Neural map organization and development in the lateral-line system

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“Knowledge is a complexity packed to break through the reality that separates two minds.” Jorge Wagensberg.

This thesis is dedicated to you, dear readers.

You were the reason for writing it.

All the effort and passion

I put into this work

is dedicated to

my family,

friends &

fellows.

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Abstract

Sensory neurons project to the central nervous system in a spatially ordered manner, assembling neural maps that represent attributes of sensory stimuli and that are thought to be essential to interpret the external world. I used the lateral-line system of the zebrafish larva as a model to study sensory neural map organization and development. Lateralis (lateral-line) sensory neurons organize a topographic neural map, called somatotopy, which encodes the position of the sensory stimulus. I demonstrated that the order of neurogenesis defines somatotopy. In addition, I identified two sub-classes of lateralis sensory neurons that differ in their central projection patterns and in their contacts with a central output neuron: the Mauthner cell. I propose that such neural-map dimorphism sub-serves appropriate behavioral reactions to the sensory context. Importantly, I also demonstrated a contribution of neuronal birth order to the assembly of the lateral-line dimorphic neural map. Finally, additional results support that the observed neuronal diversity and map topology occur normally in the absence of sensory activity.

Resum

Les neurones sensorials projecten al sistema nerviós central seguint una distribució espacial ordenada, formant mapes neuronals que representen propietats dels estímuls sensorials i que són considerats essencials per a la interpretació del món extern. He utilitzat la línia lateral de la larva del peix zebra com a model per a l'estudi de l'organització i el desenvolupament dels mapes neuronals sensorials. Les neurones sensorials de la línia lateral formen un mapa neuronal topogràfic, anomenat somatotopia, que representa la posició de l'estímul sensorial. He demostrat que l'ordre de neurogènesi defineix la somatotopia. A més, he identificat dues subclasses de neurones sensorials de la línia lateral que presenten diferències en els seus patrons de projecció central i en els contactes amb una neurona central: la cèl·lula de Mauthner. Proposo que aquest dimorfisme és important per a donar lloc a reaccions comportamentals adients al context sensorial. També he demostrat una contribució per part de l'ordre de neurogènesi a la formació del mapa neuronal dimòrfic de la línia lateral. Finalment, he obtingut resultats que mostren que la diversitat neuronal i la topologia del mapa observades ocorren amb normalitat en l'absència d'activitat sensorial.

Preface

The nervous system has seized scientists' attention throughout the ages. Anatomical methods are the oldest way for studying the nervous system and have uncovered basic principles of its organization, defining a valuable groundwork for understanding its functions. Another important source of information about nervous system function came from analyzing the consequences of damage to specific regions of the brain. More recently, the study of the brain activity has brought to light some fundamental principles of nervous system function. Nowadays, it is even possible to manipulate neuronal activity in intact behaving animals and ask for its consequences. Because of the recent technological advances and the many questions yet to be answered, these are exciting times to study one of the greatest mysteries in modern biology: how the nervous system works to exert its complex and fascintating functions.

In particular, I am attempting to understand the mechanisms that govern the communication between sensory organs and the brain. In most sensory systems, neurons project from the sensory receptors to the brain in a spatially ordered manner forming neural maps that encode stimuli attributes, such as identity or position. The formation of such precise patterns of connectivity is thought to be essential for the brain in order to process sensory information. One outstanding question for me is how a sensory system can trigger seemingly opposite behavioral responses to environmental stimuli. How sensory circuits are established during development is the other central question that receives my attention.

Most of the research on these issues has been carried out on the visual and olfactory sensory systems. I have chosen the lateral-line sensory system of the zebrafish larva because it is anatomically simple yet functionally complex, mediating contrasting behaviours that are also present in the adult fish. A decade ago, Ghysen's research group showed that the lateralis (lateral-line) sensory neurons display a topographic neural map. The same group shed some light on when and how this map is established. Since their pioneering work, more research groups have adopted the lateral-line system of the zebrafish as a model to study sensory neural map organization, function and development. During my thesis research, it has been exciting to see other laboratories' contributions to this field.

My thesis work has combined methods recently developed by other groups together with novel tools we have developed in order to examine the organization and development of the lateralis sensory neurons. I believe that my findings provide important insights on the principles of neural map organization with clear relevance to the mechanisms that govern appropriate behavioral reactions to the sensory context. This thesis also contains novel results regarding neural map development that illustrate the overwhelming importance of time as a patterning factor and provide a framework for future mechanistic interrogations. Furthermore, I believe that other researchers will profit from the novel tools we have generated.

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Chapter 1

INTRODUCTION and **AIMS**

1.1 Sensory neural maps

Animals perceive the external world by virtue of their sensory systems. Sensory systems consist in: (1) receptors that detect external stimuli; (2) neural pathways that convey sensory information to the brain; and (3) central neurons organized in a series of relay nuclei that process this information. Sensory systems ensure the interpretation of complex flows of sensory information, by translating the basic features of a stimulus -modality/identity, position, intensity and timing- into a coherent neural code. This largely relies on the highly organized patterns of connectivity between the distinct components of the sensory systems, which in many cases shape neural maps of the external world (Gardner and Martin, 2000; Kaas, 1997; Lemke and Reber, 2005).

1.1.1 Structure

Neural maps are spatial arrangements of neuronal connections that encode information. Both anatomical and physiological studies have revealed neural maps in many sensory and motor systems. Neural maps form a spectrum whose extremes are continuous neural maps, also called topographic, and discrete neural maps. The main difference between the two types is the nature of the attribute encoded by the map. In a continuous map, nearby neurons in the input region connect to nearby neurons in the target region. In other words, there is a point-to-point match of connections between input and target regions. By this arrangement, the map represents positional information. By contrast, in a discrete map, spatially dispersed neurons of the same type in the input region converge on the same cluster of neurons in the target region. In

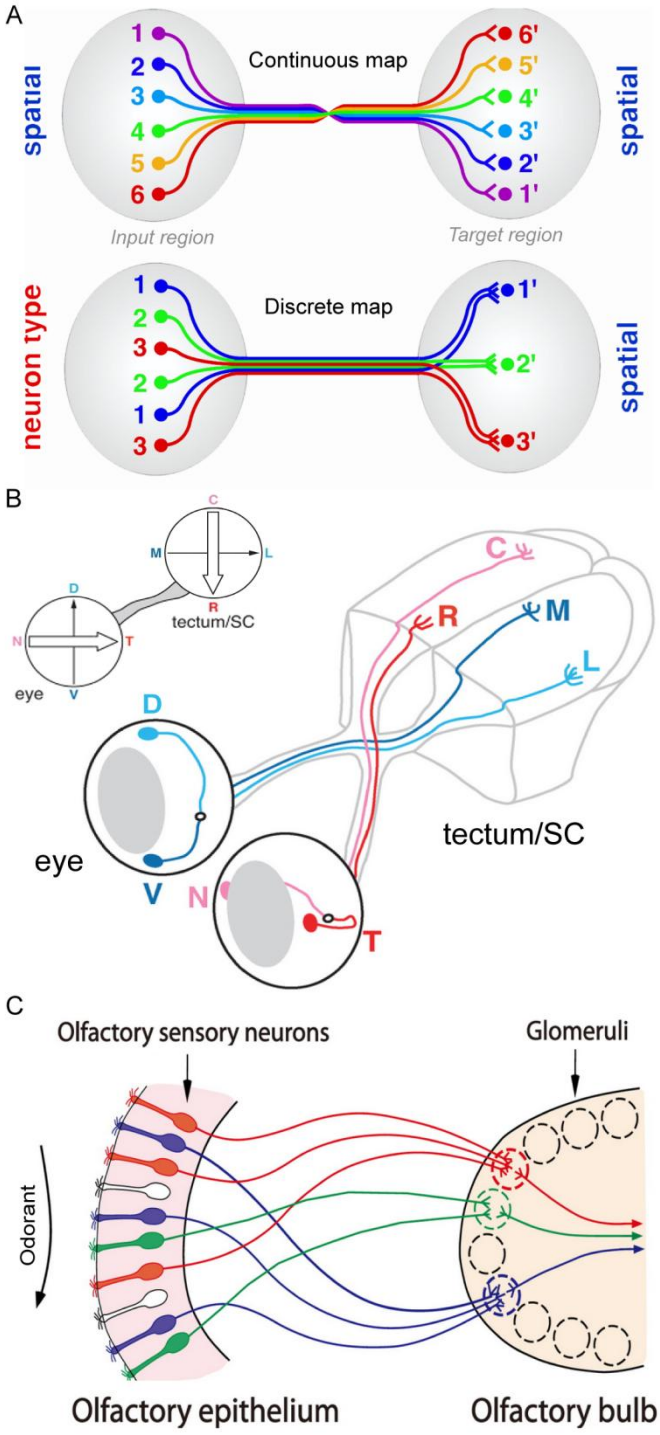
this manner, the map represents discrete information such as neuronal identity (Figure 1.1A) (Luo and Flanagan, 2007). The paradigm for continuous maps is the retinotopic map in the visual system. In vertebrates, retinal neurons convey visual information from the retina to the optic tectum forming a map where neurons from the nasal and temporal regions of the retina connect to neurons in the caudal and rostral tectum, respectively. In addition, neurons from the dorsal and ventral regions of the retina connect to neurons in the lateral and medial tectum, respectively. Thus, the brain receives an intact two-dimensional image from the retina (Figure 1.1B) (Lemke and Reber, 2005). The paradigm for discrete maps is the olfactory map. The olfactory epithelium contains olfactory sensory neurons, each of them expressing a single functional odorant receptor. Neurons expressing the same odorant receptor are spatially dispersed in the epithelium. However, central axons of neurons expressing the same odorant receptor converge on the same region, called glomerulus, in the olfactory bulb. Thus, the olfactory bulb contains an odorant receptor map that encodes the identity of the odorant signal received by the olfactory epithelium (Figure 1.1C) (Sakano, 2010).

1.1.2 Development

How are neural maps established during development? This has been a central question for neurobiologists during decades. The final patterning of neuronal connections that shape a neural map is the completion of a continuous and complex process. First, neurons extend axons which select specific paths to navigate through. Axons then recognize their correct target and establish a widespread scaffold of contacts with a set of neurons. Finally, axons select a specific subset of neurons and the initial

pattern of connections is refined into a tuned and functional circuit (Benson et al., 2001; Goodman and Shatz, 1993). How is neuronal connectivity specificity achieved during these stages? The extensive study of the retinotopic and olfactory maps, among others, has identified some common principles, as well as some particularities, of neural map development that I will next present.

One common principle of neural map development is the existence of two sets of mechanisms; one responsible for the formation of the initial coarse pattern of connections and another responsible for map refinement (Luo and Flanagan, 2007). The use of gradients as a global signal to form an initial coarse map is a well recognized mechanism. For example, both vertebrates and invertebrates use gradients to organize visual circuits into continuous or topographic maps, although the nature of the gradients differs between them (Clandinin and Feldheim, 2009). The visual system of vertebrates uses molecular gradients for the development of the retinotopic map. In order to generate a two-dimensional map, both the temporo-nasal and the dorso-ventral axes of the retina need to be represented in the tectum. The ephrin family of proteins provides a solution to this challenge. EphA signaling is required to connect nasal and temporal neurons of the retina to caudal and rostral neurons in the tectum, respectively. Similarly, EphB signaling is needed to connect dorsal and ventral neurons of the retina to lateral and medial neurons in the tectum, respectively (Figure 1.2A). In addition to the ephrin family, recent studies have shown that other families of molecules, such as Wnts and Engrailed proteins, are involved in retinotopic map formation (Lemke and Reber, 2005; Luo and Flanagan, 2007).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R
M
L
D
eye
V
N
T
tectum/SC
C
Olfactory sensory neurons
Glomeruli
Odorant
Olfactory epithelium
Olfactory bulb

Figure 1.1

Figure 1.1. Continuous and discrete neural maps. A: Scheme of a continuous (top) and a discrete (bottom) neural map. Adapted from Luo and Flanagan, 2007. **B:** Scheme of the retinotopic map, the paradigm for continuous maps. Retinal neurons from the nasal (N) and temporal (T) regions of the retina project, respectively, to the caudal (C) and rostral (R) tectum. Neurons from the dorsal (D) and ventral (V) regions of the retina project, respectively, to the lateral (L) and medial (M) tectum. Adapted from Lemke and Reber, 2005. **C:** Scheme of the olfactory map, the paradigm for discrete maps. Olfactory sensory neurons expressing the same odorant receptor (same colour) are spatially dispersed in the epithelium but converge their central axons to the same glomerulus. Adapted from Sakano, 2010.

Although the visual system of invertebrates is very different from its vertebrate equivalent, it also presents a similar retinotopic map. However, the formation of the map relies on temporal gradients in addition to molecular cues. Photoreceptors in the fly eye project axons into the lamina in the brain, where they connect to target neurons shaping a continuous map. Temporal gradients are essential for mapping the rostro-caudal axis of the retina into the lamina. Photoreceptors from the caudal region of the retina differentiate first and extend their axons to the caudal region of the lamina. There, the arriving axons promote the differentiation of target neurons and connect to them. Next, the same process occurs in photoreceptors from more rostral regions of the retina, which project axons to more rostral regions of the lamina, and so forth, until all photoreceptors have differentiated and extended their axons. By contrast, the mapping of the dorso-ventral axis of the fly retina into the lamina depends on the Wnt family (Figure 1.2B) (Clandinin and Feldheim, 2009). Temporal gradients also appear to be key factors for building the retinotopic map in the visual system of crustaceans (Flaster and Macagno, 1984).

The use of either molecular or temporal gradients is sufficient to form an initial coarse map. However, other mechanisms are required to increase the precision of the map. Neuronal activity can play a prominent role in map refinement. In the visual system of vertebrates, neuronal activity refines the retinotopic map. In fishes and amphibians, visual input evokes the firing of retinal neurons during map formation. In mammals and birds, the map forms before any visual experience has occurred. Retinal neurons, however, generate spontaneous waves of action potentials that spread across the retina during map formation. In both cases, the spatial and temporal pattern of retinal neurons firing instructs the refinement of their connections with target neurons in the tectum, in such a way that "neurons that fire together wire together". By contrast, neuronal activity seems to play no role in the formation of the retinotopic map in the invertebrate visual system (Goodman and Shatz, 1993; Luo and Flanagan, 2007). In the olfactory system, neuronal activity produced by odorant receptor signaling seems to regulate the expression of molecular cues that instruct the formation of the olfactory map (Luo and Flanagan, 2007; Sakano, 2010).

Another common principle of neural map development is the use of local interactions, both adhesive and repulsive, among axons. In the vertebrate and invertebrate visual and olfactory systems, axon-axon interactions ensure the ordering of axons during pathfinding and their proper spacing into the target region (Luo and Flanagan, 2007). In the visual system, such axon-axon interactions are mediated by cell-surface molecules like cadherins and IgCAMs (Clandinin and Feldheim, 2009). In the olfactory system, local sorting is mediated by the complementary expression of

Neuropilin-1 receptor and its repulsive ligand Semaphorin-3A (Mori and Sakano, 2011).

An important question in the study of neural map formation is where patterning instructions originate from. Three ways of establishing a neural map between two groups of neurons, input and target neurons, have been proposed. One possibility is that input neurons are prespecified and instruct their target neurons which identity to acquire. Another possibility is that input neurons are naïve and acquire their identity from the prespecified target neurons they connect to. The last possibility is that input and target neurons acquire their identities independently (Figure 1.3) (Jefferis et al., 2001). This is precisely what happens in the case of the visual system of vertebrates and the olfactory system of both vertebrates and invertebrates. In these systems, the patterning information resides in both the input and the target neurons, which are specified and sorted autonomously. By contrast, in the visual system of flies, the patterning information resides in the input neurons that eventually instruct target neurons which identity to acquire (Luo and Flanagan, 2007; Sakano, 2010).

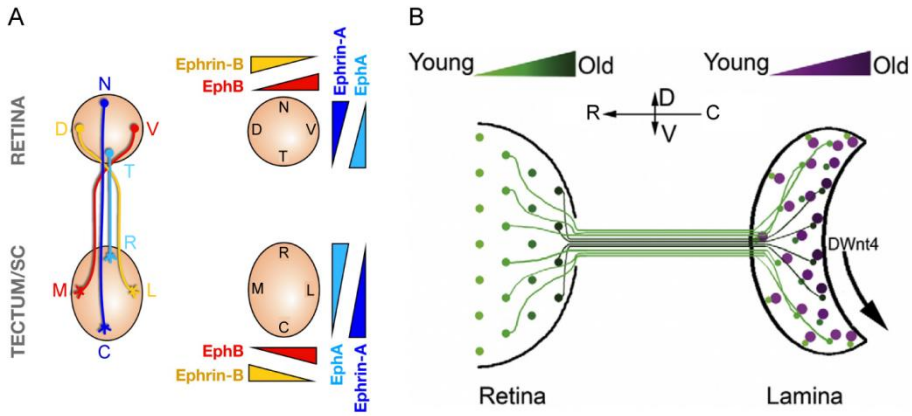


Figure 1.2. Retinotopic map formation in the vertebrate and invertebrate visual systems. A: Scheme of the retinotopic map in vertebrates is shown at the left. N:nasal, V:ventral, T:temporal, D:dorsal, R:rostral, L:lateral, C:caudal, M:medial. Ephrin ligands and their Eph receptors are expressed in complementary gradients across the retina and the tectum. The A and B subfamilies of Eph/ephrins act as mapping labels along two orthogonal axes. Neurons with high levels of Ephrin-A avoid high levels of EphA whereas neurons with high levels of Ephrin-B are attracted by high levels of EphB. Adapted from Luo and Flanagan, 2007. **B:** In invertebrates, retinotopic mapping along the rostro-caudal axis relies on temporal gradients. Photoreceptors (retina) differentiate and project into the lamina where they induce the differentiation of their targets, in a caudal to rostral wave. Wnt singaling is involved in the mapping along the dorso-ventral axis. Adapted from Clandinin and Feldheim, 2009.

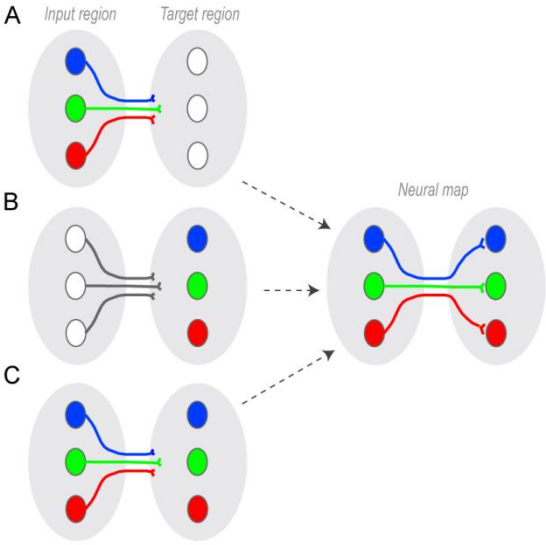


Figure 1.3. Three ways of building a neural map. A: Neurons in the input region are prespecified and instruct neurons in the target region which identity to acquire. **B:** Neurons in the input region are naïve and acquire their identity when connect to the prespecified neurons in the target region. **C:** Neurons from input and target regions are specified independently. Neurons in the same colour eventually convey the same information, such as sensory information captured at a specific location along the input field. Adapted from Jefferis et al., 2001.

1.1.3 Functional significance

Neural maps appear to be a universal solution; they are present in phylogenetically distant animals and in diverse sensory systems. Therefore, they must provide animals with some advantage. How does the brain benefit from sensory neural maps to process sensory information? What is the importance of sensory maps in guiding animal behavior? Despite the notable advances made in deciphering the mechanisms involved in neural map development, much less is known about their functional significance. Furthermore, the rules used to extract and process information from sensory maps are still obscure.

Since first evidence for neural maps came, researchers have proposed diverse hypotheses about the relevance of neural maps, especially about the importance of continuous or topographic maps for sensory processing. Some authors have claimed that such maps are the basis of perception, based on similarities between many aspects of perception and structural aspects of the maps. In addition, some theories of visual perception implicate topographic maps (Kaas, 1997). By contrast, other authors have pointed out that the particular spatial arrangement found in continuous maps may reflect an economical solution for the proper wiring of the brain, or a solution for increasing communication velocity by shortening connections between neurons (Chklovskii and Koulakov, 2004; Weinberg, 1997). With the advances in developmental and molecular neurobiology, it is now possible to disrupt sensory neural map formation and ask for its functional consequences in sensory processing and behavior.

The functional significance of the retinotopic map has been recently studied in mutant mice with disrupted projections from retinal neurons into the optic tectum; in mammals more commonly called the superior colliculus. Mutant mice with a disruption of spontaneous activity during map development show an imprecise spatial arrangement of connections between nasal/temporal retinal neurons and caudal/rostral superior colliculus neurons. In animals with such alteration, superior colliculus neurons have abnormal receptive fields along the temporo-nasal axis. In other words, these neurons respond to the presence of a visual stimulus in ectopic positions. Importantly, these animals fail to track visual stimuli moving along the temporo-nasal axis, but are able to track it without problems along the dorso-ventral axis (Wang et al., 2009). Other mutations result in some dorsal retinal neurons connecting ectopically to superior colliculus neurons that normally receive input from ventral retinal neurons. This appears to be translated into ectopic receptive fields along the dorso-ventral axis (Chandrasekaran et al., 2009). Superior colliculus neurons are known to project to the visual cortex preserving the retinotopic map. Disruptions of this map also have functional consequences such as visual cortex neurons with abnormal receptive fields and decreased visual acuity (Cang et al., 2008; Demyanenko et al., 2011).

Researchers in the field of olfactory processing have followed a similar approach. They have disrupted the olfactory map by different means and asked for the consequences in sensory processing and behavior. As mentioned previously, each olfactory sensory neuron expresses only one of many odorant receptors. Neurons expressing the same receptor project to the same spatially fixed glomerulus in the olfactory bulb. Importantly,

this anatomic map is translated into a functional map. Odorant signals received in the olfactory epithelium are translated into an odor map of activated glomeruli in the olfactory bulb (Mori and Sakano, 2011). Odor representation in the olfactory bulb is sparse since each odorant signal only activates few glomeruli (Lin da et al., 2006). Researchers have engineered a mouse in which over 95% of the olfactory sensory neurons express the same odorant receptor. This results in the activation of all glomeruli by the presence of the odor that interacts with the predominant odorant receptor. In this way, the representation of odors in the brain is severely perturbed. It appears that these mice are able to smell, but they show problems in odor discrimination and in olfactory behaviors, especially in innate behaviors (Fleischmann et al., 2008). Other approaches have consisted in the ablation of olfactory sensory neurons from specific areas of the olfactory epithelium in mice. The ablation of neurons from the dorsal region of the olfactory epithelium causes a depletion of glomerular structures in the dorsal region of the olfactory bulb. Animals with this perturbation fail to show innate responses to aversive odors, although they are able to detect them and even perform learned aversive responses to the same odors (Kobayakawa et al., 2007).

How neural maps facilitate sensorimotor transformations is another fundamental question. This issue has been examined in the oculomotor system. In many animals, eyes make very rapid movements or saccades in order to sense with high resolution regions of a visual scene and build up an internal representation of it. The superior colliculus, or tectum, contains a well-defined retinotopic map where visual space is represented. Importantly, this brain structure is involved in the transformation of visual information into orienting behaviors, including

ocular motor commands. Several studies have shown that superior colliculus neurons involved in sensorimotor transformations encode the desired motor direction based on the coordinates provided by the retinotopic map (Klier et al., 2001; Marino et al., 2008). Moreover, topographic maps from several sensory modalities (visual, auditory and somatosensory) are aligned with each other, as well as with a premotor map, in the superior colliculus. This arrangement appears an effective way to match incoming sensory information about a source with the motor outputs necessary for orientation to it (Stein et al., 2009). The analysis of some spinal cord reflex circuits similarly suggests that neural maps play a role in adapting sensory input to motor output during sensorimotor transformation (Levinsson et al., 2002).

Finally, the contribution of sensory maps to the learning of perceptual tasks has also been examined using the rat whisker sensory system. Sensory neurons innervating the whiskers on the snout project to the thalamus preserving the spatial distribution of the whiskers and thus forming a continuous map. Thalamic neurons project to the somatosensory cortex maintaining this spatial order and forming a map of the whiskers into the cortex (Petersen, 2007). Rats can cross a gap between two elevated platforms by using their whiskers to touch and locate the platform they have to reach for receiving a reward. A rat possessing a single whisker can be trained to efficiently perform this task. After, the whisker can be clipped and a prosthetic whisker can be placed on the location of the trained whisker or in the location of any other whisker. If the prosthetic whisker is placed in the location of the trained whisker, the rat can efficiently perform the gap-crossing task again. Interestingly, a period of relearning is necessary if it is placed in any

other whisker location and the duration of the relearning period is directly proportional to the distance between the locations of trained and prosthetic whiskers (Harris et al., 1999). This observation suggests that the neural modifications associated with the learning of a perceptual task are distributed according to the sensory map present in the sensory cortex (Diamond et al., 1999).

1.1.4 Summary

The use of neural maps to represent information, such as position or identity, is a fundamental organizational principle of the nervous system. Neural maps are broadly present in the nervous system, from sensory to motor components. Sensory neural maps have seized the attention of researchers for many years. How are these maps established during development? How are they used for sensory processing and for guiding behavior? These are central questions in neurobiology. Despite the notable advances in unraveling the developmental mechanisms involved in sensory map formation, there are still many open questions. For instance, the problem of how different forces act together to drive neural map development is poorly understood. In addition, most of the research has focused on a few neural maps and therefore there are still many neural maps whose assembly needs to be analyzed. Besides this, very little is known about the role of sensory maps in sensory processing and behavior.

1.2 Using the zebrafish to study sensory neural map development and function

To study sensory neural map development and function, it would be ideal to have single species in which molecular, cellular and physiological analyses, as well as perturbations, could be carried out in neurons during development and once neuronal circuits are established. The zebrafish (*Danio rerio*) has been historically used as a model for studying the basic mechanisms of development. Importantly, in the recent years, it has also become an organism of choice for analyzing how neuronal circuits function and how they mediate behavior, especially at larval stages (Fetcho and Liu, 1998).

The zebrafish compares favorably with other animal model systems to study developmental neurobiology for several reasons. The zebrafish embryo develops rapidly, externally and is optically transparent; which makes it suitable for visualization and manipulation. It is also amenable for mutational analysis. For example, it is possible to carry out large-scale screenings for developmental defects using chemical or insertional mutagenesis. Moreover, the generation of transgenic zebrafish is technically simple. Reporter transgenic methods allow in vivo time-lapse imaging of neurons. Engineered genes can also be expressed transiently, facilitating functional studies by gain- or loss-of-gene function. By using the zebrafish embryo, notable advances have been made in the fields of patterning of the nervous system, axonal pathfinding and specification of neuronal identity (Appel, 2006; Eisen, 1991; Nicolson, 2006).

The above-mentioned advantages also favor the use of the zebrafish as a model for studying neuronal circuits function and behavior. The zebrafish larva is amenable to neurophysiology, imaging and behavioral analyses. At larval stages, the zebrafish displays simple and robust behaviors that can be reliably evoked. In addition, some new tools provide the opportunity to depict the patterns of connectivity between neurons; even to reconstruct entire circuits. Other new tools allow monitoring neuronal activity during behavior, in living intact animals, as well as manipulating it; for example by activating and silencing neurons when desired. This is especially of importance since it provides a powerful way to causally relate neurons/circuits to behaviors. Last, the nervous system of the zebrafish larva is relatively small in terms of size and number of neurons; which indeed facilitates the analysis of neuronal circuits. Researchers have already benefited from these advantages to gain insight into the function of motor and sensory systems in the zebrafish larva (Del Bene and Wyart, 2011; Fetcho and Liu, 1998; Friedrich et al., 2010).

Altogether the above-mentioned qualities make the zebrafish an ideal model to close the gap between molecules, neurons, circuits and behaviors. The hope is that the findings made in the zebrafish can be extrapolated to other animals, since nervous system structure and gene function is highly conserved among vertebrates.

1.3 The lateral-line sensory system

The lateral line is a sensory system found in fish and amphibians. It was long ascribed to an auditory function since its sensory receptors, the hair cells, are the same as in the inner ear. The current view is that the lateral line mediates a 'distant-touch' sense, because it responds to water motions that occur within short-distances in the animal's surroundings (Dijkgraaf, 1963). In this chapter, I will cover aspects of the lateral line that are relevant to my thesis research focusing on our model organism: the zebrafish larva. I will also present knowledge from the adult zebrafish and from other fish species for comparisons and when there is no information from our model.

1.3.1 Distribution and morphology of the sensory organs

The lateral line comprises a set of discrete sensory organs called neuromasts. Neuromasts can occur superficially on the skin or within sub-epidermal canals open to the water through pores. In the zebrafish larva, all neuromasts are superficial and are arranged in stereotypic patterns on each side of the animal. Neuromasts on the head and trunk configure the anterior and posterior lateral-line branches, respectively. The posterior lateral line can be further divided into two sub-branches located in the lateral and dorsal aspects of the fish. In one-week-old larvæ, the posterior lateral line comprises around 14 neuromasts (Figure 1.4A) (Ghysen and Dambly-Chaudière, 2007; Metcalfe et al., 1985). The number of neuromasts dramatically increases over development. The distribution and number of neuromasts in adult fish diverge notably between different species. However, the organization of the system at

early stages of development appears to be conserved between species (Nuñez et al., 2009).

Neuromasts are small sensory patches composed of a core of 20 to 30 mechanosensory hair cells surrounded by a similar number of non-sensory supporting cells. Thus, they are structurally similar to the organs of the inner ear (Ghysen and Dambly-Chaudière, 2007). Hair cells derive their name from the hair bundle that projects from their apical domain. In the neuromast, hair bundles protrude into a gelatinous cupula that connects them to the surrounding water. The hair bundle comprises an array of stereocilia arranged in rows of increasing length, like a staircase, and a kinocilium eccentrically located adjacent to the tallest stereocilia. Therefore, each hair bundle, and thus each hair cell, is polarized within the plane of the neuromast. This represents a striking example of planar cell polarity. A water motion over the cupula that deflects the stereocilia towards the kinocilium depolarizes the hair cell. This triggers in turn an increase in neurotransmitter release at the hair cell's basal domain and a subsequent increase in the firing rate of the afferent (sensory) neuron associated to the hair-cell. By contrast, a deflection in the opposite direction hyperpolarizes the hair cell and reduces neurotransmitter release which causes a decrease of the firing rate of the associated neuron (Figure 1.4B). Therefore, the morphological polarization of the hair cell determines its axis of mechanical sensitivity (Hudspeth, 2000).

Surprisingly, each neuromast consists in two intermingled populations of hair cells, equal in number, of opposing hair-bundle polarities. Thus, neuromasts are bidirectionally sensitive; one population of hair cells is sensitive to deflections in a given direction, whereas the other population

is sensitive to deflections in the opposite direction (Figure 1.4B) (Flock and Wersall, 1962; López-Schier et al., 2004). Furthermore, two types of neuromasts have been described according to the polarization of their hair cells across the animal's body axes. Parallel neuromasts contain hair cells polarized across the antero-posterior (rostro-caudal) axis whereas perpendicular neuromasts contain hair cells polarized across the dorso-ventral axis. Consequently, parallel and perpendicular neuromasts are differentially sensitive to mechanical stimulation across two orthogonal axes (Figure 1.4C) (López-Schier et al., 2004).

1.3.2 Natural stimuli and behavior

The lateral line detects hydromechanic stimuli in the fish's surroundings, specifically low frequency (<150 Hz) water motions (Bleckmann, 2008). An important source of lateral-line stimuli is the water flow generated by a predator. Fish are able to execute an extremely fast escape response, known as the C-start reflex, after detecting the flow caused by a predator's strike. It has been shown recently that the zebrafish larva uses its lateral line to detect and to escape from a water flow that emulates a predator's strike (Figure 1.5A) (McHenry et al., 2009).

Another common source of hydromechanic stimuli is the swimming movement of an animal. For instance, the wake behind a swimming fish contains complex flow patterns and turbulences; and provides information about the wake generator, such as the size, swimming style and speed. Some fish use the lateral line to sense this information and track the trails of prey fish. The lateral line can also sense water surface waves. Surface feeding fish use their lateral line to detect preys in the

water surface, such as terrestrial insects falling into the water or animals that contact the water-air interface from below to breathe or feed (Bleckmann and Zelick, 2009).

Self-generated water motions and water currents in running water provide continuous stimulation of the lateral line. The patterns of self-generated water motions are modified when a fish approaches an object. These changes provide information about the size, shape and distance of nearby objects. This is especially used by blind cavefish to locate nearby stationary objects and avoid obstacles during navigation (Bleckmann and Zelick, 2009). Water flow information provided by the lateral line appears to be important for rheotaxis, a behavioral orientation to swim against water currents and avoid, thus, being washed out by the current (Montgomery et al., 1997). The zebrafish larva clearly exhibits a rheotactic response when exposed to a water flow (http://rubenportugues.net/). Moreover, fish use lateral-line information to make their swimming more efficient in running water (Liao, 2006).

Superficial and canal neuromasts present morphological differences which result in different respond properties to the sources of hydromechanic stimuli. The former detect flow velocity whereas the latter detect the acceleration of water motions. Thus, superficial neuromasts are efficient at detecting the flow created by a predator's strike in still water. They also sense water currents and mediate rheotaxis. By contrast, canal neuromasts are efficient at detecting small hydromechanic stimuli against a background constant water flow (Engelmann et al., 2000; Montgomery et al., 1997).

In summary, the lateral-line sensory system provides information that fish use for predator avoidance, prey detection, object discrimination and rheotaxis. Moreover, lateral-line receptors with different shapes, such as superficial and canal neuromasts, convey sensory information that might be used for different behaviors. How is the hydrodynamic information captured by the neuromasts translated into the diverse behaviors mediated by the lateral line? To comprehend this it is necessary to examine how this information is conveyed to the brain and how it is further processed centrally.

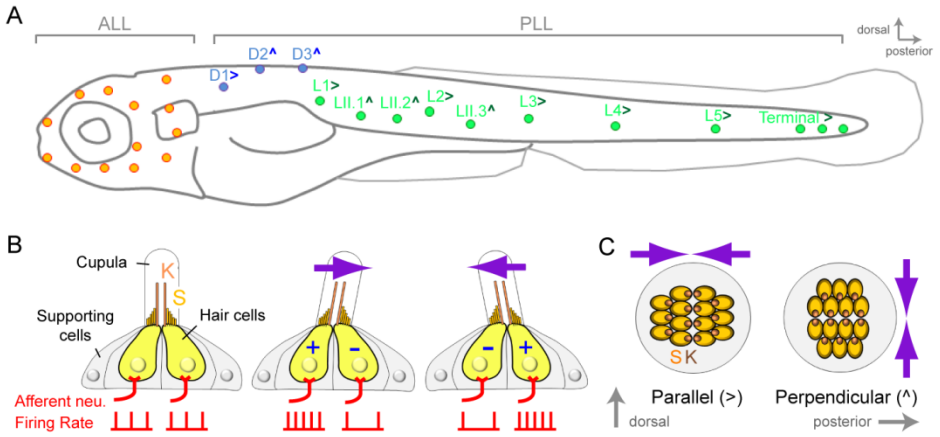


Figure 1.4. Distribution and morphology of the lateral-line sensory organs. **A:** Lateral view of a one-week-old zebrafish larva. Neuromasts on the head (orange) configure the anterior lateral line (ALL). Neuromasts on the trunk configure the posterior lateral line (PLL) which can be further subdived in its lateral (L, green) and dorsal (D, blue) branches. **B:** Lateral view of a neuromast. The hair-bundle comprises the kinocilium (K) and the stereocilia (S). A neuromast contains two populations of hair-cells of opposing hair-bundle polarities. Thus, a water movement (blue arrows) bending the cupula in a given direction depolarizes (+) one population of hair-cells whereas hyperpolarizes (-) the other one. This is translated into an increase or a decrease of the firing rate of the afferent neuron associated to each hair-cell population. **C:** Top view of a parallel and a perpendicular neuromast, which are sensitive to water movements across two orthogonal axes. (>) and (^) indicate, respectively, parallel and perpendicular neuromasts of the posterior lateral line in (A).

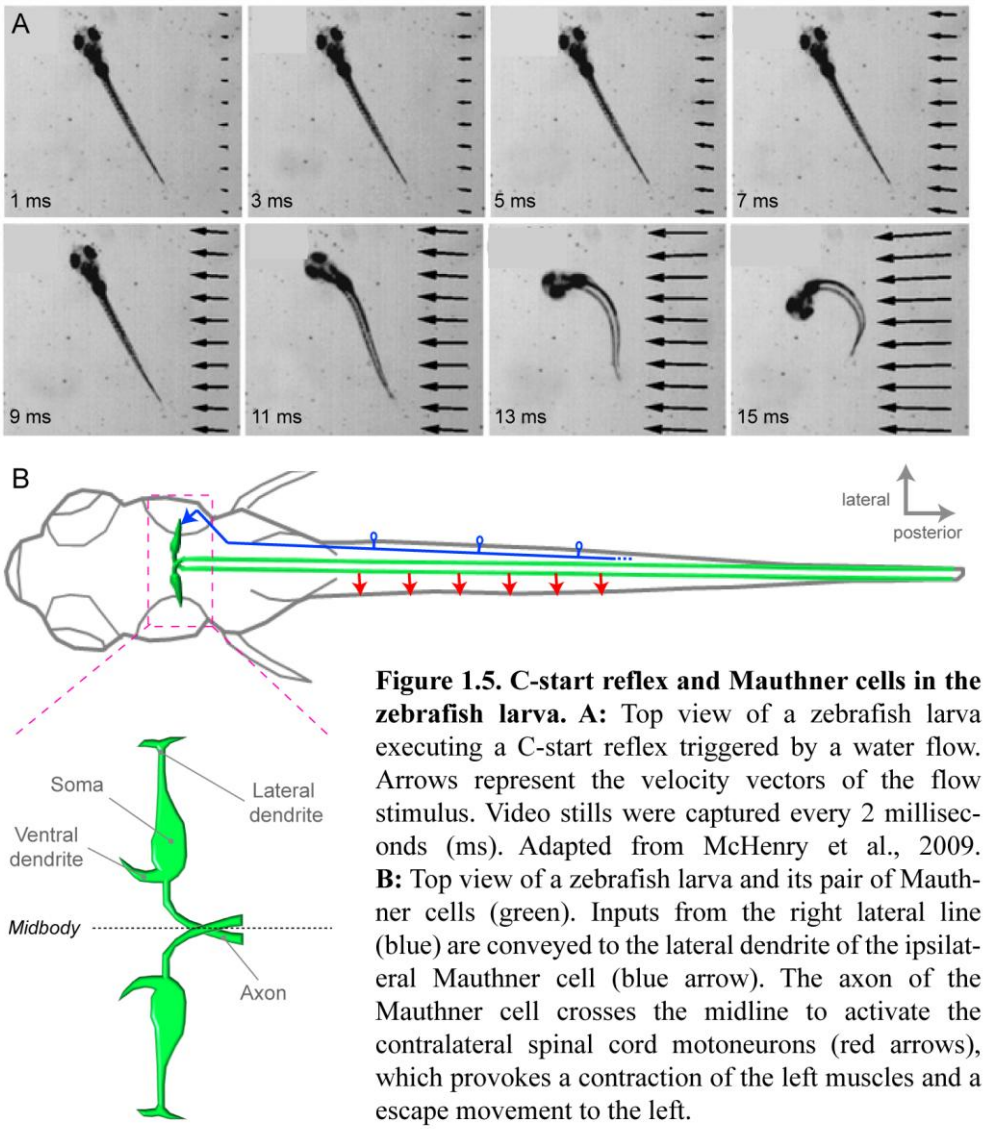


Figure 1.5. C-start reflex and Mauthner cells in the zebrafish larva. A: Top view of a zebrafish larva executing a C-start reflex triggered by a water flow. Arrows represent the velocity vectors of the flow stimulus. Video stills were captured every 2 milliseconds (ms). Adapted from McHenry et al., 2009. **B:** Top view of a zebrafish larva and its pair of Mauthner cells (green). Inputs from the right lateral line (blue) are conveyed to the lateral dendrite of the ipsilateral Mauthner cell (blue arrow). The axon of the Mauthner cell crosses the midline to activate the contralateral spinal cord motoneurons (red arrows), which provokes a contraction of the left muscles and a escape movement to the left.

1.3.3 From sensory organs to central nervous system

As mentioned previously, a water motion over the cupula that bends the stereocilia towards the kinocilium produces an increase in neurotransmitter release at the hair cell's basal domain. The neurotransmitter released by hair cells acts into the peripheral axonal endings of lateralis (lateral-line) afferent neurons, generating an action potential that travels along the neuron, from peripheral axon to central axon, passing by the neuronal soma. Axons from lateralis afferents are grouped forming nerves, whereas somata coalesce forming small cephalic ganglia near the ear. Somata from afferent neurons innervating the anterior and posterior lateral-line branches form the anterior and posterior lateralis ganglia (Figure 1.6A) (Metcalfe, 1985). In one-week-old larvæ, the number of lateralis afferents is small; the posterior lateralis ganglion comprises approximately 45 somata at around this stage (Liao, 2010). By contrast, many more lateralis afferents are found in the adult zebrafish (Metcalfe et al., 1985).

Sensory information from the lateral line arrives to the ipsilateral dorsal hindbrain through the lateralis nerves. There, central axons from anterior and posterior lateralis neurons form two contiguous yet non-overlapping columns that course rostrocaudally (Figure 1.6B). In the zebrafish larva, central axons terminate into a neuropil region ventral to the medial octavolateralis nucleus (MON) in the hindbrain. Each axon bifurcates at the level of rhombomere 6 into a rostral and a caudal branch and exhibits terminal buttons along the entire rostrocaudal extent. The rostral branch extends to rhombomere 1 whereas the caudal branch extends to rhombomere 7/8 (Figure 1.6C). Although this is the general situation, few

neurons extend their central axons further into the ipsilateral cerebellum. In the adult of several fish species, lateralis central axons end up clearly within the MON, which also receives afferent neurons from the inner ear. However, like in the larva, some central axons reach the ipsilateral cerebellum (Bleckmann and Zelick, 2009; Fame et al., 2006; Metcalfe et al., 1985).

Lateralis afferent neurons represent the first-order neurons of the lateral-line sensory system, since they conduct sensory information from the sensory receptor to the brainstem, specifically to the MON. There, they synapse with second-order neurons. It has been shown that lateralis central axons make monosynaptic contacts with the lateral dendrite of the ispsilateral Mauthner cell, a command neuron that triggers the C-start reflex, both in the zebrafish and in other species (Figure 1.5B) (Kimmel et al., 1990; Zottoli and Van Horne, 1983). In the zebrafish larva, none of the other reticulospinal neurons described, however, appear to extend dendrites near the region of lateralis central axons terminals. Dendrites of vestibulospinal neurons project into the column formed by lateralis central projections, strongly suggesting that they synapse with lateralis neurons (Metcalfe et al., 1985). The Mauthner cell and, very likely, the vestibulospinal neurons are examples of second-order neurons that pick up lateral-line information and send commands directly to motor centers in the spinal cord; avoiding high-order sensory processing.

Most of the second-order lateralis neurons described in the zebrafish larva and the adult of several fish species send lateral-line information to higher-brain centers where is further processed. In the adult, the somata from these second-order neurons are located in the MON. They send

axons largely to a midbrain nucleus called torus semicircularis, which is equivalent to the inferior colliculus of mammals, a major target of auditory information. The optic tectum, another midbrain structure, also receives projections from the MON. In both cases, projections occur bilaterally, with a contralateral predominance. In addition, second-order neurons project to the contralateral MON (Figure 1.7) (Bleckmann and Zelick, 2009). The same occurs in the zebrafish larva, although the somata from second-order neurons are not located in a nucleus but extend over a larger region, possibly over most of the hindbrain dorsal (alar) plate. Furthermore, it appears that there are projections to neurons that have not been previously identified as second-order targets in the adult fish. It has been suggested that the pattern found in the larva is an ancestral scaffold of connections from which some subsets will be selected in different groups of vertebrates at later stages of development, for their own purposes (Fame et al., 2006).

The next step in lateral-line sensory information transmission occurs from the midbrain to the diencephalon. Third-order lateralis neurons located in the torus semicircularis project axons into various diencephalic nuclei. From the diencephalon, lateral-line sensory information finally arrives to the telencephalon (Figure 1.7) (Bleckmann and Zelick, 2009).

Although we know well which the central relay stations of the lateral-line information are, their roles in sensory processing still remain largely unknown. Moreover, very little is known about the genetic identity of lateralis central neurons. In the zebrafish larva, anatomical data strongly suggest that both neurons expressing *glutamate decarboxylase 2 (gad2)* and neurons expressing *zic family member 1 (zic1)*, which are not mixed

in the hindbrain, are second-order lateralis neurons. G*ad2*(+) neurons are inhibitory and project to the contralateral hindbrain. By contrast, z*ic1(+)* neurons project to the torus semicircularis of the contralateral midbrain. To my knowledge, this is the only data regarding the genetic identity of central lateralis neurons to date (Sassa et al., 2007).

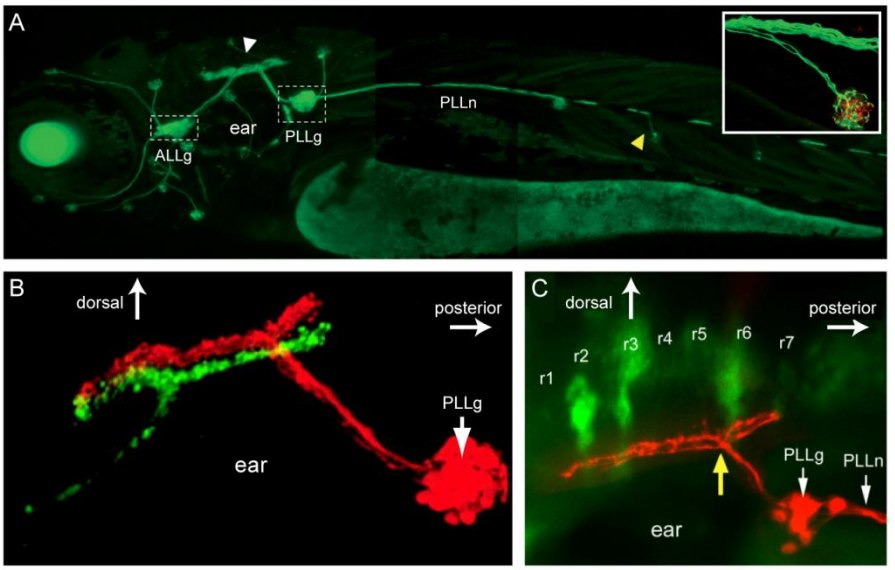


Figure 1.6. Lateralis afferent neurons in the zebrafish larva. A: Transgenic fish expressing GFP in the lateralis afferents, whose somata form the anterior and posterior lateralis ganglia (ALLg and PLLg). Peripheral axons of the posterior lateralis neurons form the posterior lateralis nerve (PLLn). White and yellow arrowheads point to the central axons and the peripheral axonal endings, respectively. The inset shows the peripheral arborization below the hair cells (red). Adapted from Faucherre et al., 2009. **B:** Central axons of the anterior lateralis afferents (green) form a column that lays ventral to the column formed by the central axons of the posterior lateralis afferents (red). Adapted from Alexandre and Ghysen, 1999. **C:** Posterior lateralis central axons are shown in red and some of the rhombomeres (r) are shown in green. Central axons bifurcate (yellow arrowhead) into two branches at the level of r6. The rostral branch extends anteriorly to the level of r1 while the caudal branch extends posteriorly to the level of r7. Adapted from Fame et al., 2006. All the pictures are lateral views.

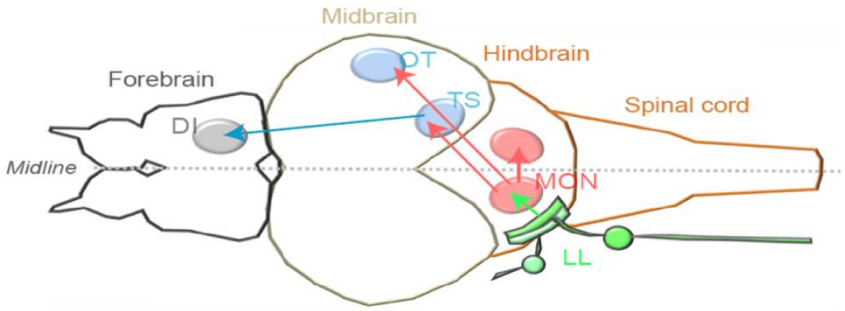


Figure 1.7. Central relay stations of the lateral-line sensory information. Top view of the adult zebrafish brain. Lateralis afferents (LL), the first-order neurons, are shown in green. Second- and third-order lateralis neurons are shown in red and blue, respectively. MON: medial octavolateralis nucleus. TS: torus semicircularis. OT: optic tectum. DI: diencephalic nuclei. Only the predominant projections from the MON to the contralateral TS, OT and MON are shown.

1.3.4 Lateral-line maps

The distribution and morphology of neuromasts provide to the lateral-line system a way to extract basic features from complex hydrodynamic stimuli; which is essential to interpret them and to react appropriately. This happens, at least, at four levels. First, each neuromast responds to stimuli in its proximity; capturing, thus, sensory information from a specific location on the animal's body. Second, superficial neuromasts appear to be specialized to detect water velocity whereas canal neuromasts are specialized to detect water acceleration. They represent two submodalities within the system. Third, hair cells of opposing polarities detect water motions in opposing directions. Fourth, parallel and perpendicular neuromasts are sensitive to water movements along the antero-posterior and dorso-ventral axes, respectively. Once these basic features –position, submodality, direction and body axis- have been extracted at the level of the periphery, they must be transmitted to the central nervous system separately in parallel pathways or channels. The

first-order neurons, the lateralis afferents, are the responsible to do so. Subsequently, this sensory information must be encoded at the level of the central nervous system. This might be achieved by means of spatially arranged neural maps, resembling what takes place in many other sensory systems.

The receptive field of an individual lateralis afferent is defined by its associated neuromasts. In the zebrafish larva, each lateralis neuron innervates a single neuromast, with some exceptions. Multiple innervation occurs largely in neurons innervating the terminal neuromasts; a group of two to three consecutive neuromasts very close to each other, located in the tip of the tail. Multiple innervation is rare in the rest of the neuromasts; and when it occurs, the innervated neuromasts are also spatially consecutive. In this way, lateralis afferents link several organs to form multi-neuromast sensory units (Faucherre et al., 2009; Nagiel et al., 2008). At a broader level, lateralis afferents are segregated into anterior and posterior ganglia and nerves that form two adjacent but segregated columns in the MON. Altogether these evidences indicate that the stimuli captured at different locations on the animal's body are relayed to the hindbrain in parallel channels. It has been revealed in several fish species, including the zebrafish larva, that these channels are arranged forming a continuous or topographic neural map. In the MON, the column formed by the central axons of the anterior lateralis afferents is always ventral to that formed by the central axons of the posterior lateralis afferents (Figure 1.6B and Figure 1.8). Moreover, within each column, lateralis afferents innervating anterior (rostral) neuromasts project central axons ventrally to those from afferents innervating posterior (caudal) neuromasts. Therefore, the distribution of neuromasts

along the antero-posterior (rostro-caudal) body axis is represented by a dorso-ventral organization of lateralis afferents' central axons, known as somatotopy (Figure 1.8A) (Alexandre and Ghysen, 1999; Bleckmann, 2008; Puzdrowski, 1989).

Is this somatotopic map maintained through the next steps of lateral-line information relay? An anatomical study in midshipman fish has shown that MON neurons project axons into the contralateral torus semicircularis forming a coarse topographic map. MON neurons contacting anterior lateralis central axons project to the rostral torus whereas those contacting posterior lateralis central axons project to the caudal torus (Figure 1.8B) (Weeg and Bass, 2000). In agreement with that, physiological data from electric fish have shown that neurons in the rostral torus respond to inputs from head neuromasts whereas neurons in the caudal torus respond to inputs from tail neuromasts (Bleckmann et al., 1987; Bleckmann and Zelick, 1993; Knudsen, 1977). Evidence for a crude topographic organization of neurons in the torus semicircularis of goldfish also exists (Engelmann and Bleckmann, 2004; Plachta et al., 2003). However, recent studies have failed to find highly space selective neurons in the MON and torus semicircularis, suggesting that single neurons do not encode the spatial location of a stimulus (Künzel et al., 2011; Voges and Bleckmann, 2011). These authors have suggested that, by contrast, populations of lateralis central neurons encode the spatial location of the stimulus. In this case, a strict topographic map would be partially lost, for example to facilitate information coding in different channels. Such a population code might be used by higher-brain centers to rebuild a spatial map; by converging inputs from many neurons into single neurons, for instance.

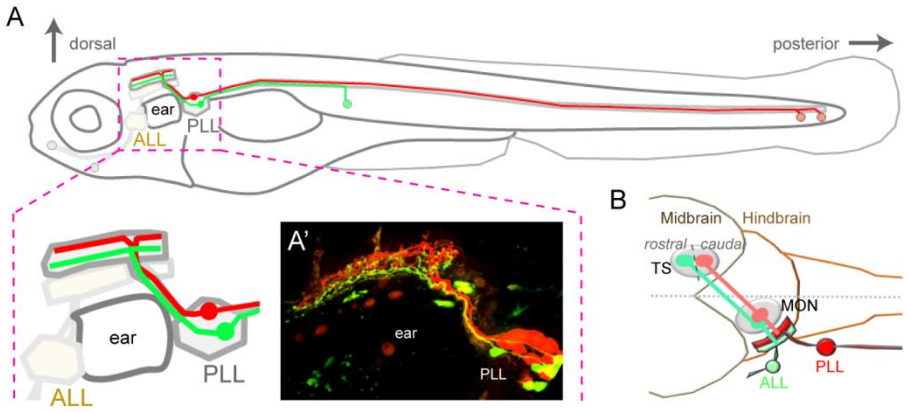


Figure 1.8. Lateral-line somatotopic map. A: Lateral view of a zebrafish larva showing the topographic arrangement of the lateralis afferent neurons, known as somatotopy. Central axons from the anterior lateral line (ALL) lay ventral to those of the posterior lateral line (PLL). Within the PLL, lateralis afferents innervating anterior neuromasts (green) project central axons ventrally to those from neurons innervating posterior neuromasts (red). (A') image is adapted from Alexandre and Ghysen, 1999. **B:** Top view of an adult fish brain showing the topographic arrangement of the second-order projections from the medial octavolateralis nucleus (MON) to the torus semicircularis (TS). MON neurons contacting ALL axons (green) project to the rostral torus whereas those contacting PLL central axons (red) project to the caudal torus.

Anatomical and physiological data have demonstrated that the information captured by superficial and canal neuromasts is transmitted separately in parallel pathways, at least until it reaches the MON. An individual lateralis afferent neuron can innervate several superficial neuromasts, but it never innervates simultaneously a superficial and a canal neuromast (Münz, 1985). Moreover, two types of lateralis afferents have been described when the lateral line is stimulated with a moving source. From their response patterns, it has been suggested that type I afferents innervate superficial neuromasts whereas type II afferents innervate canal neuromasts. In the muskellange fish, central axons from lateralis afferents innervating canal neuromasts project into a well-defined region of the MON; thus, they do not mix with lateralis afferents

innervating superficial neuromasts (Bleckmann, 2008). It is not clear yet whether there is a separation of the information coming from superficial and canal neuromasts at the torus semicircularis (Engelmann and Bleckmann, 2004; Plachta et al., 2003).

Pioneering electrophysiological analyses in cichlid fish demonstrated that all the hair cells innervated by a single lateralis afferent are functionally polarized in the same direction (Münz, 1985). Both morphological (Faucherre et al., 2009; Nagiel et al., 2008) and physiological (Liao, 2010; Obholzer et al., 2008) analyses in the zebrafish larva have shown the same recently. Each neuromast is innervated by at least two lateralis afferents. Each of these neurons synapses with hair cells of identical polarity to divide the neuromast into synaptic planar-polarity compartments (Figure 1.4B). This holds true even in the case of multiple neuromasts innervation, where the innervated hair cells belong to consecutive sensory organs. To date, there is no anatomical evidence showing that neurons innervating hair cells of opposing polarities map separately in the MON or in the torus semicircularis. Physiological studies in the adult fish of several species have shown that many lateralis central neurons from the MON and torus are sensitive to the direction of water flow. These neurons change their responses when a water flow is reversed from headward to tailward, for example. This might be explained if individual central neurons receive input exclusively from one of the two populations of hair cells of opposing polarities; in other words, if the inputs from the two populations are kept separately along the different central relay stations (Bleckmann, 2008). It might be, however, that both inputs from hair cells of opposing polarities converge on other lateralis central neurons, as recently observed in a single recording of a

neuron in the torus semicircularis of goldfish (Meyer, 2010). In any case, the direction of the water flow might be encoded in the brain by other means. Some authors have proposed that central lateralis neurons that receive inputs from different neuromasts perform spatiotemporal cross-correlations to determine water flow direction (Chagnaud et al., 2008).

In the same way as for the stimulus features addressed above, lateralis afferents transmit the inputs from parallel and perpendicular neuromasts to the brain in separate channels. In the zebrafish larva, a single lateralis afferent neuron can innervate simultaneously a parallel and a perpendicular neuromast. However, this only occurs in 10% of the cases and the general situation is that a single neuron innervating a parallel neuromast does not innervate a perpendicular one. Therefore, there is a high degree of specificity in the innervation of parallel versus perpendicular neuromasts by lateralis afferents (Sarrazin et al., 2010). There are no indications that lateralis afferents innervating parallel and perpendicular neuromasts map differentially in the brain (Bleckmann, 2008).

1.3.5 Lateral-line development

The development of the fish lateral line has been studied during the last decades mainly in the zebrafish embryo and larva. Much of the collected knowledge on this refers to the posterior lateral-line branch and their associated afferent neurons. The development of the posterior lateral line comprises several phases. First, at around 18 hours-post-fertilization (hpf), a placode appears just posterior to the otic region. Within the next hour of development, the placode splits into two groups of cells. The

rostral group consists of about 20 cells; which remain stationary and further differentiate into lateralis afferent neurons, giving rise to the posterior lateralis ganglion. The caudal group consists of about 100 cells; which form a moving primordium, known as first primordium (primI). PrimI migrates towards the tail along the horizontal myoseptum and deposits group of cells in an anterior to posterior wave; each of them eventually differentiating as a neuromast. By 48 hpf, 7 to 8 primI-derived neuromasts (L1-L5 and terminal) configure the lateral branch of the posterior lateral line (Figure 1.9) (Ghysen and Dambly-Chaudière, 2007; Metcalfe, 1985). Some of the molecular mechanisms involved in primordium migration and patterning, as well as in neuromast deposition, have been already revealed. Cxcr4b and Cxcr7b chemokine receptors are differentially expressed in primI. The former is expressed in the caudal region whereas the latter in the rostral region of primI. Directional migration of the primordium is driven by differential interactions between these two chemokine receptors and their ligand Sdf1a, which is expressed along the horizontal myoseptum. Moreover, Wnt and FGF signaling play central roles in primordium patterning and neuromast deposition (Ma and Raible, 2009).

At around 24 hpf, a second placode has appeared near the field where the first one did. Within the next eight hours of development the new placode gives rise to neurons, which are incorporated into the existing posterior lateralis ganglion, and to a group of cells called D0. These cells further split into three groups. One group of cells forms the D1 neuromast whereas the two other groups form two new primordia; called second primordium (primII) and dorsal primordium (primD). PrimII migrates and deposits neuromasts along the same trail as primI whereas primD

follows a dorsal path. In one-week-old larvæ, 3 to 4 primII-derived neuromasts (LII.1-LII.4) have been incorporated into the lateral branch and few primD-derived neuromasts (D2-D4) configure, together with the D1, the dorsal branch of the posterior lateral line (Figure 1.9). The transition from the larval to the adult lateral-line system requires further steps, such as the formation of more neuromasts from quiescent precursors dropped by the distinct primordia (Grant et al., 2005; Nuñez et al., 2009; Sapède et al., 2002; Sarrazin et al., 2010). Moreover, at the end of the larval period in zebrafish some neuromasts from the anterior lateral-line branch suffer morphogenetic changes giving rise to the canal neuromasts (Webb and Shirey, 2003).

Parallel and perpendicular neuromasts originate from different primordia. Both primI-derived neuromasts (L1-L5 and terminal) and D1 neuromast are polarized parallel to the antero-posterior body axis. By contrast, primII-derived neuromasts (LII.1-LII.4), as well as the neuromasts deposited by primD by one week of development (D2-D4), are polarized perpendicular to the antero-posterior body axis. This results in the presence of both types of neuromasts, parallel and perpendicular, in both the lateral and dorsal branches of the posterior lateral line (Figure 1.4C and Figure 1.9) (López-Schier et al., 2004; Nuñez et al., 2009).

What is known about the development of the lateralis afferent neurons? It has been recently shown that the choice of cell fate between lateralis afferent neuron or primordium cell within the placode is regulated by Notch signaling (Mizoguchi et al., 2011). Furthermore, the formation of the lateralis afferents requires the expression of the proneural gene *neurogenin1* (Andermann et al., 2002). As soon as the first posterior

lateralis afferents differentiate in the postotic region, peripheral and central axons grow out concurrently from each neuronal soma. Growing central axons extend towards the hindbrain whereas growing peripheral axons extend towards primI (Figure 1.9 and Figure 1.10). Peripheral growth cones are found within primI before the onset of migration and accompany it during the whole migratory process, eventually innervating the deposited neuromasts. They do not appear to be attached to specific cells but they move freely within primI. Moreover, they are never found more posterior (leading) than the primordium (Gilmour et al., 2004). However, other peripheral growth cones can be observed at different positions along the developing posterior lateralis nerve, associated with the axons of neurons whose growth cones accompany the primordium (Gompel et al., 2001b; Metcalfe, 1985). Therefore, it appears that during primI migration there are both 'leading' and 'following' lateralis peripheral axons, whose growth cones are, respectively, within or behind primI. It has been demonstrated that the 'leading' peripheral axons are guided by the primordium (Gilmour et al., 2004). Glial cell line-derived neurotrophic factor (GDNF) signaling is a major determinant of this process and it is thought to act at a short range (Schuster et al., 2010). During peripheral axon extension, glial cell precursors migrate along the developing axons and mediate nerve fasciculation and myelination. Peripheral axons appear to be the source of instructive cues for migrating glial precursos and s*ox10* is required for glial precursors to respond to them (Gilmour et al., 2002). Both lateralis neurons and glia are dispensable for primordium migration (Grant et al., 2005; López-Schier and Hudspeth, 2005).

Very little is known about lateral-line neural map development. In the zebrafish, research on this front has partly focused on finding out the cellular mechanisms by which the lateralis afferent neurons establish the somatotopic map. To gain insight into it, researchers have examined the development of lateralis afferents with single-cell resolution. First, they have shown that the neuromasts' constituent cells and their associated neurons are not related by fixed lineage, ruling out the possibility that a lateralis afferent neuron decides on a specific neuromast because they are siblings. Second, they have registered differences between anterior and posterior lateralis afferents and among those of the posterior lateral line well before neuromasts are innervated. Central axons from anterior and posterior lateralis neurons are topographically ordered as soon as they project into the hindbrain. Moreover, posterior lateralis afferents show differences in peripheral growth cone shape that are correlated to the positions of the neuromasts they will innervate (Gompel et al., 2001b). The authors of this work, then, have ruled out an instructive role of the neuromasts in patterning the map. Alternatively, they have proposed that each lateralis afferent neuron is somehow specified to innervate a neuromast at a given position. External cues from the hindbrain or instrinsic differences among the neurons might be key players in the process (Ghysen and Dambly-Chaudière, 2004; Gompel et al., 2001b). In agreement with this, genes differentially expressed among posterior lateralis afferents have been found (Gompel, 2001).

Furthermore, how lateralis afferents discriminate between hair cells of opposing polarities to innervate only those with the same orientation has been the other main issue recently addressed. To date, there is no evidence of molecular differences among the two populations of hair

cells. The only known difference involves evoked hair cell activity; a bending of the neuromast cupula towards a given direction depolarizes one population of hair cells whereas hyperpolarizes the other. It has been shown that evoked hair cell activity modulates peripheral axon arborization and hair cell polarity selection by lateralis afferents (Faucherre et al., 2010).

By contrast, knowledge about the development of the lateralis central neurons and the maps they shape is almost non-existent. The Mauthner cell is probably the best-studied hindbrain neuron that receives input from the lateralis afferents. In the zebrafish, the Mauthner cell is among the earliest neurons to develop in the brain. The lateral dendrite of the Mauthner cell starts growing at around 18 hpf and receives contacts from the central axons of trigeminal, acoustico-vestibular and lateralis afferents in a ventral to dorsal temporal sequence. This results in a segregation of the inputs from different sensory modalities onto the lateral dendrite. The first contacts between lateralis central axons and the lateral dendrite of the Mauthner cell are observed at around 25 hpf and occur on the most dorsal region (distal tip) of the dendrite (Kimmel et al., 1990). Concerning other hindbrain neurons which are likely to receive input from lateralis afferents, it has been demonstrated that the basic helix-loop-helix transcription factor Atonal homolog 1a (Atoh1a) is specifically required for the development of the z*ic1(+)* neurons but not for the *gad2*(+) neurons (Sassa et al., 2007).

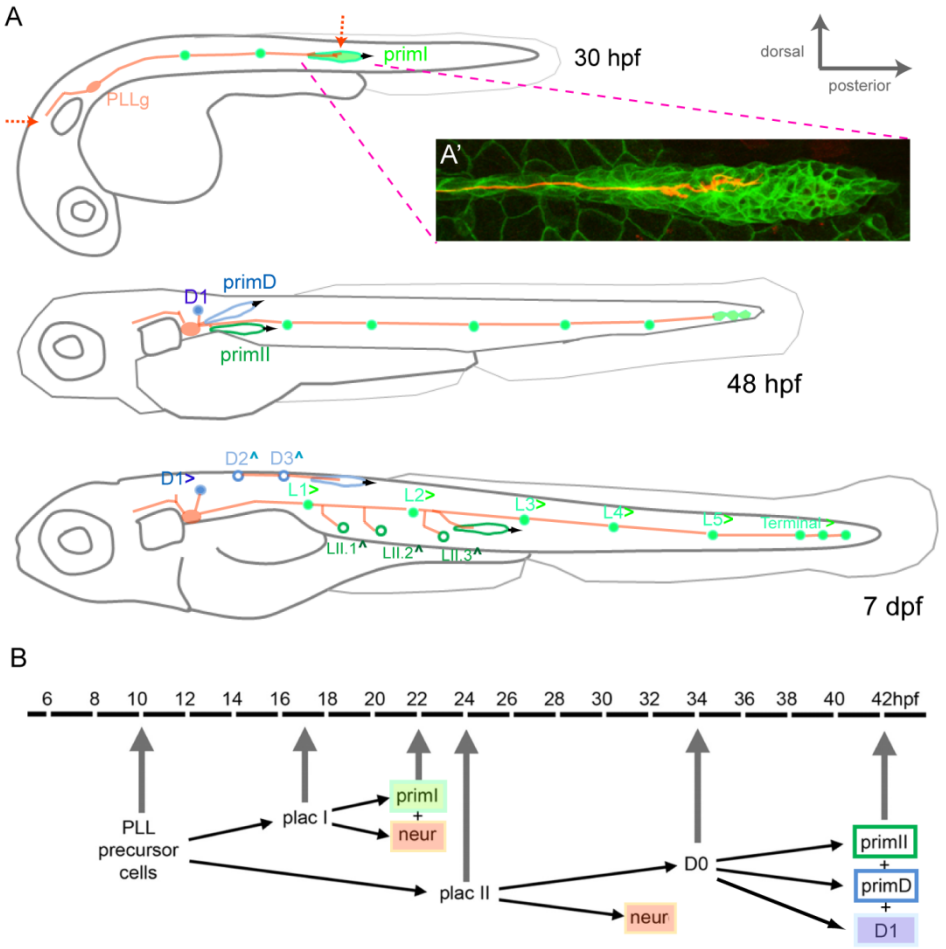


Figure 1.9. Posterior lateral-line development. A: Lateral view of a developing zebrafish at around 30, 48 hpf and 7 dpf showing the development of the peripheral components of the posterior lateral line. primI: first primordium. primII: second primordium. primD: dorsal primordium. PLLg: posterior lateralis ganglion. Arrows at 30 hpf indicate growing lateralis central and peripheral axons. (>) and (^) indicate parallel and perpendicular neuromasts, respectively. (A') A single lateralis afferent neuron is labeled in red in a transgenic zebrafish embryo expressing GFP in primI. The peripheral axonal growth cone can be observed within the migrating primordium. **B:** Development of the posterior lateral line (PLL) between 6 and 42 hpf. Vertical arrows indicate the time of appearance of the corresponding structures. placI: first placode, placII: second placode, neur: lateralis afferents. Adapted from Sarrazin et al., 2010.

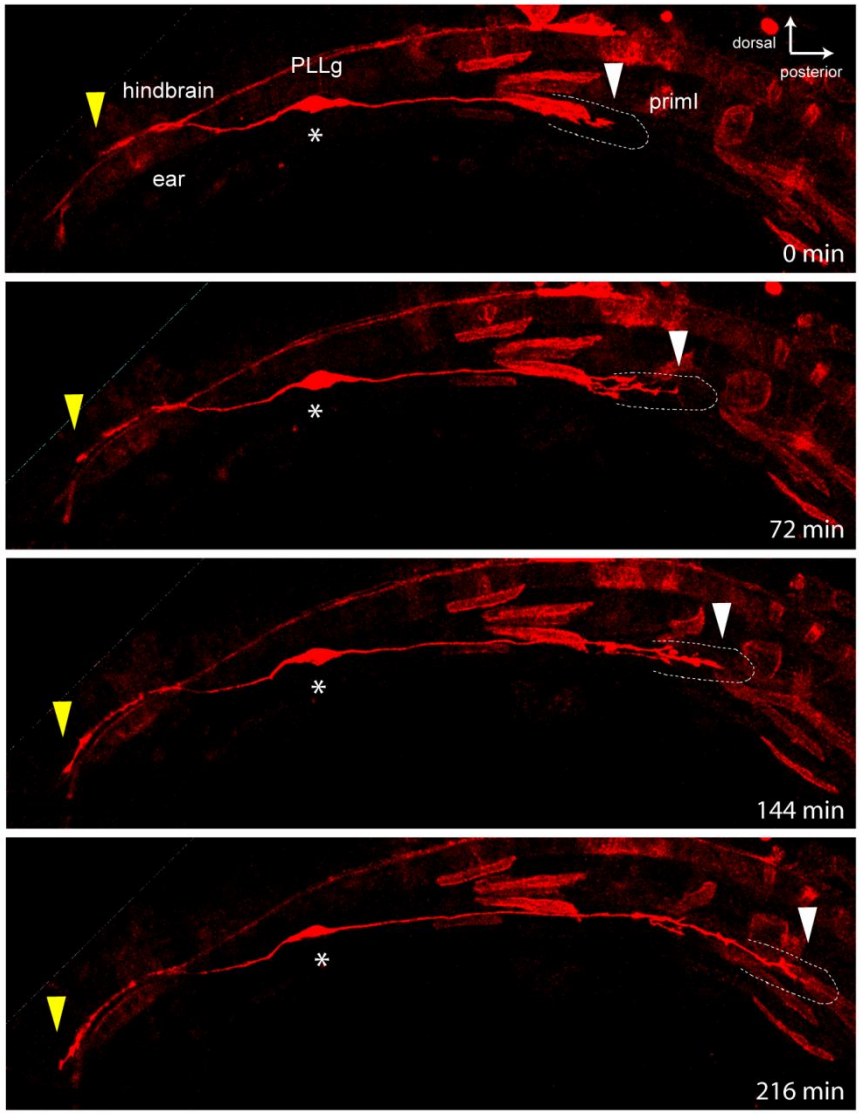


Figure 1.10. Growing of the posterior lateralis axonal projections over time. A single posterior lateralis afferent neuron is labeled in red and followed approximately from 24 hpf (0 min) to 27 hpf (216 min). PLLg: posterior lateralis ganglion. primI: first primordium (dashed line). The asterisk indicates the neuronal soma. The central axon (yellow arrowhead) grows towards the hindbrain whereas the peripheral axon (white arrowhead) accompanies primI during its migration. Note that the central axon has completely extended along the hindbrain before the peripheral axon has reached its final peripheral target, in this case the terminal neuromasts.

1.3.6 Summary

The lateral line of the zebrafish larva is anatomically much simpler than other sensory systems of vertebrates. At the peripheral level, it is composed of a few neuromasts and a few lateralis afferent neurons. In addition, the brain of the zebrafish larva is relatively small. These attributes, together with the rapid and external development of the zebrafish, facilitate the study of the peripheral and central components of the lateral-line system during development and once neural circuits are established. Despite of its anatomical simplicity, the larval lateral line shares structural features with other sensory systems. Such features are thought to be important for extracting basic attributes from complex stimuli. Importantly, the larval lateral line is already functionally complex since it appears to mediate contrasting behaviors that will be present in the adult fish, such as the C-start reflex and rheotaxis. For all the above-mentioned reasons, its study promises to shed light on a problem that still remains obscure: how the brain uses the information provided by a sensory system to generate appropriate behavioral reactions to the sensory context. Importantly, the study of the lateral line can also help to comprehend the developmental mechanisms of sensory circuits' assembly. With the excellent tools available and the advantages that the zebrafish larva exhibits, it should be possible to gain more insight into these basic and broadly interesting problems.

1.4 Aims of the thesis

1.4.1 To study the initial assembly of the somatotopic map by the posterior lateralis afferent neurons in the zebrafish larva

Since the somatotopic map was described in the zebrafish larva (Alexandre and Ghysen, 1999) to the time I started my thesis research, only one research paper focusing on its development has been published (Gompel et al., 2001b). The authors of this work suggested that each lateralis afferent is pre-specified to occupy a position in the map, either by external cues from the brain or by intrinsic determinants. Nevertheless, the results they showed are compatible with other possibilities. Neuromasts from the posterior lateral line develop progressively in an anterior to posterior wave. At the same time, lateralis afferents might be born and extend axons progressively. A synchrony of these two processes might be sufficient to generate a topographic map without the need of a neuronal pre-specification mechanism. To gain insight into the formation of the somatotopic map, I took advantage of recently developed tools for neuronal dating, new transgenic lines and live imaging of lateralis afferents during somatotopic map assembly.

1.4.2 To search for heterogeneities among lateralis afferent neurons regarding the connectivity with their central targets in the zebrafish larva

Lateralis afferents convey information about different basic features of a complex stimulus to the brain in separate channels. To understand how the brain uses this information to execute very diverse behaviors, we certainly need to know about the connectivity between the lateralis afferents and their central targets. Sensory information from different channels might be segregated at the level of the central nervous system. To test this hypothesis, I took advantage of new transgenic lines and high-resolution imaging of lateralis central axons and a known central target of the lateral line, the Mauthner cell. My hope is to set the stage for future functional studies by examining the topology of the neural maps shaped by the lateralis afferents.

Chapter 2

PROGRESSIVE NEUROGENESIS DEFINES LATERALIS SOMATOTOPY

Pujol-Martí J, Baudoin JP, Faucherre A, Kawakami K, López-Schier H. Progressive neurogenesis defines lateralis somatotopy. Dev Dyn. 2010;239(7):1919-30.

Chapter 3

NEURONAL BIRTH ORDER DELINEATES A DIMORPHIC SENSORINEURAL MAP

Pujol-Martí J, Zecca A, Baudoin JP, Faucherre A, Asakawa K, Kawakami K, López-Schier H. Neuronal birth order delineates a dimorphic sensorineural map. J Neurosci. Under review.

3.1 Abstract

Spatially distributed sensory information is topographically mapped in the brain by point-to-point correspondence of connections between peripheral receptors and central target neurons. In fishes, for example, the axonal projections from the mechanosensory lateral line organize a somatotopic neural map. The lateral line provides hydrodynamic information for intricate behaviors such as navigation and prey detection. It also mediates fast startle reactions triggered by the Mauthner cell. However, it is not known how the lateralis neural map is built to subserve these contrasting behaviors. Here we reveal that birth order diversifies lateralis afferent neurons in the zebrafish. We demonstrate that early- and late-born lateralis afferents diverge along the main axes of the hindbrain to synapse with hundreds of second-order targets. However, early-born afferents projecting from primary neuromasts also assemble a separate map by converging on the lateral dendrite of the Mauthner cell, whereas projections from secondary neuromasts never make physical contact with the Mauthner. We also show that neuronal diversity and map topology occur normally in animals permanently deprived of mechanosensory activity. We conclude that neuronal birth order correlates with the assembly of neural sub-maps, whose combination is likely to govern appropriate behavioral reactions to the sensory context.

3.2 Introduction

In aquatic environments, mechanical stimuli can convey information about the location and trajectory of obstacles, conspecifics, prey and predators, allowing animals to optimize navigation or to escape from a threat (Bleckmann and Zelick, 2009; Dijkgraaf, 1963). The spatial distribution of sensory information is topographically mapped in the central nervous system by stereotypical connections between peripheral receptors and target neurons, which is essential for the accurate transmission of environmental stimuli to processing centers in the brain (Luo and Flanagan, 2007). For example, hydromechanic variations along the body of fishes and amphibians are captured by external mechanosensory organs called neuromasts, which collectively form the lateral line (Ghysen and Dambly-Chaudière, 2007). Locally acquired mechanical signals by the sensory elements of the neuromast, called hair cells, are transmitted to bipolar afferent neurons that project central axons to the medial octavolateralis nucleus of the hindbrain (Alexandre and Ghysen, 1999; Claas and Münz, 1981; Ghysen and Dambly-Chaudière, 2007). This first mechanosensory relay contains a somatotopic neural map, in which the afferent central projections are stratified along a dorsomedial-ventrolateral axis that reflects the spatial distribution of the neuromasts (Alexandre and Ghysen, 1999; Claas and Münz, 1981; Ghysen and Dambly-Chaudière, 2007). Lateral-line somatotopy suggests that this sensory system builds a continuous neural map (Alexandre and Ghysen, 1999; Luo and Flanagan, 2007).

The lateral line possesses a dual function. It plays a major role in navigation, schooling, rheotaxis, prey detection and hunting (Coombs and Patton, 2009; Montgomery et al., 1997; Montgomery et al., 2000). This set of behaviors necessitates continuous input and involves fine and complex processing of the hydrodynamic field. The lateral line also mediates very fast responses to sudden mechanical stimuli, such as the C-start escape behavior that is triggered by the activation of a reticulospinal command neuron called Mauthner cell (McHenry et al., 2009). Because the value of the escape response is its near immediate onset after potentially threatening stimuli, it is best served by avoiding high-order processing. It is currently not understood how the lateralis neural circuit is built to sub-serve these contrasting behaviors. Here we investigate this issue by characterizing the assembly of the posterior lateralis sensorineural map.

3.3 Results

3.3.1 *HGn39D* is an insertion in c*ntnap2a*

We have previously reported that the zebrafish transgenic line *HGn39D* expresses the enhanced green-fluorescent protein (EGFP) in all the afferent neurons of the lateral line (Faucherre et al., 2009). We sought to determine the integration site in *HGn39D* by sequencing genomic DNA flanking the transgene. This revealed a single insertion on chromosome 24 within a locus coding for a zebrafish homolog of the *contactin-associated protein-like 2/Caspr2* (which we herein call c*ntnap2a* to differentiate it from a second c*ntnap2* (*cntnap2b*) located on chromosome 2) (Figure 3.1A-B). CNTNAPs are members of the Neurexin family of neuronal cell-adhesion proteins, which have been associated with autism and epilepsy in humans and in the mouse (Peñagarikano et al., 2011). Next, we used whole-mount in situ hybridization in 3 days-post fertilization (dpf) zebrafish embryos to determine the expression pattern of EGFP in *HGn39D* and of the endogenous *cntnap2a*, which revealed strong expression of both genes in the anterior and posterior lateralis ganglia (Figure 3.1C-D). Additional weak expression of *cntnap2a* may be present throughout the central nervous system. This result suggests that the specific expression in *HGn39D* may be replicated with new transgenes. Towards this aim, we characterized regulatory regions of *cntnap2a* in experimental constructs that were tested by transient transgenesis by injecting them as plasmids into fertilized zebrafish eggs. We started by cloning 2-kilobase (kb) fragments of genomic DNA flanking the insertion site in *HGn39D*. We tried several combinations of

these genomic fragments placed upstream and/or downstream of the heat-shock-70 promoter followed by a cytoplasmatic version of the monomeric red-fluorescent protein mCherry. DNA fragments upstream of the insertion site were not driving any visible transgene expression. However, a 2 kb downstream fragment placed downstream of mCherry drove robust gene expression in lateralis afferent neurons (data not shown). We named this construct SILL1 (for Sensory Innervation of the Lateral Line number 1) (Figure 3.1E). We subsequently generated a stable transgenic line bearing the SILL1 construct, which fully recapitulated the expression pattern of the original enhancer-trap line (Figure 3.1F-J). We also constructed SILL2 by replacing mCherry with a Gal4-VP16 transcription factor. Injecting the SILL2 plasmid into eggs from the *Tg[UAS:TdTomato-CAAX; HGn39D]* stable transgenic line activated mosaic expression of the red-fluorescent protein in the afferent neurons of the lateral line (Figure 3.1K-O).

3.3.2 Lateralis afferent neurons are structurally diverse and diverge in the hindbrain

Stochastic expression of SILL1 by DNA injection allowed us to obtain unprecedented resolution of individual lateralis afferent neurons (Figure 3.2A). We selected for analysis animals in which single mCherry-labeled axons were resolvable in the hindbrain. We combined this analysis with neuronal labeling with fluorescent dextrans (see below). High-resolution in vivo three-dimensional imaging and quantifications revealed that the central projection of each neuron branches into approximately 60 bulged spines or boutons (Figure 3.2A-D), which likely represent synaptic contacts with second-order targets because they enriched a well-

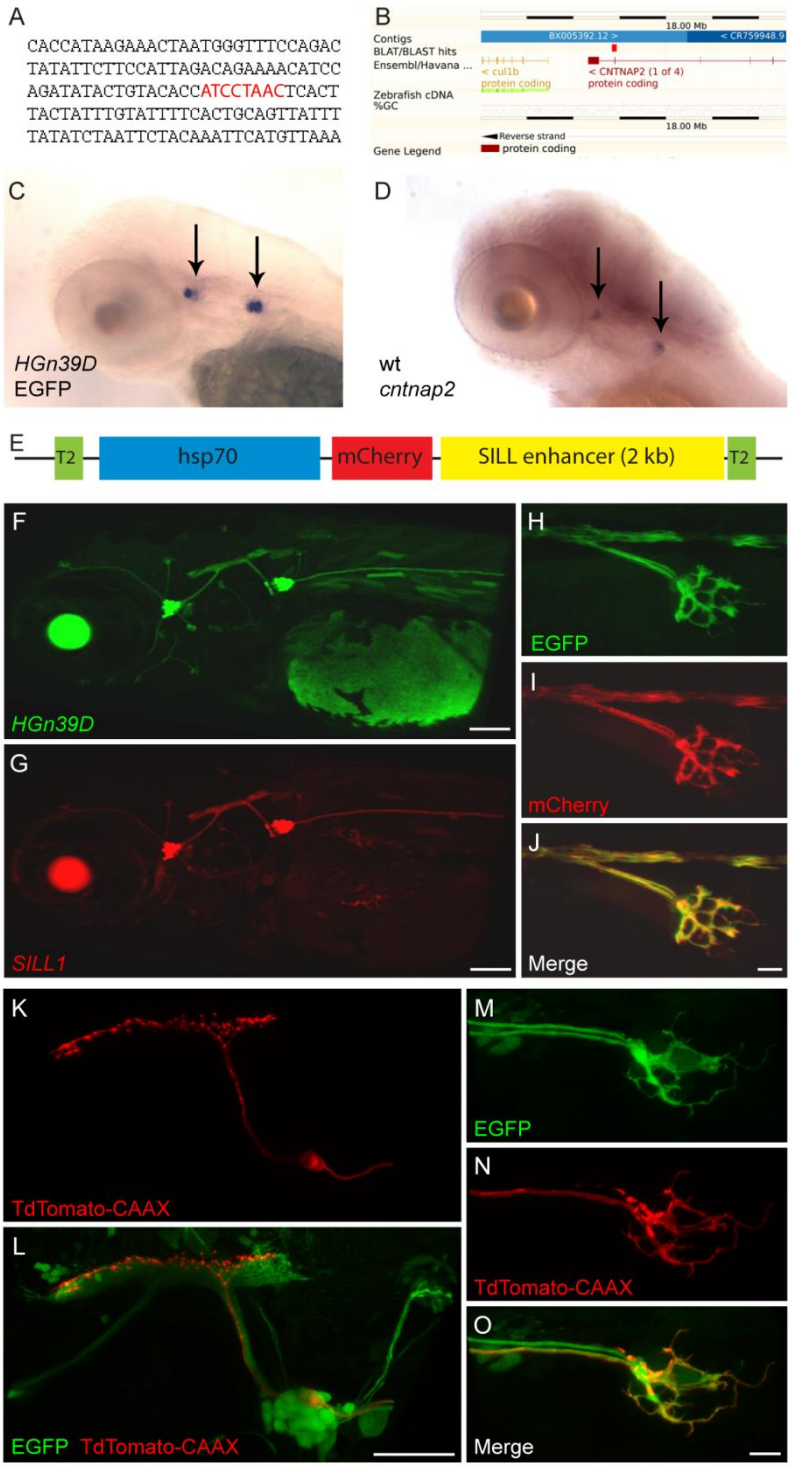

A
CACCATAAGAAACTAATGGGTTTCCAGAC
TATATTCTTCCATTAGACAGAAAACATCC
AGATATACTGTACACCATCCTAACTCACT
TACTATTTGTATTTTCACTGCAGTTATTT
TATATCTAATTCTACAAATTCATGTTAAA
B
Contigs
BLAT/BLAST hits
Ensembl/Havana ...
Zebrafish cDNA
%GC
Gene Legend
18.00 Mb
BX005392.12 >
< CR759948.9
< cul1b
protein coding
< CNTNAP2 (1 of 4)
protein coding
18.00 Mb
Reverse strand
protein coding
C
HGn39D
EGFP
D
wt
cntnap2
E
T2
hsp70
mCherry
SILL enhancer (2 kb)
T2
F
HGn39D
G
SILL1
H
EGFP
I
mCherry
J
Merge
K
TdTomato-CAAX
L
EGFP TdTomato-CAAX
M
EGFP
N
TdTomato-CAAX
O
Merge

Figure 3.1

Figure 3.1. The SILL enhancer drives gene expression in the lateralis afferent neurons. A: The *HGn39D* enhancer-trap site indicated by red nucleotides. **B:** Blast result shows the integration within *cntnap2a*. **C-D:** Whole-mount in situ hybridization of the EGFP transcripts in 3 dpf *HGn39D* transgenic fish (C), and of the *cntnap2* transcripts in a wild-type specimen (D). Arrows indicate the anterior and posterior lateralis ganglia. **E:** Schematic representation of the SILL1 construct. **F-G:** Maximal projections of lateral-views showing the expression pattern of EGFP in *HGn39D* (F) and of mCherry in *Tg[SILL1]* (G) at 3 dpf. **H-J:** Maximal projections of the peripheral axon arbor of *Tg[HGn39D; SILL1]* double transgenic fish at 3 dpf showing co-expression of EGFP (H) and mCherry (I). Merge is depicted in (J). **K-L:** Maximal projections of the posterior lateralis ganglion and central axons showing TdTomato-CAAX (K) and EGPF (L) expression in a 5 dpf *Tg[HGn39D; UAS:TdTomato-CAAX]* double transgenic fish injected with the SILL2 construct that contains a Gal4-VP16 transcriptional activator. **M-O:** *Tg[HGn39D; UAS:TdTomato-CAAX]* double transgenic fish injected with the SILL2 construct expressing EGFP (M) and TdTomato-CAAX (N). Merge is depicted in (O). Scale bars represent 100 μm in (F, G, L) and 10 μm in (J, O). In all the panels and figures, dorsal is up and anterior is left.

characterized presynaptic marker (Figure 3.2E-G) (Hua et al., 2005). In the course of this systematic anatomical study, we noticed that some central axons consistently presented a ventral-pointing indentation in their rostral ramus (Figure 3.2A). To know if this indentation identified a particular neuronal population, we labeled all the neurons from a neuromast by injecting dextrans in the vicinity of their peripheral arborization below the hair cells. Dextran uptake and retrograde transport highlights the entire neuron, including fine details of their central projections (Figure 3.2H). Injections of magenta-dextran in terminal neuromasts resulted in the labeling of up to four neurons (Figure 3.2I), whose identity as afferent was established by the localization of their soma within the posterior lateralis ganglion (Figure 3.2H). We consistently observed that only around half of the labeled axons presented an indentation. Indented axons always projected dorsally in the lateralis column of the hindbrain (Figure 3.2I-L). Neurons without the

indentation had ventrolateral projections that appeared thinner (Figure 3.2I,L). Next, we asked if the position of the indentation was conserved for all axons. For this purpose we used a triple transgenic line *Tg[SILL1; hspGFFDMC130A; UAS:EGFP]* that expressed EGFP under the control of the Gal4 in the Mauthner cell and mCherry in all the lateralis afferent neurons (Figure 3.2M). The stereotyped localization and orientation of the Mauthner cell in the hindbrain was used as three-dimensional landmark (Eaton et al., 1977; Kimmel et al., 1981). Maximal projections of confocal stacks showed that the lateral dendrite of the Mauthner cell invades the center of the lateralis column (Figure 3.2M). Medial-to-lateral progression of consecutive focal planes at higher magnification showed that the Mauthner's lateral dendrite coincides with the indentation of the dorsal-projecting axons (Figure 3.2N-Q). The indentation and projection pattern along the dorsoventral axis in the hindbrain suggest the existence of lateralis neuronal sub-classes in the zebrafish larva.

3.3.3 Neuronal sub-classification based on contacts with a central target

Lateralis afferents input monosynaptically the Mauthner cell in the zebrafish and other species (Kimmel et al., 1990; Zottoli and Van Horne, 1983). To ask if the Mauthner cell is a direct output neuron of all lateralis afferent neurons, we injected magenta-dextran in the L1 neuromast of *Tg[SILL1; hspGFFDMC130; UAS:EGFP]* triple transgenics. This experiment showed that only the dorsal-projecting axons with an indentation contact "en passant" the lateral dendrite of the Mauthner cell (Figure 3.3A-C and Supplementary Animation 3.1), whereas ventral-

projecting neurons do not, further supporting our sub-classification of neurons. In the trunk of zebrafish larva, the lateral line is formed by neuromasts that derive from at least four primordia. Two primordia give rise to the dorsal and two to the posterior neuromasts. Posterior and dorsal neuromasts are subdivided into those originating from first primordia (early-born/primary neuromasts: respectivelly L1-terminal and D1), and those that are formed by second primordia (late-born/secondary neuromasts: LII.1-LII.4 and D2-D4). Primary neuromasts contain hair cells that are plane polarized parallel to the anteroposterior body axis of the fish, whereas hair cells in secondary neuromasts are orthogonally oriented. The protracted development and structural diversity of the lateral line motivated us to ask if all the neuromasts send direct projections to the Mauthner cell. For this purpose, we systematically injected dextrans in neuromasts identified by position as early-born/primary or late-born/secondary in the posterior and dorsal branches of the lateral line. Next, we assessed their projections in the triple transgenic line *Tg[SILL1; hpsGFFDMC130; UAS:EGFP]* (Figure 3.3D-I). This experiment showed that no neuron from representative secondary neuromasts project to the Mauthner cell. However, injected primary neuromasts contained neurons that contact the lateral dendrite of the Mauthner, and neurons that do not (Figure 3.3J). Identical results were obtained by stochastic labeling of neurons by DNA injection (Figure 3.3J), suggesting that the projection differences were not due to an effect of dextran incorporation into the neurons.

There is a temporal progression of lateralis neurogenesis in the zebrafish embryo (Pujol-Martí et al., 2010; Sarrazin et al., 2010; Sato et al., 2010). Therefore, one possibility to explain neuronal heterogeneity is that

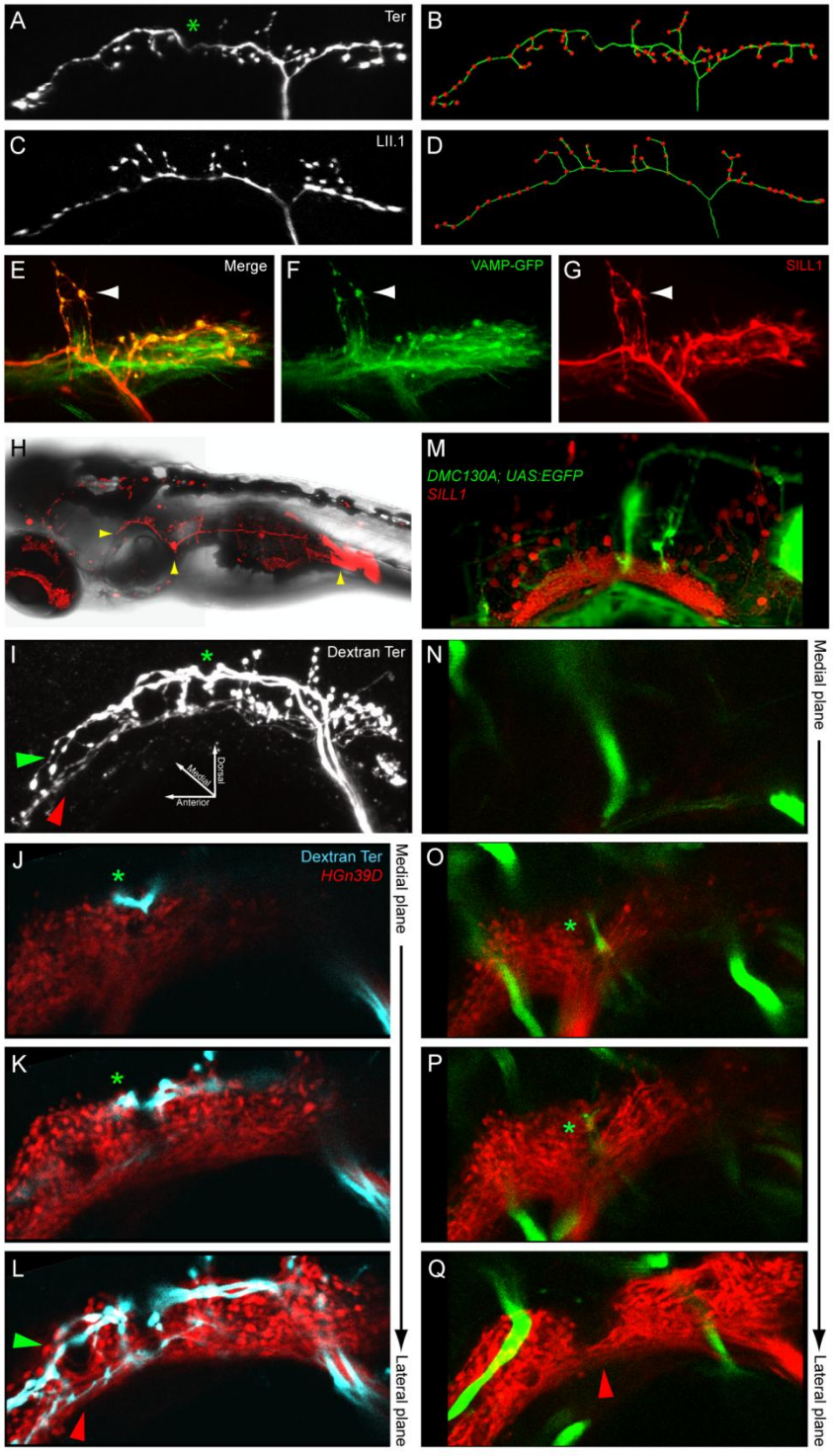
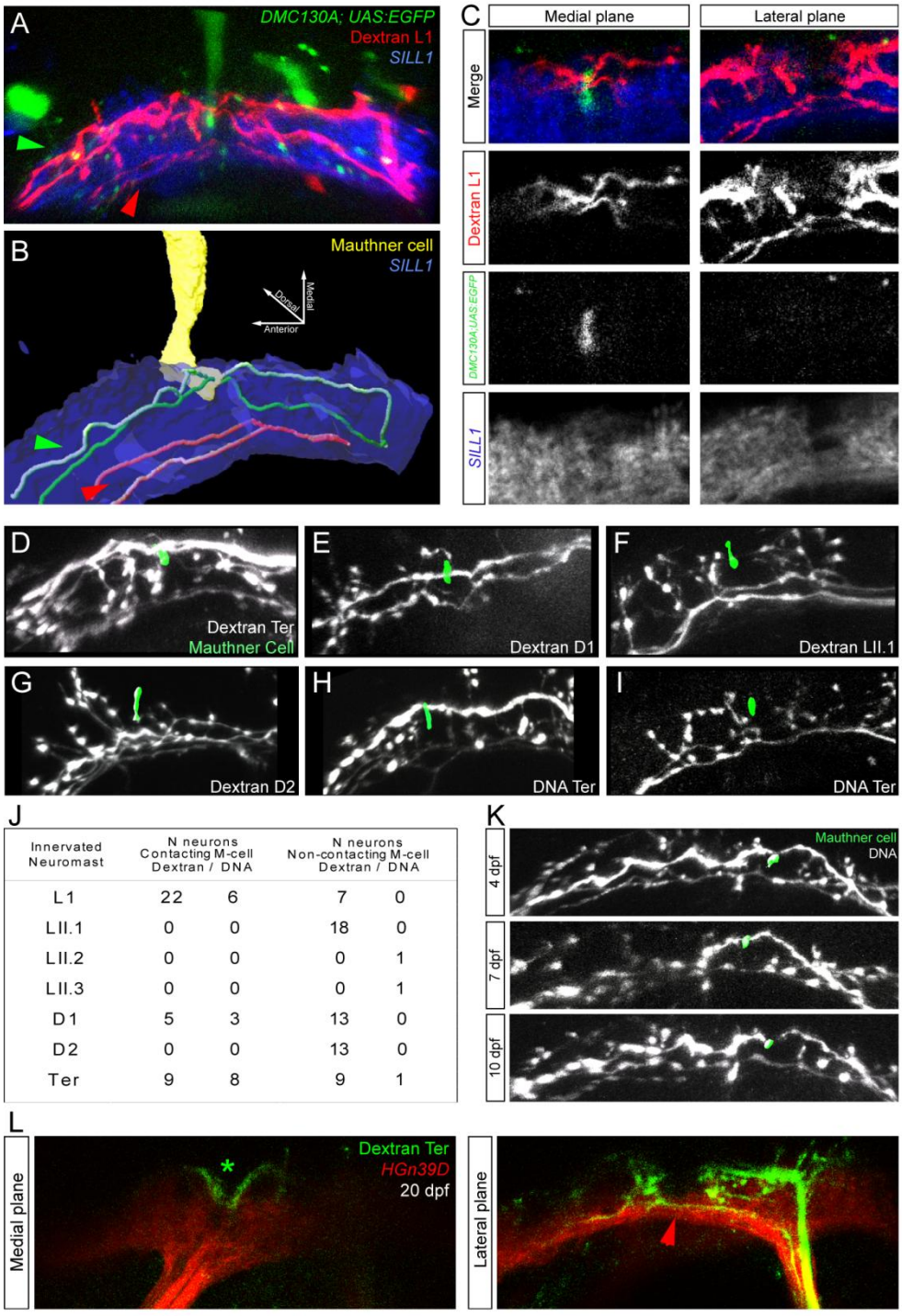

A
Ter
B
C
LII.1
D
E
Merge
F
VAMP-GFP
G
SILL1
H
M
DMC130A; UAS:EGFP
SILL1
I
Dextran Ter
Dorsal
Medial
Anterior
N
Medial plane
J
Dextran Ter
HGn39D
O
K
P
L
Q
Lateral plane

Figure 3.2

Figure 3.2. Structural diversity of lateralis afferent central projections. A-D: Central projection of an individual afferent neuron innervating a terminal neuromast (Ter) (A) or a LII.1 neuromast (C) at 6 dpf. The neurons were labeled with SILL1 DNA in (A) and by red-dextran in (C). (B) and (D) are skeletonized drawings of the central projections in, respectively, (A) and (C). The green filaments show the central axons main rami and their minor branches, the red dots represent the bulged spines. Asterisk indicates the indentation in the rostral ramus. **E-G:** Central projections of the afferent neurons co-labeled with mCherry and the presynaptic marker VAMP-GFP (by co-injection of SILL1 and SILL:VAMP-GFP constructs) at 4 dpf (Hua et al., 2005). VAMP-GFP is enriched in the bulged spines (arrowhead). **H:** A 7 dpf larva imaged with bright field and fluorescent light, showing lateralis afferent neurons innervating LII.1 labeled by red-dextran. Arrowheads indicate the central projections, neuronal soma and injection site (from left to right sequence). **I-L:** Central projections of lateralis afferent neurons from the terminal neuromasts, labeled by magenta-dextran at 6 dpf. (I) is a maximal projection. (J-L) are medial-to-lateral progression of consecutive confocal planes. The lateralis column is shown in red using *HGn39D*. The asterisk indicates the indentation. Green and red arrowheads indicate dorsal projections with indentation and ventrolateral projections without indentation, respectively. **M-Q:** Spatial relationship between the Mauthner cell and the lateralis column in a *Tg[SILL1; hspGFFDMC130A; UAS:EGFP]* triple transgenic at 6 dpf. (M) is a maximal projection. (N-Q) are medial-to-lateral progression of consecutive confocal planes. The asterisk shows the distal tip of the Mauthner cell's lateral dendrite. The red arrowhead indicates ventrolateral projections from lateralis neurons without the indentation. Dorsal is up and anterior is left.

neurons with the indentation or direct contact with the Mauthner cell are born at different times than those without it. However, over time late-born neurons may mature to resemble first-born. To test this possibility, we followed samples in which both neuronal classes from a single neuromast were labeled, and saw no differences in their central projections or contacts with the Mauthner cell over a 7-day period (Figure 3.3K). To rule out an effect of dextrans on the normal long-term behavior of the axons, we also labeled the neurons from terminal neuromasts in naïve HGn39D transgenic juveniles at 20 dpf and observed them one day later. Again, we saw the presence of both neuronal subclasses (Figure 3.3L).



Innervated Neuromast	N neurons Contacting M-cell Dextran / DNA		N neurons Non-contacting M-cell Dextran / DNA	
L1	22	6	7	0
LII.1	0	0	18	0
LII.2	0	0	0	1
LII.3	0	0	0	1
D1	5	3	13	0
D2	0	0	13	0
Ter	9	8	9	1

Figure 3.3

Figure 3.3. Neuronal sub-classification based on contacts with the Mauthner cell. **A-C:** Central projections from lateralis afferent neurons of the L1 neuromast (early born/primary neuromast) labeled by magenta-dextran at 6 dpf, in a *Tg[SILL1; hspGFFDMC130A; UAS:EGFP]* triple transgenic animal. (A) is a maximal projection, (B) is a snapshot of a three-dimensional reconstruction from the data shown in (A). Green and red arrowheads indicate, respectively, dorsal projections contacting the Mauthner cell and ventrolateral projections non-contacting the Mauthner cell. (C) A detailed view of the central projections toward the Mauthner cell are shown in a medial-to-lateral progression of confocal planes. **D-I:** Central axons from afferent neurons projecting from early born/primary neuromasts (D1, terminal) and from late born/secondary neuromasts (LII.1, D2), labeled by red-dextran (D-G) or by SILL1 DNA injection (H-I), at 6 dpf in a *Tg[hspGFFDMC130A; UAS:EGFP]* transgenic animals. All pictures are snapshots of the three-dimensional reconstruction of the central projections and the distal tip of the Mauthner cell's lateral dendrite (green). **J:** Quantification of the contacts between lateralis afferent neurons and the lateral dendrite of the Mauthner cell, sorted by labeling method and neuromast identity: early born/primary neuromasts (L1, D1, terminal) and late born/secondary neuromasts (LII.1, LII.2, LII.3, D2). **K:** Central projections from afferent neurons labeled by SILL1 DNA injection in *Tg[hspGFFDMC130A; UAS:EGFP]* transgenics, at 4, 7 and 10 dpf. Two lateralis afferent neurons are labeled; one contacts the Mauthner cell whereas the other does not. **L:** Central axons from afferent neurons projecting from terminal neuromasts and labeled by red-dextran at ~20 dpf, in the *HGn39D* transgenic line. The lateralis column is shown in red. Medial and lateral focal planes are shown. The asterisk indicates dorsal projections with an indentation. The red arrowhead indicates the ventrolateral projections without the indentation.

3.3.4 Biased axonal projection pattern of large and small neurons

Having shown neuronal sub-classification in relation to central projections and contact with the Mauthner cell, we next decided to search for additional differences between these neurons. Because dextran uptake eventually decorates the entire neuron, we looked at potential differences in the ganglion and peripheral axons. Within the ganglion we consistently found neurons with large and small somata (Figure 3.4A-B). We plotted the neurons in a two-dimensional space in which each dot represented the soma volume of individual neurons. The data was then grouped by both

the innervated neuromast and the labeling method. The distribution of neurons showed a clear distribution into two groups. We then arbitrarily set a mark of 1500 μm^3 because it is infrequently represented in the volume distribution, to classify neurons as large or small. This classification showed that large-soma neurons were always located dorsally in the ganglion, suggesting that they are older. A systematic anatomical characterization and quantification revealed that large-soma neurons projected exclusively from terminal neuromasts, whereas neurons from non-terminal neuromasts were homogeneously small (Figure 3.4C-D). We next assessed the relationship between the volume of the neuronal soma, the diameter of the axon, and contacts with the Mauthner cell in single neurons marked with the SILL1 construct. Large-soma neurons bore peripheral axons of larger diameter (Figure 3.4E-H). Additionally, the evidence from this experiment (Figure 3.4H) and that of dextran incorporation (Figure 3.3J) shows an asymmetric monosynaptic input of the lateral line to the Mauthner cell, with a disproportionate contribution from early-born neuromasts. We also found that all the large neurons made direct contact with the Mauthner cell, whereas we could further sub-classify small neurons into those that did and those that did not contact the Mauthner (Figure 3.4E-H). Because backfilling neurons with dextrans is not 100% efficient, injections in the terminal neuromasts often resulted in the exclusive labeling of large-soma neurons. In these cases, we only observed central axons contacting the Mauthner cell (N=9). By contrast, when both large- and small-soma neurons were labeled, we observe the two types of central axons, contacting and non-contacting the Mauthner cell (N=20) (Figure 3.4C). In non-terminal neuromasts, however, we only observe small neurons that display both

central-axon projection types (N=41) (Figure 3.4C). In conclusion, neuronal labeling by dextran incorporation together with single-neuron labeling by DNA injection, show that small neurons can be further subdivided into those that contact the Mauthner cell and those that do not.

3.3.5 Neuronal projections and birth date

To ask about the timing of neuromast innervation relative to neuronal size, we labeled neurons by fluorescent dextrans at different stages of zebrafish development. This experiment confirmed that large neurons arrive to the target neuromast first (Figure 3.5A-B). Lateralis neurogenesis in the zebrafish embryo occurs in two discrete waves, whose temporal sequence results in a strongly biased dorsoventral localization of neurons in the ganglion (Pujol-Martí et al., 2010; Sarrazin et al., 2010). Dorsalmost neurons belong to the first wave, suggesting that they should have bigger somata. To test this hypothesis, we developed BAIT (Birthdating combined with Anatomical analysis by Incorporation of Tracers) by which timed fluorescent-protein photoconversion dates cells, and dextran incorporation reveals their structure. BAIT showed that older neurons are larger and are mostly located in the dorsal aspect of the ganglion (Figure 3.5C-D). We have previously reported that the transgenic line *hspGFF53A* is one of the earliest markers lateralis afferent neurons, and that *HGn39D* begins to be expressed in the same neurons several hours later (Pujol-Martí et al., 2010). A re-assessment of these transgenic lines showed that the fluorescent signal from *HGn39D* persists as neurons age, whereas that of *hspGFF53A* fades with time. Neurons located in the ventral aspect of the ganglion (late-born) expressed *SILL1* (mCherry+) and *hspGFF53A* (EGFP+). However,

hspGFF53A was down-regulated in dorsally placed neurons (early-born), whereas *SILL1* expression was maintained (mCherry(+) / EGFP(-)). The combination of these transgenics is therefore complementary to BAIT to date neurons in vivo. Using the triple transgenics *Tg[SILL1; hspGFF53A; UAS:EGFP]* injected with dextrans in terminal neuromasts we analyzed the central projection of neurons of different ages by three-dimensional confocal microscopy (Figure 3.5E-G) (N=17). This experiment revealed that the oldest neurons (mCherry(+) / EGFP(-)) were larger and localized to the ganglion's dorsum. When we examined the central projections from these neurons we observed that they were always characterized by and indentation in their rostral ramus (Figure 3.5F), and projected axons dorsally in the hindbrain (Figure 3.5G). Ventrolateral projections from younger neurons (mCherry(+) / EGFP(+)) did not have an indentation. Form these results we confirm that axonal projections correlate with the sequence of neuronal birth and differentiation.

3.3.6 The lateralis neural map develops in the absence of sensory input

We wanted to know if evoked sensory activity played any role in neuronal sub-classification or instructed their projection pattern in the hindbrain. For this purpose, we combined the injection of a magenta-dextran in terminal neuromasts whose large neurons always contact the Mauthner cell, and red dextran in a secondary neuromast that are devoid of neurons projecting to the Mauthner. These injections were made in wild type animals and in mutants lacking hair-cell mechanoreception (homozygous mutant for *tmie*), or lacking hair cells in the neuromasts (homozygous mutant for *atoh1a*) (Figure 3.6A-C) (Faucherre et al.,

2010). We observed no differences in central projections or soma size between all three conditions, demonstrating that evoked sensory input does not instruct neuronal sub-classification or the pattern of central projections. Another mechanism that could control map topology is competition, whereby axons from early-born neurons repel those from neurons born later from contacting the Mauthner cell. To test this hypothesis it is useful to observe the axonal projections from late-born neurons in the absence of early-born. If competition between neuronal sub-classes does not play a role in axonal projections, late-born neurons from experimental animals should be indistinguishable from those under normal conditions. If, alternatively, interactions between axons are responsible for the differences, the projections of late-born neurons should be altered when early-born are absent. We tested this possibility by eliminating early-born neurons in *HGn39D* transgenics by laser ablation at 28-30 hpf, a stage in development between the differentiation of both neuronal sub-classes. Subsequently, magenta-dextran was injected in terminal neuromasts and central axonal projections were analyzed by three-dimensional confocal microscopy. In all cases analyzed the ablation of early-born neurons did not modify the axonal projections from late-born neurons (Figure 3.6D-E). Collectively, these results demonstrate that neither axonal competition, nor evoked sensory activity play a significant role during the assembly of the lateralis neural map.

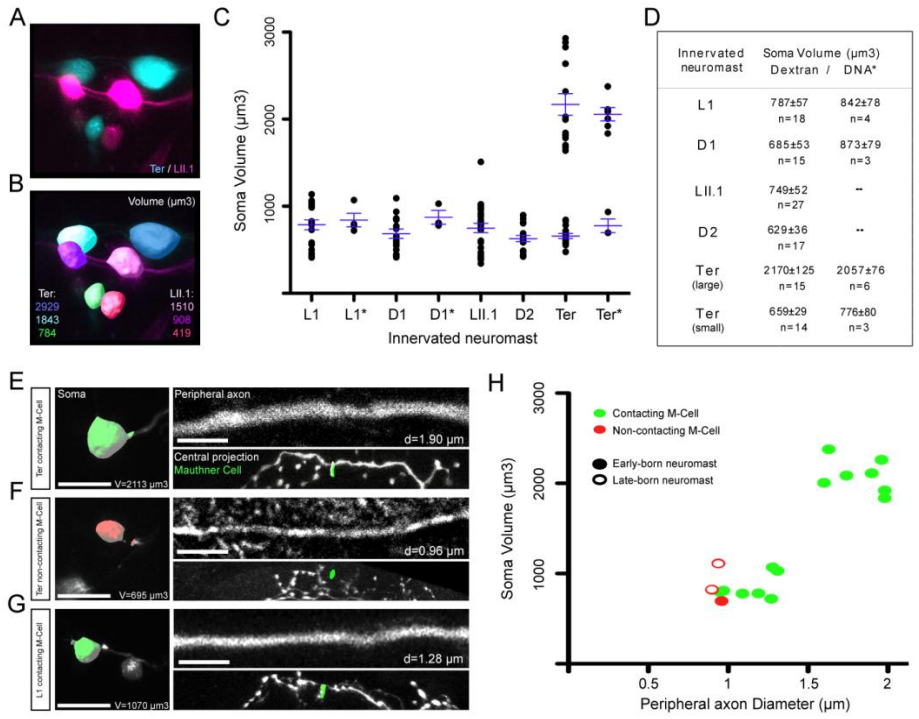


Figure 3.4. Neuronal sub-classification based on soma volume and peripheral axon diameter. A: Posterior lateralis ganglion at 7 dpf with somata from afferent neurons projecting from the terminal neuromasts labeled with red-dextran (false-colored blue) and neurons projecting from the LII.1 neuromast labeled with magenta-dextran (magenta). **B:** Surface reconstruction of each soma that was used for volumetric analysis. **C:** Quantification of soma volume (µm3) at 7 dpf. Each dot represents the soma volume of an individual neuron. Data is grouped in the X-axis by both the innervated neuromast and the labeling method (no asterisk: dextran, asterisk: DNA). Averages and error bars (SEM) are shown in blue. Neurons projecting from the terminal neuromasts were split into two categories: large-soma (>1500 µm3) and small-soma (<1500 µm3) neurons. Averages and SEM were calculated separately for each category. **D:** Soma volume averages (µm3, with SEM) at 7 dpf, grouped by both the innervated neuromast and the labeling method. **E-G:** Three examples of lateralis afferent neurons at 7dpf, labeled by SILL1 DNA injection. Surface reconstructions for each soma are shown. Green and red indicates contacting and non-contacting the Mauthner cell, respectively. Peripheral axon images are focal planes. Snapshots of the three-dimensional reconstruction of the central projection and the distal tip of the Mauthner cell's lateral dendrite (green) are also shown. **H:** Correlation between soma volume (µm3) and peripheral axon diameter (µm) as an average of ten equidistant points along the axon at 7 dpf. Each dot represents the soma volume and peripheral axon diameter of an individual neuron, labeled by SILL1 DNA injection. In all the panels dorsal is up and anterior is left. Scale bars represent 5µm for panels showing peripheral axons, and 10µm for panels showing neuronal somata.

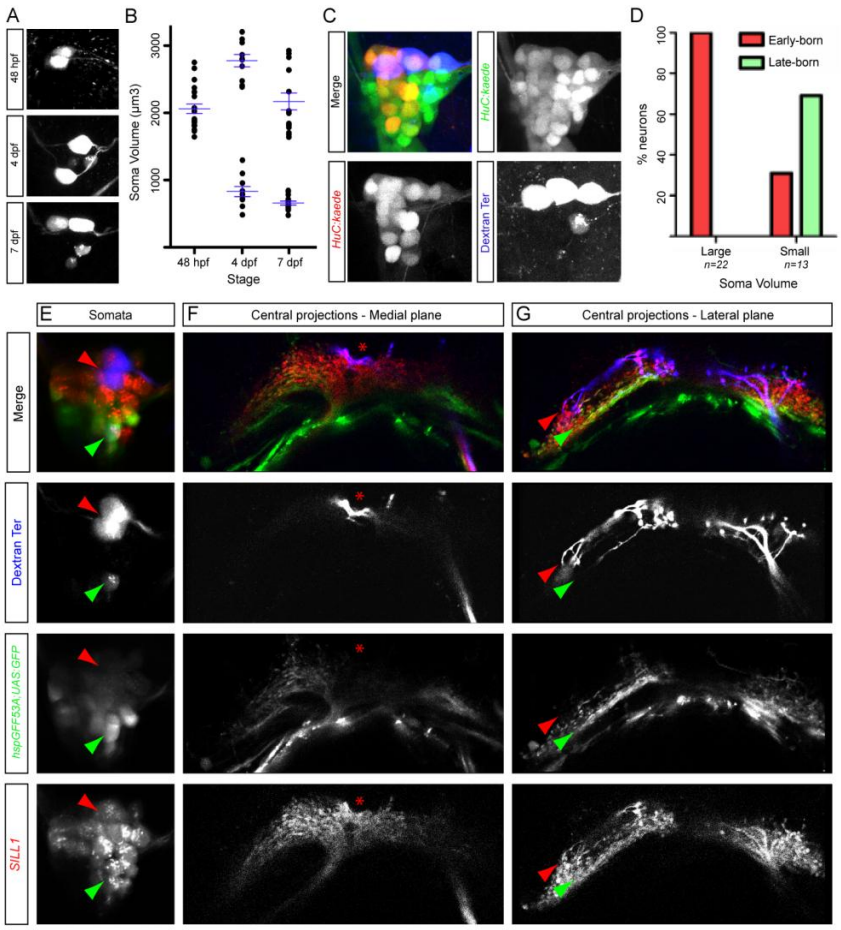


Figure 3.5. Simultaneous analysis of innervation/birthdate and soma volume of lateralis afferent neurons projecting from the terminal neuromasts. A: Posterior lateralis ganglion with somata from afferent neurons projecting from the terminal neuromasts labeled at different stages (48 hpf, 4 dpf and 7 dpf). **B:** Quantification of soma volume (µm3) from afferent neurons projecting from the terminal neuromasts labeled at different stages. Each dot represents the soma volume of an individual neuron. The data were grouped in the X-axis by the stage of labeling. Averages and error bars (SEM), shown in blue, were calculated separately for the large- and small- soma neurons categories. **C:** Maximal projection of a posterior lateralis ganglion at 5 dpf resulting from a BAIT experiment. Kaede photoconversion was performed at ~28 hpf. Early-born neurons (converted at ~28 hpf) are green and red (yellow), whereas late-born neurons appear only green. Three large and one small soma from afferent neurons projecting from the terminal neuromasts were labeled by magenta-dextran uptake at 4 dpf. **D:** Percentage of large and small somata from afferent neurons projecting from the terminal neuromasts of both types: early- and late-born. **E-G:** Confocal planes of somata (E) and central projections (F-G) from lateralis afferent neurons projecting from the terminal neuromasts labeled by magenta-dextran at 6 dpf, in a *Tg[SILL1; hspGFF53A; UAS:EGFP]* triple transgenic. The red and green arrowheads indicate two large and one small soma, respectively. Central projections pictures: the asterisk indicates the indentation. Red and green arrowheads indicate dorsal and ventrolateral projections, respectively.

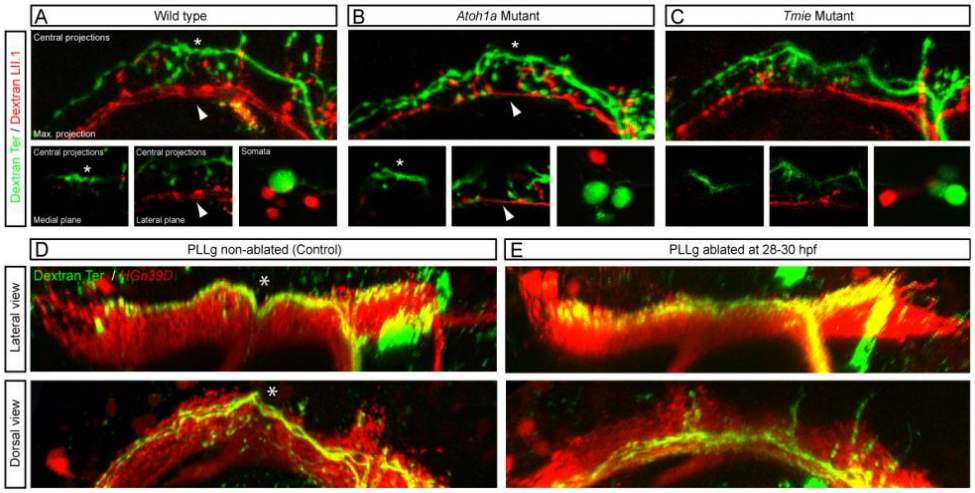


Figure 3.6. Analysis of the lateralis neural map in the absence of sensory input and in the absence of early-born neurons. A-C: Central projections and somata from lateralis afferent neurons projecting from the terminal neuromasts (in green, magenta-dextran) and the LII.1 neuromast (in red, red-dextran), in wild type (A) and homozygous mutants for *atoh1a* (B) and *tmie* (C). Neurons were labeled at 6 dpf. Detailed views of both the indentation (asterisk) and the ventrolateral projections (arrowhead) are shown in medial and lateral focal planes, respectively. Top panel in (A-C) are maximal projections. **D-E:** Central projections from afferent neurons projecting from the terminal neuromasts that were labeled by magenta-dextran in *HGn39D* transgenics, whose posterior lateralis ganglion (PLLg) was laser ablated or non-ablated (control) at 28-30 hpf. Neurons were labeled at 6 dpf by dextran uptake. Both lateral and dorsal views of the three-dimensional reconstruction of the central projections are shown. The lateralis column is shown in red. The asterisk indicates the indentation in the dorsal projections. The arrowhead indicates lateral projections without the indentation.

3.4 Discussion

The piscine lateralis afferent neurons assemble a somatotopic neural map, in which axons are reproducibly positioned along a dorsoventral axis in the hindbrain (Alexandre and Ghysen, 1999; Claas and Münz, 1981; Ghysen and Dambly-Chaudière, 2007). Although somatotopy probably forms a neuroanatomical code of the external hydrodynamic field, it is not well understood how animals use it to command behaviors. In this study we employed neuronal labeling and image registration to reveal two classes of lateralis afferent neurons defined by birth order, soma size and location, central projections and contacts with an identified output neuron, to define a dimorphic sensorineural map. Next, we discuss our results and make predictions on how lateralis map topology may underlie appropriate behavioral responses according to the biological relevance of mechanosensory input.

Lateralis afferent neurons are extremely interesting because they can shed light on the mechanisms that initiate different behavioral programs by the same sensory modality. Many studies have firmly demonstrated a role of the lateral line in navigation, schooling and prey detection (Bleckmann, 2008; Coombs et al., 1998; Coombs and Patton, 2009; Dijkgraaf, 1963; Montgomery et al., 1997; Montgomery et al., 2000) . It has also been proposed that the lateral line can send powerful inputs to the Mauthner cell to trigger the C-start escape response (McHenry et al., 2009). The optical transparency, external and fast development of the zebrafish, coupled with the accessibility and anatomical simplicity of its lateral line provides a powerful model to study the neuroanatomical bases of these contrasting behaviors. Because an essential part of investigations toward

this aim is the careful characterization of neuronal connectivity, we generated two transgenic zebrafish lines for targeted gene expression. The *hspGFFDMC130A* line contains the Gal4 transcriptional activator stably integrated in the genome (Nagayoshi et al., 2008). We demonstrate that *hspGFFDMC130A* can express fluorescent markers in the Mauthner cell and few other tissues. We also generated the *Tg[SILL1]* transgenics that expresses the mCherry red-fluorescent protein in all the lateralis afferent neurons. Single-neuron labeling with the SILL1 construct revealed unexpected neuronal diversification and neural-map dimorphism. In a divergent neural sub-map, each neuron forms around 60 boutons that concentrate a well-characterized presynaptic marker. If we assume that each bouton represents a synaptic contact with one output neuron, the posterior lateral line has no more than 2,400 second-order targets in the zebrafish larva (Fame et al., 2006; Sassa et al., 2007). In a convergent sub-map, many lateralis neurons directly contact the lateral dendrite of the Mauthner cell.

What determines map dimorphism and target selectivity? One possibility is that sensory activity plays an instructive role (Luo and Flanagan, 2007). However, our results from the analysis of two types of mutants -a strong loss-of-function in Tmie that blocks hair-cell mechanoreception, and a loss-of-function in Atoh1a that prevents the development of hair cells in neuromasts- indicate that sensory activity is not a major force in sculpting the lateralis neural map. Axonal competition has been shown to influence neural mapping in several systems. However, our selective ablation of one neuronal subpopulation did not alter the projection of the remaining neurons. Therefore, inter-class axonal competition does not appear to instruct map topology.

We combined our transgenic lines with dextran injections for anatomical tracing to develop BAIT. One caveat of BAIT is that it does not reveal the actual birth dates of cells, but their relative age. Therefore, we made the assumption that neurons differentiate at similar rates once they have fate-committed. In doing so, we demonstrate a correlation between the sequence of afferent neurogenesis and central projections, and that only early-born neurons converge on the Mauthner cell. Although we cannot currently rule out that molecular gradients in the target area refine map topology much the same way as they do in other sensory systems, our findings support a strong contribution of neurogenic timing in lateralis neural map dimorphism and projection pattern (Clandinin and Feldheim, 2009; Fariñas et al., 2001; Feldheim and O'Leary, 2010; Luo and Flanagan, 2007; Schuster et al., 2010). Such strategy is not likely to be unique to the lateral line, however. For example, mechanosensory neurons of the wing or photoreceptor neuron of the eye in Drosophila also produce tiered central projections based on their time of development (Morey et al., 2008; Palka et al., 1986; Petrovic and Hummel, 2008). Earlier studies in Xenopus tested the involvement of timing in generating the topographic organization of the neuronal projection from the retina to the tectum, and concluded that it likely plays a permissive rather than an instructive role in axonal organization (Holt, 1984). It would be interesting to test permissive versus instructive roles of timing in the lateral line.

Neuromasts are directionally sensitive by virtue of the planar polarization of their constituent hair cells. Posterior neuromasts have orthogonal planar orientations. Parallel neuromasts develop earlier from first primordia and are called “primary neuromasts”. Perpendicular

neuromasts develop subsequently from independent primordia and are called “secondary” (Ghysen and Dambly-Chaudière, 2007; López-Schier et al., 2004; Sarrazin et al., 2010). Our study demonstrates that waves of afferent neurogenesis accompany the protracted development of the neuromasts. Primary neuromasts project early-born neurons that diverge along the hindbrain but some of which also converge on the Mauthner cell. Secondary neuromasts, by contrast, only project late-born neurons that do not converge on the Mauthner. A potentially equivalent relationship between developmental timing and neuronal projection patterns has been reported for the segregation of afferent projections for the otic, lateralis and ampullary organs in the axolotl (Fritzsch et al., 2005). In this case, however, the relationship is between organs rather than within each organ.

Afferent input to the Mauthner from primary neuromasts may be an elegant strategy to couple development with behavior because the earliest born neurons and neuromasts could allow the animal to use the lateral line for escape responses before it is able to swim. We show that in the zebrafish larva, terminal neuromasts are innervated by large-diameter afferent axons. The conduction velocity of myelinated axons in vertebrates increases linearly with their diameter (Goldman and Albus, 1968; Holmes, 1941; Hursh, 1939). Therefore, early-born neurons projecting from terminal neuromasts are likely to be fast conducing, making them well suited to produce the first and fastest lateral-line stimulus for the C-start response. This also suggests that terminal neuromasts have a disproportionate relevance in the escape behavior mediated by the Mauthner cell. Interestingly, the trout also shows regional segregation of lateralis neurons relative to axon diameter and

conduction velocity, which can compensate for the increased axonal length in large animals (Schellart and Kroese, 2002).

Are terminal neuromasts enough to trigger the C-start response? Unlike neurons from other parts of the lateral line, each large neuron innervates up to three terminal neuromasts, which may increase their depolarization probability. This presents clear survival advantages because terminal neuromasts may suffice to trigger a C-start reaction by sending strong depolarizing inputs to the Mauthner cell with very short latencies. However, in general fish should not startle by non-threatening stimuli, which predicts that early-born neurons should have lower sensitivity than late-born. Behaviors such as navigation, rheotaxis and schooling necessitate continuous input and probably have lower activating thresholds that the C-start. Afferent neurons with different excitability and conduction velocities have been reported for the posterior lateral line of the goldfish (Fukuda, 1974). Also, in cichlids, lateralis afferent neurons with higher rate of spontaneous discharge are more sensitive (Münz, 1985). Interestingly, the rate of spontaneous activity of individual afferent neurons in the zebrafish larva varies along the lateral line (Liao, 2010). If these observations are extrapolated and combined with our findings, one emergent possibility is that the C-start will only be triggered by the coincident input on the Mauthner cell from “high sensitivity/low conduction velocity” and “lower sensitivity/high conduction velocity” neuronal classes. This model will safeguard the animal from startling upon stimuli that would depolarize one neuronal sub-class but not the other, and is reminiscent of the escape strategy of crayfish, in which a mechanosensory stimulus activates parallel neuronal pathways with different reaction times that trigger the startle when

arriving coincidently to an output command neuron (Mellon and Christison-Lagay, 2008; Reichert and Wine, 1982). However, more complex processing of hydrodynamic stimuli is possible, with multiple neuromasts contributing to the probability of depolarizing excitatory signals from the lateral line to the Mauthner cell (Korn et al., 1974).

Although spatiotemporal resolution may involve secondary neuromasts, they may not contribute to the C-start response because of their discrete location and their insensitivity to the direction of propagation of most of the water flow generated by submerged predators. This may explain why their afferent neurons do not form monosynaptic contacts with the Mauthner cell. This begs the question of whether somatotopy has any functional relevance to the C-start reaction. Currently, we favor the hypothesis that lateralis somatotopy does not play a major role in the escape behavior of the zebrafish larva. Thus, the lateral line would sacrifice spatial accuracy for sensitivity and response speed. For navigation, however, somatotopy is likely to be essential. Thus, the lateral line may assemble a convergent sub-map for speed and a divergent sub-map for accuracy. The combined activity of neurons can code for the entire range of stimuli (spectrum) that a sensory organ can acquire. Variation in the sensitivity of individual sensory receptors within the organ can subtract aspects of a complex stimulus to direct behavior. This subdivision of work is called range fractionation (Cohen, 1963). However, variations in sensory transducers can also impact information processing. Therefore, the next challenge is to determine if sensorineural map dimorphism in the lateral line form the bases of range fractionation by this sensory system (Braun et al., 2002; Voigt et al., 2000).

3.5 Materials and methods

3.5.1 Zebrafish strains and husbandry

Zebrafish were maintained under standardized conditions and experiments were conducted in embryos of undetermined sex in accordance with protocols approved by the PRBB's Ethical Committee of Animal Experimentation. *Tg[HuC:Kaede]*, *HGn39D* and *hspGFF53A* transgenic lines have been published previously (Faucherre et al., 2009; Pujol-Martí et al., 2010; Sato et al., 2006). The *hspGFFDMC130A* was generated by random integration of an enhancer-trap construct (Asakawa et al., 2008). The *atoh1a_fh282* mutant strain carries a missense mutation, aa126 arginine to tryptophan, and was obtained from C. Moens. The *tmie_ru01* mutant fish were described previously (Faucherre et al., 2010; Gleason et al., 2009).

3.5.2 Selection of mutants

Wild type animals and homozygous mutants for *tmie* and *atoh1a* were sorted based on DiASP incorporation into hair cells of the lateral line. Homozygous mutants for *tmie* were genotyped by amplification using the following primers and sequencing:

For: 5'-CCAGCAGCTCTCGTAACCTC-3'

Rev: 5'-CCGCCATCACCAGTCTATTT-3'

3.5.3 Plasmid DNA constructs and injections

For cloning the SILL enhancer, the 2 kb upstream of the HGn39D insertion was amplified from genomic DNA and cloned in the Tol2kit 219 plasmid using the following primers:

EnUp-attb4-For:

5'-GGGGACAACTTTGTATAGAAAAGTTGTAAAGAAATGTCAAGTGTTT-3'

EnUp -attB1-Rev:

5'-GGGGACTGCTTTTTTGTACAAACTTGGTTGGGATGGTGTACAGTAT-3'

The 2 kb downstream of the HGn39D insertion was amplified and cloned in the Tol2kit 220 plasmid using the following primers:

EnDo-attB2-For:

5'-GGGGACAGCTTTCTTGTACAAAGTGGCCATCCCAACTCACTCACTATT-3'

EnDO-attB3-Rev:

5'-GGGGACAACTTTGTATAATAAAGTTGCCTGACATTTTCCGGAACAGG-3'

The hsp70:mCherry-SILL (SILL1), hsp70:Gal4VP16-SILL (SILL2) and hsp70:MCS-SILL constructs were obtained using the ''Tol2 kit'' (Kwan et al., 2007). Entry vectors were generated as described in the Invitrogen Multisite Gateway manual. PCR were performed using primers to add att sites onto the end of DNA fragments, using Platinum Pfx (Invitrogen). The pEntry vectors containing the hsp70 promoter, the mCherry, the Gal4VP16 and the MCS (multiple-cloning site) are from the ''Tol2 kit''. To generate the SILL:VAMP-GFP construct, VAMP-GFP was amplified

using the ‘UAS:VAMP-GFP;UAS:Kir2.1’ plasmid as a template (Hua et al., 2005), with the following primers:

For: 5’-AGACGATATCATGTCTGCCCCAGATGCT-3’

Rev: 5’-TATGGATATCTTACTTGTACAGCTCGTC-3’

The PCR product was digested by EcoRV and cloned into the hsp70:MCS-SILL construct.

3.5.4 Generation of transgenic zebrafish

To generate the *Tg[SILL1]* stable transgenic line, 20 pg of the Tol2-expression clone and 20 pg of the transposase synthetic RNA were simultaneously injected into one-cell stage wild type eggs. The resulting embryos were raised to adulthood and incrossed for visually screening of germline transmission of the transgene. For mosaic expression in neurons, 25–30 pg of DNA was injected into embryos at the one- or two-cell stage.

3.5.5 Whole-mount in situ hybridization

We generated labeled RNA probes by in vitro transcription using the DIG/Fluor RNA labeling Mix (Roche). Embryos were fixed in 4% paraformaldehyde in PBS overnight at 4ºC and whole-mount in situ hybridizations were carried using standard protocols.

To characterize *cntnap2* expression, the following antisense probe was used: Ensemble Zv9 ENSDART00000081960, nucleotides 3049-3357.

3.5.6 Neuronal labeling, birthdating and imaging

Neuronal labeling by fluorescent dextrans was performed as previously described (Pujol-Martí et al., 2010). We used a red-fluorescent dextran (tetramethylrhodamine, 3000 MW, anionic, fixable, Invitrogen ref.D3308) and a magenta-fluorescent dextran (Alexa Fluor® 647, 10000 MW, anionic, fixable, Invitrogen ref.D22914). Imaging was done one day after dextran injection. In all the samples, we imaged the dextran injection site to confirm that only neurons innervating an individual neuromast were labeled. We analyzed samples in which the neuronal labeling was specific to the selected neuromast, with the exception of the dextran injections into the D2 neuromast, in which neurons innervating the more posterior and dorsal neuromasts were often also labeled.

For BAIT (Birthdating combined with Anatomical analysis by Incorporation of Tracers) experiment, *Tg[HuC:Kaede]* eggs were kept in the dark. At 28 hpf stage, embryos were exposed to 405 nm light for 2 min and subsequently kept in the dark for 3 days. Neurons innervating terminal neuromasts were labeled by magenta-fluorescent dextran and the posterior lateralis ganglion was imaged one day later.

For imaging, live samples were anæsthetized and mounted onto a glass-bottom small Petri dish (MatTek, Ashland, MA) and covered with 1% low-melting-point agarose with diluted anæsthesic. Images were acquired with a Leica TCS SP5 inverted confocal laser scanning microscope with a 20x Air objective, or a 40x Oil immersion objective. Z-stacks of central and peripheral axons consisted of 1 μm-spaced images. Z-stacks of neuronal somata consisted of 2.5 μm-spaced images. Three-dimensional

(3D) reconstructions and cropping of z-stacks were done with Imaris software (Bitplane).

3.5.7 Quantification of soma volume and peripheral axon diameter

To measure soma volume, a surface reconstruction of each soma imaged was done using Imaris, with a surface area detail level of 0.5 μm and a thresholding based on absolute intensity. Threshold was manually adjusted to optimally match the surface reconstruction with the fluorescent signal. Imaris automatically quantifies the volume of the surface reconstruction. To measure peripheral axon diameter, we imaged a ~100 μm segment of the peripheral axon just anterior to the peripheral arborization at the level of the neuromast using a Leica TCS SP5 inverted confocal micoscope with a 20x Air objective, with Zoom: 8. Diameters were measured at ten equidistant locations of the imaged axonal segment and the average was calculated.

3.5.8 Laser-mediated cell ablation

For neuronal ablation we used a Micropoint laser system (Photonic Instruments Inc.) mounted on an Olympus IX81 inverted microscope equipped with a 40x Air objective. *HGn39D* embryos at 28-30 hpf were anesthetized, mounted on a glass-bottom dish (MatTek, Ashland, MA), and covered with methylcellulose. A train of laser pulses was repeatedly applied to the posterior lateralis ganglion until all EGFP fluorescence disappeared. Embryos were allowed to recover for 2 hs and then assessed for the presence of EGFP in the region of the posterior ganglion. Total

ablation occurred in samples with no green-fluorescent signal in that region. One day later, we chose samples with new neurons forming a ganglion. We next labeled neurons projecting from the terminal neuromasts at 5-6 dpf by injecting fluorescent dextran at the neuromasts. We did the same in non-ablated controls.

3.6 Supporting information

Supplementary animation 3.1: Interactive three-dimensional reconstruction from the data shown in Figure 3.3A. Central axons from lateralis afferent neurons innervating L1 neuromast (early-born/primary neuromast) are labeled with magenta-dextran at 6 dpf, in a *Tg[SILL1; hspGFFDMC130A; UAS:EGFP]* triple transgenic animal. Dorsal projections contacting the Mauthner cell (yellow) are in green whereas ventrolateral projections non-contacting the Mauthner are in red. The lateralis column is shown in blue. The three-dimensional reconstruction can be rotated by pressing the mouse left button on the picture and moving the mouse; as well as it can be scaled by clicking on the "+" and "–" icons. Before rotation, dorsal is towards top, anterior is towards left, lateral is towards the observer.

Chapter 4

ADDITIONAL RESULTS

4.1 Lateralis afferents contacting the Mauthner cell form a somatotopic map

A previous study in the zebrafish larva showed that lateralis afferents assemble a somatotopic map where the position of each central axon represents the position of the innervated neuromast (Alexandre and Ghysen, 1999). In this study, the authors labeled neurons with fluorescent dextrans. I noticed that neuronal backfilling with dextrans is not 100% efficient; injections in terminal neuromasts often resulted in the exclusive labeling of large-soma neurons with central axons contacting the Mauthner cell. Moreover, when analyzing dextran-labeled neurons projecting from either L1 or terminal neuromasts I observed that the ones that contact the Mauthner cell are usually more strongly labeled than those that do not. The less frequent and weaker labeling of neurons that do not contact the Mauthner cell prompted me to think that the somatotopic map was originally described by analyzing only lateralis central axons from neurons that contact the Mauthner cell. By analyzing *Tg[hspGFFDMC130A; UAS:EGFP]* double transgenic animals injected with red-dextran in the terminal neuromasts and magenta-dextran in the L1 neuromast, I confirmed that central axons contacting the Mauthner cell are topographically ordered (N=5) (Figure 4.1).

4.2 The Mauthner cell receives input from hair-cells of opposing polarities

Each neuromast is innervated by at least two lateralis afferent neurons; each of them forming synapses with hair cells of identical polarity (Faucherre et al., 2009; Nagiel et al., 2008). I sought to determine whether lateralis afferents carrying inputs from hair-cells of opposing polarities converge on the Mauthner cell. For this purpose, I assessed the relationship between the presence of an indentation in the central axon, indicative of a contact with the Mauthner, and the polarity of the hair-cells innervated by single neurons marked with the SILL1 DNA construct. To visualize the hair cells I used *brn3c:memGFP* transgenic animals. Since hair cells are born in pairs of opposing polarity along a single axis creating a line of mirror symmetry, all the cells located anterior to this line are posteriorly polarized, whereas those located posterior to the line of symmetry are anteriorly polarized. As a consequence, hair-cell polarity can be predicted from the cell's position with respect to the line of mirror symmetry (López-Schier and Hudspeth, 2006). I observed the indentation in the central axons of neurons innervating posteriorly polarized hair cells (N=9) as well as in those of neurons innervating anteriorly polarized hair cells (N=6) in the terminal neuromasts (Figure 4.2). I conclude that the Mauthner cell receives sensory inputs from hair-cells with an anterior polarity as well as from hair-cells with a posterior polarity.

4.3 Peripheral arborization and neuronal sub-classes

I showed that primary neuromasts are innervated by lateralis afferents of the two sub-classes, those that contact the Mauthner cell and those that do not. I further confirmed that the former are born earlier than the latter. This was possible thanks to the combination of *Tg[SILL1]* and *hspGFF53A* transgenic lines that reveals neuronal relative age. This combination also allowed me to ask whether the two neuronal sub-classes differ at the level of the peripheral arborization of primary neuromasts. I examined terminal neuromasts and observed that the peripheral axons of early- and late-born neurons largely overlap occupying the entire neuromast (N=3) (Figure 4.3). The presence of a bulged neurite in the peripheral arbor predicts a synaptic contact between a neuron and a hair cell (Faucherre et al., 2010; Nagiel et al., 2008). Importantly, bulged neurites from early- and late-born neurons are often adjacent (N=3) (Figure 4.3). Altogether these observations strongly suggest that, within a given neuromast, neurons of the second sub-class carry sensory information from the same hair cells as neurons of the first sub-class do. This implies that late-born lateralis afferents non-contacting the Mauthner cell convey information from hair-cells of opposing polarities. Furthermore, there is evidence that strongly suggests that these neurons convey the inputs from the two populations of hair cells in separate channels. The experiments that revealed that each lateralis afferent innervates hair-cells of identical polarity were carried out by using a DNA construct encoding a fluorescent marker under the control of the HuC promoter (Faucherre et al., 2009; Nagiel et al., 2008). Due to the fact that the HuC promoter is a pan-neuronal promoter (Park et al., 2000)

and to the large amount of neurons analyzed in those experiments, it is very likely that the two neuronal sub-classes were examined to conclude that each lateralis afferent always selects hair-cells of identical polarity.

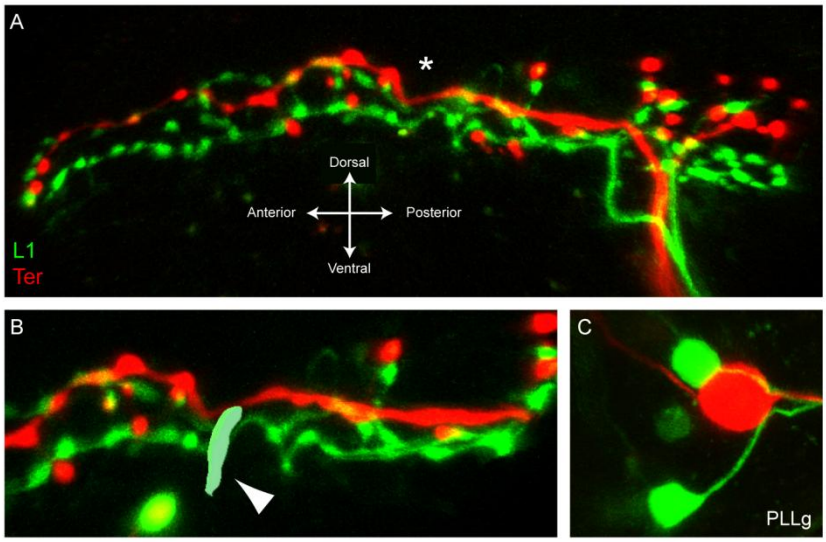


Figure 4.1

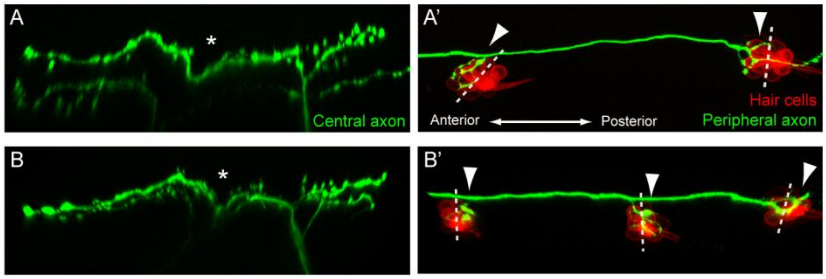


Figure 4.2

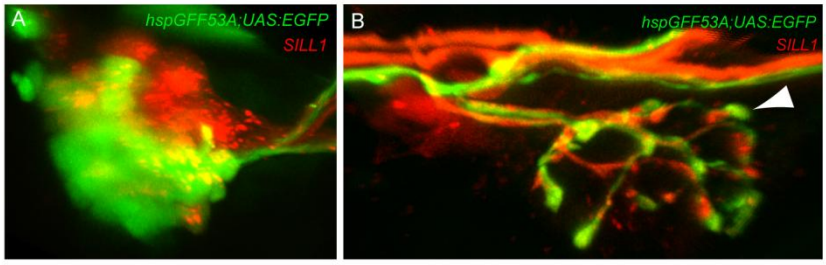


Figure 4.3

Figure 4.1. Lateralis afferents contacting the Mauthner cell form a somatotopic map. A-B: Central axons from lateralis afferent neurons projecting from the terminal neuromasts (in red, red-dextran) and the L1 neuromast (in green, magenta-dextran) in a *Tg[hspGFFDMC130A; UAS:EGFP]* double transgenic at 7 dpf. The red central axon (terminal neuromasts) locates dorsal to the green central axons (L1 neuromast) in the majority of its antero-posterior path. EGFP fluorescence is not shown in (A). (B) is a detail of the indentation (asterisk) with the surface reconstruction of the distal tip of the Mauthner cell's lateral dendrite (arrowhead), obtained from Mauthner cell's EGFP signal. All the labeled central axons contact the Mauthner. **C:** Posterior lateralis ganglion (PLLg) with labeled somata of the neurons shown in (A-B). All images are snapshots of three-dimensional reconstructions. The data used to create this figure was kindly provided by Andrea Zecca.

Figure 4.2. Polarity of the hair cells innervated by lateralis afferents with indentation projecting from the terminal neuromasts. A-B: Central and peripheral axons from individual afferent neurons (in green, labeled by SILL1 DNA injection) innervating posteriorly (A) or anteriorly (B) polarized hair cells (in red, *brn3c:memGFP* transgenic) of the terminal neuromasts at 7 dpf. The asterisk indicates the central axon's indentation, which predicts a contact with the Mauthner cell. Dashed lines indicate the line of mirror symmetry. Hair cells anterior to this line are posteriorly polarized whereas hair cells posterior to this line are anteriorly polarized. Arrowheads indicate the hair cells contacted by the lateralis afferent. All images are snapshots of three-dimensional reconstructions. In all the panels, dorsal is up and anterior is left.

Figure 4.3. Simultaneous analysis of birthdate and peripheral axon arbor of lateralis afferents. A-B: Maximal projections of a posterior lateralis ganglion (A) and the peripheral axon arbor of one of the terminal neuromasts (B) at 7 dpf, in a *Tg[SILL1; hspGFF53A; UAS:EGFP]* triple transgenic. Early-born neurons express *SILL1* but not *hspGFF53A* (Cherry(+) / EGFP(-)) whereas late-born neurons express both markers (mCherry(+) / EGFP(+)). The white arrowhead in (B) indicates adjacent bulged neurites from late- and early-born neurons. In all the panels, dorsal is up and anterior is left.

Chapter 5

GENERAL DISCUSSION

5.1 Structure and function of the lateral-line neural maps

Lateralis afferent neurons segregate the sensory inputs acquired at different locations along the animal's body and at the same time they separate the inputs from parallel and perpendicular neuromasts. Similarly, the inputs from hair-cells of opposing polarities are conveyed to the brain in separate pathways (Faucherre et al., 2009; Nagiel et al., 2008; Sarrazin et al., 2010). To better understand how the brain takes advantage of the peripheral organization of the lateral line, I reexamined the projection patterns of the lateralis afferent neurons in the zebrafish larva, with an emphasis on their connectivity with a known central target: the Mauthner cell (Kimmel et al., 1990). I will next discuss some structural and functional aspects that emerge from my findings.

5.1.1 A new view of the neural maps built by the lateralis afferent neurons

I demonstrated the existence of two sub-classes of lateralis afferents in the posterior lateral line of the zebrafish larva, which differ in their connectivity with the Mauthner cell and in their central axonal projection patterns. The first sub-class comprises neurons whose central axons contact the lateral dendrite of the Mauthner cell and are always located dorsally in the lateralis column of the hindbrain. By contrast, the second sub-class consists of neurons whose central axons do not contact the Mauthner cell and occupy a ventrolateral position in the lateralis column. Do they also differ in their peripheral axonal projection patterns? Neurons of the first sub-class convey information from anterior and posterior parallel neuromasts (Figure 5.1). Within these neuromasts, they

innervate hair cells of opposing polarities. Surprisingly, neurons of the second sub-class also innervate the very same neuromasts that are innervated by neurons of the first sub-class (Figure 5.1). Importantly, within these neuromasts there is no apparent segregation of the peripheral axon arbors of the two neuronal sub-classes; they rather overlap across the entire organ. This strongly suggests that the two sub-classes convey inputs from the same hair cells. Therefore, the second sub-class of lateralis afferents also carries information from hair-cells of opposing polarities, very likely in separate pathways. In addition, neurons of the second sub-class convey information from the perpendicular neuromasts, in contrast with neurons of the first sub-class (Figure 5.1).

A previous analysis in the zebrafish larva showed that the first lateral-line sensory information relay in the brain contains a somatotopic map. In this map, each lateralis central axon is reproducibly positioned in the hindbrain reflecting the position of the peripheral axon and thus of the innervated neuromast (Alexandre and Ghysen, 1999). However, the systematic anatomical characterization and quantification I performed revealed a more complex arrangement of the lateralis central and peripheral axons. Lateralis afferents shape a sensory neural map composed of two sub-classes of neurons that differ in their connectivity with the Mauthner cell and in their central projections' positions. Each of these sub-classes seems to convey sensory inputs from hair cells of opposing polarities and from different locations along the animal's body. With regard to this, I showed that within the first sub-class of neurons central axons are topographically ordered building a somatotopic map. Nevertheless, when one puts together the two sub-classes of neurons and correlates the positions of the central axons to those of the peripheral

axons, there is no discernible topographic ordering (Figure 5.1). Therefore, the organization of the lateralis afferents combines attributes of both discrete and continuous maps. The two sub-classes of lateralis afferents shape a dimorphic map that resembles a discrete neural map; the spatial organization of their central axons reflects a discrete quality, contacting or non-contacting the Mauthner cell, rather than the spatial organization of their peripheral axons. Embedded in this map, however, there is at least one sub-map built by the neurons of the first sub-class. This is the somatotopic map, which resembles a continuous or topographic map; the spatial organization of their central axons reflects the spatial organization of their peripheral axons. It would be interesting to examine whether there are other sub-maps embedded in the dimorphic lateral-line neural map. For instance, the second sub-class of lateralis afferents might assemble a second independent somatotopic map.

The functional and anatomical study of other sensory neural maps has previously revealed a similar degree of complexity. The visual cortex contains a continuous representation of the retina and embedded in this map there are multiple, superimposed maps of different stimulus attributes, such as eye dominance or motion direction preference (Luo and Flanagan, 2007; Swindale, 2001). Similarly, somatosensory maps normally consist of a continuous representation of the body surface in which discrete units relaying sensory inputs from different modalities, such as touch or pain, are embedded (Luo and Flanagan, 2007). In at least one species, however, the projection patterns of the somatosensory neurons appear to show a different topology. Cutaneous neurons from the chick hindlimb project to the central nervous system forming two separate bundles that represent sub-modalities of somatosensory

information. Each of these bundles assembles an independent topographic map of the skin surface (Woodbury and Scott, 1991). This organization resembles the map topology I describe here in the first step of lateral-line sensory information relay in the brain.

5.1.2 Functional implications of the lateral-line dimorphic neural map

The segregation of the lateralis central axons in dorsal and ventrolateral projections does not account for a segregation of the sensory inputs coming from different regions of the body or of the inputs coming from hair-cells of opposing polarities. Besides hair-bundle polarity, there is no evidence for other heterogeneities among the hair-cells of a given neuromast. If this was the case, however, the two sub-classes of lateralis neurons would not segregate inputs from the different receptors since they seem to contact the same hair cells within a given neuromast. Therefore, the two sub-classes of lateralis afferents rather convey the same sensory information regarding both stimulus position and direction across the antero-posterior body axis. In view of this, they might have a redundant function. However, I showed that they differ in other significant aspects. Importantly, they differ in their connectivity with the Mauthner cell. My anatomical data also argue for the existence of "low excitability/high conduction velocity" and "high excitability/low conduction velocity" lateralis neurons, as discussed previously in chapter 3. The former are only found within the first neuronal sub-class whereas the latter are found within each of the neuronal sub-classes. In addition, sensory inputs from perpendicular neuromasts are exclusively conveyed

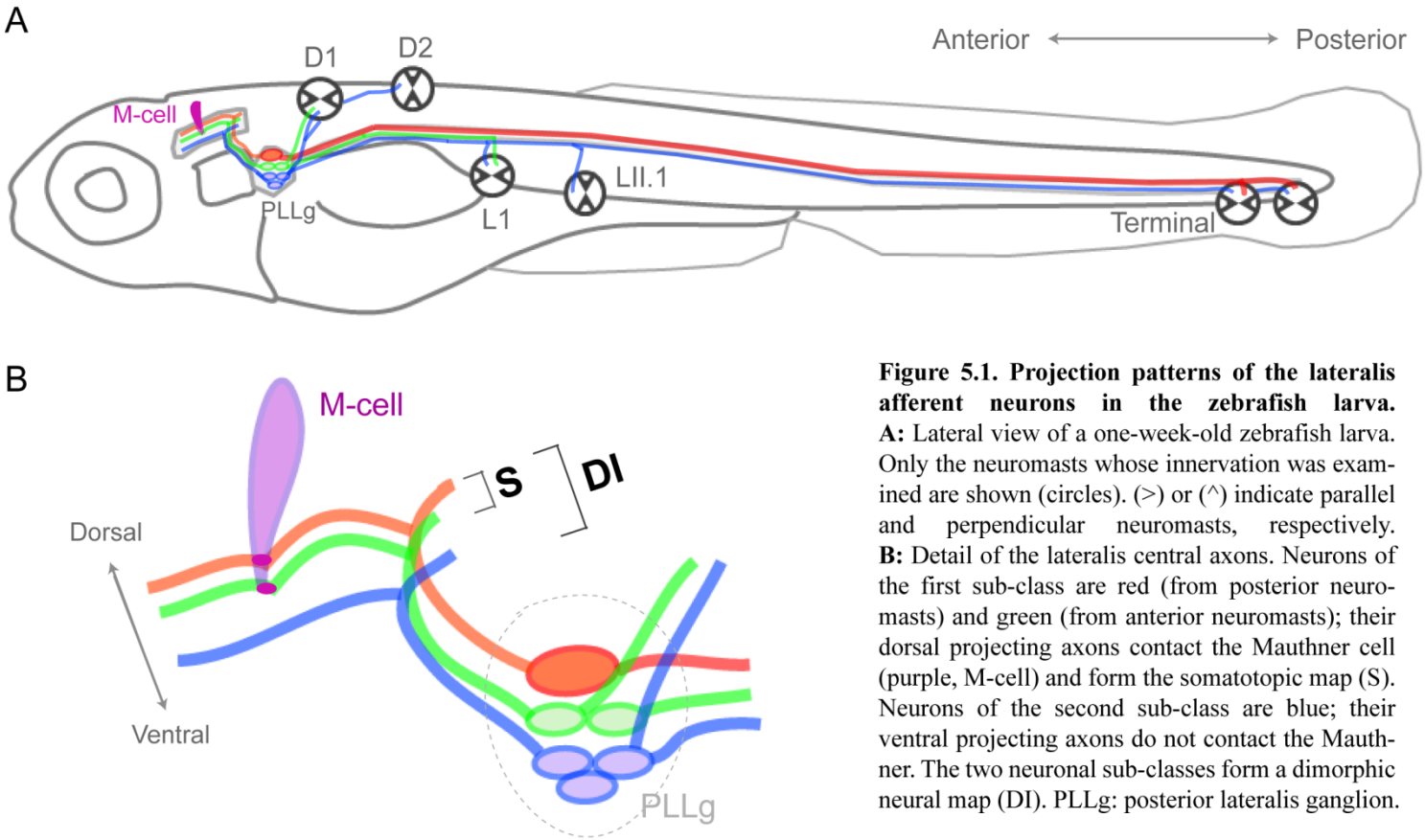


Figure 5.1. Projection patterns of the lateralis afferent neurons in the zebrafish larva. **A:** Lateral view of a one-week-old zebrafish larva. Only the neuromasts whose innervation was examined are shown (circles). (>) or (^) indicate parallel and perpendicular neuromasts, respectively. **B:** Detail of the lateralis central axons. Neurons of the first sub-class are red (from posterior neuromasts) and green (from anterior neuromasts); their dorsal projecting axons contact the Mauthner cell (purple, M-cell) and form the somatotopic map (S). Neurons of the second sub-class are blue; their ventral projecting axons do not contact the Mauthner. The two neuronal sub-classes form a dimorphic neural map (DI). PLLg: posterior lateralis ganglion.

by the second sub-class of neurons. Finally, the two neuronal sub-classes are born at different times. In light of these findings, I propose that the two sub-classes I describe here might have contrasting functions within a given neural circuit, thus sub-serving different components of the same behavior. Alternatively, they might represent parallel channels of sensory information used by different central neural circuits mediating distinct behaviors. These possibilities might be analogous to the separate central representation of submodalities observed in other sensory systems (Palka et al., 1986; Sur et al., 1984; Szwed et al., 2003) and even in the adult lateral-line system which consists in superficial and canal neuromasts (Bleckmann, 2008). However, in these cases not only the sensory neurons but also the peripheral receptors exhibit markedly physiological differences between submodalities. I will next discuss previous findings that reinforce these hypotheses and briefly propose some future investigations on it.

The lateral line mediates very fast responses to abrupt stimuli, such as the C-start reflex (McHenry et al., 2009). This escape behavior is triggered by the activation of the Mauthner cell, which together with some excitatory and inhibitory interneurons in the hindbrain constitute the escape circuit. Importantly, a balance between direct sensory excitation of the Mauthner and a feed-forward inhibition regulates the onset of the C-start reflex (Faber et al., 1989; Faber et al., 1991; Koyama et al., 2010). This is known to occur for the sensory inputs from the inner ear in the zebrafish larva (Takahashi et al., 2002). Physiological data from the goldfish also showed that the Mauthner cell receives both monosynaptic excitatory and polysynaptic inhibitory inputs from the lateral line (Korn and Faber, 1975). The lateralis afferents that contact the lateral dendrite

of the Mauthner cell must account for the monosynaptic excitatory input. These very same neurons, however, might also account for the polysynaptic inhibitory input by exciting interneurons that further inhibit the Mauthner cell (Figure 5.2A). Alternatively, this could be done by the lateralis afferents that do not contact the Mauthner cell (Figure 5.2B) (Faber and Korn, 1975). Therefore, the heterogeneous anatomical connectivity between lateralis afferents and the Mauthner cell that I found might reflect a functional specialization where (1) only a subset of the neurons participates in the escape circuit or (2) the two sub-classes of neurons play opposite roles, excitatory versus inhibitory, within the same escape circuit. To distinguish between these possibilities, it will be necessary to dissect the connectivity between the two sub-classes of lateralis afferents and the feed-forward inhibitory interneurons, which have been recently identified in the zebrafish larva (Koyama et al., 2010). Besides this, it would be very interesting to examine how the activities of these two neuronal sub-classes influence the Mauthner cell firing and the C-start behavior. This can be done by means of the recently developed optogenetic tools, that provide a convenient way to manipulate and monitor neuronal activity (Del Bene and Wyart, 2011).

Lateralis afferents synapse with hundreds of hindbrain second-order neurons beyond the Mauthner cell. The hindbrain of the zebrafish larva contains functional circuits that control diverse motor outputs, including swimming and escape behaviors. Interestingly, many of these circuits appear to be built from a basic ground plan. They are made of a set of dorso-ventral stripes which consist of neurons that use the same neurotransmitter. Neurons within a given stripe are arranged by structural and functional properties, as well as by age. Dorsal neurons show high

excitability, are involved in slow swimming movements and are young. By contrast, ventral neurons show low excitability, are involved in fast swimming movements and are old (Kinkhabwala et al., 2010). Such spatial organization of the hindbrain neurons, arranged by age and degree of excitability, strikingly resembles that of the lateralis central axons. This prompts me to speculate that lateralis afferents synapse with second-order neurons in the hindbrain with similar properties that are well-suited for a particular motor output (Figure 5.2C). For instance, an escape behavior would take advantage of sensory and central neurons with low excitability (high thresholds for activation) in order to exclusively detect and respond to sudden and powerful threatening stimuli. By contrast, rheotaxis would take advantage of neurons with high excitability (low thresholds for activation) in order to detect and react to finer changes in the environment. There is already some indirect evidence for a synaptic match between lateralis afferents and central targets that share functional properties. Dorsal hindbrain neurons (high excitability/young) send dendrites to the ventral neuropil, where the lateralis ventrolateral axons (high excitability/young) presumably reside. Ventral hindbrain neurons (low excitability/old) project instead to the dorsal neuropil, where lateralis dorsal axons (low excitability/old) are supposed to locate (Kinkhabwala et al., 2010). It will be possible to further confirm this by means of neuroanatomical methods and optogenetic tools. Moreover, it would be exciting to directly test the role of the two sub-classes of neurons in sub-serving different motor outputs. This can be done, for instance, by using optogenetics or by cell ablations. These experiments will certainly shed more light on the significance of the heterogeneities among lateralis afferents delineated by my work.

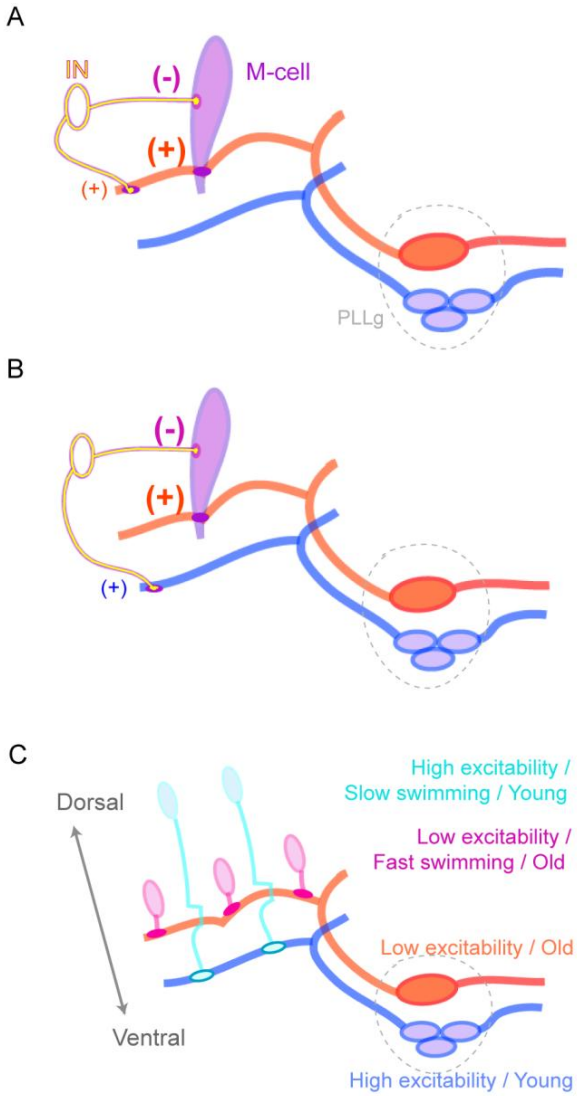


Figure 5.2. Hypothetical connectivity between the lateralis afferent neurons and their central targets. A-B: Possible roles of the two neuronal sub-classes within the escape circuit. (A) Neurons of the first sub-class (red) might account for both monosynaptic excitatory (+) and polysynaptic inhibitory (-) input to the Mauthner cell (purple, M-cell). IN: interneurons. (B) Neurons of the first sub-class (red) account for the monosynaptic excitatory input (+) whereas neurons of the second sub-class (blue) might account for the polysynaptic inhibitory input (-) to the Mauthner. **C:** Hypothetical synaptic match between lateralis afferents and second-order neurons with similar functional properties and relative age.

5.2 Neural map formation in the lateral-line system

The development of the neural maps assembled by the lateralis afferents has been the other central subject of my thesis research. I initially asked how posterior lateral-line somatotopy is established. I demonstrated that among the lateralis afferents that shape a somatotopic map, the ones with dorsal projecting central axons and innervating posterior neuromasts are older than those with ventral projecting central axons and innervating anterior neuromasts. Therefore, there is a correlation between the time at which a lateralis afferent neuron differentiates and its projection patterns. I further found that these lateralis afferents make contacts with the Mauthner cell and originate from a first wave of neurogenesis. Importantly, a second wave of neurogenesis gives rise to another sub-class of lateralis neurons, characterized by central axons that do not contact the Mauthner cell and locate more ventrally in the lateralis column. I propose a twofold contribution of progressive neurogenesis to the diversification and patterning of the lateralis afferents. First, it arranges a somatotopic map that encodes the position of the sensory stimulus. Second, it delineates a dimorphic neural map with interesting potential functional implications. Timing of neurogenesis is known to contribute to sensory neural map assembly in other sensory systems, such as the visual and olfactory systems of both invertebrates and vertebrates (Clandinin and Feldheim, 2009; Holt, 1984; Imamura et al., 2011; Jefferis et al., 2001; Petrovic and Hummel, 2008). Moreover, neurogenic timing contributes to connectivity diversity in motor systems as well as in other regions of the brain (Deguchi et al., 2011; McLean and Fetcho, 2009; Tripodi et al., 2011).

5.2.1 How can progressive neurogenesis build the lateral-line neural maps?

Lateralis afferents face a considerable challenge during development; they must connect the peripheral neuromasts to the central neurons in a spatially orderly manner. How can the progressive birth of lateralis afferents contribute to this task? One possibility is that lateralis afferents are conferred with different properties on the basis of their differentiation dates. Temporal fate or identity is well-known for bearing instructions to create not only cell type heterogeneity but also neuronal projection patterns diversity (Jefferis et al., 2001; Pearson and Doe, 2004; Petrovic and Hummel, 2008). Each lateralis afferent could have an intrinsic temporal identity that determines its final projection patterns. For instance, lateralis neurons born at different times could express different combinations of proteins (molecular codes) that might account for connectivity specificity in the context of a Sperry-type chemoaffinity mechanism (Sperry, 1963). A similar mechanism, based on molecular heterogeneities within the retina and tectum governs retinotopic map formation (Lemke and Reber, 2005). In the lateral-line sensory system, contrary to what happens in the visual system, afferent neurons and sensory receptors develop far from each other. In this scenario the problem is more complicated and a chemoaffinity mechanism would require not only the target hindbrain neurons (central target field) but also the neuromasts (peripheral target field) to obey the same molecular code. Temporal identity could also set up the distance at which a lateralis peripheral axon extends along the antero-posterior axis and thus the choice of neuromast, for instance through the *slit/robo* system (Ghysen

and Dambly-Chaudière, 2004). Alternatively, a complementary expression of a guidance receptor and its repulsive ligand might occur in neurons born at different times; which could account for a segregation of their axons even before reaching their target fields (Imai et al., 2009). So far, there is no evidence of molecular heterogeneities with a potential role in neural map formation among lateralis afferents. Candidate genes involved in the development of other neural maps need to be tested in the future.

Another possibility is that temporal identity plays no role in neural map formation in the lateral line. Progressive neurogenesis might instruct the process without creating other differences among neurons than those in differentiation and axogenesis timing. Topographic map formation in the visual system of arthropods appears to occur in this way, without any genetic contribution (Clandinin and Feldheim, 2009; Flaster and Macagno, 1984). In the case of the lateral line, progressive neurogenesis could simply give rise to neurons that do not differ in properties instructing projection patterns diversity. However, these 'identical' neurons appear progressively in a changing environment since neuromasts are sequentially deposited by several primordia (Ghysen and Dambly-Chaudière, 2007) and the hindbrain is under continuous growth (Kinkhabwala et al., 2010). The final projection patterns of each neuron might exclusively count on the interactions between its growing axons and their surroundings. In such case, the position of each neuron within the map would be circumstantial rather than an intrinsic property of the neuron. This hypothesis is supported by the flexibility exhibited by lateralis afferents in neuromast selection. First, when a neuron innervates more than one neuromast there is no consistent pattern of coinnervation,

except for the terminal neuromasts (Nagiel et al., 2008). Second, lateralis neurons coinnervate neuromasts originating from different primordia in 10% of the cases in normal conditions. Moreover, when early-born neurons are ablated soon after their birth, the next differentiating neurons show a very marked decrease of specificity in the innervation process: around 80% of these neurons coinnervate neuromasts with different origins (Sarrazin et al., 2010).

5.2.2 A 'circumstantial' assembly of the lateral-line neural maps

Based on my observations and on those from others (Gompel et al., 2001b; Sarrazin et al., 2010; Sato et al., 2010; Schuster et al., 2010) I propose the following model to explain the final projection patterns of the lateralis peripheral axons (Figure 5.3). The earliest differentiating lateralis neurons extend their peripheral axons when the first migrating primordium (primI) is still adjacent to them. Therefore, their axons are exposed to high levels of GDNF produced by primI which keeps them growing as the primordium migrates, towing them to the posterior primary neuromasts. Next neurons to differentiate extend their axons when primI has migrated some distance; their peripheral axons are thus less exposed to GDNF produced by primI, since GDNF is thought to act at a short range. As a consequence, their towing by the migrating primordium is weaker and, attracted by the already deposited neuromasts, they abandon it earlier than the oldest axons. The following neurons appear and extend axons when primI is very far from them. Their axons do not sense GDNF from primI but follow instead the already existing peripheral components of the lateral line. These neurons end up

innervating anterior primary neuromasts, which have not been innervated by any neuron yet. A second wave of neurogenesis occurs when new migrating primordia (primII and primD) arise. The axons of these youngest neurons are towed by the new primordia and reach the secondary neuromasts. Some of the youngest neurons, however, innervate primary neuromasts although these have been already innervated by older neurons.

On the other hand, almost nothing is known about the interaction of the lateralis central axons and their targets in the hindbrain during development. I have suggested above a synaptic match between lateralis afferents and central targets with similar relative ages. This holds true at least for the Mauthner cell, which is among the earliest neurons to develop in the brain (Kimmel et al., 1990) and is exclusively contacted by the central axons of early-born lateralis afferents. If this is the general situation, a simple temporal code might match lateralis afferents with the second-order neurons in the hindbrain (Figure 5.3). The earliest differentiating lateralis afferents extend central axons that reach the hindbrain first. These axons would occupy the most dorsal region of the neuropil and associate with the earliest born second-order neurons. Next lateralis afferents to differentiate extend central axons that would occupy an adjacent ventral position to that of the earliest axons and associate with younger second-order neurons. Lateralis and second-order neurons that are born subsequently would synapse progressively, repeating the process. The generation of transgenic animals that highlight second-order neurons in the hindbrain and the use of neuroanatomical tools will allow for the testing of this possibility.

In summary, I propose that neural map assembly in the lateral line largely results from the coincident progressive development of each of its components: the lateralis afferents and their central and peripheral targets. Furthermore, it is very likely that the control of the progressive development that takes places within one component is independent of that taking place within the others. To test these hypotheses it will be necessary to alter the development of the lateralis afferents, for instance, without interfering with the development of their targets or with other functions. Besides neurogenic timing, however, other factors might influence to different extents neural map formation in the lateral line. It has been recently shown that retinotopic map formation needs the simultaneous action of molecular gradients, neural activity and interaxon competition (Triplett et al., 2011). Moreover, olfactory map assembly relies on the sequential arriving of axons to their target plus the complementary expression of a guidance receptor and its repulsive ligand by these axons (Takeuchi et al., 2010). My results indicate that sensory or evoked activity does not play an instructive role in the coarse arrangement of the lateralis central axons within the hindbrain. In addition, lateralis axons of early-born neurons do not appear to repel those from late-born neurons from contacting the Mauthner cell. However, the role of these elements in connectivity specificity and map refinement, if there was, still needs to be tested. Other factors, such as intrinsic neural activity, might also be involved in the assembly of the lateral-line neural maps.

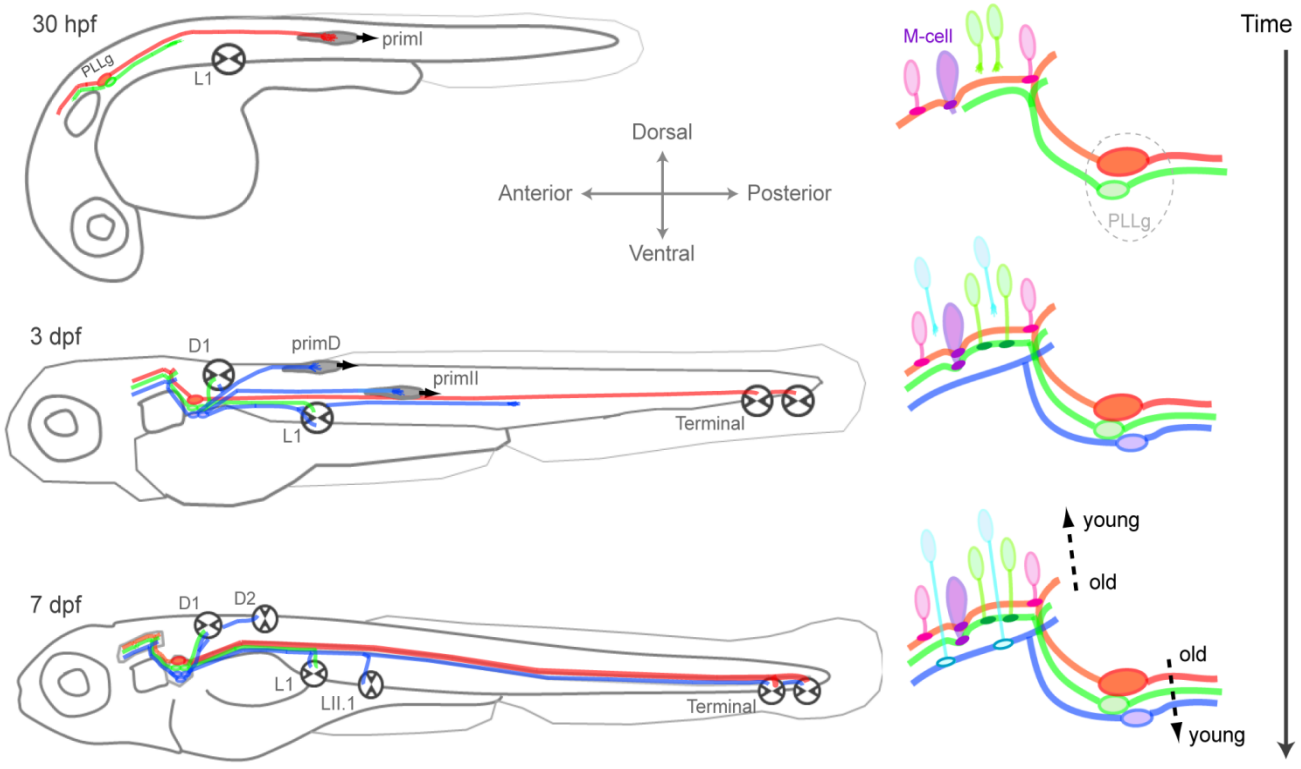


Figure 5.3. An hypothetical 'circumstancial' assembly of the lateral-line neural maps. Lateral view of a developing zebrafish at around 30 hpf, 3 and 7 dpf showing the coincident progressive development of the lateralis afferents and their peripheral and central targets. Neurons of the first sub-class are red (to posterior primary neuromasts) and green (to anterior primary neuromasts). Neurons of the second sub-class are blue. primI: first primordium, primD: dorsal primordium, primII: second primordium. Only the neuromasts whose innervation was examined are shown (circles). L1, Terminal and D1 neuromasts are primary (early-born) and parallel (>). LII.1 and D2 neuromasts are secondary (late-born) and perpendicular (^). M-cell: Mauthner cell. PLLg: posterior lateralis ganglion.

Chapter 6

CONCLUSIONS

[1]

I characterized two transgenic lines that contain the Gal4 transcriptional activator stably integrated in the genome and can drive expression of UAS-driven transgenes in the lateralis afferent neurons (*hspGFF53A*) and in the Mauthner cell (*hspGFFDMC130A*). Moreover, *hspGFF53A* represents the earliest known marker with specificity for the lateralis afferents.

[2]

My anatomical data revealed two sub-classes of lateralis afferent neurons which differ in their connectivity with the Mauthner cell and in their axonal projection patterns, defining a dimorphic neural map (discrete map). Embedded in this map, there is at least one sub-map: the somatotopic map (continuous map).

[3]

My anatomical data revealed that lateralis neuronal somata are heterogeneous in size. Furthermore, large-soma neurons project exclusively from terminal neuromasts whereas neurons from non-terminal neuromasts are homogenously small.

[4]

I demonstrated growth anisotropy of the posterior lateralis ganglion that occurs because new neurons are preferentially added to its ventromedial side. This determines a conserved topological organization of the neuronal somata within the ganglion that reflects the differentiation order of the neurons.

[5]

The order of differentiation predicts the position of the neuron's central axon along the somatotopic axis in the hindbrain and the neuron's choice of peripheral target. I concluded that neuronal birth order defines lateral-line somatotopy.

[6]

The order of differentiation predicts the position of the neuron's central axon within the dimorphic neural map and the establishment of a contact with the Mauthner cell. I concluded that neuronal birth order defines the lateral-line dimorphic neural map. In addition, birth order also defines the heterogeneity in neuronal soma size.

[7]

My results from the analysis of *atoh1a* and *tmie* mutants indicate that sensory activity is not a major player in the formation of the lateral-line dimorphic neural map. Moreover, competition among lateralis central axons does not seem to influence the establishment of contacts with the Mauthner cell.

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Appendix

OTHER CONTRIBUTIONS

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