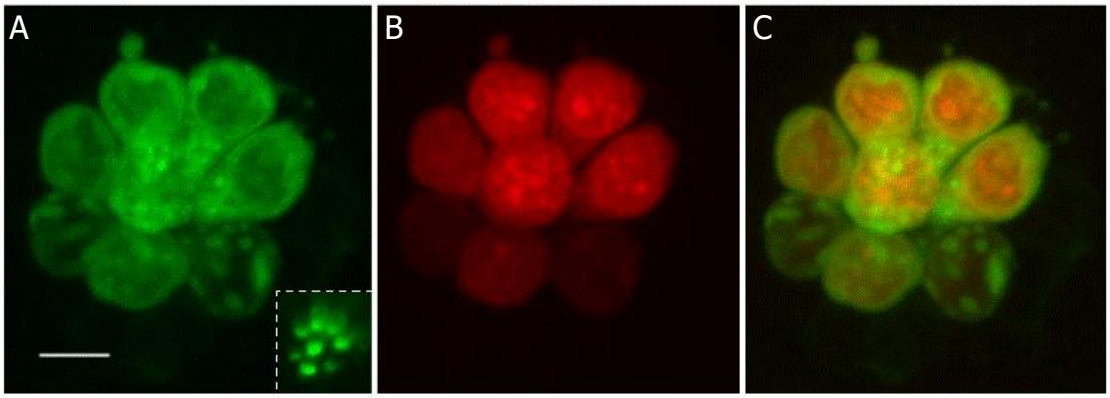

Figure 19. DiASP staining in Emx2 mutants. A: Tg (myo6b:actb1-EGFP) labels hair cells in green and we can see the polarity in a different plane (dashed-line square). **B**: DiASP staining labels functional hair cells in red. **C**: Merged image shows how all hair cells overlap with DiASP positive hair cells, what reveals a completely functional neuromast. Scale bar: 5 µm.

3.5.2 AFFERENT INNERVATION OF THE EMX2 CRISPANT MUTANTS

To investigate their innervation pattern of the Emx2 CRISPant fish I sparse-labelled afferent neurons by injecting Sill:mCherry DNA in one-cell stage eggs of the transgenic line Tg(myo6b:actb1-EGFP; pou4f3:ctbp21-mKOFP), which allows the visualization of both, polarity of the hair cells and synaptic components. The injection of Sill:mCherry was performed in combination with sgRNA and Cas9 following the CRISPant protocol to obtain mutated neuromasts at the F0 stage.

I could observe two scenarios: one where several hair cells are innervated by the neuron (innervation of rostral/dorsal hair cells) (Figure 20) and a second one in which no hair

cells were innervated by the labelled neuron (innervation of caudal/ventral hair cells) (Figure 21).

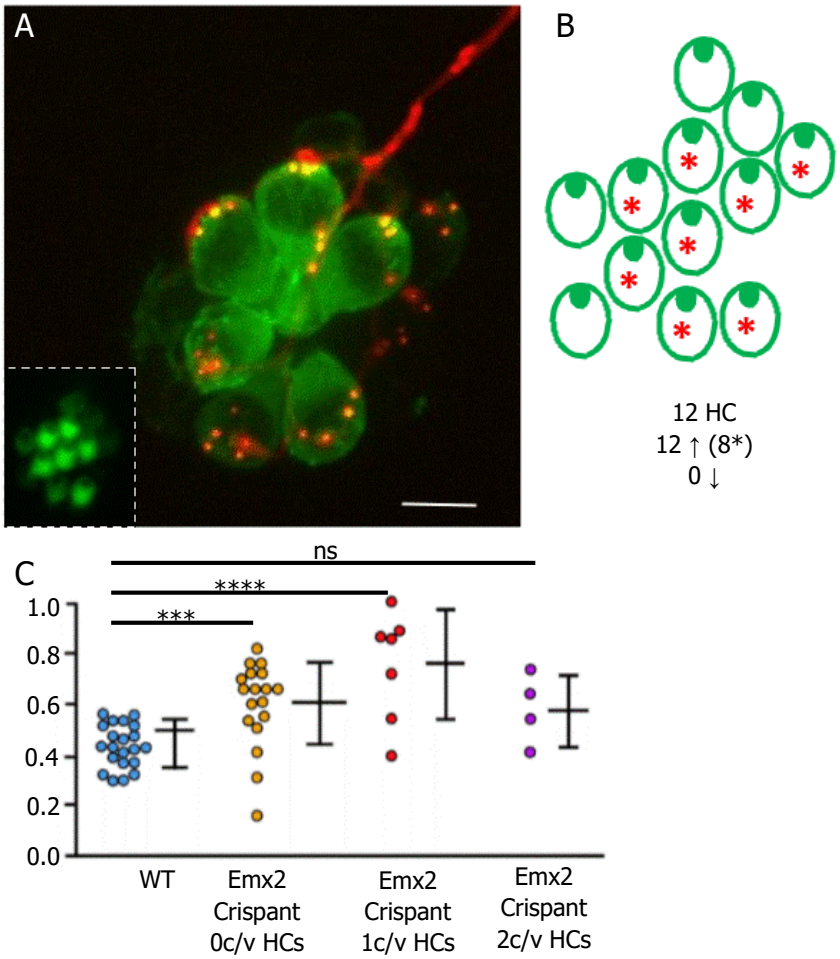

Figure 20. Rostral/dorsal afferent innervation in Emx2 mutants. **A:** Single neuron labelled innervating dorsally oriented hair cells in a vertical neuromast of a Emx2 loss-of-function mutant fish with transgenic background Tg(myo6b:β-actin-GFP; pou4f3:ctbp21-mKOFP). **B:** Schematic representation of A. **C:** Statistical comparison of the fraction of hair cells innervated in a neuromast in wild type fish, completely mutated neuromasts (Emx2 CRISPant_0c/vHCs) and partially mutated neuromasts (Emx2 CRISPant_1c/vHCs and Emx2 CRISPant_2c/vHCs). Scale bar: 5 µm. Adapted from Lozano-Ortega, Valera, et al., 2018, with permission.

The first situation shows a neuron that recognizes hair cells oriented rostrally or dorsally in a fully mutated neuromast (Emx2 CRISPant 0c/vHCs) and innervates a higher proportion of hair cells than in a wild type situation (Figure 20C). This corroborates the idea that Emx2 is the reference landmark for the neurons independently of the number of hair cells innervated. This is also true in not-fully mutated neuromasts, where there is still one cell oriented in caudal or ventral direction (Emx2 CRISPant_1c/vHCs). When there are two caudal or ventral hair cells (Emx2 CRISPant_2c/vHCs), the situation becomes much closer to a wild type situation and we can see no significant differences in the proportion of innervated hair cells (Figure 20C).

In the second case, a neuron that recognizes hair cells oriented caudally and ventrally reaches a fully mutated neuromast and it does not innervate any of the hair cells (Figure 21A). The neurite does not establish any stable synapsis because that particular neuron innervates Emx2-positive (caudal/ventral) hair cells and it is not able to find any target of the right identity in that neuromast. When the mutation is not complete and there are one or two cells oriented in caudal/ventral direction, the neuron remains selective (Figure 21B-C) resulting in a lower ratio of innervation compared to a wild type fish, where the proportion of innervated hair cells is always close to 50%.

These results confirm that selective innervation of the afferent neurons is determined by the Emx2 expression status of the hair cells of the neuromast.

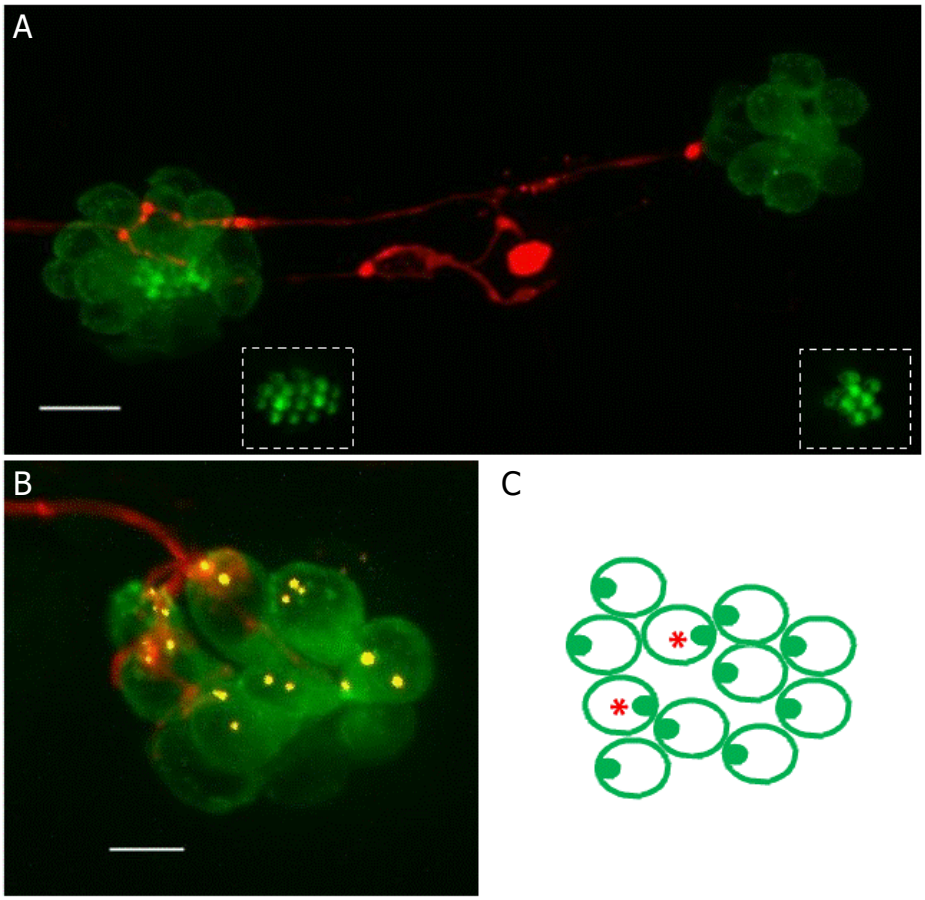

Figure 21. Caudal/ventral afferent innervation in Emx2 mutants. A: By injection of Sill:mCherry DNA and the CRISPant mixture in Tg(Myo6b:β-actin-GFP) we can see a single neuron that develops normally towards these two terminal neuromasts but it does not stablish any stable synapsis, suggesting that it has a preference for Emx2 positive hair cells. **B:** In a CRISPant Tg(Myo6b:β-actin-GFP; pou4f3:Rib-Kus) fish with a single neuron labelled in a partially mutated neuromast we can see how the only two cells not affected by the mutation are innervated. **C:** schematic representation of the polarity of the neuromast described in B. Scale bar: 5 µm.

3.5.3 CONCLUSION

The pattern of innervation observed in neuromasts with homogeneously oriented hair cells in Emx2 loss-of-function mutants demonstrates the role of Emx2 expression status in afferent innervation selectivity.

4. DISCUSSION

The mammalian utricular and saccular maculae are the inner ear organs in charge of detecting linear acceleration and gravity. The planar cell polarity of the hair cells in those organs enables detection of movement in opposite directions (Deans, 2013). Similarly, the two subpopulations of hair cells based on polarity differences in a fish lateral line allow the animal to detect directionality of the water flow movements and it is indispensable to transmit that information properly in order to read environmental cues of water flow (Bleckmann, 2008; Faucherre et al., 2009).

Polarity-specific afferent innervation of the hair cells in the zebrafish lateral line is crucial for the correct transmission of the mechanosensory information of the surroundings of the fish to the brain. The organization of the afferent pathways in the lateral line is similarly organized as in other structures, such as the mammalian utricular and saccular maculæ and the vestibular apparatus of amphibians and birds (Maklad et al., 2010).

In this study, I have investigated neuronal tuning of mechanical cues and directionality using the zebrafish lateral line as animal model because of its transparency, rapid development and manipulability, which are very advantageous features in observational studies and experimental research.

This work explores the mechanism that governs the development and maintenance of the sensory circuits and shows how the establishment of the coherent circuit is mediated by asymmetric expression and selective recognition of the transcription factor Emx2.

Polarity selective afferent innervation of the zebrafish has been widely investigated in the past looking at the different possible mechanisms that may be involved. Whereas some of the proposed hypotheses have been previously ruled out by investigators, some other models remain to be further tested. After reviewing the evidence that is known

up to date, I considered that the most relevant models to focus on, were the investigation of pre-synaptic projections and hair cell identity or molecular code.

4.1 HAIR-CELL PROJECTIONS DO NOT INSTRUCT SELECTIVITY

When the projections scaffolds model was proposed, it was based on the observation of the dynamic filopodia that appear on hair cells at early stages of their development (Dow et al., 2015). Nevertheless, this model was neither quantified over time nor manipulated experimentally. By resolving these two limitations, it was possible to prove that even when these structures may have an aid role in terms of expanding hair cells' surface, they are dispensable for selectivity.

I first observed that projections are indeed present during development and they overlap in time with the process of neuron-hair cell synaptic partner matching, but the contacts between projections and neurites did not always lead to the establishment of a stable synapsis.

Second, I tested this hypothesis in regenerative conditions, which is especially important when studying this and other sensory models given their exposure to the environment and therefore, to potential damage. The results of this experiment showed how re-innervation occurs in the absence of projections. This confirms that projections are restricted to early stages of hair cell development and that they are not required for innervation.

These two experiments conclude that young hair cells have a very dynamic soma that extends and retracts filopodial projections that may facilitate the encounter with afferent axons by increasing the hair cells' surface area but they do not instruct polarity-selective innervation.

4.2 HAIR CELL GLOBAL POLARITY AND SELECTIVITY ARE SEPARABLE

In order to study the relationship between polarity and selectivity, I took advantage of the Vangl2 mutants, which lack the core planar-polarity protein and produce a phenotype where hair cells are randomly oriented in all directions. The idea behind these experiments was that if polarity is really the cue that instructs neurons for selective innervation, these mutants would have random ratios of hair cell innervation. Interestingly, in Vangl2 mutants a single neuron innervates approximately half of the population of hair cells, just the same as in a wild type situation. From this result we can conclude that hair cell planar polarity and synaptic selectivity are separable.

Further evidence supporting that polarity and selectivity are separable is provided by the results obtained from re-innervation experiments in which neurons switch from horizontal to vertical neuromasts. These experiments show consistent recovery of a selective innervation after neuronal or hair cell damage even when the regenerating axons target a different neuromast than original, which is an interesting phenomenon given the somatotopic organization of the lateral line. In cases where a neuron moves from innervating a horizontal neuromast to a vertical, we can also see a consistent correlation between caudal-ventral and rostral-dorsal orientations suggesting that there is an element that labels them as equivalent. This is consistent with the process of development of the lateral line, during which neuromasts deposited by primII rotate 90º and give origin to the vertical orientation, suggesting that caudal-ventral and rostral-dorsal hair cells are the same in terms of identity but rotated 90º.

Although polarity and selectivity are separable, we cannot conclude that they are completely independent because in normal conditions, there is an obvious correlation between innervation and hair cell orientation. My work aims to determine the nature of this correlation and to understand the mechanism behind this phenomenon.

4.3 HAIR CELL SUBPOPULATIONS HAVE DIFFERENT MOLECULAR IDENTITY BASED ON EMX2 EXPRESSION

Just as in the mammalian saccula, Emx2 is a transcription factor expressed in half of the hair cells population of the organ (Holley et al., 2010; Jiang et al., 2017). In the lateral line, the expression of Emx2 correlates with the caudal orientation of the hair bundle in horizontal neuromasts and ventral orientation in the vertical neuromasts. This pattern of expression is consistent with the afferent partner choice in the case of a regenerating neuron that switches from innervating horizontal to vertical neuromasts.

Looking at the Emx2 expression in Vangl2 mutants, I observed that it is not affected by the loss of coherent planar polarity and is limited to half of the population of the neuromast. Furthermore, I also tested the innervation pattern in this setting and found that afferent neurons selectively innervated hair cells with the same Emx2 expression status despite the different orientation of the hair bundles. These results demonstrate that Emx2 provides hair cells with an asymmetric molecular identity code that is consistently recognized by the afferent neurons regardless the polarity.

I have studied how the Emx2 loss-of-function affects hair cells and afferent innervation by inducing directed mutagenesis. I have observed that the lack of Emx2 led to neuromasts in which all hair cells were oriented in the same direction, rostrally in horizontal neuromasts and dorsally in vertical neuromasts, as predicted from the results obtained in the Emx2 immunostainings. I have found that axons innervating the mutated neuromasts either synapse with a significant higher proportion of hair cells than half population or do not contact any hair cell at all, corroborating the results that point at Emx2 as responsible of the selective afferent innervation.

My observations demonstrate that selectivity and polarity are not directly related but they are both interconnected and governed by the expression of Emx2. The transcription factor Emx2 establishes an identity code that enables polarity determination in hair cells and instructs selective innervation for afferent neurons.

In the Emx2 loss-of-function mutants, in both inner ear of mice and neuromasts of zebrafish, the global axis of polarization is not altered. In the zebrafish, this means that there is no distinction between the two different polarities but neuromasts maintain their horizontal or vertical directionality. The reason for this is that the global axis of polarization is not controlled by Emx2, but by other proteins such as Vangl2 and Fz6. Based on the described experiments, we can conclude that once the axis of polarization is established, Emx2 instructs the hair cells about how to read it and therefore, about how to orientate along the axis in one of the two possible directions, giving origin to the two different polarity subpopulations.

Afferent neurons are able to recognize hair cell identity regardless polarity and global orientation and also after neuronal or hair cell injury. In juvenile and adult fish, the lateral line expands and neurons co-innervate clusters of neuromasts (Ledent, 2002; Wada et al., 2013). The fact that this recognition mechanism is based on Emx2 expression, allows the fish to maintain selective innervation in later stages of development independently of the global orientation of the neuromast and the hair cells.

Although there is no evidence to date of a molecular asymmetry between neurons, it is something that may require further investigation in order to elucidate the mechanism that allows neurons to recognize the different hair cell identities. These differences among the neurons would support the results of axonal regeneration where we can see how neurons always re-innervate hair cells that are equal in terms of Emx2 expression. In the case of auditory afferent neurons in the mouse, it has been shown that there are subpopulations with different connectivity pattern in the inner ear, which might be a helpful insight and set the basis for further investigation in other models. Nevertheless, considering the promiscuity results obtained in the solitary neuron experiments, it seems that neuronal identity alone it is not sufficient for selectivity, which relies on the presence of both types of neurons.

4.4 SELECTIVITY ALGORITHM CONTROLLING AFFERENT INNERVATION OF THE ZEBRAFISH LATERAL LINE

For a better understanding of the connectivity process in the afferent innervation of the zebrafish lateral line, I have outlined an algorithm that allows to explain the different interactions between neurons and hair cells. This algorithm explains how innervation is established in both situations, development and recovery after damage, and how directional mechanosensation can be maintained throughout the whole life of fish (Figure 22).

Selectivity is based on the existence of two subpopulations of hair cells and two subpopulations of neurons. The asymmetry of the hair cells is determined by the positive or negative expression of the transcription factor Emx2 while in the case of the neurons the distinction between subpopulations remains unknown.

During development, hair cells attract both types of axons in a non-selective manner establishing transient contacts with them (Faucherre et al., 2009), nevertheless, depending on the Emx2 expression status, these contacts will stabilize and become synapse or not (Figure 22A). Nonselective attraction of afferent axons can also happen in later stages when there is only a solitary neuron innervating the neuromast (Figure 22B, Pujol-Martí et al., 2014). In both situations, once a hair cell has been innervated by the right neuron, it will repel axons of the other subtype.

This algorithm describes a mechanism that also explains transient promiscuity when there is a solitary neuron innervating the neuromast (Figure 22C). This neuron (neuron A) will synapse with hair cells of the wrong polarity because repelling effects of the right neuron (neuron B) have been lost and therefore, they cannot prevent misinnervation between neuron A and hair cell B. Once the neuron B re-growths, it recovers the innervation and promiscuous neuron A retracts the axons from the wrong type of hair cells.

In a scenario where neurons are damaged, they will regenerate and re-innervate hair cells maintaining selectivity as originally. Neurons preserve selectivity memory even when they re-grow to innervate a different neuromast than originally and even when they switch from innervating a horizontal neuromast to a vertical one. Considering the obtained results, this algorithm can explain the memory phenomenon based on the fact that as Emx2 expression is inherent to each hair cell and does not change over the course of the animal's life (Figure 22D). Hence, neurons are able to recognize their appropriate synaptic partners throughout continuous cycles of damage and recovery that occur normally in sensory systems during fish life. The same result in the recovery of selective innervation is observed when hair cells regenerate after damage (Figure 22E).

This model also explains why selectivity is also maintained in fish with non-functional hair cells (Faucherre et al., 2010). Regardless the mechanorreceptive activity of the hair cell, the neuron will always be able to make the right choice when connecting with a synaptic target because this process is based on recognizing the Emx2 expression and not on activity pattern. The solitary neuron experiments are the only exception because in that case the repulsive effect exerted by the correct neuron is lost and promiscuity is not prevented.

When the fish are experimentally manipulated and lose expression of Emx2, one of the required elements of selectivity is lost. In this situation, instead of two subpopulations of neurons and hair cells, the hair cells population becomes uniformly oriented and only one type of neuron will be able to find its synaptic target. Only neurons that match with rostrally-/dorsally-oriented hair cells will form synapses and will have therefore an inhibitory effect on the other neuronal subpopulation, which will not be able to establish connections with any mechanorreceptive cell (Figure 22F).

As a final remark on the proposed model, it is important to notice that Emx2 is the real factor to consider in terms of selectivity and not polarity. This outcome is confirmed by the experiments carried out with Vangl2 mutants where it is confirmed that neurons keep the same pattern of innervation when the natural axis of planar polarity is lost and

it corresponds to the Emx2 expression status of the hair cells, following the structure predicted by the model even when coherent polarity is lost. (Figure 22G).

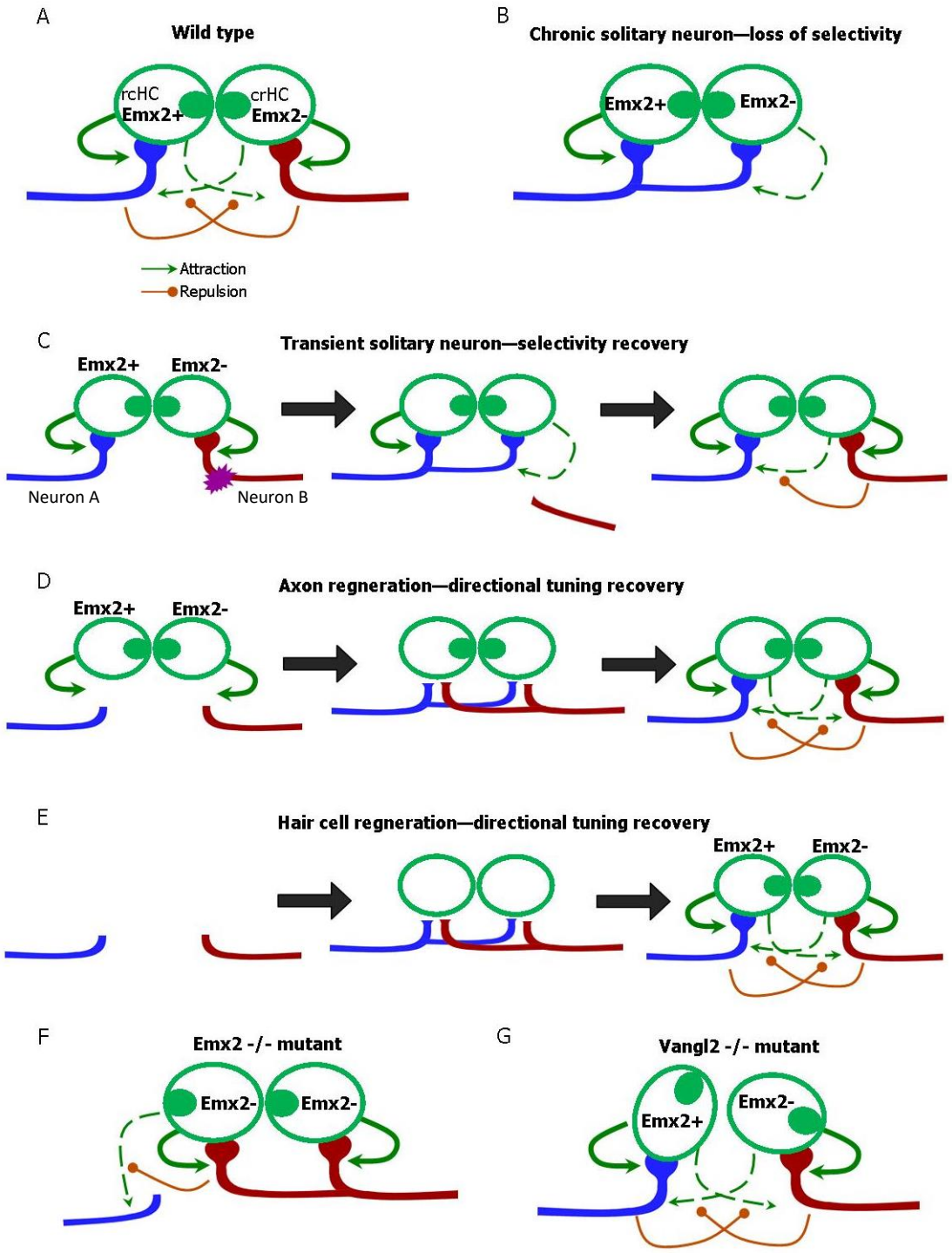

Figure 22. Wiring algorithm underlying polarity-selectivity synaptogenesis and neuronal directional tuning A: Afferent innervation in a wild type situation. The innervation of a hair cell A by a neuron A exerts a repulsive effect on a neuron type B. **B:**

In the case of a solitary neuron innervating the neuromast, the repulsive effect is lost and the remaining neuron is able to synapse all hair cells regardless their polarity and identity. **C:** As in B, the solitary neuron A innervates both polarities because there is not any inhibitory effect from the neuronal subtype B. When the damaged neuron B re-growths, it recovers innervation of hair cell type B and exerts an inhibitory effect that pushes neuron A away. **D:** Similarly to development, in the re-innervation situation after neuronal damage, the two neuronal subtypes synapse with their right targets and prevent the other type of neuron to make wrong connections. **E:** after hair cell damage neurons are able to recognize newborn hair cells in a similar way as in development and axonal regeneration. **F:** In loss-of-function Emx2 mutants rostral and dorsal neurons will innervate all the hair cell population whereas caudal and ventral neurons will not be able to find any appropriate synaptic partner. **G:** Vangl2 mutants have the same innervation pattern as wild type fish but in this case, the polarity phenotype does not allow to recognize the two hair cell subpopulations. rcHC = rostral-caudal hair cell (hair cell oriented caudally), crHC = caudal-rostral hair cell (hair cell oriented rostrally). Adapted from Lozano-Ortega, Valera, et al., 2018, with permission.

4.5 CONCLUSIONS

The general conclusions that can be extracted from this work are two: 1) selective innervation of identically oriented hair cells of the lateral line by afferent neurons is independent of hair cells projections and is separable from hair cell coherent planar polarity and global orientation; 2) the developed wiring algorithm explains the mechanism behind selectivity and sets the basis for neuronal directional tuning.

This model is simple but consistent. It is valid despite the continuous cycles of hair cell death and regeneration that occur throughout the life of the fish. Also, the model remains accurate not only for larval fish, but also at later stages of development, when the lateral line expands and neurons co-innervate multiple neuromasts with different global orientation (Ledent, 2002) and in cases of peripheral damage.

Considering the similarities between the zebrafish lateral line and the mammalian vestibular system, and how in both cases Emx2 is involved in hair cell orientation, this model could serve as a reference for further investigation of innervation patterns in other vertebrates in the field of sensory neurobiology.

5. MATERIALS AND METHODS

5.1 ETHICS STATEMENT

Experiments with wild-type, mutant, and transgenic embryos of undetermined sex were conducted under a protocol approved by the Ethical Committee of Animal Experimentation of the Parc de Recerca Biomedica de Barcelona (Spain), and protocol number Gz.:55.2-1-54-2532-202-2014 by the “Regierung von Oberbayern” (Germany).

5.2 ZEBRAFISH STRAINS AND HUSBANDRY

Zebrafish eggs were collected and appropriately cleaned with Danieau. Embryos were maintained and raised at standard conditions of 28,5 °C with a maximum density of 50 animals per Petri dish (85 mm).

All the transgenic lines used in this study have been previously described: Tg(pou4f3:GAP-GFP) (Xiao et al., 2005), Tg(pou4f3:ctbp21-mKOFP) (Pinto-Teixeira et al., 2015), Tg(myo6b:actb1-EGFP) (Kindt et al., 2012), Tg(hsp701:mCherry-2.0cntnap2a) (Pujol-Martí et al., 2014), Tg(nkhgn39d) (Faucherre et al., 2009), Et(krt4:EGFP)sqet4 (López-Schier and Hudspeth, 2006), Tg (Islet 1:GFP) (Higashijima, 2000).

Mutant lines have also been previously characterized: *neurogenin1* line is neurog1^{hi1059} (Pujol-Martí et al., 2014) and the *trilobite*/Vangl2 is Df (Chr07:stbm)^{vu7} (Jessen et al., 2002, López-Schier and Hudspeth, 2006).

5.3 DNA CONSTRUCTS

The hsp70:mCherry-SILL (SILL:mCherry) construct was generated using the Tol2 kit, using entry vectors that were generated as described in the Invitrogen Multisite Gateway manual. PCRs were performed using primers to add att sites onto the end of DNA fragments, using Platinum Pfx (Invitrogen). The pEntry vectors containing the UAS sequence, hsp70 minimal promoter, mCherry, and polyA are from the Tol2 kit. The pEntry vector containing the SILL enhancer was previously generated in the laboratory (Pujol-Martí et al., 2014).

5.4 MOSAIC NEURONAL LABELING

Sparse labeling of lateralis afferent neurons was achieved by injecting 15-20 pg of a DNA plasmid containing the construct hsp70:mCherry_SILL-enhancer (SILL:mCherry) in 1-4-cell stage eggs. Injection needles were made by one-stage pull of borosilicate glass capillaries.

Injected eggs were cleaned and the resulting embryos were anesthetized and screened for red fluorescence at 3-5 dpf using a Zeiss stereomicroscope. Embryos that had one single neuron or one single neuron labelled per neuromast were selected for the experimental procedures.

5.5 EMX2 LOSS-OF-FUNCTION

In order to induce targeted somatic mutagenesis of Emx2, I used the CRISPant strategy described by Burger et al., 2016. Following the protocol, I incubated for 5 minutes a solution containing sgRNA (160 ng/µl) and Cas9 (760 ng/µl) at 37 °C, and injected in one-

cell stage eggs of the transgenic lines Tg(Myo6b:βactin-GFP) or Tg(Myo6b:βactin-GFP; Brn3c:Kus-Rib).

In the case of experiments that also required the visualization of the afferent innervation of the Emx2 mutants, the injected solution also contained SILL:mCherry DNA (20ng/µl).

For the design of the sgRNA, I used the online tool CCTop – Crispr/Cas9 target online predictor (crispr.cos.uni-heidelberg.de) and the selected target sequence used was GGAGGAGGTACTGAATGGACTGG.

5.5 IMMUNOHISTOCHEMISTRY

After fluorescence screening when required, larvae were first fixed overnight at 4 °C in a solution of 4% PFA. After fixation, fish were washed with PBST (phosphate-buffered saline (PBS) solution containing 1% Tween-20) and permeabilized in acetone at -20 °C for 5min. Samples were then washed with miliQ water 5min and incubated for 1-2 hours at room temperature with blocking solution (1%BSA, 2%NGS, 1% DMSO). After blocking, larvae were incubated at 4 °C in PBS with 0.2% Tween-20 overnight with primary Ab (Ctbp2 1:100) or for 48 hours in the case of Ab (EMX2 1:250). After that, fish were washed with PBST 5-6 times and incubated overnight at 4 °C with secondary Ab (GaRb 633, GaRb 555). Then samples were washed with PBST and mounted for imaging.

The EMX2 antibody was acquired from Trans Genic Inc (Fukuoka, Japan) and Ctbp2 was obtained from Proteintech (Manchester, UK).

5.6 IMAGING AND VIDEOMICROSCOPY

For in vivo imaging, laser-mediated axotomy and the visualizations of some of the Emx2 immunostainings, I used a custom-built inverted spinning disc microscope (Zeiss

Axioscope). In the case of Emx2 immunostainings in wild type fish with a single neuron labelled and Ctbp2 immunostainings, imaging was done using a Zeiss inverted confocal microscope with a 40x water immersion objective.

Embryos and larvae used for in vivo imaging were anesthetized in MS-222 (3-aminobenzoic acid ethyl ester) and mounted in 1% low melting point agarose on the coverslip of a glass-bottom dish (MatTek, Ashland, MA). When the agarose was dry, imaging dishes were bathed in Danieau with MS-222 0,16 g/L (reduced to 0,08 g/L for time-lapse imaging) and imaged at 28,5 °C using a 63x water immersion objective.

5.7 ASSESSMENT OF SYNAPSES

Synaptic connectivity was assessed in transgenic fish expressing different fluorescent markers in hair cells and axons. Synapses were identified as bulged postsynaptic endings adjacent to the base of hair cells. The assessment was always done by progressing through individual focal planes of Z-stacks, from the apical end of the epithelium (to determine the planar polarization of each hair cell) to the most basal area of the epithelium (where apposition of neuronal endings and hair cells are found).

5.8 STATISTICAL ANALYSES

Because the total number of hair cells is not uniform across different neuromasts, quantification of the innervation in wild type, Vangl2 and Emx2 mutants was done using the fraction of innervated hair cells divided by the total number of hair cells in the neuromast. Then the obtained fractions in the different groups were compared using a one-way analysis of variance (ANOVA) test.

For analysing the Emx2 expression in wild type and Vangl2 mutants, the same procedure was applied, I normalized the samples by dividing the number of positive hair cells by

the total number of hair cells. With the resulting data, I then compared the wild type and Vangl2-mutant groups using an unpaired t-test.

5.9 NEOMYCIN TREATMENT

Larvae were incubated in a 250 µM solution of neomycin (dissolved in Danieau) at room temperature for pharmacological ablation of the hair cells. After 60 minutes, larvae were incubated for 1 hour at room temperature and then rinsed with Danieau medium (Pinto-Texeira et al., 2015).

5.10 AXONAL SEVERING

Similarly to the procedure describe for imaging and videomicroscopy, larvae were incubated with MS-222 and mounted in 1% low melting point agarose on a glass-bottom Petri dish. Axons were severed using a iLas-pulse laser system (Roper Scientific SAS, Evry, France) that includes a ultraviolet laser (355 nm; 400 ps/2,5 µJ per pulse) and is integrated in the spinning disc microscope (Zeiss Axioscope). After this procedure, larvae were recovered from the agarose and left to recover in fresh Danieau medium and posteriorly used for imaging or time-lapse experiments.

5.11 PHALLOIDIN STAINING

Larvae were fixed in 4% PFA overnight at 4 ºC and washed 4-6 times in 0,1% PBST. Then, samples were incubated overnight at 4 ºC in phalloidin-Alexa 568 or Alexa 488 (Invitrogen) diluted 1:20 in 0,1% PBST. The next day, samples were washed 6 times in

0,1% PBST and mounted for visualization with Vectashield 1/100 (Vector Labs, Burlingame, CA, USA) or, in the case of *trilobite* mutants with deformed body shapes, samples were mounted in agarose low melting point on glass-bottom dish.

6. REFERENCES

Axelrod, J. D. (2008). Basal bodies, kinocilia and planar polarity. *Nature Genetics, 40,* 10-11. doi: 10.1038/ng0108-10

Basaldella, E., Takeoka, A., Sigrist, M. and Arber, S. (2015). Multisensory Signaling Shapes Vestibulo-Motor Circuit Specificity. *Cell 163,* 301–312. doi: 10.1016/j.cell.2015.09.023

Bleckmann, H. (2008). Peripheral and central processing of lateral line information. *Journal of Comparative Physiology A, 194*(2), 145-58. doi: 10.1007/s00359-007-0282-2

Bleckmann, H. and Zelick, R. (2009). Lateral line system of fish. *Integrative Zoology, 4,* 13-25. doi: 10.1111/j.1749-4877.2008.00131.x

Bricaud, O., Chaar, V., Dambly-Chaudière, C., and Ghysen, A. (2001). Early efferent innervation of the zebrafish lateral line. *The Journal of Comparative Neurology, 434*, 253-261. doi:10.1002/cne.1175

Burger, A., Lindsay, H., Felker, A., Hess, C., Anders, C., Chiavacci, E., Zaugg, J., Weber, L. M., Catena, R., Jinek, M., et al. (2016). Maximizing mutagenesis with solubilized CRISPR-Cas9 ribonucleoprotein complexes. *Development, 143*(11), 2025–37. doi: 10.1242/dev.134809

Clause, A., Kim, G., Sonntag, M., Weisz, C. J., Vetter, D. E., Rübsamen, R., and Kandler, K. (2014). The precise temporal pattern of prehearing spontaneous activity is necessary for tonotopic map refinement. *Neuron, 82*(4), 822-835. doi: 10.1016/j.neuron.2014.04.001

Coombs, S., Mogdans, J., Halstead, M. and Montgomery J. (1998). Transformation of peripheral inputs by the first-order lateral line brainstem nucleus. *Journal of Comparative Physiology A 182*(5):606-26. doi: 10.1007/s003590050207

Copley, C. O., Duncan, J. S., Liu, C., Cheng, H., and Deans, M. R. (2013). Postnatal refinement of auditory hair cell planar polarity deficits occurs in the absence of Vangl2. *The Journal of Neuroscience*: the official journal of the Society for Neuroscience, 33(35), 14001–14016. doi:10.1523/JNEUROSCI.1307-13.2013

Cruz, I. A., Kappedal, R., Mackenzie, S. M., Hailey, D. W., Hoffman, T. L., Schilling, T. F., and Raible, D. W. (2015). Robust regeneration of adult zebrafish lateral line hair cells reflects continued precursor pool maintenance. *Developmental biology*, *402*(2), 229–238. doi:10.1016/j.ydbio.2015.03.019

Dambly-Chaudière, C., Sapède, D., Soubiran, F., Decorde, K., Gompel, N., and Ghysen, A. (2003). The lateral line of zebrafish: a model system for the analysis of morphogenesis and neural development in vertebrates. *Biology of the Cell, 95*(9), 579-87. doi: 10.1016/j.biolcel.2003.10.005

Deans, M. R., Antic, D., Suyama, K., Scott, M. P., Axelrod, J. D., and Goodrich, L. V. (2007). Asymmetric distribution of prickle-like 2 reveals an early underlying polarization of vestibular sensory epithelia in the inner ear. *The Jounal of Neuroscience, 27(*12), 3139-47. doi: 10.1523/JNEUROSCI.5151-06.2007

Deans, M. R. (2013). A balance of form and function: planar polarity and development of the vestibular maculae. *Seminars in cell & developmental biology*, *24*(5), 490–498. doi:10.1016/j.semcdb.2013.03.001

Denman-Johnson, K and Forge, A (1999). Establishment of hair bundle polarity and orientation in the developing vestibular system of the mouse. *Journal of Neurocytology, 28*(10-11), 821-35. doi: 10.1023/a:1007061819934

Dijkgraaf, S. (1963). The functioning and significance of the lateral-line organs. *Biological Reviews, Soc 38,* 51-105. doi: 10.1111/j.1469-185X.1963.tb00654.x

Dow, E., Siletti, K., and Hudspeth, A. J. (2015). Cellular projections from sensory hair cells form polarity-specific scaffolds during synaptogenesis. *Genes & development*, *29*(10), 1087–1094. doi:10.1101/gad.259838.115

Eatock, R. A. and Songer, J. E. (2011). Vestibular hair cells and afferents: two channels for head motion signals. *Annual Review of Neuroscience 34*, 501-34. doi: 10.1146/annurev-neuro-061010-113710

Einhorn, Z., Trapani, J. G., Liu, Q., and Nicolson, T. (2012). Rabconnectin3α promotes stable activity of the H+ pump on synaptic vesicles in hair cells. *The Journal of neuroscience: the official journal of the Society for Neuroscience*, *32*(32), 11144–11156. doi:10.1523/JNEUROSCI.1705-12.2012

Fame, R. M., Brajon, C., and Ghysen, A. (2006). Second-order projection from the posterior lateral line in the early zebrafish brain. *Neural development*, *1*, 4. doi:10.1186/1749-8104-1-4

Faucherre, A., Pujol-Martí, J., Kawakami, K., and López-Schier, H. (2009). Afferent neurons of the zebrafish lateral line are strict selectors of hair-cell orientation. *PloS one*, *4*(2), e4477. doi: 10.1371/journal.pone.0004477

Faucherre, A., Baudoin, J. P., Pujol-Martí, J., and López-Schier, H. (2010). Multispectral four-dimensional imaging reveals that evoked activity modulates peripheral arborization and the selection of plane-polarized targets by sensory neurons. *Development, 137,* 1635-1643. doi: 10.1242/dev.047316

Flock, A. and Russell, I. (1976). Inhibition by efferent nerve fibres: action on hair cells and afferent synaptic transmission in the lateral line canal organ of the burbot Lota lota. *The Journal of physiology*, *257*(1), 45–62. doi:10.1113/jphysiol.1976.sp011355.

Flock, A. and Wersäll, J. (1962). Synaptic structures in the lateral line canal organ of the teleost fish Lota vulgaris. *Journal of Cell Biology 13*(2):337-43. doi: 10.1083/jcb.13.2.337

Friedrich, R. W., Jacobson, G. A., and Zhu, P. (2010). Circuit neuroscience in zebrafish. *Current Biology, 20*(8), R371-81. doi: 10.1016/j.cub.2010.02.039

Ghysen, A. and Dambly-Chaudière, C. (2004). Development of the zebrafish lateral line. *Current Opinion in Neurobiology, 14,* 67-73. doi: 10.1016/j.conb.2004.01.012

Ghysen, A. and Dambly-Chaudière, C. (2005). The three-sided romance of the lateral line: glia love axons love precursors love glia. *Bioessays, 27*(5), 488-494. doi: 10.1002/bies.20225

Ghysen, A. and Dambly-Chaudière, C. (2007). The lateral line microcosmos. *Genes and Development, 21,* 2118-2130. doi: 10.1101/gad.1568407

Gompel, N., Dambly-Chaudière, C., and Ghysen, A. (2001). Neuronal differences prefigure somatotopy in the zebrafish lateral line. *Development, 128,* 387-393

Haehnel, M., Taguchi, M., and Liao, J. C. (2012). Heterogeneity and dynamics of lateral line afferent innervation during development in zebrafish (Danio rerio). *The Journal of comparative neurology*, *520*(7), 1376–1386. doi:10.1002/cne.22798

Hale, R. and Strutt, D. (2015). Conservation of Planar Polarity Pathway Function Across the Animal Kingdom. *Annual Review of Genetics, 49*, 529-51. doi: 10.1146/annurev-genet-112414-055224

Higashijima, S., Hotta, Y., and Okamoto, H. (2000). Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer. *The Journal of Neuroscience, 20*(1), 206-218. doi: 10.1523/JNEUROSCI.20-01-00206.2000

Holley, M., Rhodes, C., Kneebone, A., Herde, M. K., Fleming, M., and Steel, K. P. (2010). Emx2 and early hair cell development in the mouse inner ear. *Developmental biology*, *340*(2), 547–556. doi: 10.1016/j.ydbio.2010.02.004

Hudspeth, A. J. (1989) How the ear's works work. *Nature, 341(6241):*397-404. doi: 10.1038/341397a0

Jessen, J. R., Topczewski, J., Bingham, S., Sepich, D. S., Marlow, F., Chandrasekhar, A., and Solnica-Krezel, L. (2002). Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. *Nature cell biology, 4*(8), 610–615. doi:10.1038/ncb828

Jiang, T., Kindt, K., and Wu, D. K. (2017). Transcription factor Emx2 controls stereociliary bundle orientation of sensory hair cells. *eLife*, *6*, e23661. doi:10.7554/eLife.23661

Julien, D. P. and Sagasti, A. (2014). Synaptic specificity: when the neighbors are away, sensory axons turn promiscuous. *Current Biology, 24*(24), PR1168-R1170. doi: 10.1016/j.cub.2014.11.004

Kalmijn, A. J. (1988). Electromagnetic orientation: a relativistic approach. *Progress in Clinical Biological Research, 257,* 23-45

Kindt, K. S., Finch, G., and Nicolson, T. (2012). Kinocilia mediate mechanosensitivity in developing zebrafish hair cells. *Developmental cell*, *23*(2), 329–341. doi: 10.1016/j.devcel.2012.05.022

Kishore, S. and Fetcho, J. R. (2013). Homeostatic regulation of dendritic dynamics in a motor map in vivo. *Nature communications*, *4*, 2086. doi:10.1038/ncomms3086

Ledent, V. (2002). Postembryonic development of the posterior lateral line in zebrafish. *Development 129*(3), 597-604

Liao, J. C. (2010). Organization and physiology of posterior lateral line afferent neurons in larval zebrafish. *Biology letters*, *6*(3), 402–405. doi:10.1098/rsbl.2009.0995

López-Schier, H., Starr, C. J., Kappler, J. A., Kollmar, R., and Hudspeth, A. J. (2004). Directional cell migration establishes the axes of planar polarity in the posterior lateral-line organ of the zebrafish. *Developmental Cell, 7*(3), 401-412. doi: 10.1016/j.devcel.2004.07.018

López-Schier, H. and Hudspeth, A. J. (2006). A two-step mechanism underlies the planar polarization of regenerating sensory hair cells. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(49), 18615–18620. doi:10.1073/pnas.0608536103

Maklad, A., Kamel, S., Wong, E., and Fritzsch, B. (2010). Development and organization of polarity-specific segregation of primary vestibular afferent fibers in mice. *Cell and tissue research*, *340*(2), 303–321. doi:10.1007/s00441-010-0944-1

Metcalfe, W. K. (1985). Sensory neuron growth cones comigrate with posterior lateral line primordial cells in zebrafish. *The Journal of Comparative Neurology, 238*(2), 218-024. doi: 10.1002/cne.902380208

Mirkovic, I., Pylawka, S., and Hudspeth, A. J. (2012). Rearrangements between differentiating hair cells coordinate planar polarity and the establishment of mirror symmetry in lateral-line neuromasts. *Biology open*, *1*(5), 498–505. doi:10.1242/bio.2012570.

Mizoguchi, T., Togawa, S., Kawakami, K., and Itoh, M. (2011). Neuron and sensory epithelial cell fate is sequentially determined by notch signaling in zebrafish lateral line development. *The Journal of Neuroscience, 31*(43), 15522-15530. doi: 10.1523/JNEUROSCI.3948-11.2011

Mogdans, J. and Bleckmann, H. (2012). Coping with flow: behavior, neurophysiology and modeling of the fish lateral line system. *Biological Cybernetics, 106,* 627-642. doi: 10.1007/s00422-012-0525-3

Nagiel, A., Andor-Ardó, D., and Hudspeth, A. J. (2008). Specificity of afferent synapses onto plane-polarized hair cells in the posterior lateral line of the zebrafish. *The Journal of neuroscience: the official journal of the Society for Neuroscience*, *28*(34), 8442–8453. doi:10.1523/JNEUROSCI.2425-08.2008

Obholzer, N., Wolfson, S., Trapani, J. G., Mo, W., Nechiporuk, A., Busch-Nentwich, E., Seiler, C., Sidi, S., Söllner, C., Duncan, R. N., et al. (2008). Vestibular glutamate transporter 3 is required for synaptic transmission in zebrafish hair cells. *The Journal of Neuroscience, 28*(9), 2110-2118. doi: 10.1523/JNEUROSCI.5230-07.2008

Oteiza P, Odstrcil I, Lauder G, Portugues R, Engert F. (2017) A novel mechanism for mechanosensory-based rheotaxis in larval zebrafish. *Nature, 547*(7664):445-448. doi: 10.1038/nature23014

Pinto-Teixeira, F., Viader-Llargués, O., Torres-Mejía, E., Turan, M., González-Gualda, E., Pola-Morell, L., and López-Schier, H. (2015). Inexhaustible hair-cell regeneration in young and aged zebrafish. *Biology open*, *4*(7), 903–909. doi:10.1242/bio.012112

Pujol-Martí, J., Baudoin, J. P., Faucherre, A., Kawakami, K., and López-Schier, H. (2010). Progressive neurogenesis defines lateralis somatotopy. *Developmental Dynamics: an official publication of the American Association of Anatomists, 239*(7), 1919-1930. doi: 10.1002/dvdy.22320

Pujol-Martí, J., Zecca, A., Baudoin, J. P., Faucherre, A., Asakawa, K., Kawakami, K., and López-Schier, H. (2012). Neuronal birth order identifies a dimorphic sensorineural map. *The Journal of neuroscience: the official journal of the Society for Neuroscience*, *32*(9), 2976–2987. doi:10.1523/JNEUROSCI.5157-11.2012

Pujol-Martí, J. and López-Schier, H. (2013). Developmental and architectural principles of the lateral-line neural map. *Frontiers in Neural Circuits, 7*:47. doi: 10.3389/fncir.2013.00047

Pujol-Martí, J., Faucherre, A., Aziz-Bose, R., Asgharsharghi, A., Colombelli, J., Trapani, J. G., and López-Schier, H. (2014). Converging axons collectively initiate and maintain synaptic selectivity in a constantly remodeling sensory organ. *Current Biology, 24*(24), 2968-2974. doi: 10.1016/j.cub.2014.11.012

Raible, D. W. and Kruse, G. J. (2000). Organization of the Lateral Line System in Embryonic Zebrafish. *The Journal of Comparative Neurology, 421*(2), 189-198. doi:10.1002/(SICI)1096-9861(20000529)421:2<189::AID-CNE5>3.0.CO;2-K

Sapède, D., Rossel, M., Dambly-Chaudière, C., and Ghysen, A. (2005). Role of SDF1 chemokine in the development of lateral line efferent and facial motor neurons. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(5), 1714–1718. doi:10.1073/pnas.0406382102

Shi, D., Usami, F., Komatsu, K., Oka, S., Abe, T., Uemura, T., and Fujimori, T. (2016). Dynamics of planar cell polarity protein Vangl2 in the mouse oviduct epithelium. *Mechanisms of Development, 141,* 78-89. doi: 10.1016/j.mod.2016.05.002

Simmons, D. D. (2002). Development of the inner ear efferent system across vertebrate species. *Journal of Neurobiology, 53*(2), 228-250. doi: 10.1002/neu.10130

Steiner, A. B., Kim, T., Cabot, V. and Hudspeth, A. J. (2014). Dynamic gene expression by putative hair-cell progenitors during regeneration in the zebrafish lateral line. *Proceedings of the National Academy of Sciences of the United States of America, 111*(14):E1393-401. doi: 10.1073/pnas.1318692111

Strutt, D. (2003). Frizzled signalling and cell polarisation in *Drosophila* and vertebrates. *Development, 130*(19), 4501-4513. doi: 10.1242/dev.00695

Suli, A., Watson, G. M., Rubel, E. W. and Raible, D. W. (2012) Rheotaxis in larval zebrafish is mediated by lateral line mechanosensory hair cells. *PLoS One, 7*(2):e29727. doi: 10.1371/journal.pone.0029727

Tomchik, S.M. and Lu, Z. (2006). Auditory physiology and anatomy of octavolateral efferent neurons in a teleost fish. *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology, 192*(1), 51-67. doi: 10.1007/s00359-005-0050-0

Trapani, J. G. and Nicolson, T. (2010). Physiological recordings from zebrafish lateral-line hair cells and afferent neurons. *Methods in Cell Biology, 100,* 219-231. doi: 10.1016/B978-0-12-384892-5.00008-6

Wada, H., Dambly-Chaudière, C., Kawakami, K., and Ghysen, A. (2013). Innervation is required for sense organ development in the lateral line system of adult zebrafish. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(14), 5659–5664. doi:10.1073/pnas.1214004110

Wedemeyer, C., Vattino, L. G., Moglie, M. J., Ballestero, J., Maison, S. F., Di Guilmi, M. N., Taranda, J., Liberman, M. C., Fuchs, P. A., Katz, E., et al. (2018). A Gain-of-Function Mutation in the α9 Nicotinic Acetylcholine Receptor Alters Medial Olivocochlear Efferent Short-Term Synaptic Plasticity. *The Journal of neuroscience: the official journal of the Society for Neuroscience*, *38*(16), 3939–3954. doi:10.1523/JNEUROSCI.2528-17.2018

Xiao, T., Roeser, T., Staub, W., and Baier, H. (2005). A GFP-based genetic screen reveals mutations that disrupt the architecture of the zebrafish retinotectal projection. *Development, 132*(13):2955-2967. doi: 10.1242/dev.01861

Xiao, Y., Tian, W., and López-Schier, H. (2015). Optogenetic stimulation of neuronal repair. *Current Biology, 25*(22):R1068-1069. doi: 10.1016/j.cub.2015.09.038.

Zimmerman, D.M. (1979). Onset of neural function in the lateral line. *Nature, 282*(5734):82-4. doi: 10.1038/282082a0

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7. APPENDIX

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EIDESSTATTLICHE ERKLÄRUNG

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

Marta Lozano Ortega

München, den 28/02/2022

Erklärung

Hiermit erkläre ich,

☒ dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.

☒ dass ich mich anderweitig einer Doktorprüfung ohne Erfolg nicht unterzogen habe.

Marta Lozano Ortega

München, den 28/02/2022