Uncovering the interplay between cell fate specification and progenitor dynamics during the development of the lower rhombic lip

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“The only thing that makes life possible is permanent, intolerable uncertainty: not knowing what comes next.”

— Ursula K. Le Guin, *The Left Hand of Darkness*

“What I've started I must finish. I've gone too far to turn back. Regardless of what may happen, I have to go forward.”

— Michael Ende, *The Neverending Story*

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ABSTRACT

The Lower Rhombic Lip (LRL) is a transient neuroepithelial structure of dorsal hindbrain that gives rise to deep brainstem nuclei like the vestibular, auditory and precerebellar nuclei. In this work, we have followed the LRL-progenitor cell population through early steps of neurogenesis and hindbrain morphogenesis to understand proneural function and progenitor dynamics during neuronal specification. We provide information about the *atoh1* gene regulatory network operating in the specification of LRL cells, and the kinetics of cell proliferation and behaviour of *atoh1a* derivatives by using functional and *in vivo* imaging strategies in the zebrafish embryo.

We propose that *atoh1a* and *atoh1b* have sub-functionalized: *atoh1a* acts as the fate selector gene in LRL progenitors, whereas *atoh1b* acts as the downstream neuronal differentiation gene carrying out the neurogenic program. Moreover, our *in vivo* cell lineage approaches revealed a regionalization of modes of division within the LRL, orchestrating the balance between neuronal differentiation and progenitor self-renewal.

RESUM

El Llavi Ròmbic Inferior (LRI) és una estructura neuroepitelial transient del romboencèfal dorsal que genera nuclis profunds del tronc de l'encèfal, com ara els nuclis vestibulars, auditius i precerebel·lars. En aquest treball hem seguit la població progenitora del LRI durant els primers estadis neurogènics i de morfogènesi per entendre la funció proneural i la dinàmica dels progenitors durant l'especificació neuronal. Informem sobre la xarxa genètica reguladora depenent d'*atoh1* que opera al LRI, així com del comportament proliferatiu i migratori de les cèl·lules derivades del LRI servint-nos d'experiments funcionals i d'imatge d'embrions de peix zebra *in vivo*.

Proposem que *atoh1a* i *atoh1b* estan subfuncionalitzats: *atoh1a* actua com a gen selector dels progenitors LRI, mentre que *atoh1b* funciona sota *atoh1a* mantenint el seu programa neurogènic. A més, els estudis de llinatge cel·lular *in vivo* mostren la regionalització dels diferents modes de divisió, orquestrant així l'equilibri entre la diferenciació neuronal i l'auto-renovació progenitora.

PREFACE

A single fertilized egg holds the potential to generate the vast array of cellular phenotypes that populate an adult multicellular organism. Besides, in order to generate function, the embryo not only faces the challenge of specializing functional cells, but also to coordinate growth with the generation of shapes that will ultimately generate organs. In short, we can say that embryonic development is the most important period in the life of any animal.

The adult rhomboencephalon or hindbrain, which is the most posterior part of the brain, serves as the perfect example of such developmental challenge. It houses the neuronal circuits implicated in key autonomous processes, such as heartbeat, respiration and wakefulness cycles. Moreover, it also serves as a relay station for sensory information that allows the fine tuning of motor coordination. Each one of the circuits aforementioned are constituted by neurons that bear unique characteristics in terms of morphology and gene expression, being specialized for the function they are meant to fulfil. After characterizing the function of the neuronal circuits that populate the adult hindbrain, we keep stumbling upon the same fundamental question: how are such unique and diverse neuronal identities generated during embryogenesis?

In this work, we wanted to elucidate the genetic players behind the generation of diverse neuronal lineages within the hindbrain. In the past decades, many efforts have been devoted at understanding the molecular and genetic codes that confer specific identities to progenitor cells within the embryo, restricting the potential of said progenitors and instructing cell fate choices. Thanks to all the work that came before us, most of such molecular mechanisms are well defined today. However, there is still a gap between our knowledge on the gene regulatory networks active during neurogenesis and the dynamic cellular events that progenitor cells undergo during the process of differentiation. Once a progenitor cell has been recruited to a given progenitor population through patterning cues, how does this progenitor domain cope with cellular proliferation, differentiation and apoptosis? How do the dynamics of progenitor cells shape the neural tissue during

neurogenic stages? And, most importantly, how are these processes coordinated with the changing neural tube architecture upon morphogenesis?

To shed some light into these questions, it was of special interest to us to investigate the proneural gene code instructing neurogenesis within the very dynamic context that is the developing hindbrain. In this regard, a particular hindbrain lineage caught our attention: the Lower Rhombic Lip (LRL), which presented the challenge of being a neurogenically active progenitor population at the time of extensive hindbrain morphogenesis. Thus, we thought of LRL-progenitors as the suitable cellular candidates in which to study proneural function along with cellular dynamics. However, in order to grasp the dynamism that is so characteristic to neural progenitors at the onset of hindbrain neurogenesis, we needed to quantify cellular kinetics of proliferation and differentiation. It was in this thought process that it became evident how important *in vivo* imaging was for the fulfilment of these work and for modern developmental neurobiology in general. Hence, the zebrafish embryo felt as the more natural model in which to perform our research.

In all, the work presented in this thesis combines the use of classical developmental biology approaches as well as more advanced imaging techniques. Such procedures allowed us to identify different modes of clonal growth according to position of progenitor cells, an early exploratory work that already revealed that the LRL behaved in different ways than the rest of hindbrain progenitors. Moreover, we inform of the sub-functionalization of the *atoh1* genes driving neurogenesis within the LRL, which define different progenitor states within the neurogenic process. Lastly, we provide information on the different modes of division that LRL progenitors undergo, as well as the location preferences of LRL-derived neurons within the differentiation domain of the developing hindbrain.

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CHAPTER 1: INTRODUCTION

FROM PROGENITORS TO NEURONS: THE GENERATION OF NEURONAL DIVERSITY

1.1. ON EMBRYOGENESIS AND CELL LINEAGES

Embryonic development is a key period in the life of every multicellular organism. During this process, a single cell, the zygote, gives rise to the whole cellular diversity present in an adult individual. Cell diversification begins with a single cell that holds the potential to generate an entirely functional multicellular organism. Not only a huge number of cellular types must be generated, but also arranged in discrete blocks or units following specific patterns. By doing so, the embryo will acquire specialized structures and shapes that will ensure and enhance its functional capabilities.

Historically, developmental biology aimed to understand how organisms form in space and time by dissecting the succession of events during embryogenesis at different levels. At the tissue level, morphogenesis takes a stand-alone role, as the generation of shape during embryogenesis is crucial to generate organs with the proper physiological functions. At the cellular level, embryonic stem cells or progenitors proliferate and, upon differentiation, produce specific morphologies that account for specific functions. Lastly, we can dissect developmental processes at a molecular and genetic level. Prominently, cell fate determination is governed by the activation of different transcriptional programs that provide progenitors with a given cell identity[1]. Moreover, transcription factors (TFs) usually work as hubs that integrate a vast array of inputs, such as active signalling pathways or other transcriptional regulators. The collection of TFs and signalling pathways ruling over fate determination has been conserved along evolution and is known as the developmental “toolkit”.

These processes, enclosed within these three main layers of complexity, are interwoven in both cell autonomous and non-autonomous ways, which results in great challenge for developmental biologists to dissect and organize them hierarchically. Within this intellectual framework, we aim at investigating a fundamental question in the field: how is cellular diversity achieved during

[1]The term “cell identity” refers to the collection of features that define a progenitor at a given developmental time, such as transcriptional footprint or morphology. Thus, we can define “fate” as the definitive identity that progenitors acquire after differentiation.

embryogenesis and, in particular, in the Central Nervous System (CNS)? In this section, we will discuss the central role of cell-lineage tracing approaches in elucidating the interactions between cell fate determinants and progenitor cell dynamics in generating diverse neuronal lineages.

During the embryonic development of the CNS, a vast array of neuronal types, as well as glial cells, must be generated at specific positions along the CNS primordium, known as the neural tube. Not only that, but these phenotypically diverse neurons must organize in functional units and connect with each other by extending axonal trajectories and establishing synaptic contacts. This wiring process will eventually generate the functional circuits that underlie both simple and complex behaviours of adult individuals, such as locomotion, feeling and thought. However, how do we reach such level of cellular and functional complexity during embryogenesis?

As we will see throughout this manuscript, the mechanisms by which neuronal diversity is generated are diverse and depend on the brain region and developmental time. However, we can divide the determination of specific neuronal fates into two fundamental mechanisms.

The first mechanism is the inheritance by progenitor cells of a restricted developmental potential by its parents or ancestors. In simpler words, the determination of cell fate by lineage. In the case of the developing CNS, cellular linages influence cell fate by the segregation of progenitor potentialities in space and time. Hence, we can have two possible scenarios: distinct progenitors, segregated in space and with specific positional identities that generate distinct classes of neurons; and the same multipotent progenitor that generates an array of diverse neuronal phenotypes upon time following an intrinsic plan that generates a predetermined pattern (McConnel, 1995).

The second general mechanism governing the specification of distinct neuronal fates is the interaction of progenitor cells with the local environment or developmental niche. In the end, multipotent progenitors choose a pathway in favour of other possible outcomes, and the chosen pathway arises as the result of interactions with inductive signals. Such signals can be provided to progenitor

cells by cell-to-cell interactions or by the establishment of local gradients of diffusible morphogens.

In conclusion, cell lineage trees alone cannot reveal the mechanisms by which a cell acquires its final phenotype, as fate selection is a decision governed by: a) the inherited intrinsic signals that inform of a given developmental potential and spatiotemporal context, and b) the extrinsic environmental cues and cell-to-cell interactions that progenitor cells sense during the neurogenic process. Thus, lineages put cells at the right place and at the right time to facilitate these interactions and provide a given developmental niche with the right cell fate determinants (Greenwald, 1989; Stent, 1985; McConnel, 1995).

In this light, the recording of cellular linages during development arises as a powerful tool to investigate the generation of diverse neuronal phenotypes, especially when it is combined with experimental manipulation of both intrinsic and extrinsic signals regulating cell-fate decisions.

1.1.1. Cell-lineage tracing approaches for the reconstruction of neural lineages

Since the determination of neuronal fates is intrinsically tied to spatiotemporal cues, following the dynamic behaviour of every cell at every point in time and space throughout the development of the CNS is one of the central goals of developmental neurobiology. Thus, reconstructing cell lineages must be placed at the centre of any research that aims to unveil how progenitor dynamics are coupled with cell fate determination. In this light, cell lineage relationships are experimentally revealed through cell-fate mapping methods; when fate mapping is carried out at single-cell resolution it is known as cell-lineage tracing.

A milestone in cell-lineage analysis was the reconstruction of the entire lineage of all cells present in an adult organism: the *C. elegans*. By direct visualization and manual annotation of all divisions, the complete genealogy of all cells in the nematode was traced (Sulston *et al*., 1983). This feat informed the comprehensive lineage relationships between cells and served as a fundamental framework instructing subsequent experimental design and data interpretation.

However, the *C. elegans* complete cellular lineage is an example of a pure deterministic, invariant model of cell fate determination.

Therefore, in order to unravel significant non-autonomous cell fate decisions, vertebrate model organisms have been favoured. Inconveniently, direct visualization without any kind of cellular label is only possible under specific conditions of embryo size, accessibility and transparency such as those offered by the nematode. Thus, classical cell-lineage tracing experiments done in frog and avian embryos often involved transplantation of cells and tissues, which offers several possibilities for donor cell detection (Kretzschmar and Watt, 2012). This experimental embryology approach has provided extremely valuable insights, such as the identification of the Spemann's organizer during neural induction in amphibian embryos (Spemann and Mangold, 1924). Nonetheless, single-cell resolution was never achieved in this kind of experiments, which is a requisite when aiming at describing cellular processes such as proliferation, mode of division and differentiation.

With the advent of the parallel advances in molecular, genetic and imaging technologies, many new methods with increasing cell resolution have emerged to allow cell tracking within the developing embryo. The available rainbow of fluorescent proteins has increased the number of founder cells that can be labelled and tracked (Cai *et al*., 2013). Labels can be delivered at different stages of development by viral infection, in *utero/ovo* electroporation or direct injection into the embryo in mice, chick and zebrafish (Woodworth *et al*., 2017).

Genome-editing technologies have allowed to introduce cellular tracers directly into the genome, where they will be known as reporter genes. This allows to permanently mark cellular lineages without the drawback of the marker dilution over time. Not only that, but reporter genes can be tissue and time specific if regulated by the genomic environment of relevant developmental genes. Thus, with recombination-based approaches like the Cre-loxP system (Awatramani *et al*. 2003, Yamamoto *et al*., 2009), the directed insertion of genetic material by CRISPR-Cas9 (Albadri *et al*., 2017; Raveux *et al*., 2017), or transcriptional control systems like the Gal4-UAS (Osterwalder *et al*., 2001; Distel *et al*., 2009), we have

a collection or approaches to specifically label progenitors of our interest and analyse clonally-related cells in a given developmental context *in vivo*.

Combining the genetic tools aforementioned with last generation *in vivo* imaging techniques has proven to be of great use in order to obtain relevant biological insights from cell-lineage tracing experiments. In that sense, the zebrafish embryo emerges as a more than suitable model for this kind of approach, due to the transparency of embryo, rapid generation time when compared to other models, as well as fairly easy to handle and manipulate genetically.

Even with all its potentialities, the tracking of every cell at every point of time and space during zebrafish CNS development is still a great challenge. In the case of the nervous system, cell-lineage tracing of whole structures by simultaneously tracking all of their individual cells has been achieved in smaller, more accessible neural tissues such as sensory placodes (Dyballa *et al*., 2017). However, in order to achieve single-cell resolution, more complex structures like the retina or CNS regions partial labelling approaches have been taken such as the photoconversion of reporter proteins (He *et al*., 2012), multicolour mosaics (Pan *et al*., 2013; Brockway *et al*., 2019) or direct genetic targeting of specific progenitors by taking advantage of relevant developmental genes expressed or active signalling pathways in the population of interest (Satou *et al*., 2013; Voltes *et al*., 2019). This leads to a scenario in which, even if we are able to obtain valuable biological insights on specific progenitor populations in specific brain regions, we are still unable to fully reconstruct cell lineages.

To overcome the lack of context, some zebrafish-based experiences exist generating brain atlases that integrate several modalities of information: either fixed or *in vivo* expression data of endogen and reporter genes (Ronneberger *et al*., 2012; Tabor *et al*., 2019), brain activity (Portugues *et al*., 2014; Randlett *et al*., 2015) and even axonal wiring (Kunst *et al*., 2019). Although useful to compare expression patterns across brain regions and active circuits, these atlases were generated at late stages in development -from 5 to 6 days post-fertilization (dpf)- and do not take into account developmental time. Thus, we are still far from a

comprehensive platform that integrates all information modalities generated in lineage tracing experiments.

In spite of the limitations, cell lineage-based reconstruction of embryonic development offers a great opportunity to address questions at the system and cell-population level and to assess whether cell behaviour follows recurrent patterns that scaffold specific developmental processes like the acquisition of cell identity during neurogenesis. With this in mind, the CNS serves a thrilling context in which the knowledge and the tools are already set for us to start unravelling the intricate relationship between progenitor dynamics, cell fate determination and cell lineage.

1.2. ON CELL FATE ACQUISITON AND NEUROGENESIS

A multitude of neurons of different types, as well as oligodendrocytes and astrocytes, are generated as the vertebrate CNS develops. These different neural cells are generated at defined times and positions by multipotent progenitors of the embryonic neural tube. Before going into the general mechanisms that produce neuronal diversity during the development of the CNS, we first need to understand how neural progenitors behave during the neurogenic process.

1.2.1. Neural progenitors within the developing CNS

During embryogenesis, the neural tube consists of a pseudostratified epithelium populated by neuroepithelial cells (NECs). NECs are highly polarized cells, with an apical pole facing the lumen of the neural tube and a basal pole in contact with the basal lamina. Before the onset of neurogenesis, there is a phase of expansion of the progenitor pool where NECs divide symmetrically, giving rise to two daughter cells with the same progenitor potential (PP divisions). These mode of division accounts for the overall growth of the neural tube. However, at neurogenesis onset, NECs will commit to neurogenic progenitors (NPs) and will start dividing asymmetrically generating two daughter cells: one cell that will remain as an NP, and a second cell that will constitute a neuronal precursor that will exit the cell cycle and become a post-mitotic neuron (NP divisions). In the

mammalian cortex, these neurogenic progenitors are known as radial glial cells (RGCs) and a key step in the initiation of neurogenesis is the so-called NEC-to-RGC transition (Martynoga *et al*., 2012; Paridaen and Huttner, 2014). As this distinction between progenitor types is not apparent in other CNS regions and model organisms, we will favour the general term "neural progenitors" throughout the manuscript. At late stages of neurogenesis, neural progenitors divide symmetrically and give rise to two neurons, which results in the exhaustion of the progenitor pool (NN divisions; Figure 1.1A).

Balancing these different modes of division (symmetric proliferative, asymmetric neurogenic and symmetric neurogenic) is of key importance in order to maintain the homeostatic growth of the structure and to produce the proper number of neurons: many early neurogenic divisions will exhaust the progenitor pool before the neural tube had time to grow, whereas failing to exit the cell cycle will result in the lack of neuronal differentiation.

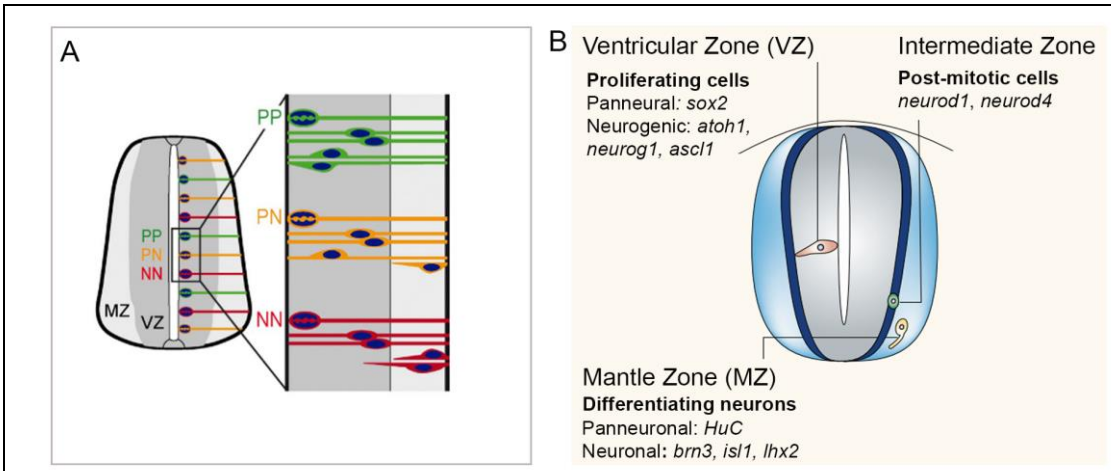

Figure 1.1. Neural progenitors within the developing CNS. **A**) Schematic representation of the different modes of division neural progenitors undergo during CNS development. As a reference, a transverse section of the chick spinal cord is represented, with dorsal to the top. PP divisions are represented in green, PN divisions represented in yellow and NN divisions represented in red. Note how mitoses only occur in close contact with the VZ. Adapted from Le Dréau *et al*., 2014. **B**) Schematic representation depicting the layered organization of the developing spinal cord. Proliferating neural precursors are present in the VZ and can be defined by panneural markers such as *sox2*, or neurogenic markers such as proneural genes. Newly postmitotic precursors are positioned in the intermediate zone and can be defined by the expression of neuronal differentiation genes such as *neurod1* and *4*. Differentiating neurons in the MZ can be assessed by the panneuronal differentiation marker *HuC* or by markers of specific neuronal subpopulations such as *isl1* or *brn3*. Adapted from Corral and Storey, 2001.

Lastly, there is a differential distribution of cellular types within the apico-basal axis of the neural tube. In this sense, neural progenitors are located in close contact with the tube's lumen, in an area known as the ventricular zone (VZ). As neural progenitors commit to neurogenesis and start generating neuronal precursors, they will detach from the apical surface and lose their polarity as they migrate towards the basal pole. The accumulation of neurons as neurogenesis proceeds in the basal half of the neural tube will eventually generate a thickening of the structure known as mantle zone (MZ). Since each one of these steps is regulated by different sets of transcription factors, we can take advantage and use them as markers of the different neurogenic steps (Figure 1.1B).

1.2.2. General mechanisms that generate neuronal diversity

The process by which a multipotent progenitor cell generates a particular type of neuron can be subdivided into a series of sequential steps. First, neural progenitors acquire positional identity through patterning cues, which regionalize the neural tube according to the anteroposterior (AP) and dorsoventral (DV) axes. As an example, the vertebrate spinal cord is patterned due to the establishment of two opposing morphogen gradients: BMPs and Wnts, which are produced at the dorsal pole by the roof plate; and Shh, which is produced at the ventral pole by the floor plate and the notochord. The different levels of morphogen signals will induce the expression of downstream patterning genes, which will regionalize the spinal cord in different DV domains of expression, as extensively studied in mouse and chick embryos (Wilson and Madden, 2005; Le Dréau *et al*., 2012). The patterning proteins induced by the signals aforementioned are transcription factors of the homeodomain (HD) and basic Helix Loop Helix (bHLH) family, such as *pax6, nkx2.2* and *olig2*, which activate transcriptional programs responsible for the acquisition of a given neuronal fate. Broadly, dorsally-located progenitors will give rise to sensory interneurons, whereas ventrally located progenitors will generate motoneurons.

After positional identity has been stablished, proneural genes start to be expressed in proliferative progenitors promoting cell cycle exit and neuronal differentiation, and cooperating with patterning genes in order to assign neuronal

subtype (see next section, 1.3.3). Lastly, neuronal HD proteins will start to be expressed in post-mitotic neurons conferring specific neuronal features such as neurotransmitter properties, axonal pathfinding and wiring specificity (Guillemot, 2007).

In conclusion, the neural tube arises as a grid of Cartesian coordinates where every cell can interpret their position within the structure and generate a specific neuronal lineage due to the sequential (and partially overlapping) function of specific transcriptional codes classified in patterning proteins, proneural proteins and neuronal proteins (Figure 1.2).

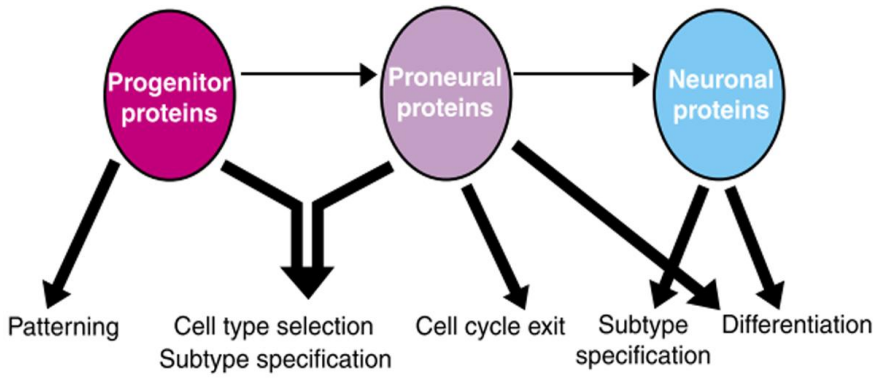

Figure 1.2. The differentiation of multipotent neural progenitors into specific classes of neurons involves transcriptional cascades in which patterning proteins induce proneural proteins, which in turn induce neuronal HD proteins. These factors regulate different phases of neural development. Subtype specification is initiated in dividing progenitors coordinated by progenitor proteins and proneural proteins and further promoted by neuronal proteins after cell cycle exit (see 1.3.3). Adapted from Guillemot, 2007.

An additional and equally important mechanism for the generation of neuronal diversity is the temporal control of neuronal fate specification. In other words, the sequential production of different neuronal types by the same progenitor population at distinct temporal windows during neurogenesis. There are many examples of such lineage segregation in time, such as (a) the different layers of the mammalian cortex (McConnel *et al*., 1995); and (b) the gliogenic switch that

previous neuron-generating progenitors undergo in the ventral spinal cord and in the mammalian cortex (Bansod *et al*., 2017).

We have seen how neural progenitors integrate spatiotemporal cues in order to generate specific neuronal lineages, which raises the following question: is the acquisition of fate a purely deterministic process? In other words, is every step of the neurogenic process ruled over deterministic cues, or can cell fate be selected stochastically?

Such questions have been investigated in the vertebrate retina. Rat retinal progenitors grown *in vitro* generate clones in a big variety of sizes, as well as cellular types, which are difficult to explain only with deterministic molecular steps (Gomes *et al*., 2011). In zebrafish, clonal analysis of individual retinal progenitors *in vivo* shows the same phenomenon: retinal progenitors' growth rate, as well as the pattern in which different cell fates are generated vary considerably from one clone to another (He *et al*., 2012). These observations have favoured the stochastic model, in which retinal progenitors make cell-fate choices due to an accumulation of fixed probabilities provided by transcription factor codes that allow a certain level of stochasticity. Indeed, although it still remains to be elucidated, the stochastic model faithfully recapitulates the observations made *in vitro* and *in vivo* (Boije *et al*., 2015).

1.3. PRONEURAL GENES AND NEURONAL DIVERSITY

Once a neural progenitor has acquired a given positional identity, the next step in the generation of a given neuronal lineage is to initiate a program for neuronal differentiation. In this sense, expression of a collection of different proneural basic Helix-Loop-Helix (bHLH) genes in discrete domains of the developing CNS ensures the generation of neurons as well as the assignment of specific neuronal subtypes. As we will see throughout this section, the mechanisms by which these factors initiate and maintain the neurogenic program are largely shared among different proneural genes, whereas the adscription of specific neuronal identities is unique to each one of them. This is the basic principle of how proneural genes

contribute to the generation of neuronal diversity: by defining different neurogenic regions that will generate distinct neuronal identities. Therefore, when aiming to unveil how a particular neuronal lineage chooses its fate, analysing proneural function is crucial.

From a definition point of view, the proneural gene concept refers to a transcription factor from the bHLH family that is both necessary and sufficient for the generation of neurons. In *Drosophila*, where they were first identified, proneural genes are initially expressed by ectodermal cells that are not yet committed to a neural fate. These groups of proneural-expressing ectodermal cells are called “proneural clusters” and are distributed in patterns that foreshadow the distribution of neural progenitor cells in the peripheral and central nervous systems (PNS and CNS) (Skeath and Carrol, 1992; Cubas *et al*., 1991; Hartenstein and Wodarz, 2013).

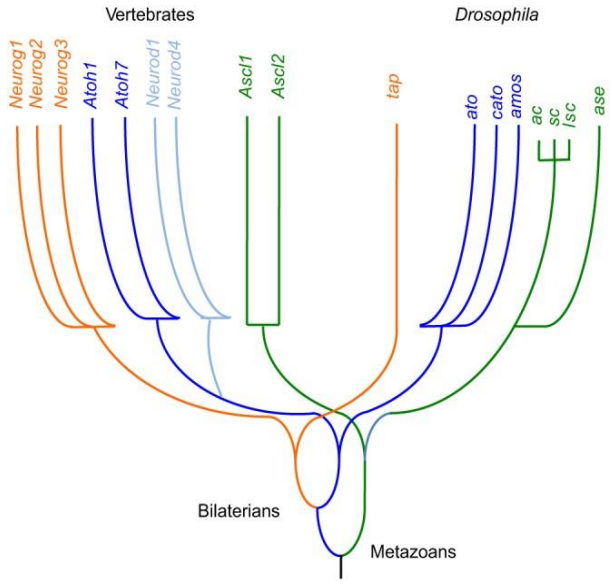

Figure 1.3. Schematic phylogeny of proneural bHLH genes. The relationships and ancestry of proneural genes in vertebrates and *Drosophila*. Each colour represents a related subfamily. In vertebrates, *ascl2* is only expressed in the gut, while *atoh7* is specific for retinal development. The rest of the proneural genes, as well as the neuronal differentiation genes *neurod1* and *neurod4*, are expressed in different regions of both vertebrate CNS and PNS. Baker and Brown, 2018.

In vertebrates, proneural genes can be divided into three different families according to their similarity to their orthologs in *Drosophila*: the *atoh*, the *neurog* and the *ascl* families (Figure 1.3). However, and as the major difference with invertebrate neurogenesis, proneural genes start to be expressed in progenitors

that have already acquired a neural character (Bertrand, *et al*., 2002). Thus, and taking the murine CNS as a reference, *atoh1* is expressed in the dorsal hindbrain and spinal cord, *neurog1* and *neurog2* are expressed in the dorsal telencephalon and in different domains of the spinal cord, *neurog3* is expressed in the spinal cord and in the hypothalamus, and *ascl1* is expressed in the ventral telencephalon and the dorsal spinal cord. There are several differences among vertebrate species, being worth mentioning the lack of *neurog2* in zebrafish, as well as the presence of extra paralogues for *atoh1* and *ascl1* in teleost fish that are absent in amniotes (Baker and Brown, 2018).

The developing vertebrate spinal cord serves as a very informative paradigm in order to understand proneural function. As explained in previous sections, the patterning of the spinal cord along the DV axis greatly determines the fate of the newly generated neurons. Accordingly, proneural gene expression in the spinal cord is restricted to discrete DV domains (Figure 1.4A). In turn, these domains will generate a specific set of neurons defined by the expression of different HD neuronal transcription factors and neurotransmitter properties (Figure 1.4B).

For example, progenitors that will generate motoneurons are located in the presumptive motoneuron domain (pMN) and express *neurog2* at the onset of neurogenesis. However, more dorsal progenitor domains, characterized by the expression of *atoh1* or *ascl1*, will generate sensory interneurons that express *lhx2/9* and *lhx1/5*, respectively (Figure 1.4). Thus, by analysing both proneural and neuronal genes expression, we can infer lineage relationships between neural progenitors and differentiated neurons according to their relative position within the apico-basal axis of the neuroepithelium.

The distribution of different proneural genes along the DV axis of the neural tube already hints that these factors are able to integrate spatial cues and collaborate with patterning genes in order to determine neuronal identity (Guillemot, 2007). However, before going into the specificities of how proneural genes generate diverse neuronal lineages, we first need to understand the basics of proneural function.

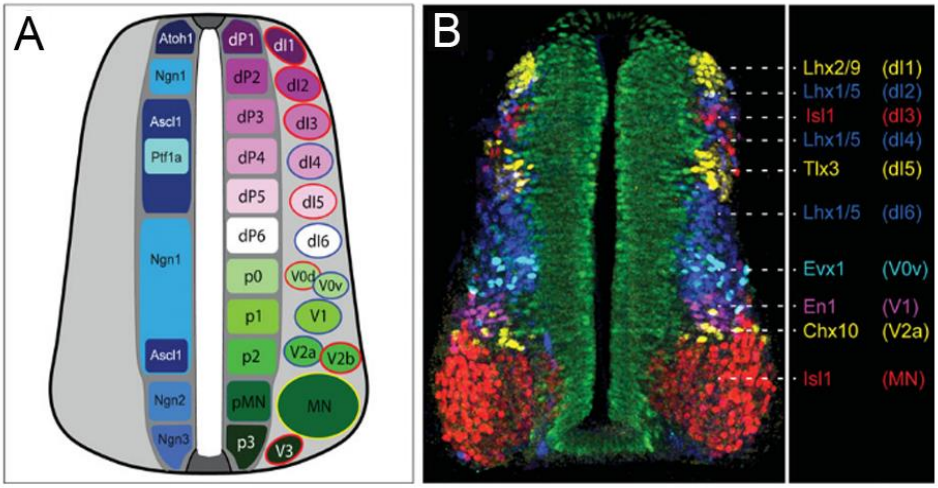

Figure 1.4. Dorsoventral patterning of the chick developing spinal cord at HH24. **A**) Schematic representation of a transverse section of a chick neural tube. Left side shows the expression profile of proneural factors. Right side presents the subdivision of the neural tube into the 11 distinct domains of neural progenitors and their differentiating progeny which first migrate laterally and thus form the MZ. With the exception of the pMN which generate acetylcholinergic-secreting motoneurons (yellow circle), all these domains will generate diverse populations of glutamatergic (red circles) or GABAergic/Glycinergic (blue circles) interneurons. **B**) Photographic reconstruction of immunostainings performed on chick neural tube sections showing *sox2*-expressing neural progenitors located in the VZ (green) and various subpopulations of differentiating neurons according to the expression of HD neuronal factors indicated in red, yellow, cyan, blue and magenta. Adapted from Le Dréau and Martí, 2012.

Proneural genes code for transcription factor proteins that bind DNA through their basic domain and heterodimerize with ubiquitously expressed bHLH proteins, known as E proteins, through the HLH domain. Since this interaction is instrumental for DNA binding, there are several factors that act at this level as passive inhibitors of proneural function. Some of these regulators are HLH proteins that lack the basic domain, known as Id proteins. Hence, they do not bind DNA but sequester the available E protein, rendering proneural proteins inactive (Wang and Baker, 2015).

Functional proneural - E protein dimers bind DNA sequences that contain a core hexanucleotide motif, CANNTG, known as E-box, where NN are variable nucleotides (Bertrand *et al*., 2002). In general, all proneural genes are able to bind the consensus E-box sequence and activate target gene transcription, which would largely explain why different proneural genes initiate a common neurogenic

program. This program consists in the promotion of cell cycle exit, neuronal differentiation and maturation, plus non-cell-autonomous progenitor maintenance through the control of the Notch signalling pathway (Bertrand *et al*., 2002; Ross *et al*., 2003; Wilkinson *et al*., 2013; Imayoshi and Kageyama 2014). In the following sections, we will go through each one of these functions in more detail.

1.3.1. Proneural genes and the Notch pathway

The function of Notch signalling in neurogenesis is to single-out neuronal precursors from an equipotent field of neural progenitors confined within the neuroepithelium. This singularization is based on cell-to-cell communication: the signalling cell, which displays the ligand in its membrane, signals to neighbouring cells, which expresses the Notch receptor also in the cellular membrane. In mammals, we find four Notch paralogues (Notch1 - 4) and various ligands in the Delta-like (DLL1, DLL3, and DLL4) and Jagged (JAG1 and JAG2) protein families. However, only Delta-like ligands have been described to be involved in neurogenesis (Bray, 2016).

The singularization of neuronal precursors occurs through the process denominated lateral inhibition, which operates as follows. Proneural genes, which start to be expressed in neural progenitors, induce the expression of Delta ligands by direct transcriptional activation (Seo *et al*., 2007; Castro *et al*., 2011). The binding of Delta induces a change in the conformation of the Notch receptor, which exposes a specific site that is cleaved by the activity of the metalloprotease ADAM10. Cleavage renders the remaining transmembrane intracellular fragment a substrate for the γ-secretase complex, which catalyses intramembrane proteolysis to release the Notch intracellular domain (NICD). NICD translocates into the nucleus and associates with the DNA-binding protein CBF1 (also known as RBPJ) and the coactivator Mastermind (MAM) to activate gene transcription (Bray, 2016). Among all Notch targets, *hes/her* genes play a major role in neurogenesis. They encode for bHLH transcription factors that, unlike proneural genes, act as transcriptional repressors. More specifically, Hes/Her proteins homodimerize and bind to specific DNA sequences known as C, E or N-boxes

and inhibit proneural gene transcription by the recruitment of corepressors (Ross *et al*., 2003; Imayoshi and Kageyama, 2014).

As a result, progenitors with high and sustained proneural activity and, consequently, high Delta expression, which will be engaged into the neurogenic program surrounded by progenitor cells with high Notch activity that will remain as cycling progenitors in the VZ (Figure 1.5). The outcome of the lateral inhibition process is made evident at the tissue level, as proneural genes and Notch-dependent *hes/her* genes are expressed in the same neurogenic domains but in a salt-and-pepper pattern, as observed in *Drosophila* proneural clusters.

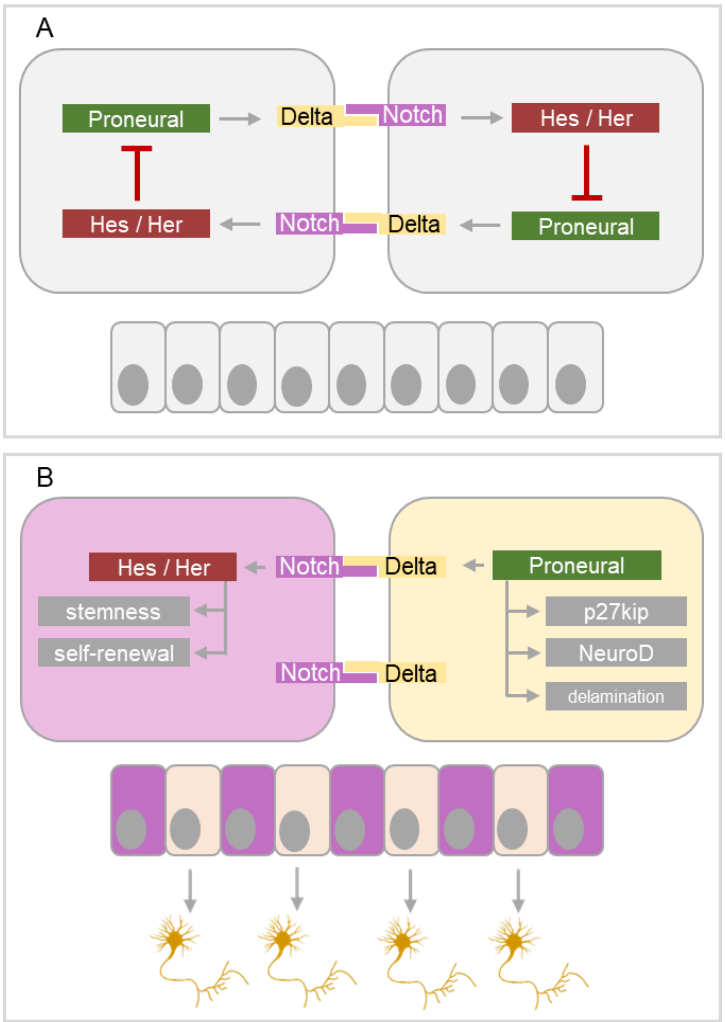

Figure 1.5. Proneural genes control neuronal commitment through Notch signalling. A) In a field of equipotent and proliferative neural progenitors (grey), Notch signalling maintains a state of symmetry between cells. Through lateral inhibition, a regulatory loop takes place between the cells, involving the upregulation of Delta expression via proneural gene and downregulation of proneural gene expression via Notch signalling pathway. As a result of the symmetric bidirectional signalling, neuronal commitment does not occur. **B**) Proneural gene expression is induced at a high level in the neurogenic progenitor (orange), which initiates a programme that leads to neuronal differentiation. In neighbouring cells (purple), Notch signalling represses the expression of proneural genes, resulting in the maintenance of progenitor features such as proliferative self-renewing and expansion.

However, in the light of more recent research, salt-and-pepper expression pattern of proneural and *hes/her* genes is most likely to be a snapshot view of a dynamic process of the oscillatory expression of these factors in the same cell rather than a product of a fixed, sustained expression of mutually exclusive factors.

For example, in the telencephalon of mouse embryos, *hes1* represses its own promoter through N-boxes in a negative feedback mechanism, which associated with short-lived transcript and protein, results in autonomous oscillations with 2-3h period in radial glial cells. Since *hes1* inhibits proneural expression, this oscillatory behaviour causes the oscillation of proneural genes like *neurog2* and *ascl1* as well in an opposing manner (Figure 1.6A), which in turn causes the oscillation of Delta ligands in a symmetric loop (Shimojo *et al.*, 2008; Imayoshi *et al*., 2013; Shimojo *et al*., 2016).

Still in the mammalian cortical context, *ascl1* and *neurog2* switch their mode of expression from oscillatory to high and stable at the onset of neuronal production, which suggests that neurogenic engagement occurs as a consequence of breaking the oscillation. On the other hand, the oscillatory expression enables cell cycle progression and proliferation. Thus, proneural gene expression is not necessarily indicative of differentiation, as it could just reflect a peak on the oscillation (Shimojo *et al.*, 2008; Imayoshi *et al*., 2013).

Eventually, the symmetry will be broken and the neural progenitor cell will select a fate: sustained proneural expression will lead to neuronal differentiation, whereas proneural downregulation due to active Notch signalling will keep the neural progenitor in an undifferentiated state, which will eventually differentiate into glial cells (Figure 1.6B). However, the mechanism behind the symmetry break and hence, cell fate selection, is still unknown.

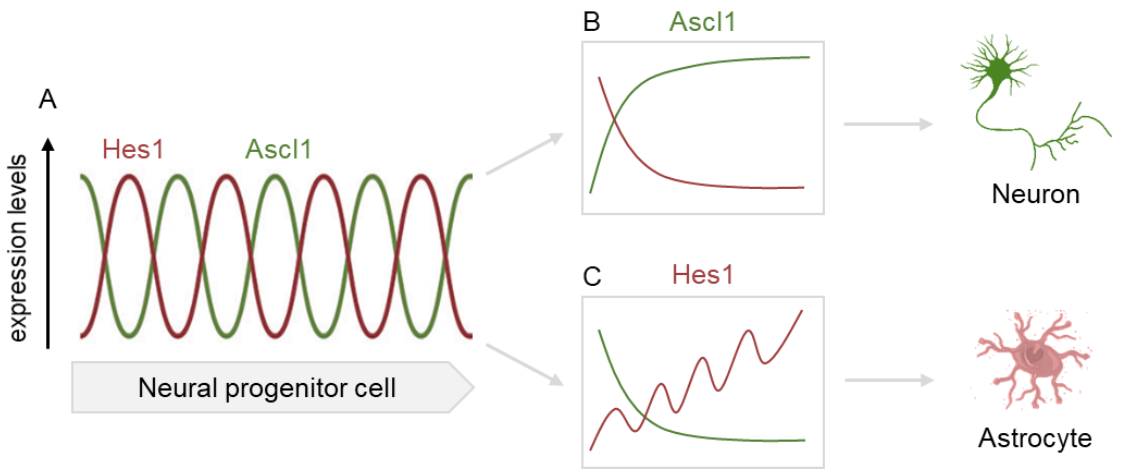

Figure 1.6. Expression dynamics of bHLH factors in multipotency and cell fate choice. **A**) Hes1 and Ascl1 oscillate in neural progenitors with a 2-3h period. **B**) Sustained expression of Ascl1 leads to neuronal differentiation. **C**) Low levels of Ascl1 allows Hes1 protein to accumulate, which will keep the neurogenic progenitor as a cycling progenitor that will eventually give rise to glial cells after the neurogenic wave. Adapted from Vasconcelos and Castro, 2014 and Imayoshi and Kageyama, 2014.

1.3.2. Proneural genes and the progression of neurogenesis

Neurogenesis involves three sequential steps: cell cycle arrest, apical detachment and migration, and terminal differentiation. Proneural gene expression starts to be downregulated once neuronal precursors detach from the VZ as observed in the telencephalon, the thalamus and the cerebellum in mouse embryos (Fode *et al*., 2000; Ben-Arie *et al*., 1997). Therefore, in order to carry on with neuronal differentiation, there must be a sequence of downstream activated genes that maintain the initiated transcriptional program.

In vertebrates, *neurod1* and *neurod4* are the main neuronal differentiation genes downstream of proneural genes. They are very similar to proneural genes in sequence and structure, as they also are bHLH transcription factors with high homology within the bHLH domain of proneural genes (Bertrand *et al*., 2002). Nevertheless, since they act under the transcriptional control of proneural genes, they are a step below in the hierarchy of transcription factors regulating neurogenesis. Therefore, we expect to find proneural genes expressed in proliferative neural progenitors, whereas neuronal differentiation genes will be

expressed in neuronal precursors with no mitotic capacity. Accordingly, the *neurod* genes are expressed in a multitude of contexts in the vertebrate developing CNS and PNS and they are always expressed under the control of proneural function (Fode *et al*., 1998; Park *et al*., 2003; Mattar *et al*., 2004; Schuurmans *et al*., 2004; Bae *et al*., 2006; Seo *et al*., 2007). However, how functionally distinct are proneural and neuronal differentiation genes?

Like proneural genes, ectopic expression of neuronal differentiation genes promotes neuronal differentiation, as demonstrated in early gain-of-function studies in *Xenopus* embryos and cultured mammalian cells (Lee *et al*., 1995; Farah *et al.*, 2000). Moreover, *neurog1* and *neurod1* can bind to the same E-box sequences in common target genes, as demonstrated during *Xenopus* primary neurogenesis, which would explain the ectopic expression phenotype (Seo *et al*., 2007). Loss-of-function studies in zebrafish sensory placodes, as well as in the zebrafish neural tube, suggest that *neurod1* and *neurod4* can function, to some extent, independently from proneural function and can account for neuronal differentiation. Thus, although classically hierarchized, proneural and neuronal differentiation genes cooperate in the same neurogenic processes in order to ensure the robustness of the neurogenic system (Sapede *et al*., 2012; Madelaine *et al*., 2011; Park *et al*., 2003; Halluin *et al*., 2016).

In summary, proneural genes act like cell fate determinants that choose a neuronal fate over a glial fate, while neuronal differentiation genes are expressed later in time and maintain the differentiation program initiated by proneural genes, which involve cell cycle arrest, apical detachment and neuronal migration.

a) Promotion of cell cycle exit

Neural progenitors progress through the cell cycle in order to proliferate. However, neuronal differentiation occurs concomitantly with cell cycle exit into the quiescent G0 phase. Therefore, there must be a link between cell cycle arrest and neuronal differentiation.

Indeed, proneural genes are required for the expression of inhibitors of cell cycle progression like p27Kip (Farah *et al*., 2000; Castro *et al*., 2006). This factor is a

cyclin-dependant kinase inhibitor (cdki) that has been shown to interact and stabilize the *neurog2* protein, as an example of mechanistic coupling between neuronal differentiation and cell cycle arrest (Nguyen *et al*., 2006). Moreover, p27Kip has also been reported to be a part of the repressive complex on the *sox2* promoter (Li *et al*., 2012), indicating an active role in downregulating progenitor maintenance and promoting neurogenic differentiation. Nevertheless, upregulation of cdkis is likely to be indirect, since the transcriptional profiles of cells overexpressing proneural genes do not identify cdkis as direct downstream targets of proneural proteins (Castro *et al*., 2011; Seo *et al*., 2007).

These very same studies and others reveal non-canonical functions of proneural genes in promoting proliferation and antagonizing neurogenesis in a context-dependent manner, such as *neurog1* in the dorsal telencephalon (Han *et al*., 2018) and *ascl1* in the ventral telencephalon and in cultured NSCs (Castro *et al*., 2011). Although this apparent dual function might be explained by the oscillatory mode of expression previously reviewed, these findings stress the need to consider developmental time in order to fully understand proneural function in the generation of neuronal lineages.

b) Progenitor delamination and migration

Proneural genes are involved in neuronal precursors' delamination and migration, as some of their targets regulate many steps of these processes. For example, in the vertebrate spinal cord, *neurog2* function is required for N-cadherin downregulation through the activation of the transcription factor *foxp2*, allowing apical detachment (Rousso *et al*., 2012; Das and Storey, 2014). Similarly, in the mammalian forebrain, proneural genes promote progenitor delamination by activating transcription factors of the Scratch family, which downregulate E-cadherins (Itoh *et al*., 2013). After delamination, neurons migrate in order to integrate into functional circuits. This is a phenomenon well studied in the developing cortex, where late-born neurons migrate through the basal process of radial glial cells to reach the outer layers. Thus, in the mammalian forebrain, *neurog1, neurog2* and *ascl1* control neuronal migration through the transcription

regulation of target genes that include the Rho GTPases *rnd2 and rnd3* (Heng *et al*., 2008; Pacary *et al*., 2011).

1.3.3. Proneural genes and the specification of neuronal identity

Up to know, we have been discussing the mechanisms by which proneural function engages neural progenitors into neuronal differentiation, which are common for all proneural genes. However, as beautifully characterized in the vertebrate spinal cord, proneural genes delimitate different progenitor populations that will give rise to specific neuronal types (Figure 1.5). Proneural-linked neuronal specification occurs by the regulation of DNA-binding specificity for lineage-specific target selection, the cooperation with factors that provide with spatiotemporal context and the cross-regulation of proneural activity.

Previous evidence in *Drosophila* showed that different proneural gene / E protein dimers display differential binding preference for the two variable nucleotides in the E-box sequence, as well as the flanking nucleotides (Powell *et al*., 2004). Indeed, more recent ChIP-seq based studies revealed the specific E-box based motifs for proneural *atoh1*-binding in the murine cerebellum and spinal cord, *ascl1*-binding in the murine telencephalon, and *neurog1*-binding in *Xenopus* and zebrafish (Lai *et al*. 2011; Klisch *et al*., 2011; Castro *et al*., 2011; Seo *et al*., 2007; Madelaine and Blader, 2011). Thus, each proneural gene bears preference for specific *cis*-regulatory sequences, which might be regulating lineage-specific target gene selection.

In addition, the selection of cell lineage-specific targets must occur in coordination with spatiotemporal cues, as different neuronal lineages arise at specific spatiotemporal coordinates. In this sense, proneural proteins are able to interact with patterning and HD neuronal transcription factors, which allow them to interpret the specific spatiotemporal context and generate the suitable kind of neuron. As a reminder, patterning proteins provide with positional identity to neural progenitors, whereas HD neuronal proteins are involved in neuronal differentiation by controlling axon path-finding, neurotransmitter properties and circuit assembly in newly differentiated neurons (Guillemot, 2007; see 1.2.1, Figure 1.2).

These cooperative relationships can occur in different ways. First, patterning and proneural proteins can interact indirectly by activating their own sets of transcriptional targets, being both necessary for the specification of a given neuronal identity, as would be the case for *olig2* and *neurog2* in the production of motoneurons in the ventral spinal cord (Sugimori *et al*., 2007). Secondly, proneural and HD neuronal proteins can bind to distinct sites in the promoter of a common target gene and synergistically activate its expression, as described for *neurog2/neurod4* and *lhx3/isl1* in motoneuron generation in the spinal cord (Lee and Pfaff, 2003). As a third kind of interaction, proneural and neuronal proteins can act as cofactors taking part in the same transcription-activator complex that bind to the enhancer of target genes, as reported for Ascl1 and Brn3 proteins on the *delta1* enhancer (Castro *et al*., 2006). This last observation highlights that, apart from the *cis*-regulation based on E-box preferences, target selection is also regulated by context-dependent cofactors. In this sense, recent research suggests that the ubiquitously expressed class II bHLH factors, the E proteins, also play a role in selecting cell lineage-specific targets and regulating neurogenic capacities (Flora *et al*., 2007; Le Dréau *et al*., 2018, see 1.6.3 for the *atoh1* specific example).

To finish, proneural genes are able to cross-regulate each other in order to refine their domains of expression, as well as to ensure neuronal specification. In this sense, loss of proneural function does not usually result in a defect of neuronal production, but rather causes a change in neuronal subtype. For example, in the dorsal spinal cord in chick and mouse embryos, loss of *atoh1* causes *neurog1* expression to be dorsally-expanded, compensating for neuron production but changing neuronal fate (Gowan *et al*., 2001). A similar mechanism occurs between the posterior hindbrain and the anterior spinal cord, where *neurog3* establishes the posterior boundary of the serotonergic system by actively suppressing serotonergic specification in the spinal cord and favouring glutamatergic differentiation (Carcagno *et al*., 2014). Thus, proneural genes ensure fate selection by two major principles: the activation of cell linage-specific transcriptional programs and the cross-regulatory activity that represses the expression of neighbouring proneural genes.

As a concluding remark, proneural genes are pivotal factors not only because they drag progenitors out of cycle and commit them to neuron production, but because they act as an integrative hub that ultimately selects the neuronal type that is best suited for a specific set of spatial coordinates within the CNS (Powell *et al*., 2008). Therefore, when aiming to unveil how a particular neuronal lineage chooses its fate, analysing proneural function is crucial.

1.4. COUPLING FATE DETERMINANTS WITH PROGENITOR DYNAMICS

The genetic regulators and signalling pathways governing progenitor specification and differentiation have been well characterized. The neural tube ends up regionalized in different progenitor populations defined by the combinatorial expression of patterning, specification and differentiation genes. However, how is the pattern generated by these transcriptional factor codes maintained when challenged by cellular events such as proliferation or differentiation? Are there any signals coordinating progenitor dynamics with tissue patterning? In other words, how is cell specification coordinated with tissue growth and differentiation?

The main cellular events that influence tissue architecture in the vertebrate CNS are proliferation, mode of division and neuronal differentiation that we put under the umbrella term of “progenitor dynamics”. Apoptotic events have also been reported to occur, but at negligible levels (Cayuso *et al*., 2006; Kicheva *et al*., 2014). Then, balancing the rate of differentiation and proliferation during CNS development is of key importance in order to ensure the production of the necessary type and number of neurons, as well as allowing tissue growth. Progenitor proliferation adds new cells, whereas differentiation eliminates cells from the progenitor cell population. In this sense, mode of division rises as the key balancer between tissue growth and neuronal production. As such, any signals regulating these events are expected to be very important in coupling tissue pattern with progenitor cell dynamics.

1.4.1. Mode of division regulation

Numerous works have focused in understanding what are the intrinsic cellular factors regulating the mode of division in neurogenic progenitors, such as centrosome asymmetry, inheritance of membrane determinants and mitotic spindle orientation (Das and Storey, 2012; Ghosh *et al*., 2008; Lesage *et al*., 2010). However, since mode of division ultimately balances progenitor proliferation and neuronal production, it must be tightly coordinated in space and time at a tissue scale.

A remarkable example of this tight regulation occurs in the pMN domain of the ventral spinal cord in chicken embryos. During the early phase of progenitor specification, when Shh signalling is active in the ventral pole, cells divide symmetrically giving rise to two daughter cells with the same progenitor potential (PP divisions). However, as the structure grows, progenitors of the pMN domain switch their mode of division progressively from PP divisions to asymmetric PN (progenitor and neuron) divisions to eventually symmetric neurogenic divisions (NN) (Saade *et al*., 2013). Interestingly enough, the switch in the mode of division coincides in time with the peak of Shh activity. Maintenance of Shh activity beyond this point prevents the switch in the mode of division from happening. Thus, Shh maintains progenitors in a PP dividing fashion.

Dorsally derived signals, such as BMP, have also been described to be regulating mode of division in the chick spinal cord, as its effectors' activity is required for maintaining PP divisions during the generation of spinal interneurons (Le Dréau *et al*., 2014). The different extrinsic signals regulating mode of division at distinct domains of the spinal cord highlight the necessity to elucidate the molecular mechanisms regulating this context-dependency, as well as the identification of common downstream effectors regulating progenitor maintenance.

1.4.2. The differentiation rate patterns the tissue

As mentioned before, the different modes of division challenge tissue pattern, since the addition or removal of progenitor cells during proliferation and differentiation could change the proportion of the different progenitor populations specified by cell fate determinants.

How these cellular processes change tissue pattern has been investigated within the different DV domains of the murine spinal cord. It has been proposed a two-phase model for tissue growth that explains the differences in pattern proportion as development proceeds. First, there is an initial phase where morphogen gradients activate the gene regulatory networks necessary for progenitor specification. During this early phase, progenitor specification and recruitment into the different DV domains is the main factor shaping the tissue pattern. As the neural tube grows in size, diffusive molecules no longer account for progenitor specification since they cannot reach their target cells. Secondly, the tissue enters a phase where the differentiation rate of neurogenic progenitors is the one shaping the pattern within the spinal cord. Proliferation rates are constant throughout the whole DV longitude of the structure. However, the pMN generates neurons at a higher rate than the rest of the progenitor populations. Not only that, but pMN clones grow with no preferential direction, when the rest of spinal cord clones grow preferentially in the DV axis (Kicheva *et al*., 2014; Kicheva and Briscoe 2015; Zagorski *et al*., 2017). This work highlights a key observation that needs to be taken into account in order to understand the generation of neuronal lineages during CNS development: pattern changes with time due to differences in differentiation rate and clonal growth directionality. Although the authors propose that different proneural genes might be promoting different differentiation rates along the spinal cord DV territories, the direct cause of such differences, especially in growth directionality, is unknown.

1.4.3. Cell shape and tissue packing regulate neurogenesis

Other factors that influence the differentiation rate of progenitor cells are cell shape and tissue packing. The nuclei of neural progenitors change position in

coordination with the cell-cycle phases, in a dynamic process known as inter-kinetic nuclear migration (INM). This influences the overall shape of the neural cells and, as a consequence, the overall tissue architecture. When in mitosis, progenitors round up in the apical surface of the VZ and generate two daughter cells. This “rounding up” perturbs cellular shape on neighbouring cells, pushing their cell bodies towards more basal positions. Thus, decisions on the mode of division might depend on tissue restrictions due to progenitor or neuronal crowding.

Indeed, cell shape and tissue packing regulate neurogenesis in an interesting feedback mechanism, as demonstrated in the zebrafish spinal cord: they observed that progenitors with cell bodies biased towards the basal pole were more prone to differentiate than progenitors closer to the apical pole (Hiscock *et al*., 2018). Accordingly, when apical crowding of mitotic progenitors was ectopically induced neurogenesis was promoted in the surrounding, basally-displaced cells. Therefore, inducing neuronal differentiation in neighbouring cells is a way to empty the neuroepithelium and leave space for cellular divisions to occur when they are occurring at high numbers. When the number of mitoses is low, progenitors’ cell bodies have enough room to remain close to the apical side and neurogenesis is then slowed down. Although it is yet to be completely elucidated, Notch signalling has been proposed to regulate this feedback mechanism and mediate between cell shape and neurogenesis. It has been proposed that neural progenitors contain an NICD gradient from apical to basal. This is due to the presence of the Notch receptors and ligands to be restricted to the cellular apical surface. Then, the released NICD is more likely to reach the nucleus if the nucleus is positioned closer to the apical side than the basal side (Del Bene *et al*., 2008; Aggarwal *et al*., 2016). This would explain why neuronal differentiation depends on the distance between cell body and apical surface.

However, there are other ways in which cell shape and Notch signalling might be impacting neurogenesis. For example, the size of the apical contact between the cell and the lumen of the neural tube (from now on apical foot) is crucial, since it is within the apical foot where cell-to-cell contacts occur. Hence, the bigger the apical foot, the more Delta-Notch interactions will be established and more active

NICD will be present within the cell, preventing it from differentiating. Accordingly, cells basally-displaced present a smaller apical foot due to the membrane requirement (Hiscock *et al*., 2018).

The last insights about cell shape and tissue packing regulating differentiation might be just the initial observations of a greater picture, in which not only local cues but general tissue rearrangements like the ones occurring during morphogenesis influence how neuronal lineages acquire fate. Signalling pathways mediated by diffusible signals have been determined key in regulating progenitor modes of division and instructing tissue growth (Saade *et al*., 2013; Zagorski *et al*., 2017). However, what happens to molecular, diffusible cues when changing the shape of the whole structure, and, therefore, cellular location and disposition? Thus, it is imperative that in our studies regarding neuronal lineage specification we take into account not only the transcriptional regulators of cell identity, but also the shape and the disposition of cell neighbours, as well as the morphogenetic movements occurring in the niche tissue where progenitors are being specified. In brief, we need to account for the positional history of cells throughout embryogenesis.

1.5. THE HINDBRAIN AS A MODEL FOR STUDYING NEURONAL LINEAGES.

The generation of diverse neuronal lineages throughout development relies on the segregation of progenitor potentialities in space and time, which is orchestrated by the interplay of intrinsic transcription factor codes with extrinsic signalling cues. The interaction between neural progenitors and the environment generates diverse progenitor identities depending on the spatiotemporal context, which results in the adscription of diverse neuronal phenotypes. However, how is the function of cell fate determinants coordinated with neural progenitor proliferation, differentiation, and, ultimately, tissue morphogenesis?

To understand such fundamental questions, we resorted to the zebrafish embryonic hindbrain. Its segmented nature serves as a long-used strategy in

evolution to generate repeated structures that can act as compartments. Moreover, there are further segregation mechanisms within each segment that allow the disposition of neurogenic and non-neurogenic fields. During early embryonic development, concomitantly with cell fate specification, the hindbrain generates a big, dorsal lumen that impacts progenitor and neuronal position. Thus, the embryonic hindbrain serves as a very interesting playground to unravel the molecular and mechanical mechanisms that generate neuronal diversity and control the balance of progenitor cells versus differentiated neurons.

1.5.1. Hindbrain anatomy, function and basic body plan

The hindbrain or rhomboencephalon is the most posterior vesicle of the embryonic brain and the most conserved among vertebrates (Kiecker and Lumsden, 2005). In mammals, the embryonic hindbrain gives rise to three main adult structures: the cerebellum, the medulla and the pons. These structures function as a relay station for sensory information that modulate several motor behaviours, such as locomotion, posture and facial expression; as well as autonomous functions such as breathing, swallowing, and even REM/non-REM sleep regulation (Bonis *et al*., 2013; Hayashi *et al*., 2015; Hernandez-Miranda *et al*., 2017). However, how is this functional diversification achieved during hindbrain embryogenesis?

During development, the vertebrate hindbrain relies on the process of segmentation or compartmentalization, which is an important developmental mechanism for generating iterative units that respond to axial patterning signals to create regional diversity. The separation of adjacent cell populations in an underlying segmental ground plan enables their independent divergence along distinct developmental pathways, a concept known as metamerism (Parker *et al*., 2016).

The embryonic hindbrain is transiently segmented along the into different 7-8 segments known as rhombomeres (r1-r8; Figure 1.7). Each one of these segments is a unit of gene expression, meaning that they display a unique gene transcriptional signature that confers a given molecular identity to the segment (Figure 1.7A; for review see Kiecker and Lumsden, 2005). They are also cell

lineage-restricted compartments, as cells confined within a rhombomere do not cross to adjacent rhombomeres, as demonstrated in chick and mouse embryos (Fraser *et al*. 1990; Jimenez-Guri *et al.*, 2010).

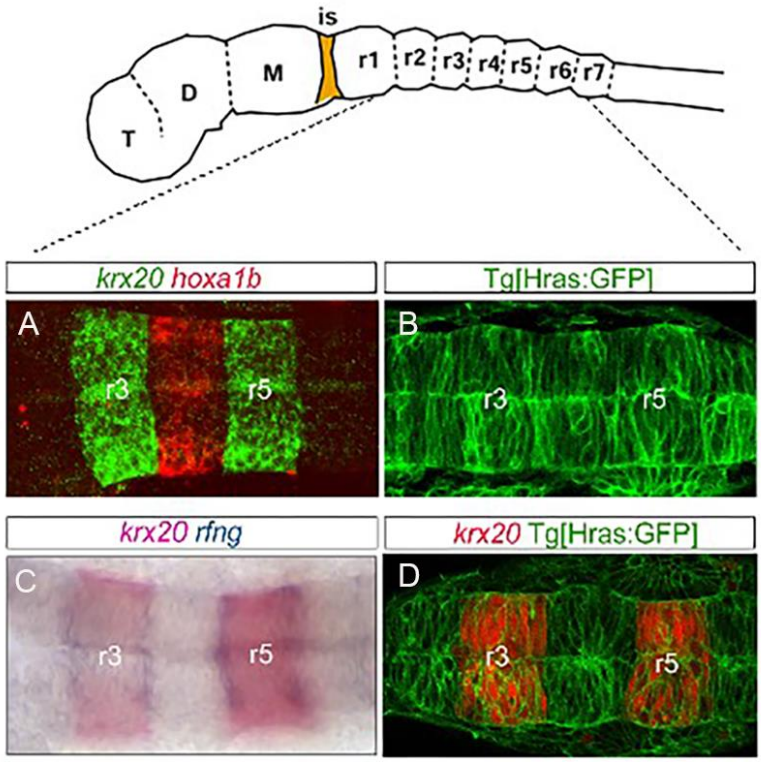

Figure 1.7. Rhombomeres constitute units of gene expression. Upper scheme depicts the embryonic brain (T, telencephalon; D, diencephalon; M, mesencephalon; r, rhombomeres). **A**) Fluorescent *in situ* hybridization for *krx20* (green) and *hoxa1b* (red) displaying non-overlapping rhombomere-restricted expression as observed with sharp interfaces of gene expression. **B**) Transgenic embryo displaying GFP in the plasma membrane. Hindbrain segmentation results in the appearance of morphological bulges that correspond to rhombomeric segments. **C**) *In situ* hybridization of *kox20* (red) and *rfng* (blue). Note that at the interface of two adjacent rhombomeres the boundary cell population (blue) is specified. **D**) Fluorescent *in situ* hybridization for *krx20* (red). Note that the borders of *krx20* expression coincide with morphological bulges. Cell membranes are seen in green. All panels show flat-mounted embryos with anterior to the left.

However, even though different rhombomeric cell lineages do not intermingle during neurogenesis in the proliferating VZ, post-mitotic neurons of the MZ are able to cross rhombomere boundaries during programmed neuronal migration (Wingate and Lumsden, 1996). This indicates that cell-tight boundaries might

only be required in proliferating cell populations with labile cell fates that are still subject to specification. Hence, positional restriction is likely to become dispensable for post-mitotic cells, as their fates are already specified (Kiecker and Lumsden, 2005).

Segmental patterns of proliferation and neurogenesis generate reiterated populations of neurons, which differentiate in a rhombomere-specific manner (Wingate and Lumsden, 1996). This is exemplified by reticulospinal neurons (RSNs) in zebrafish, which differentiate early during hindbrain development and retain their segmental pattern long after morphological segments have disappeared. Moreover, RSNs are found in similar numbers in each rhombomere, but their specialization in morphology and function is unique for each segment (Moens *et al*., 2002; Figure 1.8). Thus, segmentation is involved in the establishment of fundamental neuronal identity and connectivity between the hindbrain, other brain centres and the periphery. Disruption of segmentation results in neuronal patterning defects, such as fusion of motor nuclei and loss of specificity in target innervation (Guthrie, 2007; Parker *et al*., 2016).

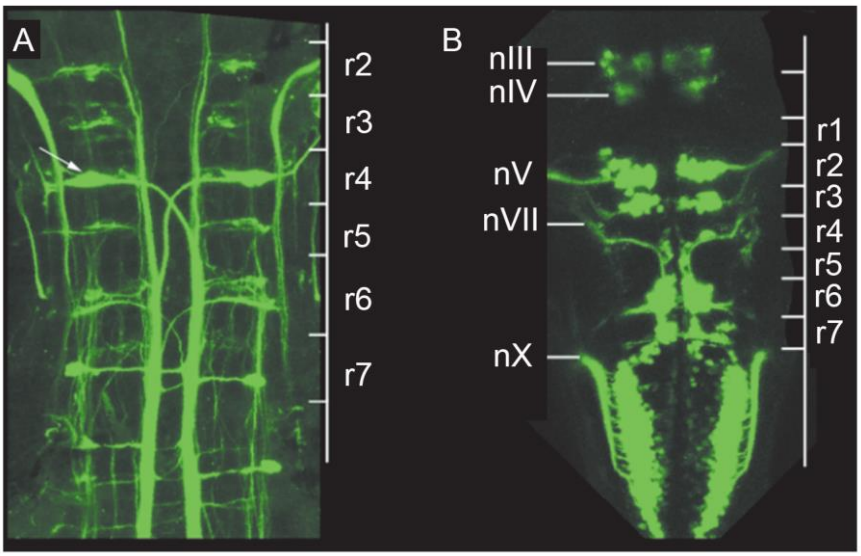

Figure 1.8. Cranial neuroanatomy reflects the segmental organization of the zebrafish hindbrain. **A**) The individually identified RSNs are organized in a ladder-like array along the AP extent of the hindbrain. The arrow points the large bilateral Mauthner neuron in r4 with its contralateral projections. **B**) The branchiomotor neurons of the cranial nerves (n) also have a rhombomere-specific disposition revealed in live embryos by using the transgenic line Tg[isl1:GFP], which drives GFP expression to motoneurons. Anterior is to the top. Adapted from Moens *et al*., 2002.

What are the molecular mechanisms underlying hindbrain segmentation? In early vertebrate CNS development, the prospective hindbrain becomes progressively partitioned into rhombomeric compartments by the restricted expression of transcription factors along the AP axis. The expression of these transcription factors is promoted by the interplay of Fibroblast Growth Factor (FGF) and Retinoic Acid (RA) opposing gradients, which are generated from the anterior MHB and the ventral hindbrain and anterior presomitic mesoderm, respectively. In zebrafish, the action these morphogen gradients initiate the expression of patterning genes, which are *krox20*, *vhnf1*, *mafb*, *iro7*, *cdx1* and PG1 *Hox* genes (Hernandez *et al*., 2004; Sadl *et al*., 2003; Lecaudey *et al*., 2004; Skromne *et al*., 2007; Choe *et al*., 2004).

Following the establishment of *krox20* and *mafb* rhombomere-restricted expression (r3 and r5; and r5 and r6, respectively), further downstream *Hox* genes of the PG1-4 groups start to be expressed. The *Hox* PG1-4 gene expression domains prefigure and respect rhombomeric territories, with each rhombomere expressing a different combination of *Hox* genes, thus providing a mechanism for specifying unique segment identities and providing with AP positional information to neural progenitors confined within each segment (Parker *et al*., 2016).

In all, through the interaction between signalling gradients, cross-regulatory activities and positive feedback loops, the initial domains of expression that present fuzzy interfaces are sharpened into rhombomere-restricted territories, which correspond with morphological bulges (Figure 1.7).

Downstream of the AP determinants, there are further mechanisms reinforcing the sharpening of rhombomeric interfaces. For example, Eph and ephrins are expressed in complementary rhombomeres, regulating cell sorting by dictating differential cell adhesion properties between different rhombomeres (Xu *et al*., 1999). In zebrafish, the hindbrain BCP generate actomyosin cables that serve as an elastic mesh to generate tension into the BCP to prevent cell intermingling when boundaries are challenged by cell divisions (Calzolari *et al*. 2014, Letelier *et al*., 2018). The tension generated by the actomyosin cable in the BCP is

sensed by the mechano-transducers YAP/TAZ, which form a transcription-activator complex with TEAD and keep the BCP in a proliferative state when the rest of the hindbrain is actively engaged into neurogenesis (Voltes *et al*., 2019). Thus, hindbrain segmentation is a paradigm of the interaction between molecular and mechanical cues that shape the tissue and instruct cell fate.

1.5.2. Hindbrain morphogenesis and tissue architecture

Morphogenesis can be defined as the generation of form of tissues and organs. During embryonic development of the CNS, the brain primordium converts from a simple tubular structure into a highly convoluted brain. Developmental neurobiologists have recognized that mechanics must be playing a major role when generating brain shape. However, the physical mechanisms of brain morphogenesis remain poorly understood. In the case of the hindbrain, the two main morphogenetic events occurring at early stages of embryonic development are tissue segmentation and lumen formation. Since hindbrain segmentation has already been discussed, in this section we will focus on the generation of the fourth ventricle and its impact on neurogenesis.

Ventricle development is a late stage of the conserved morphogenetic process of neurulation that produces the lumen of the brain and the spinal cord. Neurulation begins as the neural plate transitions into the neural tube. During this process, cell movement and rearrangement is coupled with regulated growth and patterning of the tissue. In amniotes, neurulation occurs through the uprising of lateral neural folds that meet at the dorsal midline to form the hollow neural tube (Lowery and Sive, 2004). However, in teleost fish such as the zebrafish, neurulation occurs by the transient generation of the neural keel, as the left and right sides of the neural plate converge towards the dorsal midline to form the neural keel without a clear midline (Kimmel *et al*., 1994; Papan and Campos-Ortega, 1994). This transient stage is followed by the formation of the neural rod, in which cells become more organized and start to express junctional complexes (Geldmacher-Voss *et al*., 2003). After the formation of a clear midline at the neural rod stage, the lumen of the hindbrain starts to form.

A specific characteristic of the hindbrain is its large and dorsally located lumen called the fourth ventricle. The fourth ventricle is covered by a roof plate that is induced from the lateral edges of the neural tube and expands dorsally to form a single-cell layer membrane (Chizhikov and Millen, 2004). In zebrafish, lumen formation is initiated at early stages by the generation of an epithelial seam at the midline of the neural rod, followed by a rapid opening of the ventricle as right and left sides of the rod pull apart to produce a hollow neural tube that is covered by the dorsal roof plate (Tawk *et al*., 2007; Campo-Paysaa *et al*., 2019; Figure 1.9, white arrows). The roof plate seems to be instrumental for hindbrain lumen formation, as *zic1* and *zic4* zebrafish mutants, which display a complete loss of roof plate epithelium, do not generate the fourth ventricle (Elsen *et al*., 2008). Therefore, the roof plate acts as a local organizer that is involved in both morphogenesis and the induction of dorsal cell fates.

In zebrafish, the first opening point of the tube appears at 18hpf between r1 and the midbrain-hindbrain boundary (MHB). As development proceeds, other opening points start to appear more posteriorly and at the level of rhombomeric boundaries (Gutzman and Sive, 2010; Campo-Paysaa *et al*., 2019; Figure 1.9C). As a result of the sequentially appearing opening points, the ventricle grows bigger and it displaces the dorsal pole of the hindbrain away from the midline and towards the lateral side. More ventral regions of the hindbrain now become exposed to the ventricle, as apical feet of neural epithelial cells become visible (Figure 1.9G).

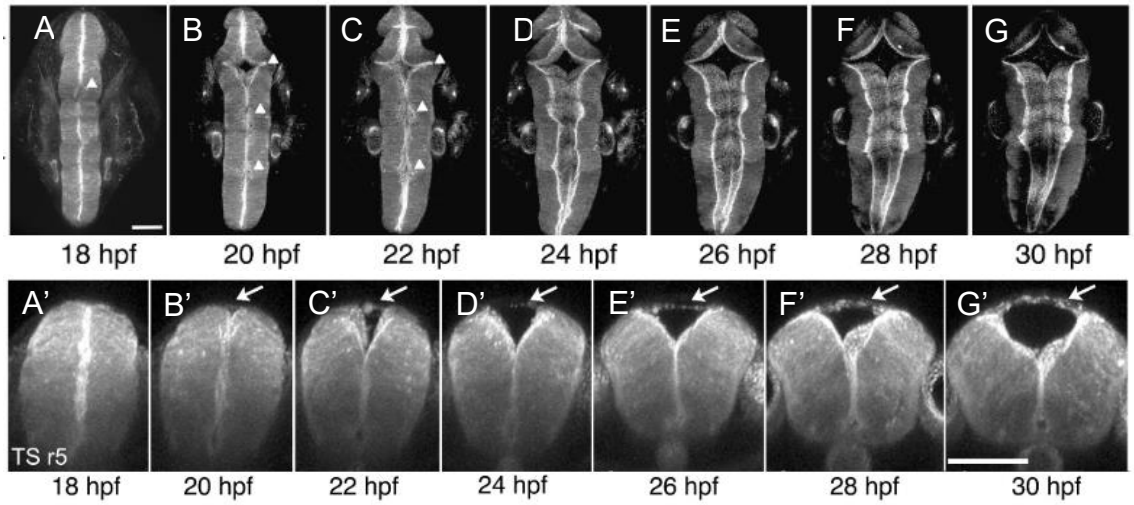

Figure 1.9. Morphogenetic progression of the formation of the fourth ventricle. A-G) Selected frames from time-lapse of Tg[Cdh2:(Cdh2-tFT)] embryo tracking opening and expansion of fourth ventricle from 18-30hpf. White arrowheads on photomicrographs indicate the three points at which widening commences. Roof plate widening is initiated in r2, 4, 6. Anterior is to the top. **A'-G'**) Transverse optical reconstructions of equivalent panels above at the level of r5. White arrows indicate the roof plate epithelium. Dorsal is to the top. Scale bars correspond to 50 µm. Adapted from Campo-Paysaa *et al*., 2019.

Morphogenesis is, essentially, the generation of shape. Hence, in order to shape the tissue, mechanical forces are expected to be involved. In this case, we have two sources of opposing forces: the cerebro-spinal fluid (CSF) flow and the tension of the neuroepithelial wall. Pressure exerted by the CSF is necessary for lumen formation, as demonstrated in zebrafish mutants lacking CSF production (Lowery and Sive, 2005). In turn, there is a certain degree of tension exerted by neuroepithelial cells that is needed to oppose CSF pressure. However, if this tension increases over a certain degree, the lumen will not be generated either. This is demonstrated in zebrafish, as mutants of *mypt1* myosin II phosphatase, an activator of actomyosin structures, show aberrant cell shape and increasing tension in neural progenitors. This leads to defects in neural tube opening (Gutzman and Sive, 2010). Hence, CSF flow, accompanied by epithelial relaxation mediated by cell shape, maintain the proper force equilibrium in order to ensure the formation of the fourth ventricle without compromising epithelial architecture. Nevertheless, the tissue does change its shape overtime, changing the orientation of neural progenitors along the way. Therefore, what are the

consequences of the hindbrain morphogenetic process on neurogenesis and circuit assembly?

As pointed out previously, the opening of the neural tube displaces the most dorsal part of the hindbrain towards the lateral side, while more ventral domains end up exposed to the lumen. Lumen formation occurs alongside extensive neurogenesis, generating a size-increasing MZ that pushes and confines progenitors from the VZ zone even further (Figure 1.10). However, these morphological changes are unequally distributed along the DV of the hindbrain. For example, *atoh1a*-expressing domain is dramatically displaced towards the lateral side (Figure 1.10A-C). In contrast, *neurog1* expression domain becomes exposed to the lumen but remains in close contact with the midline in its ventral half (Figure 1.10D-F). This change will surely impact differently on the location of neurons derived from these progenitor populations.

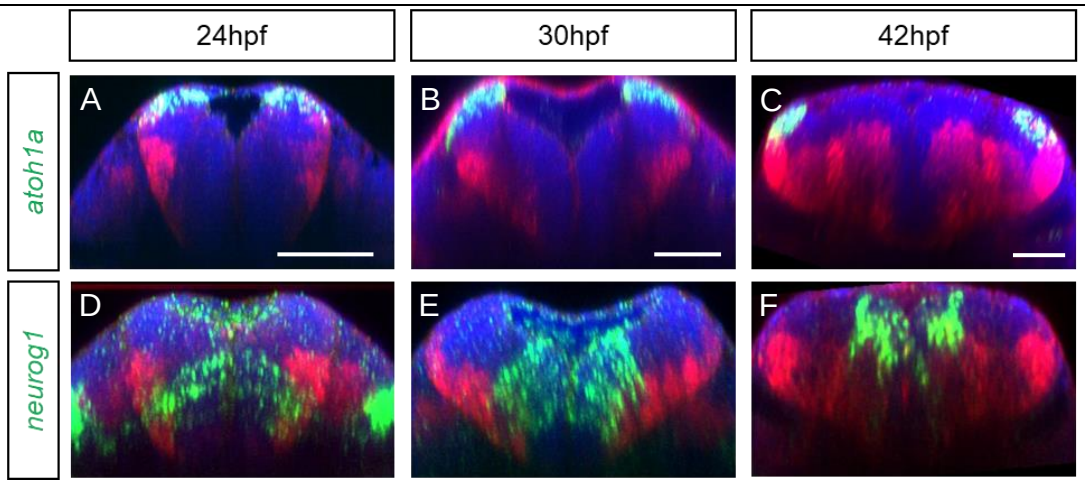

Figure 1.10. Lumen formation shifts the apico-basal axis of hindbrain progenitors. **A-F**) Fluorescent *in situ* hybridizations with *atoh1a* (A-C) and *neurog1* (D-F), both in green in Tg[HuC:GFP] embryos, which display GFP in differentiated neurons of the MZ (red staining). In blue, DAPI nuclear staining. Confocal images were obtained and processed in order to reconstruct the transverse views shown here as maximum intensity projections (MIP). All sections shown are at the level of r4. Dorsal is to the top. Collapsed roof plate is a consequence of staining procedures. Scale bars correspond to 50μm.

In conclusion, the apico-basal axis, which indicates differentiation directionality during neurogenesis, is shifted due to morphogenesis from being horizontally oriented to perpendicular to the midline, ending being almost vertical.

Accordingly, studies on the expression pattern of neurotransmitter phenotypes and HD neuronal proteins in the larval zebrafish hindbrain showed a columnar organization of mature and functional neurons that participate in escape motor-responses mediated by the Mautner cell (Koyama *et al*., 2011). These neuronal columns appear within the DV axis of the hindbrain, organized according to birth date. Each neuron within the DV column participates in the same circuit of motor-escape behaviour. Moreover, neuron position within such columns depends on the time of differentiation: ventrally-located neurons are the first to be differentiated and followed by the most dorsal ones (Kinkhabwala *et al*., 2010). These observations point out that the verticality of the apico-basal axis acquired during ventriculogenesis is favouring the neuronal organization in columns, where neurogenic progenitors generate neurons that “fall” through this axis like droplets on a stalagmite. Nonetheless, the birthdating observations in this study were performed after 2dpf, when ventricle formation is more than complete. To unravel how progenitor position evolves through morphogenesis and during active neuronal production, more detailed cell-lineage tracing analysis are needed and they would provide information on how newly born neurons integrate into the MZ upon differentiation.

1.5.3. Regionalization of hindbrain neurogenesis and progenitor capacities

The vast array of hindbrain neuronal lineages discussed in previous sections is generated from neuronal progenitors that acquire a given fate according to spatial and temporal coordinates and differentiate into neurons. In the embryonic hindbrain there is not a continuous field of differentiating progenitors, but rather a succession of well-organized neurogenic and non-neurogenic territories along the AP axis. Before going into the temporal sequence of hindbrain neurogenesis, we first need to understand the general mechanisms operating in neurogenesis regionalization.

The large-scale organization of the developing CNS into neurogenic versus non-neurogenic zones is a recurring mechanism regulating the specific location of neurogenesis (Corral and Storey, 2001; Bally-Cuif and Hammersmith, 2003). For

instance, neurogenesis does not occur in the midbrain-hindbrain boundary (MHB), nor along the AP axis of the posterior neuroectoderm in zebrafish, where we find in inter-proneuronal domains where neurogenesis is actively supressed (Bae *et al*., 2006; Geling *et al.*, 2003, Ninkovic *et al*., 2005; Figure 1.11).

Formally, these findings coined the terms “proneural cluster” and “progenitor pool” to refer to two types of progenitor populations with different neurogenic properties during the developing CNS of both mice and zebrafish (Baek *et al*., 2006; Shimojo *et al*., 2008, Dirian *et al*., 2014). Hence, proneural clusters are defined by the expression of proneural genes and the Notch targets *hes/her* in a salt-and-pepper manner. Therefore, cells within a proneural cluster are actively engaged into neurogenesis due to the expression of proneural genes and Notch activity directly activates the expression of *hes/her* genes in neighbouring cells in order to prevent them to undergo neurogenesis. Progenitors within these so-called proneural clusters present oscillatory and complementary expression of proneural genes and *hes/her* genes (see 1.3.1).

On the other hand, progenitor pools do not express proneural genes, display a different set of *hes/her* genes in high and stable levels that does not depend on Notch signalling. The expression of these factors prevents neurogenesis by inhibiting the expression of proneural genes (Baek *et al*., 2006; Geling *et al*., 2003). These territories, such as the MHB or the *zona limitans intrathalamica* often act as signalling centres (Kiecker and Lumsden, 2005; Scholpp *et al*., 2009). Thus, inhibition of neurogenesis by Notch-independent *hes/her* genes in certain areas of the developing brain is necessary in order to keep progenitor cells from differentiating. How do these mechanisms apply to zebrafish hindbrain neurogenesis?

In the zebrafish prospective hindbrain, as early as 11hpf, neurogenic regions express the proneural gene *neurog1* (Blader *et al*., 1997), the neuronal differentiation gene *neurod4* (Park *et al*., 2003), and the Notch-dependent *her4* (Bae *et al.*, 2006; Figure 1.11A) in a salt-and-pepper manner, as expected from a Notch-mediated lateral inhibition mechanism (Figure 1.11).

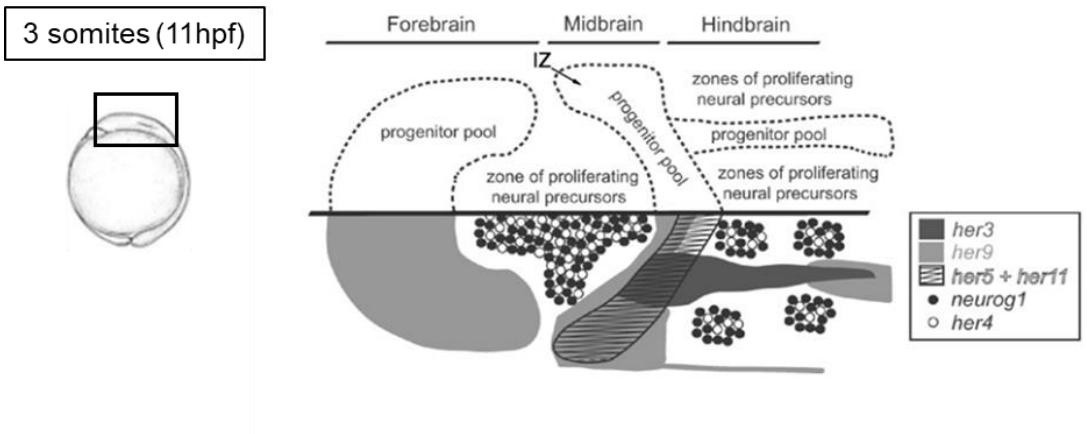

Figure 1.11. Zebrafish primary neurogenesis. Scheme depicting an enlargement of the anterior neural plate area, showing that proneural clusters contain a salt-and-pepper distribution of proliferating neural precursors (*her4*-positive, white dots) and neuroblasts undergoing neurogenesis (*neurog1*-positive, black dots), while progenitor pools are entirely composed of cells that are refractory to neurogenesis and express various combination of the *her* genes such as *her3*, *5*, *9* and *11* (grey scale). Note that half of the neural plate indicate the corresponding progenitor domains, and the other half the genes expressed. Anterior is to the left. Adapted from Stigloher *et al*., 2008.

a) Hindbrain boundary-flanking regions are proneural clusters

Once the neural tube is formed, from early segmentation stages (18hpf) onwards, the proneural genes, *ascl1a*, *ascl1b* and *neurog1* start to be expressed in rhombomeric compartments. These factors are initially expressed in the whole rhombomeric segment but as development proceeds their expression confines to the boundary flanking region. This regionalization generates reiterative neurogenic stripes along the AP axis of the hindbrain and boundaries and rhombomeric centres are devoid of neurogenesis (Amoyel *et al*., 2005; Cheng *et al*., 2004; Gonzalez-Quevedo *et al*., 2010) (Figure 1.12). Accordingly, the expression of ligands and receptors of the Notch pathway, as well as the Notch target *her4* (Nikolaou *et al*. 2009, Figure 1.13) reinforces the idea that neurogenic regions within the hindbrain behave as proneural clusters. Indeed, Notch activity within the hindbrain at these stages controls neurogenesis, as demonstrated in zebrafish *mindbomb* mutant embryos or by Notch-activity inhibition that results in an increase of neurons within rhombomeres (Bingham *et al*., 2003; Nikolaou *et al*., 2009).

Another interesting feature of hindbrain proneural clusters is the expression pattern of the neuronal differentiation gene *neurod4* (Wang *et al*., 2003; Park *et al*., 2003). It is expressed in the same spatiotemporal pattern as proneural genes, as it appears to be confined to boundary-flanking regions. However, *neurod4* expression domain is broader than any of the proneural genes and seems to engulf them all (Figure 1.12E-H; Figure 1.13D). This observation already hints that *neurod4* might be acting downstream of proneural genes as a common trigger of differentiation in the hindbrain, whereas the expression of the different proneural genes might underlie the generation of diverse neuronal lineages.

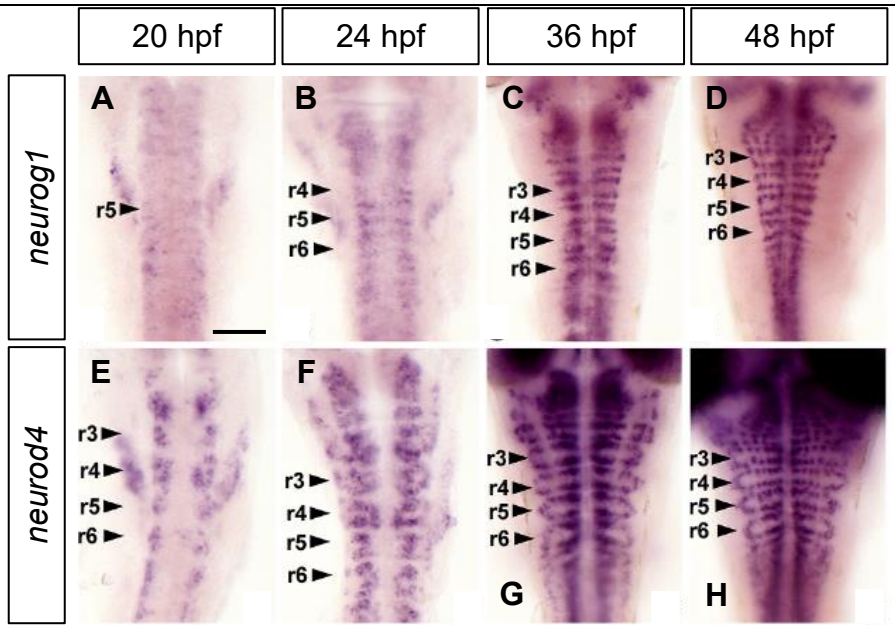

Figure 1.12. Hindbrain neurogenesis becomes restricted to boundary-flanking regions over time. **A-H**) *in situ* hybridization detecting *neurog1* (A-D) and *neurod4* (E-H) expression in the developing hindbrain of zebrafish embryos at the indicated developmental stages. Dorsal views of flat-mounted embryos with anterior to the top. Arrowheads indicate rhombomeric centres. Scale bar corresponds to 50 µm. Adapted from Gonzalez-Quevedo *et al*., 2010.

If we were to describe the sequence of events occurring during hindbrain neurogenesis, we would expect to have proliferative proneural-expressing progenitors in boundary-flanking regions (either *neurog1*, *ascl1a* or *ascl1b*, according to position in the DV axis). These genes would activate the expression of *neurod4*, promoting cell cycle exit and delamination in neuronal precursors, which would abandon the VZ and differentiate into HuC-expressing, post-mitotic

neurons of the MZ. In turn, the proneural-mediated expression of *Delta* genes (*dl*), also found within boundary-flanking regions such as *dla*, would activate the expression of *her4* in neighbouring cells, impairing neurogenesis by inhibiting the expression of proneural genes and accounting for the salt-and-pepper pattern observed in hindbrain proneural clusters (Figure 1.12 and 1.13).

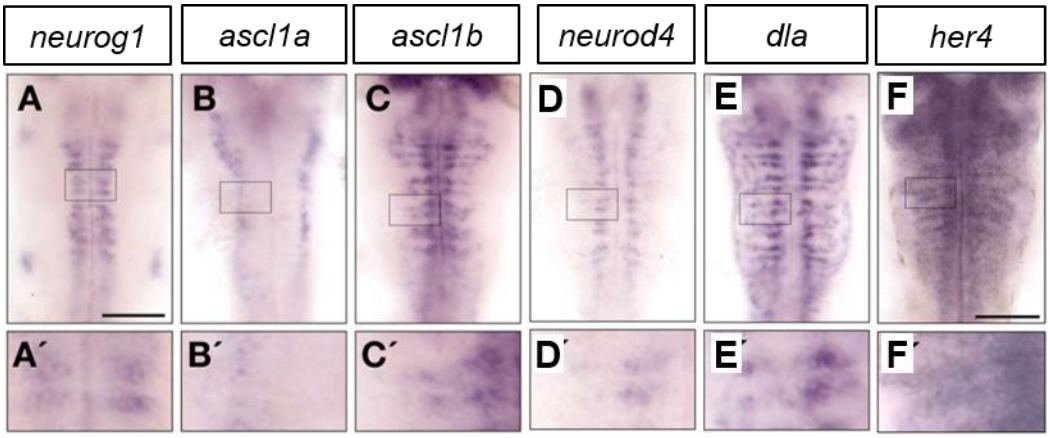

Figure 1.13. Boundary-flanking regions express the genes involved in proneural cluster behaviour. **A-F**) *in situ* hybridization for the proneural genes *neurog1*, *ascl1a* and *ascl1b* (A-C), and the neuronal differentiation gene *neurod4* (D), the Notch ligand *dla* (E) and the Notch target *her4* (F) in the zebrafish hindbrain at 3 hpf. **A'-F'**) Magnifications of regions framed (A-F), which correspond to rhombomeric boundaries. Scale bars correspond to 100 µm. Adapted from Nikolaou *et al*., 2009.

Nevertheless, there are a few missing pieces in this puzzle. We still lack an extensive analysis of the spatiotemporal pattern of proneural gene expression, as well as neuronal differentiation genes and Notch pathway elements during the zebrafish hindbrain morphogenesis. Such topological map of the hindbrain proneural diversity would allow us to set the starting point for dissecting the different mechanisms that co-exist in order to generate different neuronal lineages through development.

b) Hindbrain boundaries are progenitor pools

As previously stated, boundary cells show an increased proliferation rate compared to the rest of the tissue, which is indicative of a higher "stemness" state (Voltes *et al*., 2019). This and other observations have led to postulate that

boundary cells are indeed progenitor pools. First, rhombomeric boundaries are devoid of proneural gene expression (Figures 1.12 and 1.13). Second, hindbrain boundaries act as signalling centres instructing neuronal rearrangement of the adjacent rhombomeres (Terriente *et al*., 2012) and serve as physical anti-intermingling barrier (Calzolari *et al*., 2014; Letelier *et al*., 2018). Third, boundary cells express the bHLH transcription factor *her9*, which does not depend on Notch signalling. Hence, hindbrain boundary cells fulfil the requisites for being considered progenitor pools. Interestingly enough, these behaviour as a progenitor pool seems to be transient, as late boundary cells downregulate *her9* expression and become neurogenic, generating neurons that integrate within the MZ (Voltes *et al*., 2019; Engel-Pizcueta, preliminary results).

c) What about rhombomeric centres?

From the expression of the panneuronal marker HuC*,* expressed in post-mitotic, immature neurons, we know that first differentiated neurons in the zebrafish hindbrain are located in rhombomeric centres (Park *et al*., 2003). Not only that, but these neurons express the signalling factor *fgf20* that is needed to restrict rhombomeric centres devoid of neurogenesis (Gonzalez-Quevedo *et al*., 2010; Terriente *et al*., 2012). Accordingly, *sox9a* and *b*, which are transcription factors known to downregulate neurogenesis and are necessary to initiate the transition towards gliogenesis (Poché *et al*., 2008; Yokoi *et al*., 2009; Kang *et al*., 2012), are expressed in these territories. Moreover, and *sox9a*-expressing progenitors give rise to GS-positive astroglial-like cells and oligodendrocytes from the *olig2* lineage. The expression of *sox9a/b*, as well as the generation of their associated lineages, is dependent on active FGF-signalling (Esain *et al*., 2009). Thus, rhombomeric centres do not harbour neurogenic capacity but rather neural progenitor cells that may give rise to glial lineages.

In summary, the hindbrain contains a collection of distinct progenitor populations that display different capacities and functions depending on position and developmental time. When aiming to characterize the different neuronal lineages produced in this brain structure, we need to understand how the spatial distribution of proneural clusters changes over time. Interestingly, different

proneural genes are expressed in a restricted pattern along the AP axis (with the notable exception of *atoh1a*, see 1.6.3). However, there are two pieces of information that we still need to elucidate: (a) the DV distribution of proneural gene expression, and (b) the dissection of the differentiation domain into specific, neuronal subtype populations by the expression of HD neuronal proteins. The topological disposition along the apico-basal axis of progenitor and neuronal populations is the starting point in order to determine cell lineage relationships among them. While work on these issues is scarce in zebrafish, we can gain valuable insights from the research done in the murine dorsal hindbrain and spinal cord.

1.5.4. The dorsal hindbrain and the generation of neuronal diversity

Even when restricting ourselves to the development of the dorsal hindbrain, we find a stunning diversity of neuronal types. As in the spinal cord, the murine dorsal hindbrain is divided into different DV progenitor domains that will give rise to neuronal populations defined by the combinatorial expression of HD neuronal proteins (Hernandez-Miranda *et al*., 2017). The hindbrain HD neuronal code will assign a neurotransmitter phenotype, a migration trajectory and circuit assembly to neurons (Guillemot, 2007; Kohl *et al*., 2015). The most dorsal progenitor cells, which are known as class A progenitors, express the bHLH transcription factor *olig3* that acts together with proneural genes (such as *atoh1*, *ptf1a*, *ascl1*) to define three or four subdomains, depending on the specific rhombomere. Loss of *olig3* leads to vast miss-specification of dorsal cell fates (Liu *et al*., 2008; Storm *et al*., 2009). Class B progenitors are located more ventrally and are characterized by generating neurons that express the HD neuronal protein *lbx1.* However, there is no common molecular marker for all class B progenitors, as *olig3* is for class A progenitors. *olig3* and *lbx1* have opposing and antagonistic roles in the specification of their respective fates (Liu *et al*., 2008; Storm *et al*., 2009; Sieber *et al*., 2007). Hence, the class A and B nomenclature only describes the exclusive requirements of these two factors. However, there are other

proneural genes implicated in the generation of the different class B neurons such as *ascl1*, *ptf1a* and *neurog1* (Sieber *et al*., 2007; Storm *et al*., 2009; Figure 1.14).

Whereas DV patterning of the spinal cord has been extensively studied, the knowledge about the hindbrain DV patterning is still scarce. Many progenitor populations defined by a specific set of transcription factors extend from the spinal cord into the hindbrain, suggesting that general patterning mechanisms are conserved. For example, the *olig3/atoh1*-expressing progenitors extend along the entire AP longitude of the spinal cord and the hindbrain. Hence, the *lhx2/9*-expressing neurons are also common in both structures. Accordingly, BMPs and Shh signals are necessary for proper hindbrain DV patterning (Arkell and Beddington, 1997; Echelard *et al*., 1993). Nevertheless, there are as well some differences. For example, the *neurog1* expression domain within the class A progenitors is only found in the spinal cord and r7, which results in the loss of *lhx1/5* expressing neurons in more anterior territories. Furthermore, an additional progenitor domain exists in rhombomeres r2 to r6 that is characterized by the expression of *phox2b*.

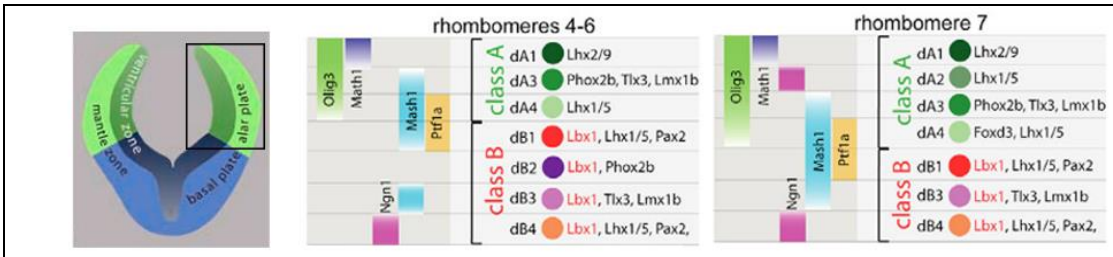

Figure 1.14. Neuronal cell types emerging from the dorsal spinal cord and hindbrain. The left scheme shows a transverse view of the hindbrain indicating the alar (green) and basal (blue) plates. Middle and right schemes display the distinct alar plate expressed genes (left side) and neuronal subtypes (right side) of rhombomeres and 4-6 and 7. Adapted from Storm *et al*., 2009 and Hernandez-Miranda *et al*., 2017.

1.6. THE RHOMBIC LIP: WHERE GENE FUNCTION MEETS PROGENITOR DYNAMICS.

The rhombic lip constitutes a hindbrain progenitor population that gives rise to neuronal structures controlling essential functions such as motor coordination, respiration, proprioception and wakefulness cycles. Each one of these functions are executed by different neuronal lineages generated from the same progenitor population in a sequential, timely manner (Machold and Fishell, 2005; Farago *et al*., 2006; Rose *et al*., 2009; Hayashi *et al*., 2015). Notoriously, rhombic lip neuronal derivatives are known to migrate away from the progenitor source in order to generate the definitive neuronal nuclei. Not only that, but the rhombic lip is highly impacted by one morphogenetic event occurring in the hindbrain: the generation of the fourth ventricle. Thus, the opening of the neural tube displaces this progenitor population laterally at the same time that progenitors are engaged in neurogenesis (Campo-Paysaa *et al*., 2019).

Therefore, the rhombic lip provides a platform to further elucidate how gene regulatory networks dictating neuronal fate and progenitor dynamics are intertwined in a context where brain shape changes dramatically in a very short period of time.

1.6.1. Rhombic lip anatomy and derivatives

The rhombic lip (RL) is a transient neuroepithelial structure that lies in the most dorsal part of the hindbrain. RL progenitors will give rise to different neuronal lineages according to their AP position. The most anterior region of the RL, which coincides with the dorsal pole of r1, is known as Upper Rhombic Lip (URL) and gives rise to all granule cells of the external and internal granular layers of the cerebellum. The rest of the RL, which expands from rhombomeres 2 to 8, is known as Lower Rhombic Lip (LRL) and gives rise to deep nuclei of the brainstem, such as the precerebellar system and the vestibular and auditory nuclei (Wullimann *et al*., 2011) (Figure 1.15A).

In molecular terms, the RL is defined by the expression of several factors, such as the diffusible molecule *wnt1* and the bHLH transcription factors *olig3* and

atoh1. These factors have been used as genetic drivers in order to generate genetic fate maps of the RL derivatives and to unveil their contribution to cerebellar and brainstem nuclei (Machold and Fishell, 2005; Wang *et al*., 2005; Rose *et al*., 2009; Fujiyama *et al*., 2009; Hayashi *et al*., 2015).

In addition, to understand the different RL cell contributions depending on their AP position, intersectional genetic fate-mapping strategies were developed by the combination of two different recombinase systems: a Cre recombinase that is under the control of *wnt1*, which is specific for RL progenitors; and a Flp recombinase under the control of AP hindbrain determinants such as *krox20* (which is specific for r3 and r5). The combination of the different reporters allowed for the generation of a LRL fate map along the different AP hindbrain compartments and showed that the LRL is regionalized into an anterior “auditory lip”, which generates mainly the cochlear and vestibular nuclei, and a posterior “precerebellar lip” that gives rise to precerebellar nuclei like the inferior olive (Farago *et al*., 2006; Figure 1.15B).

Rhombic lip derivatives are amongst the best studied paradigms of tangential cell migration within CNS development, as migration of neuronal precursors is a key step in cerebellar and brainstem differentiation. Genetic fate-mapping studies revealed not only the final destination of RL derivatives, but also their migratory routes. For example, *atoh1*-expressing cells migrate out of the cerebellar primordium and into the rostral hindbrain to populate specific nuclei of the tegmental system in a migratory stream known as rostral rhombic-lip migratory stream (RLS). In turn, the caudal migratory route derived from the LRL that generates the cochlear nuclei is known as the cochlear extramural stream (CES) (Machold and Fishell, 2005; Wang *et al*., 2005).

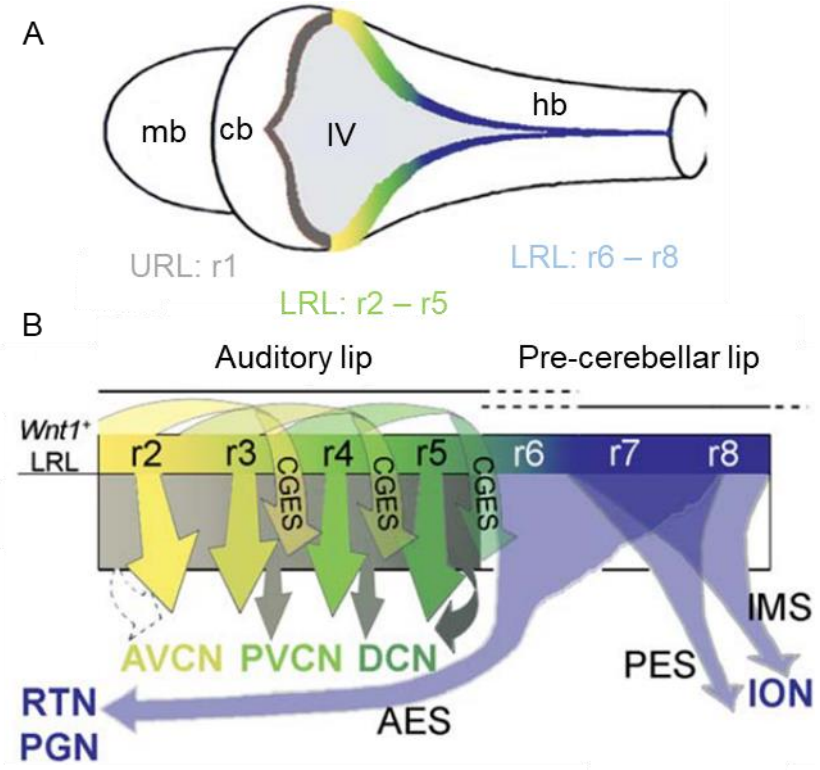

Figure 1.15. **Multiple genetically separable progenitor populations within the RL contribute to the cerebellar, auditory and precerebellar systems. A**) Scheme of a dorsal view of a hindbrain showing the RL, which is partitioned along its AP axis, with the cerebellar URL (grey); auditory LRL (yellow-green) generating neurons of the cochlear nuclei, and the precerebellar LRL (blue) generating neurons of the precerebellar nuclei. **B**) Scheme of a lateral view of a hindbrain showing the LRL derivatives (coloured in yellow-green-blue depending on the derivatives), and VZ ventral to the rhombic lip in r2 to r5 is shaded grey. LRL r2-r5, comprising the auditory rhombic lip, generates the anteroventral, posteroventral and dorsal cochlear nuclei (AVCN, PVCN and DCN, respectively), as well as the cochlear granule cell extramural migratory stream (CGES). The cochlear nuclei also receive contributions from territory ventral to the lip (grey arrows). LRL r6-r8, containing the precerebellar rhombic lip, generates the anterior extramural, posterior extramural, and intramural migratory streams (AES, PES, and IMS, respectively) to form the precerebellar nuclei. IV, fourth ventricle; mb, midbrain; cb, cerebellum; hb, hindbrain; RTN, reticulo-tegmental nucleus; PGN, pontine grey nucleus; ION, inferior olive nucleus. Anterior is to the left. Adapted from Farago *et al*., 2006.

Another remarkable example of neuronal migration are the neurons from the pontine nuclei (PN), which are part of the so-called precerebellar nuclei. These neurons are generated from LRL progenitors located in rhombomeres 6 to 8 (Kratochwil *et al*., 2017). The migratory pathway of these cells is outstanding, as they first migrate ventrally, and then anteriorly through rhombomeres 5 and 4. Migration takes a ventral turn at the level of the trigeminal nerve root and

eventually migrating cells settle down in between rhombomeres 3 and 4 floor plate (Farago *et al*., 2006; Okada *et al*., 2007; Figure 1.16A-B). Several guidance molecules and transcription factors have been implicated in regulating the migratory stream generated by PN neurons that are attracted by the floor plate. This attraction is mediated by the pathway Netrin/DCC, with the ligand Netrin (*ntn1*) being expressed in the floorplate and the receptor *dcc* in migrating precerebellar neurons (Fazeli *et al*., 1997; Yee *et al*., 1999; Alcántara *et al*., 2000; Zelina *et al*., 2014). In *dcc* and *ntn1* null mutants, PN neurons do not reach the midline and are stuck in a mediolateral position (Yee *et al*., 1999; Zelina *et al*., 2014; Figure 1.16E).

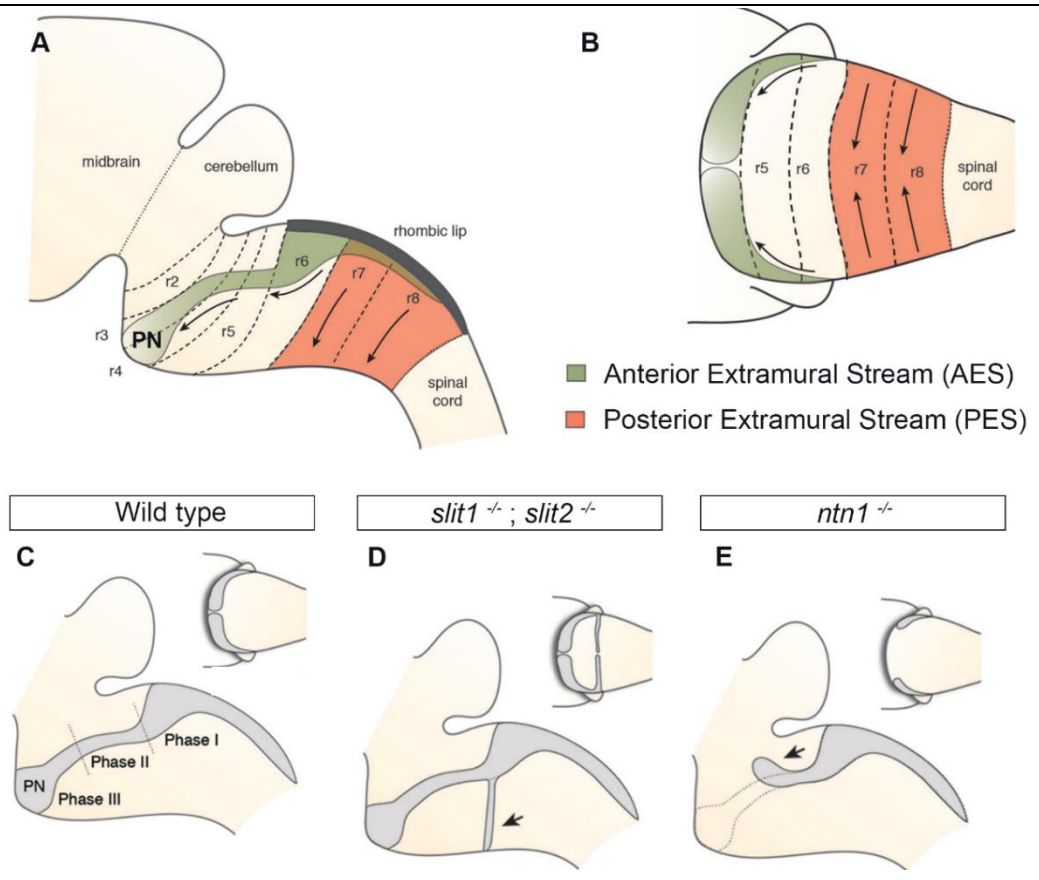

Figure 1.16. Migratory pathways of LRL cells and guidance signalling molecules in the generation of ventral neuronal nuclei. A-B) Lateral and dorsal views of the murine hindbrain with anterior to the left. LRL-derived neurons from r6-r8 migrate following the AES (green) and settle in the ventral half of r3 and r4 constituting the pontine nuclei (PN). LRL-derived neurons from r7-r8 that migrate following the PES (orange) generate other nuclei like the reticular and the cuneate. **C-E**) Schemes depicting lateral (big) and dorsal (small) views of the hindbrain. **C**) Dissection of the AES in three phases in a wild type embryo. **D**) Double *slit* mutant embryo displaying an ectopic migratory stream (arrow). **E**) *ntn1* mutation results in preventing the AES from reaching its destination point (arrow). Adapted from Kratochwil *et al*., 2017.

1.6.2. Proneural function in the specification of cerebellar lineages

There are two main progenitor cell populations that contribute to cerebellar development, the ventricular neuroepithelium and the cerebellar rhombic lip or URL[2]. The proneural gene *atoh1* is expressed in the URL and is involved in cerebellar granule cell generation as demonstrated by loss-of-function experiments and genetic fate-mapping. In fact, *atoh1*-derived cells in mutant conditions are unable to migrate away from the RL and generate defined neuronal structures (Ben-Arie *et al*., 1997 and 2000; Bermingham *et al*., 2001; Machold and Fishell, 2005; Wang *et al*., 2005). Thus, promotion of neuronal migration seems to be a conserved mechanism among different proneural genes in order to drive neuronal differentiation away from the proliferative, progenitor-harbouring VZ (see 1.3.2). However, *atoh1* is not the only proneural gene important for the development of cerebellar structures. The proneural gene *ptf1a* is expressed in the ventricular neuroepithelium, immediately bellow of the URL. The *ptf1a* null mutant in mice, called eloquently *cerebelless*, display a rudimental cerebellum with no Purkinje cells (Hoshino *et al*., 2005). Thus, we can classify the cerebellum into two different lineages: one that derives from the *atoh1a*-expressing URL and another that derives from the *ptf1a*-expressing cerebellar VZ.

This is not exclusive of cerebellar lineages, as *atoh1a* and *ptf1a* are also expressed in the dorsal posterior hindbrain. The cochlear nuclei (CN) present an example of brainstem nuclei where dual contribution of *atoh1a* and *ptf1a* also occurs. Thus, as in the cerebellum, CN excitatory neurons derive from *atoh1a*-expressing progenitors, while *ptf1a*-expressing progenitors generate neurons of the inhibitory phenotype and the function of both transcription factors is needed

[2] URL progenitors are also apico-basally polarized, as any other neuroepithelial progenitor. However, due to the morphogenetic folding that the cerebellum undergoes during development, URL progenitors end up confined in an anatomically distinct compartment than the VZ, where *ptf1a* progenitors are located. Thus, these anatomically distinct progenitor fields have been defined as the cerebellar rhombic lip or cerebellar plate, and the more ventrally located VZ (Figure 1.19D).

in order to generate their respective lineages (Fujiyama *et al*., 2009). Interestingly, loss of *ptf1a* function transforms cerebellar lineages into more ventral, "brainstem-like" lineages (Millen *et al*., 2014). In fact, in *ptf1a*-null conditions, progenitors of the cerebellar VZ produce extracerebellar *lmx1b* neurons, which are typically specified by the more ventrally-located *ascl1,* at the expense of Purkinje cells. Thus, *ptf1a* expression delimits the ventral cerebellar boundary. Loss of *ptf1a* function in a more posterior region of the hindbrain generates a similar misspecification phenotype. Hence, in r4, *lhx1/5* dorsal interneurons turn into more ventrally-located *lmx1b* neurons (Iskusnykh *et al*., 2016). Interestingly, *lmx1b* neurons also originate from *ascl1* progenitors, suggesting a putative cross-regulatory activity between *ptf1a* and *ascl1* in these contexts. These dorsal-to-ventral fate switches remind to the cross-regulatory activities between proneural genes observed in the spinal cord (Gowan *et al*., 2001; Carcagno *et al*., 2014; see 1.3.3). Nevertheless, no mutual cross-inhibition mechanism has been described either for *ptf1a* and *ascl1,* or *ptf1a* and *atoh1*.

In summary, the mammalian rhombic lip gives rises to three main hindbrain structures: the cerebellum, the cochlear nuclei and the precerebellar nuclei. In turn, each one of these organs is populated by diverse neuronal lineages that are produced at different AP locations and time windows. The proneural gene *atoh1* is critical in order to select cell fate in RL progenitors and specify neuronal subtype. However, how does a single transcription factor, such as *atoh1*, regulate fate acquisition in such a broad array of neuronal types?

1.6.3. *atoh1*: function, regulation and context dependency

atoh1 is also involved in cell fate selection and terminal differentiation of proprioceptive neurons of the spinal cord (Lai *et al*., 2011), secretory cells in the gut (Tomic *et al*., 2018) and sensory hair cells of the inner ear (Millimaki *et al*., 2007). Not only that, but *atoh1* is also involved in regulating early specification events during neurogenesis, cell cycle progression and tissue homeostasis (Mulvaney and Dabdoub, 2012). In order to understand how *atoh1* acts in such diverse contexts, we need information about *atoh1* protein function, transcriptional targets and activity regulation.

atoh1 was first described in *Drosophila* (*ato*) as a bHLH transcription factor both necessary and sufficient for the generation of the chordotonal organ (Jarman *et al*., 1993). *ato* has been conserved along evolution and *atoh1* is its vertebrate orthologue (Figure 1.17, grey box).

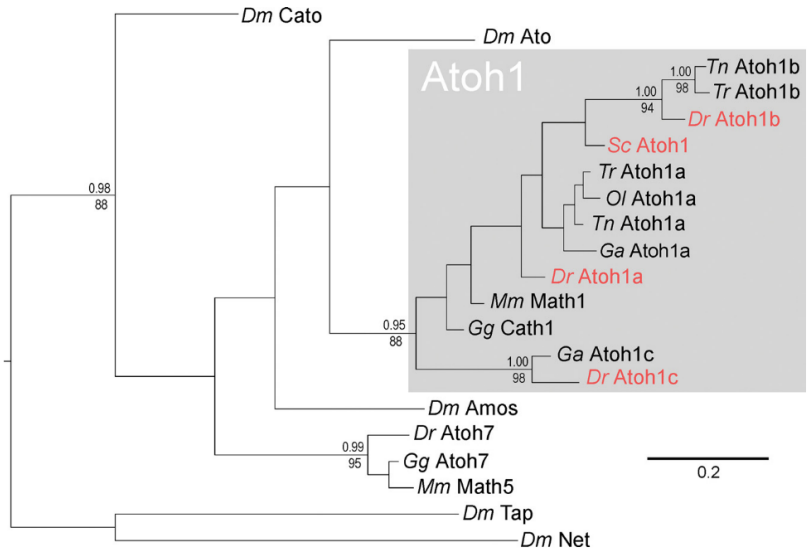

Figure 1.17. Phylogenetic relationships of *atoh* genes. The phylogenetic tree was derived by Bayesian analysis of the aligned bHLH region and is rooted with the *Drosophila* bHLH genes *tap* and *net*. The grey box highlights *atoh1* paralogues found in vertebrates. In red, *atoh1* paralogues found in the zebrafish genome. Support values above nodes represent Bayesian posterior probabilities, and those below nodes represent bootstrap percentages of 1000 pseudoreplicates. *Dm*, *Drosophila melanogaster*; *Dr*, *Danio rerio*; *Ga*, *Gasterosteus aculeatus*; *Gg*, *Gallus gallus*; *Mm*, *Mus musculus*; *Ol*, *Oryzias latipes*; *Sc*, *Scyliorhinus canicula*; *Tn*,*Tetraodon nigroviridis*; *Tr*, *Takifugu rubripes*. Adapted from Chaplin *et al.*, 2010.

As any other proneural gene, *atoh1* consists of a basic domain, which is necessary for DNA binding, and two helixes separated by a variable loop, which mediates the dimerization with the E protein E47. This interaction is required for the heterodimer to bind *cis*-regulatory regions of target genes and activate their transcription (Aguado-Llera *et al*., 2010). The specific *cis*-regulatory region that is preferred by *atoh1* is known as Atoh1a E-box associated Motif (AtEAM), and consists of a 10-nucleotide sequence based on the E-box consensus sequence (Klisch *et al*., 2011). The identification of the AtEAM motif facilitated genome-wide screenings in order to unveil *atoh1* targets. In the cerebellum and in the dorsal spinal cord, *atoh1* directly activates the expression of proneural common

effectors, such as regulators of neuronal migration, cell cycle and Notch signalling (Klisch *et al*., 2011). As examples of the *atoh1* targets we find: (a) the HD neuronal proteins *bahrl1* and *2* in the cerebellum and dorsal spinal cord, which are necessary for terminal differentiation (Lai *et al*., 2011); (b) *atoh1* itself (Helms *et al*., 2000), and (c) the effectors of the Shh pathway *gli1* and *gli2* (Flora *et al*., 2009; Klisch *et al*., 2011), which are specific for cerebellar lineages. Thus, *atoh1* renders cerebellar granule cells competent to the Shh signal derived from Purkinje cells, activating pro-proliferative genes. Although contradictory to canonical proneural functions, Shh-mediated proliferation of granule cell precursors is of key importance in order to generate a homeostatic cerebellum in postnatal mice (Chang *et al*., 2019).

Context-dependent functions of *atoh1* rely as well on differential regulation. At the transcriptional level, murine *atoh1* is regulated by a 3' enhancer responsible to drive its expression to the dorsal neural tube and the inner ear (Helms and Johnson, 1998; Helms *et al*., 2000). This enhancer contains an E-box that can be bound by *atoh1*, activating its own expression through a positive feedback loop (Ebert *et al*., 2003), as well as an N-box that can be bound by proteins of the Hes/Her family, inhibiting *atoh1* transcription as demonstrated in the vertebrate inner ear (Mulvaney and Dabdoub, 2012). At the protein level, *atoh1* activity can be modulated by (a) the formation of heterodimers that change or restrict its activity, (b) competitive binding to the AtEAM by Id and Hes/Her proteins, and (c) post-translational modification based on the phosphorylation of a serine residue in the N-terminus domain necessary to promote Atoh1 function, as demonstrated in murine cerebellar and hair cell development (Xie *et al*., 2017).

As an example of the importance of the specificity of *atoh1* heterodimers in functional regulation, *atoh1* can heterodimerize with the E protein TCF4, which activates the specific transcriptional program that assigns cell fate to pontine neurons during murine hindbrain development (Flora *et al*., 2007). This interaction is linage-specific, since *tcf4* mutant embryos only showed deficiencies in the pontine nuclei. Interestingly, E47 partnership does not result in the differentiation of this specific neuronal lineage, suggesting that Atoh1 dimerization partners can determine the selection of target genes and, ultimately,

cell fate. Moreover, *atoh1* activity in generating specific neuronal populations greatly depends on E47 and ID2 activities *in vivo* (Le Dréau *et al*., 2018). Thus, E protein partner selection does not seem to be a passive regulatory step, as initially believed, but a step in regulating *atoh1* cell lineage-specific functions.

In conclusion, *atoh1* function is tightly regulated at multiple levels in order to ensure the activation of the proper set of *atoh1* target genes in specific contexts. Thus, knowing the aspects involving *atoh1* function and regulation is of core importance when aiming to understand the generation of neuronal lineages from *atoh1*-expressing progenitors.

1.6.4. Insights from the zebrafish rhombic lip

The visualization of cellular lineages *in vivo* is a core procedure when studying progenitor dynamics and cell fate. The combination of genetic manipulation tools with last generation imaging techniques has made the zebrafish embryo a popular model for cell-lineage tracing and cell tracking experiments (Khairy and Keller, 2011; Olivier *et al*., 2011). This, together with the fact that the RL is a very superficial structure within the embryo, has led to the generation of many transgenic reporter lines that allowed the monitoring of RL progenitors *in vivo*. Thus, zebrafish transgenic reporter lines with fluorescent proteins to allow visualization of RL lineages *in vivo* were favoured over the genetic recombination approaches as the ones performed in mice (Wullimann et al., 2011). However, most of the work about RL lineages in zebrafish has been focused on cerebellar development (URL lineages), which share several commonalities with the amniote developmental plan.

The URL is spatially patterned in a similar way in zebrafish and mammals and it houses the same neuronal types, granular and Purkinje cells (Volkmann *et al*., 2008; Bae *et al*., 2009). Furthermore, zebrafish URL progenitors give rise to tegmental hindbrain nuclei after ventral migration equivalent to the ones found in mice. These tegmental nuclei, namely, the superior reticular nucleus and the secondary viscero-sensory nucleus, are generated from URL progenitors in a sequential manner that is also conserved among other vertebrates (Volkmann *et al.*, 2010), and are, in fact, the first neuronal nuclei generated from the URL

(Figure 1.18). In this same study, the migration behaviour of URL cells was assessed by following the trajectories of individual cells, which occurred tangentially and into deeper layers of the developing hindbrain.

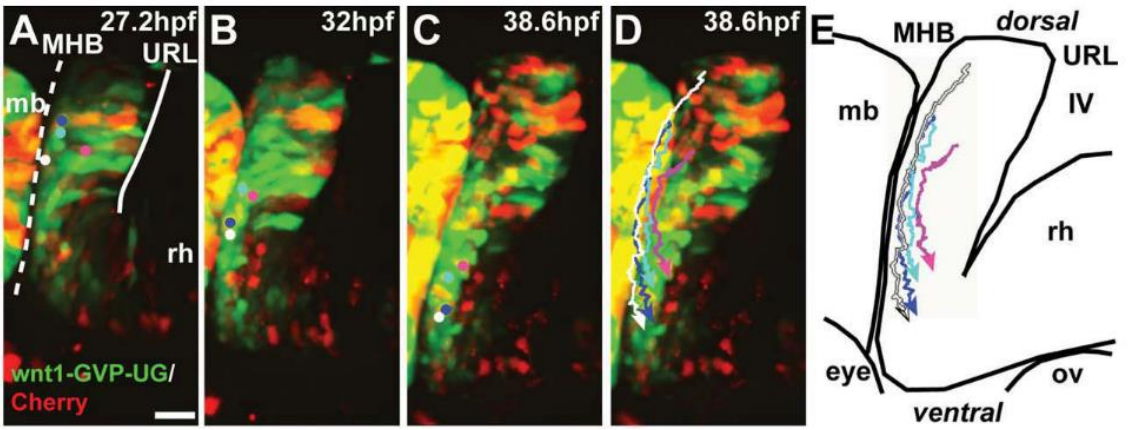

Figure 1.18. Migratory characteristics of *wnt1*-expressing cells when generating hindbrain ventral nuclei. **A-D**) Maximum brightness projections from individual time points of a time-lapse study of a transgenic Tg[wnt1-GVP-UG] embryo after injection with UAS:mCherry construct are shown. The embryo displays URL cells in green and overall mCherry expression (red). Double-positive cells were traced manually in lateral views and marked with coloured dots. **D**) Migratory routes were visualized by manual cell tracing and overlaid onto pictures of individual time points at which tracing has been finished. Arrowheads indicate direction of movements. **E**) Schematic drawing depicting the individual migratory routes in lateral view in the context of other brain structures. All images are lateral views of r1. IV, forth ventricle; CC, corpus cerebelli; CeP, cerebellar plate; EG, eminentia granularis; mb, midbrain; MHB, midbrain-hindbrain boundary; ov, otic vesicle; rh, rhombencephalon; URL, upper rhombic lip. Scale bar corresponds to 20 µm. Adapted from Volkmann *et al*., 2010

Not only this, the zebrafish cerebellum development also mirrors amniotes when focussing on progenitor contribution: URL-derived *atoh1a* progenitors are the ones giving rise to glutamatergic granular cells; whereas VZ-derived *ptf1a* progenitors give rise to GABAergic Purkinje cells (Kani *et al.*, 2010; Figure 1.19).

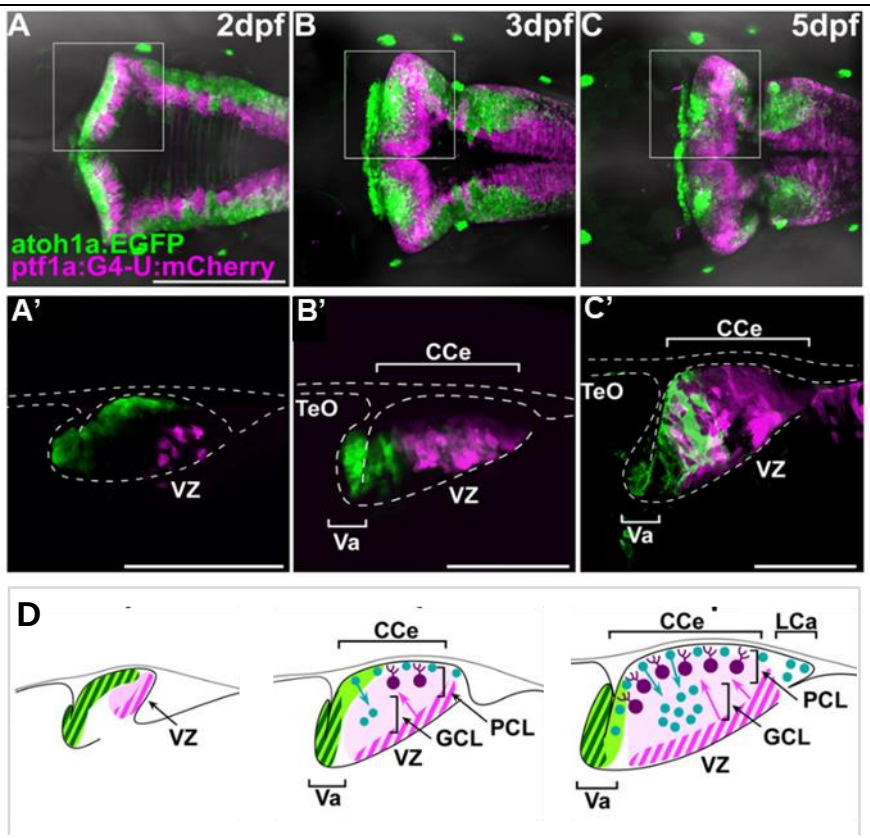

Figure 1.19. Contribution of two progenitor cell populations to the main cellular types in the zebrafish cerebellum. **A-C**) Dorsal views of the anterior hindbrain at 2, 3 and 5 days post-fertilization (dpf) with their corresponding sagittal optical sections (A'-C'). **D**) Scheme representing the cell cerebellar development in the zebrafish embryo and larva. At 2 dpf, *atoh1a* (in green) glutamatergic and *ptf1a* (in magenta) GABAergic progenitors occupy, the anterior and posterior domains of the cerebellum, respectively. From 3 to 5 dpf, *atoh1a* progenitors generate *atoh1a*-derived granule cells, which become *neurod*-immature granule cells and migrate to the GCL; *ptf1a*-derived cells migrate dorsally from the VZ and become differentiated into Purkinje cells at the PCL. These processes generate the three cerebellar layers visible at 5 dpf. CCe, corpus cerebelli; GCL, granular cell layer; LCa, lobus caudalis cerebelli; PCL, Purkinje cell layer; TeO, tectum opticum; Va, valvula cerebelli; VZ, ventricular zone. Scale bar corresponds to 50 µm. Adapted from Kani *et al*., 2010.

However, the zebrafish RL opens a new scenario concerning gene sub-functionalization in neuronal specification, since teleost fish underwent a whole genome duplication (WGD) that generated a great number of paralogues absent in their terrestrial relatives (Amores *et al.*, 1998; Meyer and Van de Peer, 2005; Hoegg *et al.*, 2004). Thus, the zebrafish arises as a very interesting model in order to study novelty and diversification of gene function through evolution. Some of these teleost-specific paralogues have already been proven to be the

substrate for co-opting old functions to participate in new developmental processes, such *rac3b* in hindbrain segmentation (Letelier *et al*. 2018).

The *atoh1* gene has three paralogues in zebrafish, *atoh1a*, *b* and *c*. In all fish sequenced to date, *atoh1a* is present in all of them and is the orthologue of *atoh1* gene in amniotes. However, *atoh1b* is only found in pufferfish and zebrafish, while *atoh1c* is only found in zebrafish in stickleback (Chaplin *et al*., 2010, Figure 1.17). All three zebrafish *atoh1* genes are expressed in the URL, although in a different spatiotemporal manner (Kani *et al.* 2010; Kidwell *et al*., 2018; Figure 1.20A-C). Cell-lineage tracing experiments revealed that *atoh1a* and *atoh1c* progenitors generated distinct cellular lineages, as their derivatives never overlapped and appeared in different positions. Therefore, additional *atoh1* genes that arose during the teleost WGD seem to be contributing to granule cell diversity in cerebellar structures (Kidwell et al., 2018; Iulianella *et al*., 2019).

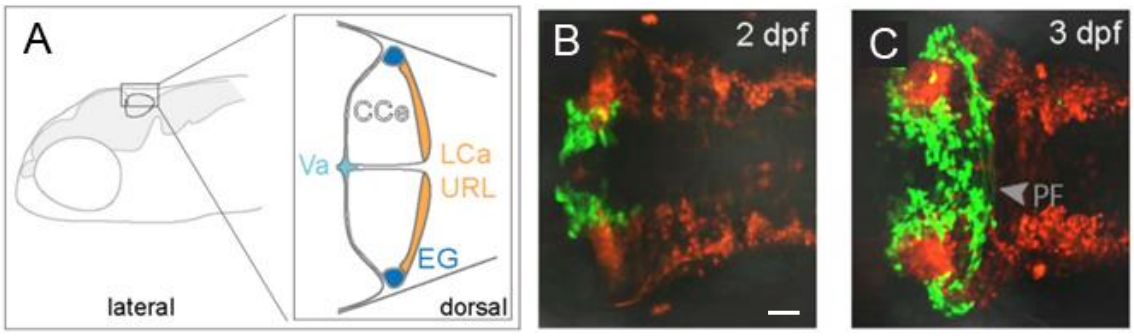

Figure 1.20. *atoh1a* and *atoh1c* progenitors contribute to cerebellum development by generating distinct populations of granular cells. **A**) Schematic representation of a lateral view of the zebrafish larva (left) and a dorsal view focusing on the cerebellar primordium (right). **B-C**) Stills at indicated times of live imaging movies of Tg[atoh1c:KAEDE] (green) and Tg[atoh1a:dTomato] (red) embryos indicating that *atoh1a* and *atoh1c*-derived neurons are distinct cerebellar populations. Maximum projections of z-stacks. CCe, corpus cerebelli; LCa, lobus caudalis cerebelli; Va, valvula cerebelli; EG, eminentia granularis; URL, Upper Rhombic Lip. Anterior is to the left. Scale bar corresponds to 50µm. Adapted from Kidwell *et al*., 2018.

In summary, amniote and anamniote URL lineages share a common developmental plan, generating three fundamental neuronal types segregated in a timely sequence. First, *atoh1* progenitors confined in the cerebellar lip migrate ventrally to generate the tegmental nuclei. Second, rostral migration of URL-derived neuronal precursors generate cerebellar nuclei. Lastly, granular cells are

generated, which contribute to the granular layer of the cerebellum. Interestingly enough, the presence of multiple *atoh1* genes in the zebrafish seems to have favoured the diversification of neuronal types in all of the lineages of the URL temporal sequence (Figure 1.21).

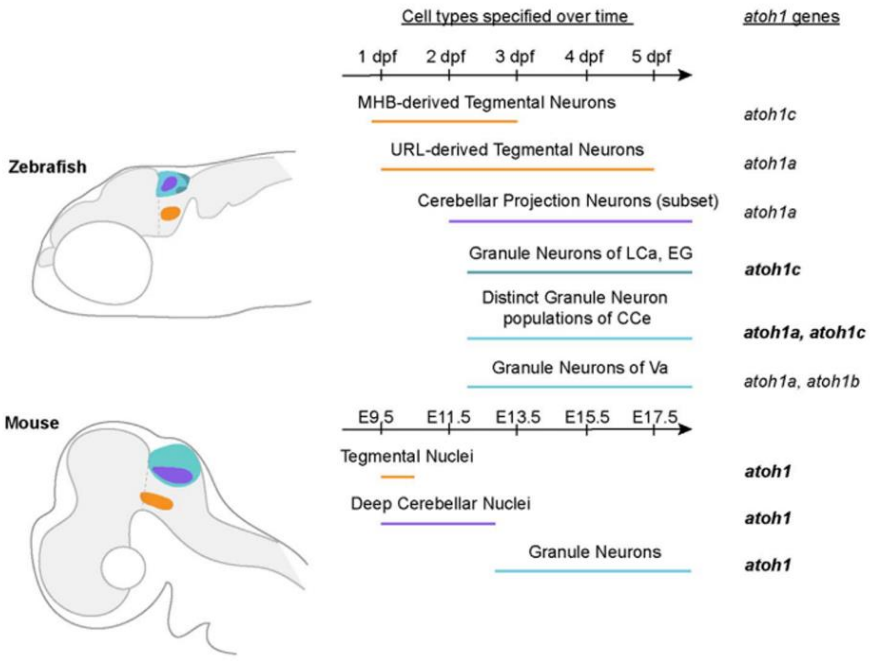

Figure 1.21. *atoh1* derivatives in zebrafish and mouse. Schematic outlines of *atoh1* derivatives generated over time in the anterior hindbrain of zebrafish (top) and mouse (bottom). Bold text indicates the gene required for development of the neuronal population. Homologous neuronal populations are indicated within the same the colour in the brain domains and as lines over time. Adapted from Kidwell *et al*., 2018.

However, the cellular contributions and epistatic relationships between the different *atoh1* genes expressed in more posterior, LRL progenitors has not been addressed yet. In the same line, derivatives of the LRL in the deep zebrafish hindbrain, as equivalents to mammal brainstem nuclei, have not been studied in detail. Complementary to this, it would be interesting to investigate whether the medial octavolateral nucleus (MON), a neuronal nucleus carrying vestibular information from the lateral line, is a rhombic lip derivative with glutamatergic cells coming from *atoh1a*-expressing progenitors, similar to the observations made in

mammal auditory structures (Fujiyama *et al*., 2009). All anamniotes have this sensory organ and the related CNS structures, needed for aquatic navigation and lost in amniotes. However, LRL derived structures have not been revealed by genetic fate mapping in teleosts so far (Wullimann *et al*., 2011).

1.7. SUMMARY

The zebrafish developing hindbrain provides us a model system in which diverse neuronal lineages are being specified during extensive cell proliferation and tissue morphogenesis resulting in the displacement of the LRL, the most dorsal neurogenic population within the hindbrain, upon brain ventricle formation.

The previous research devoted to fate-map URL progenitors in the zebrafish cerebellum has provided with a collection of transgenic reporter lines proven useful for cell-lineage tracing studies. Since the zebrafish LRL expresses two *atoh1* related genes (*atoh1a* and *atoh1b*), this raises the question whether the same scenario is replicated within the LRL. As described before, *atoh1* genes have been sub-functionalized during zebrafish cerebellar development.

Thus, by combining classical developmental biology tools with high-resolution *in vivo* imaging, we aim at dissecting the different mechanisms that co-exist within the very same structure in order to generate different neuronal lineages from LRL progenitors during hindbrain morphogenesis.

CHAPTER 2: AIMS AND OBJECTIVES

In this project we aim to unveil how neuronal lineages are specified in the highly dynamic context of hindbrain development. To do so, we will address the following objectives:

Objective 1. To generate a topological map of neurogenic progenitors and neuronal populations at different stages of the developing zebrafish hindbrain. In order to understand which neuronal lineages are generated within these structure, we first need to understand the location of the neurogenic capacity, as well as the specific neuronal populations within the mantle zone (MZ). In this sense, we will:

1.1. Examine the spatiotemporal expression pattern of the proneural genes *atoh1a*, *ascl1a/b ptf1a*, *neurog1,* and the neuronal differentiation gene *neurod4* at different stages of the developing hindbrain.

1.2. Study the spatiotemporal expression pattern of different HD transcription factors defining specific neuronal populations within the MZ.

1.3. Explore the proneural gene requirements for hindbrain neuronal specification using loss-of-function approaches.

Objective 2. To analyse of the overall hindbrain growth. In order to understand the proliferative and neurogenic potentials of hindbrain progenitors, we aim at unveiling different growth patterns by clonal analysis. For this, we will:

2.1. Randomly label hindbrain progenitors by genetic recombination induced with a Cre-LoxP system combined with *in vivo* imaging.

2.2. Quantify the number of progenitor versus differentiated cells within each induced clone at different developmental stages.

2.3. Investigate different growth patterns of hindbrain progenitors according to spatiotemporal coordinates.

To unveil the dynamic processes that neurogenic progenitors undergo during cell fate specification and differentiation, we will focus on the *atoh1a*-neuronal lineage derived from the LRL, and will follow two complementary lines of research (see Objectives 3 and 4).

Objective 3. To characterize the function of *atoh1a* and *atoh1b* in LRL-derived neurogenesis. We will unveil the proneural hierarchy between *atoh1a* and *atoh1b* and address their contribution to the same/different neuronal population. In order to answer these questions, the objectives proposed are:

3.1. To characterize the spatiotemporal expression domains of *atoh1a* and *atoh1b* within the LRL.

3.2. To trace the positioning of LRL derivatives using reporter transgenic lines.

3.3. To assess the putative hierarchy among *atoh1a* and *atoh1b* with functional experiments.

3.4. To uncover the functional requirements of *atoh1a* and *atoh1b* in specification and differentiation of LRL-derived neurons.

3.5. To investigate the role of the Notch signalling in LRL-derived neurogenesis.

Objective 4. To reconstruct the cell lineage within the LRL of *atoh1a* progenitors by high resolution *in vivo* imaging. To decipher the dynamic events that LRL progenitors undergo during fate specification and neurogenesis and to assess the importance of LRL-progenitor location on the final destination of their neuronal derivatives we will focus on the following objectives:

4.1. To assess the birthdate of *atoh1a* progenitors by photoconversion experiments.

4.2. To follow the *atoh1a*-progenitor dynamics (proliferation, mode of division and differentiation) by cell tracking.

Overall, by combining developmental biology tools with imaging-based approaches we aim to uncover LRL progenitor behaviour during cell fate specification and differentiation to provide further insights into the functional diversification of bHLH proneural genes during the neurogenic process.

CHAPTER 3: RESULTS (I)

HINDBRAIN GROWTH AND NEUROGENIC CAPACITY

3.1. Introduction and aims

The generation of neuronal diversity during embryonic development relies on the patterning of the neural tube into discrete domains populated by progenitor cells that restrict their potential to specific neural types. Such dorsoventral (DV) regionalization is achieved through the activity of local morphogen gradients that induce the expression of patterning genes that convey positional information to neural progenitors. Therefore, the generation of specific neuronal lineages relies on the molecular identity of neural progenitors, which, in turn, depends on progenitor position. Concomitantly to the neural tube growth, new progenitor cells are generated and added to the pre-existing progenitor domains, and a balance between progenitor proliferation, specification and differentiation needs to be fine-tuned to properly shape the tissue. However, progenitor cells within the neural tube are not a homogenous population, as we find cells displaying different gene expression signatures according to their position. All these observations pose several questions, such as: how tissue patterning is maintained during cell specification, proliferation and differentiation? Do different progenitor cells display distinct cellular dynamics and, as a consequence, generate different growth patterns?

In this first results chapter, we aim at addressing these questions. In order to do so, we first characterized the hindbrain neurogenic domains by examining the expression of proneural genes by *in situ* hybridization. To identify putative lineage relationships between hindbrain progenitors and specific neuronal populations, we assessed the expression pattern of neuronal HD differentiating genes, and subsequently we sought for the proneural requirements for proper neuronal differentiation by loss-of-function experiments.

In parallel, we assessed the contribution of the different progenitor and differentiation domains to the size of the embryonic hindbrain over time through area quantification. Complementarily, we analysed the overall hindbrain growth by randomly inducing genetic clones to label hindbrain progenitors and followed them upon morphogenesis.

3.2. The neurogenic hindbrain is organized in distinct progenitor domains according to proneural gene expression

Proneural gene expression is delimited to discrete progenitor domains that will give rise to specific neuronal lineages, generating neuronal diversity within the developing brain (Guillemot, 2007). Therefore, in order to identify the putative different neuronal populations that arise within the embryonic hindbrain, we first sought to examine the expression of the proneural genes *atoh1a*, *ascl1a*, *ptf1a*, *ascl1b* and *neurog1* along the anteroposterior (AP) and DV axes by mRNA expression detection through *in situ* hybridization.

The most dorsally-expressed proneural gene was *atoh1a*, and its expression was continuous throughout the whole hindbrain AP axis from 18hpf onwards. This expression remained at least until 48hpf (Figure 3.1A-C; Figure 3.2A-D). Two proneural genes were expressed immediately ventral to *atoh1a* expression domain, *ptf1a* and *ascl1a*. Their expression mostly overlapped, defining the same progenitor domain as observed by double *in situ* hybridisation (see transverse sections in Figure 3.1P-Q). However, even if expressed in the same DV domain, their onset of expression within the AP axis differed: *ptf1a* started to be expressed at 18hpf in rhombomere 3 (r3) and expanded first towards r1 and r2, and then posteriorly as development proceeded (Figure 3.1D-F). *ascl1a* expression was already present in r2 to r6 at 18hpf, very similar to the expression pattern of *ascl1b* (Figure 3.1G-L). Nonetheless, the expression of *ascl1a* and *ascl1b* along the DV axis differed, since they did never overlapped and *ascl1b* was ventral to the *ptf1a*/*ascl1a* domain (Figure 3.1R). Lastly, *neurog1* and *ascl1b* displayed a similar expression pattern along the AP domain, being restricted to the rhombomeres with slightly different intensities. Moreover, as observed in the transverse sections, *neurog1* expression was confined within *ascl1b* domain (Figure 3.1L'-O'; Figure 3.2E'-L'). Overall, by 24hpf hindbrain boundaries were devoid of proneural gene expression, except for *atoh1a*.

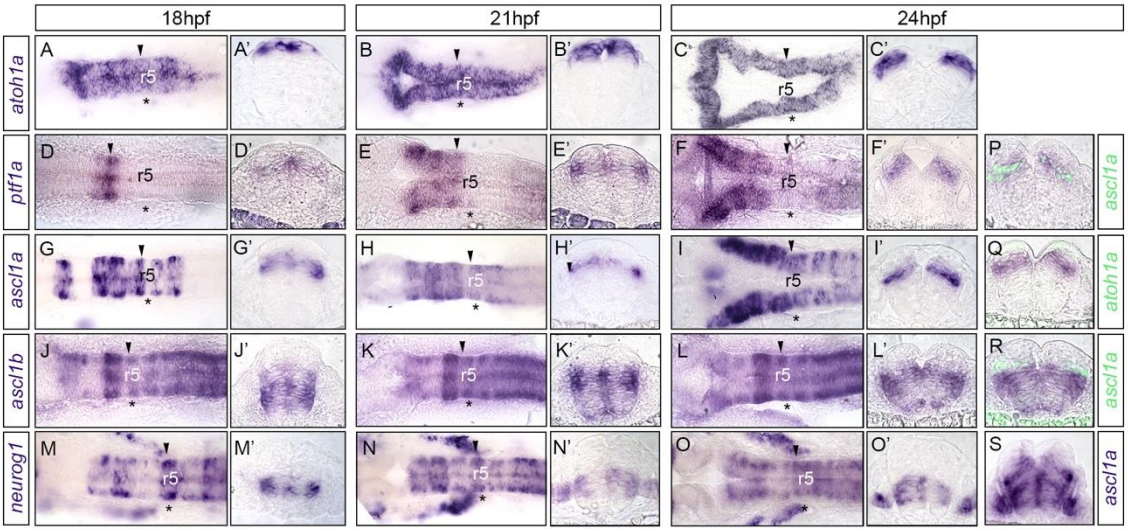

Figure 3.1. Proneural gene expression within the zebrafish embryonic hindbrain. A-O) Whole-mount *in situ* hybridization at indicated developmental stages using: *atoh1a* (A-C, Q), *ptf1a* (D-F, P), *ascl1a* (G-I, P-S), *ascl1b* (J-L, R) and *neurog1* (M-O, S) probes. **A'-O'**) Transverse views of embryos displayed in (A-O) at the level pointed by the black arrowhead. **P-S**) Transverse views of double *in situ* hybridized embryos using FLUO- (green) or DIG-labelled (blue) probes. Dorsal views of flat-mounted hindbrains with anterior to the left (A-O) with asterisks indicating the position of the otic vesicle. Transverse sections are 20 µm thick. r, rhombomere.

At 24hpf, *ascl1b* and *neurog1* expression was widespread within rhombomeres. However, rhombomeric boundaries did not contain neurogenic progenitors or differentiated neurons since they were devoid of proneural gene expression and did not display HuC at 24 and 30hpf (Figure 3.2E-F, I-J; blue arrowheads). As development proceeded and neurons accumulated within the mantle zone (MZ), *ascl1b* and *neurog1* expression became confined to boundary-flanking regions, clearly apparent from 42hpf onwards. At these stages, both rhombomeric boundaries and centres contained differentiated neurons, according to HuC expression (Figure 3.2G-H, K-L; orange arrowheads). Interestingly, *atoh1a* was never regionalized in such pattern, as it was expressed continuously along the AP axis of the hindbrain (Figure 3.2A-D).

During this particular time window, the hindbrain underwent a morphogenetic process resulting in the formation of the fourth ventricle by an enlargement of the neural tube's lumen in its dorsal half. As a consequence of this morphogenetic event, the *atoh1a* domain was displaced towards a lateral position and away from

the midline (Figure 3.2A'-D'). The more ventrally-located proneural gene domains were also impacted by the formation of the lumen. This resulted in the dorsal half of *ascl1b*/*neurog1* domains that were in contact with the midline at 24hpf positioning dorso-laterally at 48hpf (Figure 3.2E'-L').

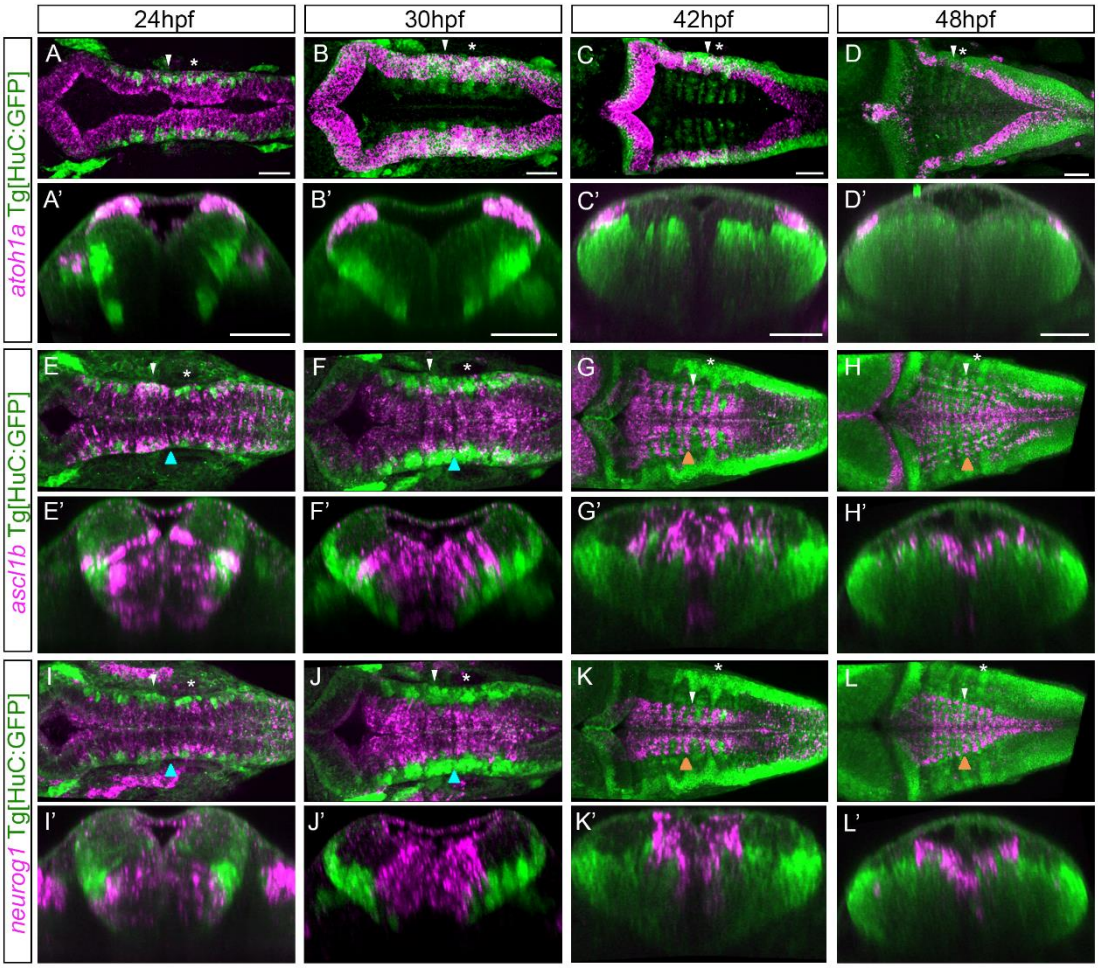

Figure 3.2. Expression of *atoh1a*, *ascl1b* and *neurog1* proneural genes in the context of the neuronal differentiation domain. **A-L**) Tg[HuC:GFP] embryos were *in situ* hybridized at indicated developmental stages with: *atoh1a* (A-D), *ascl1b* (E-H) and *neurog1* (I-L). **A-L**) Dorsal views with anterior to the left. Images are maximum intensity projections of confocal stacks. **A'-L'**) Transverse views of embryos displayed in (A-L) at the level pointed by the white arrowhead. Transverse views are maximum intensity projections of 10 consecutive images reconstructed from a confocal stack. Blue arrowheads indicate the rhombomeric boundary between r4 and r5 (r4/r5). Orange arrowheads point to the proneural expression on the anterior flanking region in r5. Asterisks indicate the position of the otic vesicle. Scale bars correspond to 50µm.

In summary, the zebrafish embryonic hindbrain contains different proneural progenitor pools ordered along the DV axis as follows: *atoh1a*, *ptf1a/ascl1a*, *ascl1b* and *neurog1*.

Progenitor cells within the ventricular zone (VZ) give rise to post-mitotic precursors that detach from the apical surface and differentiate into neurons that are located within the MZ. Accordingly, all proneural genes assessed were expressed in progenitor cells, as none of these factors co-localized with early differentiated neurons, assessed by the expression of GFP derived from the transgene Tg[HuC:GFP] (Park *et al*., 2000; Figure 3.2).

According to previous research, *neurod4* is widely expressed in the developing zebrafish hindbrain (Park *et al*., 2003; González-Quevedo *et al*., 2010); whereas *neurod1* is not expressed during early steps of hindbrain neurogenesis (Zecca *et al*., 2015). Little is known on the proneural hierarchy controlling *neurod4* expression in this context. In this sense, we decided to examine the expression of *neurod4* through *in situ* hybridization and compare it to the previously described proneural domains. *neurod4* expression recapitulated the flanking boundary pattern in an identical fashion as proneural genes (Figure 3.3A-B). However, *neurod4* was never expressed in the VZ nor co-localized with HuC (Figure 3.3A'-B'). When comparing *neurod4* with *ascl1b* expression, we observed that although the expression of both factors overlapped along the AP axis (Figure 3.3C-E) it differed along the DV axis, since *ascl1b* expression was restricted to the VZ and *neurod4* was biased toward the basal HuC domain (Figure 3.3C'-E'). Taken together, these observations hint that *neurod4* expression may define an intermediate zone of neuronal precursors that are no longer ventricular but not yet fully differentiated. Interestingly, *neurod4* was never expressed within the *atoh1a* domain, suggesting that *neurod4* was not the differentiation gene within this population (compare Figure 3.2B', D' with Figure 3.3A'-B').

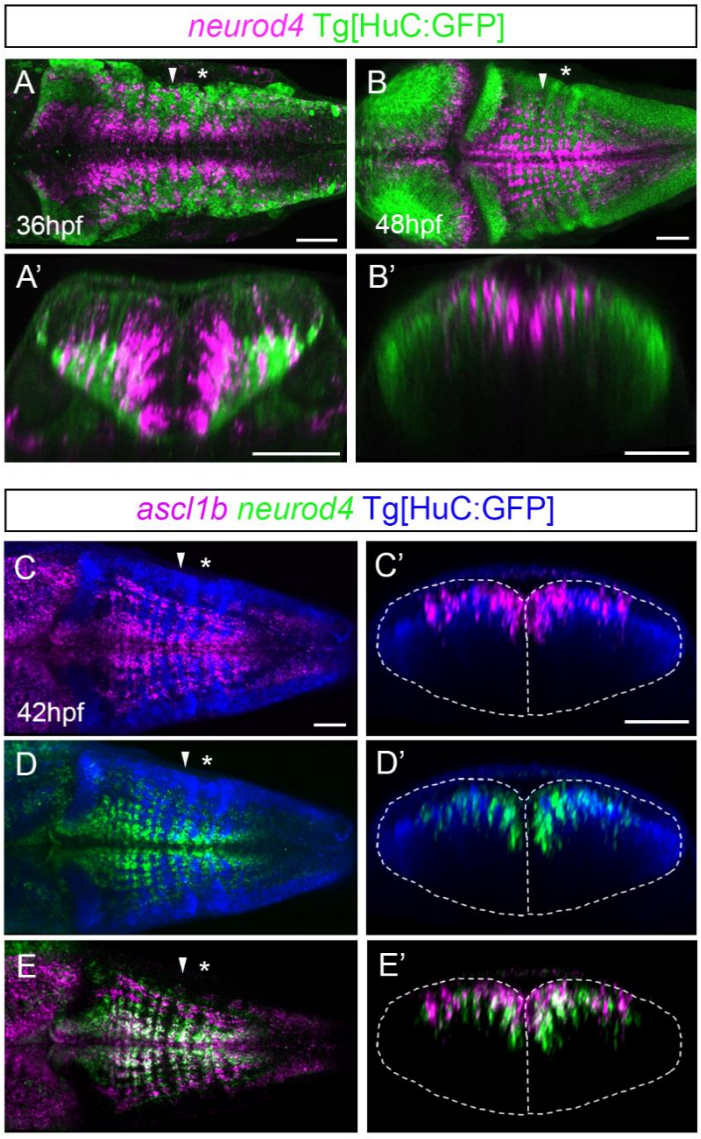

Figure 3.3. *neurod4* expression defines an intermediate zone of neuronal precursors. A-B) *In situ* hybridization of *neurod4* at 36hpf and 48hpf in dorsal view. **A'-B'**) Transverse views of (A-B). **C-E**) Double *in situ* hybridization of *neurod4* and *ascl1b*. Transverse views (C'-D') show the expression of *neurod4* in an intermediate zone between the VZ (*ascl1b* in magenta) and the MZ (HuC in green). Arrowheads indicate the point of transverse view reconstruction. Asterisks indicate the position of the otic vesicle. Scale bars correspond to 50 µm.

3.3. The hindbrain proneural network: proneural requirements for neuronal differentiation

The movement of neuronal precursors upon differentiation is directional and follows the apico-basal (AB) axis. Thus, we sought to unveil putative lineage relationships focusing on the relative position of different progenitor domains and specific committed neuronal populations along the AB axis. To do so, we performed double fluorescent *in situ* hybridization of the proneural genes and the differentiation HD neuronal genes *lhx2b* and *lhx1a*, which are factors known to define specific neuronal subtypes (Guillemot, 2007).

As expected, expression of both *lhx2b* and *lhx1a* was always confined within the HuC-expressing domain, confirming that they were specific of differentiated neurons (Figure 3.4A'-C'; 3.4A'''-C'''). *lhx2b* displayed two domains of expression: one lateral all along the AP axis, and one medial located only in r4-r6 (Figure 3.4A'). Double fluorescent *in situ* hybridization of *lhx2b* and *atoh1a* revealed that the most lateral *lhx2b* territory fell just underneath the *atoh1a* domain, suggesting that it most likely derived from *atoh1a*-expressing progenitors (Figure 3.4A'''). However, this would not be the case for the more medial domain of *lhx2b* expression, as it was far located from *atoh1a* progenitors and only in the r4-r6 region.

lhx1a also displayed two domains of expression according to the mediolateral (ML) axis (Figure 3.4B'-C'). There was a small *lhx1a*-neuronal population located lateral within the HuC-expressing domain and adjacent to the most lateral domain of *lhx2b* neurons, most probably deriving from *ascl1a*-expressing progenitors according to their relative position (Figure 3.4B'''). The second pool of *lhx1a*-neurons was located medially within the MZ and was bigger in size, which might be derived from *ascl1b* progenitors (Figure 3.4C'''). At this stage, 42hpf, the fourth ventricle was already formed with the consequent progenitor displacement. Therefore, we could clearly appreciate the change in the AB axis, from being perpendicular (Figure 3.2E', I') to being parallel to the midline (Figure 3.4A'''-C'''). In contrast with the spinal cord, which does not undergo morphogenetic changes due to lumen formation, hindbrain differentiated neurons ended up in a ventral position, whereas progenitor cells ended up positioning dorsally and in close contact with the lumen. Altogether, our observations highlight the impact of hindbrain morphogenesis on progenitor and neuronal position.

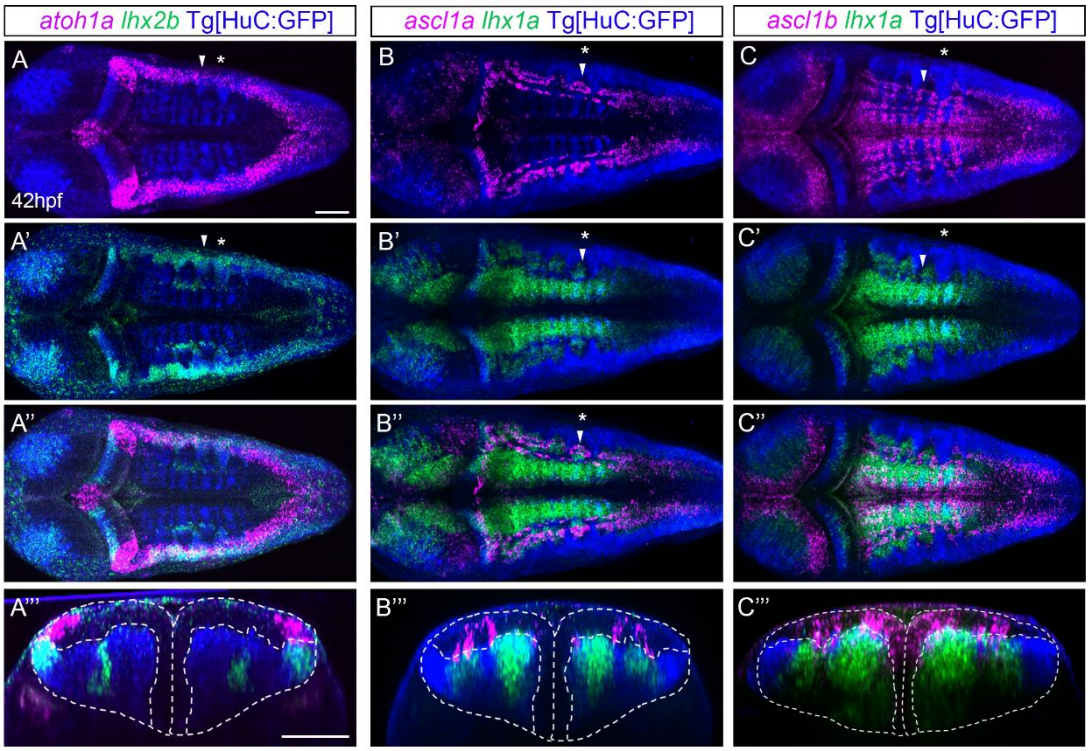

Figure 3.4. Comparison of the expression domains of proneural and neuronal factors. A-C) Tg[HuC:GFP] embryos at 42hpf. Double *in situ* hybridization of the proneural genes *atoh1a* (A-A'''), *ascl1a* (B-B''') and *ascl1b* (C-C''') with the neuronal genes *lhx2b* (A-A''') and *lhx1a* (B-B''', C-C'''). **A'''-B'''**) Transverse views of (A-B) show the apico-basal relationship of proneural and neuronal cell populations. Arrowheads indicate the point of transverse view reconstruction. Asterisks indicate the position of the otic vesicle. Scale bars correspond to 50 µm.

The mapping of proneural gene expression with specific neuronal populations provided a starting point for elucidating the proneural hierarchy governing neurogenesis in the embryonic hindbrain. In order to dissect the proneural requirements for the generation of neuronal diversity within the hindbrain, we assessed the impact in the expression of the neuronal differentiation gene *neurod4* and the neuronal factor *lhx1a* upon loss-of-function of proneural genes by the use of translation-blocking morpholinos (MOs).

Neither downregulation of *ascl1a*, *ascl1b* or *neurog1* affected the expression of the *neurod4* domains (n=15, n=10, n=14, respectively, where n is the number of embryos analysed per condition). Note that, upon *neurog1* downregulation, *neurod4* expression in the cranial ganglia was lost (Figure 3.5D), which

demonstrated the efficiency of the morpholino as described in Andermann *et al*., 2002 (n=12/14; where n is the number of embryos with a morphant phenotype in the cranial ganglia versus the total number of analysed embryos). Same results were obtained when we looked at the proneural requirements for neuronal differentiation, as HuC staining was not affected upon downregulation of any of the three proneural genes (Figure 3.5E-H; n=13, n=11 and n=16, respectively). Since proneural genes generate specific neuronal subtypes and can cross-inhibit, the lack of phenotype could be explained by a switch in the fate of neuronal populations. However, when the expression of *lhx1a* was assessed, none of the proneural-morphants displayed ectopic activation of *lhx1a* (Figure 3.5J-L; n=8, n=12 and n=9). These results suggested that there was proneural gene redundancy to ensure the production of given cell fates.

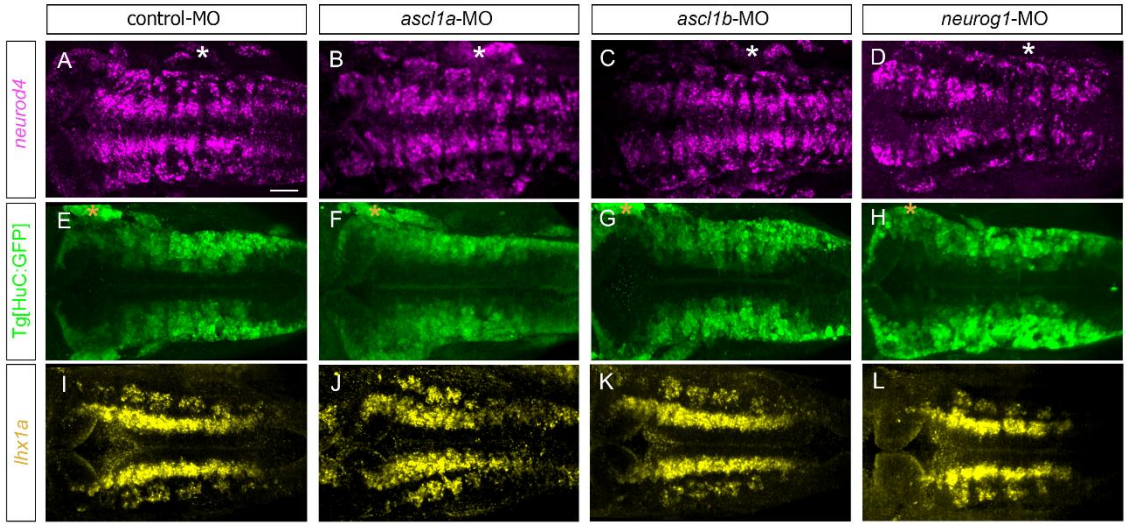

Figure 3.5. The downregulation of a single proneural gene does not impair neuronal differentiation or the acquisition of neuronal subtype. A-L) *In situ* hybridization of *neurod4* (A-D), GFP expression in Tg[HuC:GFP] embryos (E-H), and *in situ* hybridization of *lhx1a* (I-L) in injected embryos with the following MOs: control (A, E, I), *ascl1a* (B, F, J), *ascl1b* (C, G, K), and *neurog1* (D, H, L). Embryos were analysed at 30hpf. White asterisks in (A-D) indicate the position of the statoacoustic ganglion. Orange asterisks in (E-H) indicate the position of the trigeminal ganglion. Scale bar corresponds to 50 µm.

Since loss-of-function experiments did not result in any phenotypes in *neurod4* expression or neuronal production, we verified the efficiency of the previously published MO. Due to the small size of proneural genes with one or no introns,

we favoured the use of translation-blocking MOs as previously published (Pogoda *et al*., 2005; Flasse *et al*., 2013; Cornell *et al*., 2002). For assessing the efficiency of *ascl1a*-MO, we used as a read-out the *neurod1* expression in the adenohypophysis, since *neurod1* was described to be an *ascl1a* target in this context. One-cell stage embryos were injected with 5ng of either control or experimental MOs. The expression of *neurod1* in the adenohypophysis was assessed at 30hpf as described in (Pogoda *et al*., 2006). As expected, *neurod1* expression in the adenohypophysis was downregulated in *ascl1a* morphants (Figure 3.6B; n=14/16), whereas no effect was observed in embryos injected with the control MO (Figure 3.6C; n=14). In the case of the *ascl1b*-MO, we examined the expression of *neurod1* in the developing pancreas at 18hpf, as described in Flasse *et al*., 2013. In the control situation, *neurod1* expression in the pancreas resembled a “Y” shape (Figure 3.6D; n=3). In morphant conditions, the pool of *neurod1*-expressing cells was reduced or aberrant in more than a half of the embryos (Figure 3.6E; n=15/26). For the *neurog1*-MO, we assessed the expression of *vglut2a*, a maker for glutamatergic differentiation (Higashijima *et al*., 2004). In the control situation, both the trigeminal and statoacoustic ganglia expressed *vglut2a*, as well as the rhombomeric centres (Figure 3.6F; n=10). In *neurog1*-morphant conditions, the expression in the sensory ganglia was lost, although it remained in the rhombomeres (Figure 3.6G; n=16/20). All of the MOs aforementioned were co-injected with the standard p53-MO to diminish any morpholino artefact (Robu *et al*., 2007).

Taken together, our results suggested that downregulation of single proneural function in the embryonic zebrafish hindbrain was not enough to impair *neurod4* expression or neuronal production. This can be explained by several possibilities. On one hand, proneural genes function redundantly and can back each other up, since most of them are expressed in pairs in the same progenitor domains, such as *ascl1a*/*ptf1a* and *neurog1*/*ascl1b*. In this scenario, downregulation of *ascl1a* could be compensated by *ptf1a,* and *neurog1* could be compensated by *ascl1b*. These compensatory relationships among proneural genes have already been described in the mammalian cortex and spinal cord in double knock-out mice (Tomita *et al*., 2000; Nieto *et al*., 2001; Gowan *et al*., 2001). Another possibility is

that *neurod4* expression was independent of proneural function, as described in other contexts such as the zebrafish olfactory bulb and habenula, where *neurod4* expression was independent of *neurog1* function (Madelaine *et al*., 2011; Halluin *et al*., 2016).

A

MOs	Sequence	Reference
ascl1a	5'-ATC TTG GCG GTG TGA TGT CCA TTT CGC-3'	Pogoda *et al*., 2006
ascl1b	5'-TCG TAG CGA CAG TTG CCT CCA T-3'	Amoyel *et al*., 2005
neurog1	5'-ACG ATC TCC ATT GTT GAT AAC CTG G-3'	Cornell *et al*., 2002

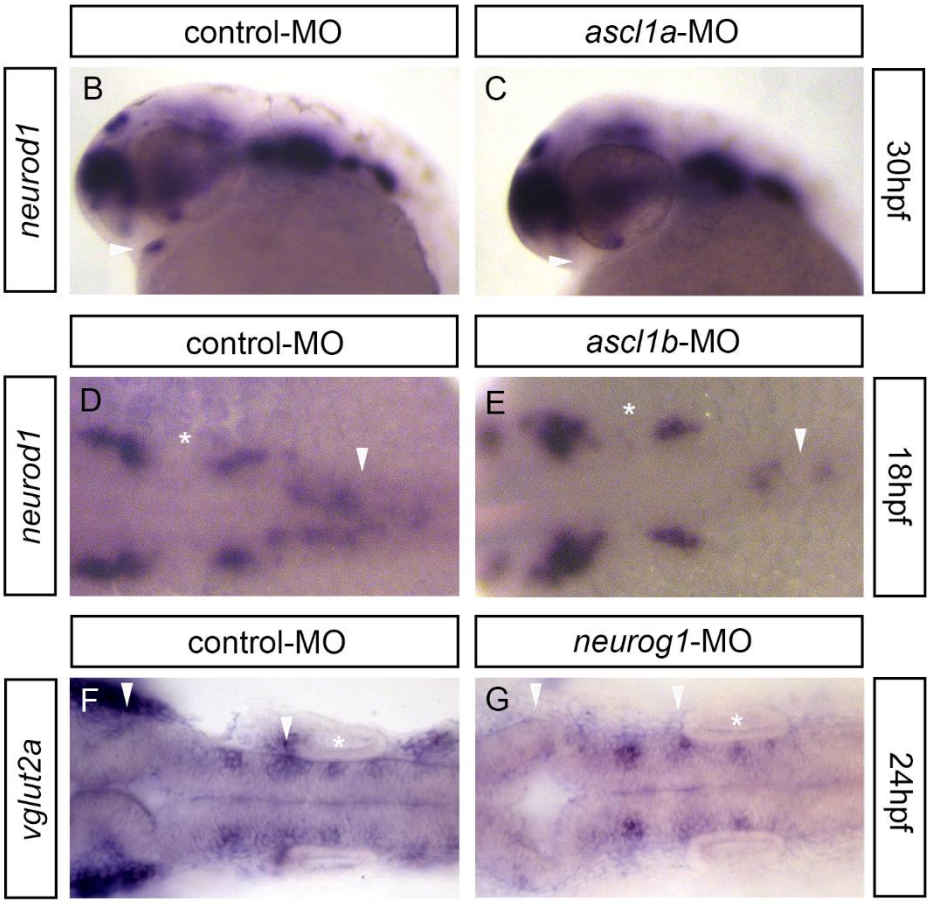

Figure 3.6. Efficiency of morpholinos. **A**) Morpholino sequences used in LOF experiments with respective references. **B-C**) *In situ* hybridization of *neurod1* in whole-mounted embryos injected with control-MO (B) and *ascl1a*-MO (C). **D-E**) *In situ* hybridization of *neurod1* in whole-mounted embryos injected with control-MO (D) and *ascl1b*-MO (E). **F-G**) *In situ* hybridization of *vglut2a* in flat-mounted embryos injected with control-MO (F) and *neurog1*-MO (G). Lateral (B-C) or dorsal (D-G) views with anterior to the left. Arrowheads in (B-G) point to the patches of *neurod1* or *vglut2a* expression. Asterisks indicate the position of the otic vesicle.

3.4. The growth of neurogenic and differentiation domains upon morphogenesis.

Up to now, our experiments have informed us about a) the spatiotemporal organization of proneural progenitor domains; b) putative lineage relationships between progenitor cells and differentiated neurons; and c) the proneural requirements for neuronal differentiation. However, and as highlighted during the introductory chapter, little is known on how progenitors behave during tissue growth and morphogenesis.

In order to have a general overview on how the hindbrain grows during embryonic neurogenic stages, we took advantage of the expression data generated in our previous experiments. We evaluated the growth of the proneural progenitor regions and the neuronal differentiation domains and compared them with the overall growth of the hindbrain. For this, we measured the MZ growth by quantifying the area occupied by the neuronal differentiation domain (HuC-positive) and the progenitor territory (proneural gene domains) upon embryonic development and compared them with the total neural tube area (Figure 3.7).

The neuronal differentiation territory grew dramatically during the first neurogenic wave transitioning from approximately 20% of the hindbrain area at 24hpf to almost 80% at 48hpf (Figure 3.7B). Concomitantly, the areas occupied by the proneural genes *atoh1a*, *ascl1b* and *neurog1* decreased over time (Figure 3.7C-E). This decrease in size seemed constant for all of the three proneural populations assessed, which could be explained either by similar differentiation rates or by an increase in tissue packing. To confirm that indeed proneural domains behaved similarly, we assessed their dynamics in the context of the whole progenitor domain (VZ). To do so, we obtained the area values of the whole progenitor domain (progenitor area) by subtracting the neuronal differentiation area (HuC-positive) from the total hindbrain area. The relative sizes of the *atoh1a*, *ascl1b* and *neurog1* domains remained constant within the progenitor area from 24hpf to 48hpf, suggesting that the proneural pattern of progenitor cells was maintained even after extensive neurogenesis (Figure 3.7F-H). In the mammalian spinal cord, the different progenitor populations grew equally due to similar

differentiation and proliferation rates across domains. However, the differentiation rate in the domain harbouring motoneuron progenitors was higher than the rest of progenitor populations, which altered the pattern (Kicheva *et al*., 2014). We did not observe such differences in the zebrafish hindbrain's progenitor domains, either because our analysis did not focus on the number of progenitor versus differentiated cells in each population but in the overall size of expression domains, or because the ratio of motoneurons generated in the hindbrain and in the spinal cord is different. Nonetheless, we cannot exclude that the morphogenetic events taking place in the embryonic hindbrain alongside neurogenesis might be impacting the differentiation rate of hindbrain progenitors. Thus, since these observations are only snapshots of a very dynamic process, these results indicate us that to fully understand the growth pattern of hindbrain progenitors we need to follow their dynamics. Therefore, our next approach was the study of the behaviour of individual cells during neuronal specification and tissue morphogenesis *in vivo*.

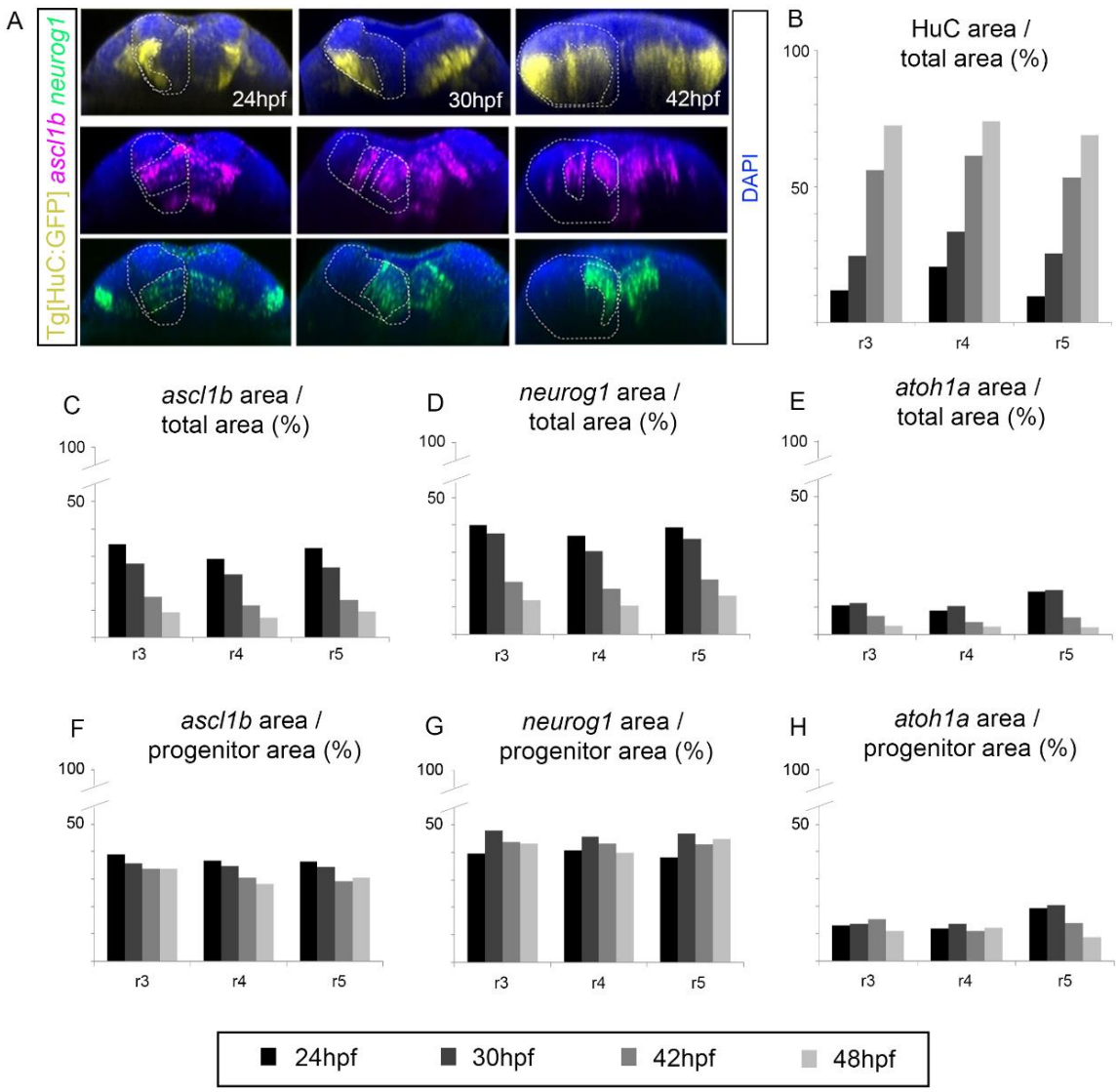

Figure 3.7. Quantification of proneural and differentiation domains growth over time. A) Transverse views of representative examples of expression data used to quantify the area of the domains of interest. Dashed lines highlight the contour of the measured areas. **B-E**) Graphical representation of the proportion (%) of a given gene expression domain over the total area of the embryo (assessed by DAPI staining) in rhombomeres 3, 4 and 5 at indicated stages with different hues. The domains represented are: neuronal differentiation HuC domain (B), *ascl1b* (C), *neurog1* (D) and *atoh1a* (E). **F-H**) The area sizes of each proneural domain, being *ascl1b* (F), *neurog1* (G) and *atoh1a* (H) are represented as a proportion within the total progenitor area. The progenitor area is obtained from the subtraction of the HuC area from the total area. Each area measurement was taken in five different embryos for stage and staining. The domains of interest were measured in reconstructed transverse views of confocal stacks of whole-mounted embryos using the imaging software Fiji. r, rhombomere.

3.5. The hindbrain displays different patterns of progenitor behaviour

The different proneural expression domains are populated by progenitor cells that proliferate and give rise to newly generated progenitors, which are added to the pool. This process is counterbalanced by neuronal differentiation, which subtracts cells from the VZ. Thus, cell proliferation and differentiation rates shape the tissue, as well as the specification of neural progenitors that are recruited to the pre-existing expression domains (Kicheva *et al*., 2014). Thus, to understand the growth patterns of hindbrain progenitors according to differential proneural expression, we analysed the clonal growth of hindbrain progenitors by tracking individual progenitors and recording their growth patterns according to position and developmental time.

To analyse individual progenitor cells upon time we randomly labelled progenitor cells, and then *in vivo* imaged these clones with high spatial and temporal resolution. To meet such requirements, we used the inducible Ubi-Switch system (Figure 3.8A) (Mosimann *et al*., 2011), which required two zebrafish transgenic lines: i) Tg[Ubi:CreERT2] transgenic line that expressed an inducible form of Cre under the control of the *ubiquitin B* promoter, and contained a *cmlc2*:GFP reporter allele for the green heart that facilitated the offspring screening (Mosimann *et al*., 2011); and ii) Tg[Ubi:loxP-EGFP-loxP-mCherry] or Tg[Ubi:Switch], which contained a floxed EGFP ubiquitously expressed under the control of the same promoter and labelled the whole fish green. mCherry was only expressed after Cre-recombinase activity, which excised EGFP.

Double transgenic Tg[Ubi:Switch]Tg[Ubi:CreERT2] embryos were injected at one-cell stage with 5ng of lyn-GFP mRNA to mark the cell contours. Embryos were dechorionated with pronase treatment at 11hpf and incubated with 5µM Endoxifen during 30 minutes (Felker *et al*., 2016), and grown at 23ºC until they reached the desired developmental stage. Then, they were whole-mounted in 1% agarose and imaged with a confocal microscope. Such short treatment at early stages induced recombination at a desirable rate, and their fluorescence was stable at least up to 72hpf (Figure 3.8C-E). Living embryos upon clonal induction

were imaged every three hours (from 21hpf to 72hpf), in order to follow the behaviour of the clones within the hindbrain. The experiments regarding the imaging of induced embryos, as well as the analysis of the clonal growth, were performed in collaboration with Christian Cortés Campos, PhD.

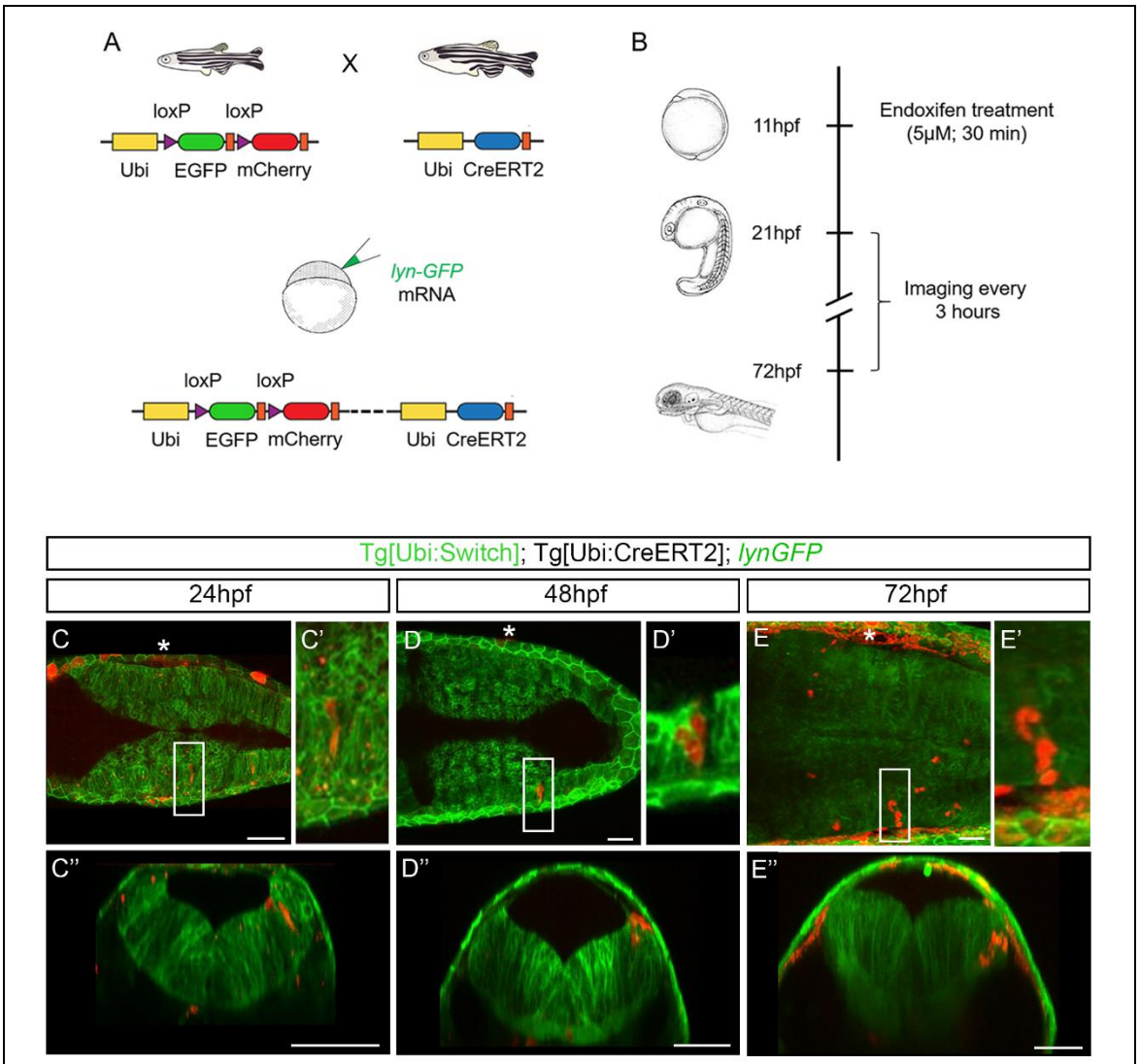

Figure 3.8. Clonal growth analysis within the hindbrain (I). **A**) Schematic representation of the two transgenic alleles carried by the Tg[Ubi:Switch] and Tg[Ubi:Cre-ERT2] zebrafish lines, used for the induction of random genetic recombination. **B**) Pipeline of clone induction and *in vivo* imaging experiments. **C-E**) Confocal images of the same living embryo at 24, 48 and 72hpf after clone induction. See how framed clones grow in size upon time. Dorsal view of hindbrains with anterior to the left. **C'-E'**) Magnifications of framed areas in (C-E). **C''-E''**) Transverse views depicting the DV positioning of the clones highlighted in the dorsal views. Asterisks indicate the position of the otic vesicle. Scale bars correspond to 50µM.

In order to understand the growth dynamics of hindbrain progenitors during morphogenesis, we quantified the number of cells per clone at three different embryonic stages, 24, 48 and 72hpf, which cover from early to late and residual neurogenesis (Figure 3.9). The normal distribution of clone sizes (number of cells per clone) revealed that the growth dynamics of hindbrain progenitors was diverse and that the proliferative capacity concentrated between 24 and 48hpf (Figure 3.9A-C). Most of the clones at 24hpf were constituted by one or two cells, which corresponded to the peak of the normal distribution. However, the distribution of clone sizes by 48hpf unveiled that hindbrain clones grew unequally, with clone size spanning from one cell to seven cells. At 72hpf, the clone size distribution was very similar to the one at 48hpf, with the exception of some outliers. This suggested that clones induced early in embryonic development exhausted their proliferative capacity and differentiated before 48hpf (Figure 3.9C).

The diversity in clone sizes observed when comparing 24hpf with 48hpf could be explained by the heterogeneous behaviour of progenitor cells: those that underwent early differentiation and did not have time to proliferate generated small clones, and progenitor cells that remained within the cell cycle for a longer time generated big clones. Moreover, the presence of even and odd-numbered clones at 48 and 72hpf also suggested a diverse proportion of modes of division, since the only way to generate odd-numbered clones from once-cell clones is through asymmetric cell divisions (He *et al*., 2012). Thus, hindbrain progenitors displayed heterogeneous proliferative capacity.

We next investigated the differentiation rate by examining the position of the former clones along the AB axis: cells in contact with the VZ most likely corresponded to progenitor cells in a proliferative state, whereas cells located close or within the MZ corresponded to neuronal precursors or differentiated neurons. Hence, we referred to the fate of the cells as either ventricular cells with spindle-like shape and an apical contact with the ventricle (Figure 3.9D-D', 3.9F-F'), or mantle cells characterized by a round shape and a close contact with the basal domain, especially at early stages (Figure 3.9E-E'; 3.9G-G'). Following these criteria, we observed that almost 90% of all analysed cells were ventricular

(n=71/79) at 24hpf, whereas they were only a 32% of the total (n=46/144) at 48hpf and 13% by 72hpf (n=17/135; Figure 3.9H). From the pool of labelled progenitors, 90 newly generated neurons were added to the MZ during the first time window. However, from 48hpf to 72hpf, only 20 neurons were born. These results confirmed our previous observations suggesting that neurogenesis occurred mainly from 24 to 48hpf.

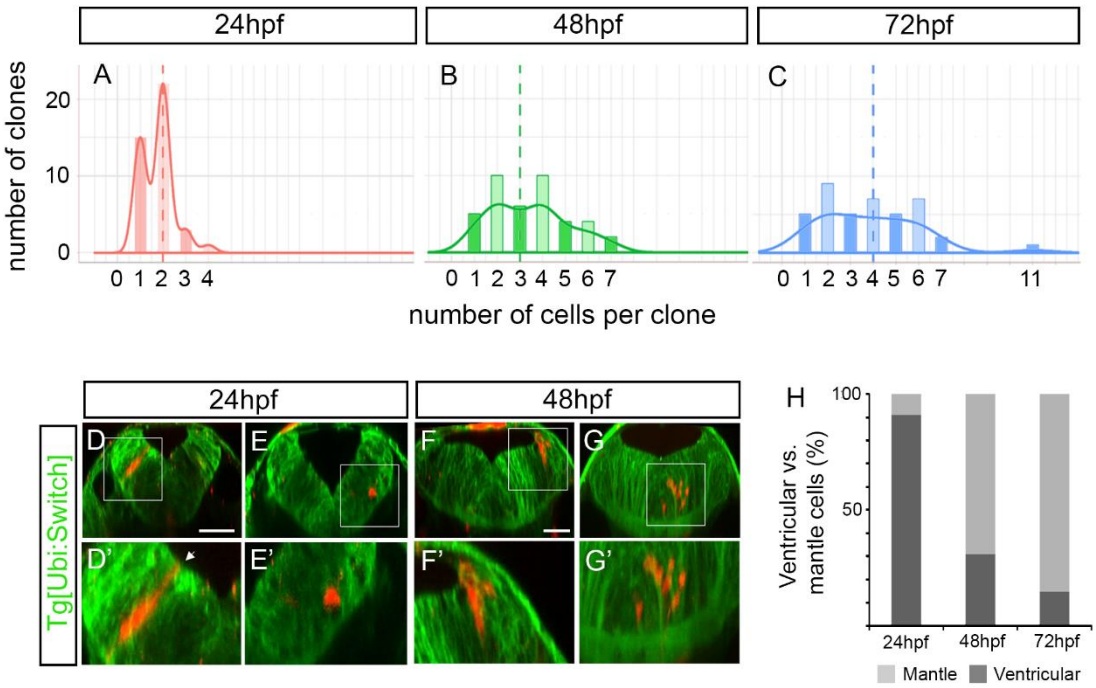

Figure 3.9. Clonal growth analysis within the hindbrain (II). A-C) Normal distribution of clone sizes (number of cells) at indicated stages. Each bar represents the number of clones found within the total pool of embryos that contain the given number of cells. Even- and odd-number of cells per clone is displayed with different hues. The dashed line for each graph represents the mean absolute number. Overall, 42 clones were analysed of 10 embryos. **D-G**) Representative examples of clones located in the ventricular and mantle zones. **D'-G'**) Magnifications of framed regions in (D-G). The arrowhead in D' points to the apical contact with the ventricle of the represented cell. **H**) Growth of the mantle zone at expense of the progenitor domain over time. Graphical representation showing the proportion of ventricular cells (dark grey) versus mantle cells (light grey) at indicated stages. Proportions are shown as percentages. For quantifications, only clones spanning from r2 to r6 were considered. Scale bar corresponds to 50µM.

Taken together, these results support the concept that progenitor expansion and neuron production occur at the same time, and that different modes of division coexist during hindbrain growth. These observations raised the question of whether the different progenitor growth patterns, understood as the balance between proliferation and differentiation rates, were differentially located in time and space.

We observed that the smallest clones, containing one or two cells by the end of the *in vivo* imaging, derived from the most dorsally located progenitors (Figure 3.10A). After losing the apical contacts, these dorsal cells migrated ventrally, usually between 20 and 30hpf, and differentiated. Since the ventral migration and subsequent differentiation occurred very early, these cells generated small clones, which represented 24% of the analysed clones (n=10/42). In addition, we identified a second type of progenitor cells that behaved in an opposing manner and they were also located in the dorsal pole of the hindbrain but in a more ventral position. These cells remained as progenitor cells for long time, which allowed them to proliferate and generate clones with high number of cells. As an example of such behaviour, the clone shown in Figure 3.10B started with two initial cells detected at 30hpf that became six cells by 48hpf. At 54hpf, some of these clonally related cells started to migrate towards the MZ and differentiated. By the end of the time lapse experiment, most of the cells produced by this particular clone had differentiated except from two cells that remained ventricular. This type of cell behaviour was observed in 14% of the analysed clones (n=6/42). The rest of the induced clones in the hindbrain were located in medial to ventral positions when compared to the first two types of progenitors, with no stereotyped growth pattern. Nonetheless, as a general observation, medioventral cells showed a moderate growth rate, generating clones of intermediate size when compared to the clones generated from dorsal progenitors (Figure 3.10C, 62% n=26/42 clones).

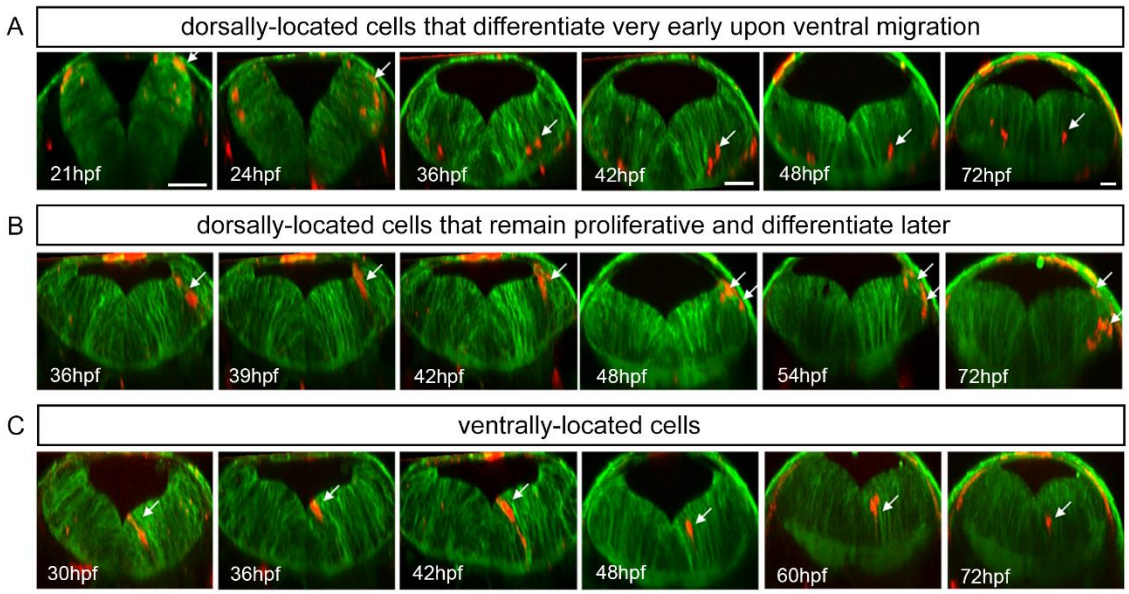

Figure 3.10. Clonal growth analysis within the hindbrain (III) A-C) Different growth patterns of hindbrain progenitors according to DV position. Each row shows a given clone and its progeny through successive time points in transverse views, which are indicated with white arrows. Scale bars correspond to 50 µm.

In conclusion, these observations suggested that the proliferative and neurogenic capacities of hindbrain progenitors were differentially located within the DV or ML axes. In the mammalian spinal cord, progenitor clonal growth was directed towards the DV axis and restricted in the AP axis (Kicheva *et al*., 2014). Growth directionality in the zebrafish hindbrain was mostly occurring in the AB axis, as newly generated cells in each clone rarely moved along the AP or the DV axis. As suggested from the research done in the spinal cord, the growth differences between the DV domains of the neural tube could be explained by their specific transcriptional signature, which would confer specific proliferative and/or neurogenic properties. With this in mind, and in line with our proneural expression profiles, we could assign the identity of *atoh1a* progenitors to the dorsal progenitor cells, whereas more medial progenitors would belong to the *ascl1b/neurog1* domain. However, to understand the relationship between proneural function and progenitor dynamics, we focused on a specific progenitor population, the lower rhombic lip (LRL), which constitutes the most dorsal progenitor population of the developing hindbrain.

CHAPTER 4: RESULTS (II)

THE ROLE OF *ATOH1* GENES IN THE DEVELOPMENT OF THE LOWER RHOMBIC LIP DURING ZEBRAFISH HINDBRAIN MORPHOGENESIS

This chapter has been submitted for publication and it is under revision. The original manuscript can be found in bioRxiv as following:

Belzunce I., Pujades C. The role of *atoh1* genes in the development of the lower rhombic lip during zebrafish hindbrain morphogenesis. *bioRxiv*, 2019.

https://www.biorxiv.org/content/10.1101/719997v1.full

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CHAPTER 5: GENERAL DISCUSSION

ON PRONEURAL FUNCTION AND PROGENITOR DYNAMICS DURING THE SPECIFICATION OF HINDBRAIN NEURONAL LINEAGES

5.1. The proneural gene hierarchy during cerebellar and hindbrain neurogenesis

During embryonic brain development, the generation of neuronal diversity relies on the restriction of progenitor potentials to specific spatiotemporal coordinates. Hence, neural progenitors become confined within different territories defined by the specific expression of transcriptional programs. Such gene regulatory networks confer positional identity to progenitor cells, which in turn become specified and give rise to specific types of differentiated neurons. However, how these discrete populations balance their different modes of cell division and cope with tissue morphogenesis is still unclear. In this work, we have followed the Lower Rhombic Lip (LRL) progenitor cell population through the early steps of neurogenesis and hindbrain morphogenesis in order to understand proneural function and progenitor dynamics during neuronal specification. In this section, we will discuss the role of *atoh1* genes in the generation of RL-derived lineages.

In amniotes, *atoh1* has been long known as the proneural gene responsible for engaging rhombic lip progenitors into neuronal differentiation. Self-renewing *atoh1* progenitors give rise to post-mitotic granule cell precursors that are characterized by the expression of *neurod1*. This transcription factor is necessary for the generation of radially-migrating, post-mitotic granule cells (Miyata *et al*., 1999). Interestingly, *neurod1* overexpression in cerebellar progenitors causes a premature downregulation of *atoh1* expression and precocious radial migration of granule cell precursors (Butts *et al*., 2014). This paradigm follows the classical proneural hierarchy, where the cell fate selector gene *atoh1* is expressed in proliferative apical progenitors that divide asymmetrically giving rise to post-mitotic precursors that will turn on the neuronal differentiation gene *neurod1*.

In the case of the embryonic zebrafish cerebellum, *atoh1a* and *atoh1c* seem to be the homologs of *atoh1* in amniotes, promoting progenitor cell delamination and neuronal differentiation. Moreover, they are expressed in different sets of progenitors that contribute with different neuronal populations to cerebellar development (Kidwell *et al*., 2018). However, what are the downstream activated neuronal differentiation genes in this context? In the zebrafish Upper Rhombic

Lip (URL) the mechanism seems to be analogue to amniotes, since *atoh1a* generates intermediate progenitors that will express *neurod1* (Kani *et al*., 2010). On the other hand, our results in the anterior LRL show that the onset of LRL neurogenesis is around 24hpf, before the onset of *neurod1* expression, which starts to be detected in LRL-derived precursors around 48hpf (Rauch *et al*., 2003), suggesting that a different neuronal differentiation gene rather than *neurod1* is functioning in early LRL-derived neurogenesis. *neurod4* is another bHLH transcription factor classically described as a neuronal differentiation gene that is expressed in the hindbrain at the onset of neurogenesis. We show that *neurod4* is expressed in basally-biased neuronal precursors. Even though it is expressed in a more basal location, the *neurod4* territory would cover all proneural gene expression domains as if *neurod4* was a convergence node for all proneural genes to promote hindbrain neurogenesis. However, *neurod4* is never expressed in LRL-derived cells. These observations pose the question of what is the proneural hierarchy during early stages of LRL-derived neurogenesis.

According to our gene expression data and functional experiments, *atoh1a* is expressed in proliferating, apical progenitors of the LRL as early as 14hpf, and it is necessary and sufficient for *atoh1b* expression and neuronal differentiation, as *atoh1a*^{fh282} mutants show defects in progenitor delamination and neuronal production. a*toh1b* is expressed later in time, in neuronal precursors with narrow apical contacts and cell bodies that are basally biased. Taking into account the work described in Hiscock *et al*., 2018, *atoh1b* cells would be more prone to engage into differentiation than the apically located *atoh1a* progenitors. This observation places *atoh1b* as the reasonable candidate for playing the role of neuronal differentiation gene in the LRL.

Our functional experiments show that both *atoh1a* and *atoh1b* are able to induce neuronal differentiation by activating the *lhx2b* specific program. The neurogenic role of *atoh1,* as well as the contribution to the *lhx2b* population, had already been described in the cerebellum and the spinal cord in chick and mouse embryos (Helms and Johnson, 1998; Gowan *et al*., 2001; Bermingham *et al*., 2001). However, we demonstrate for the first time that *atoh1b* functions as a neurogenic factor that can also specify neuronal identity in a cell-autonomous manner. In this

sense, we can conclude that *atoh1a* and *atoh1b* functions are very similar, as it is to be expected from closely related genes. Nevertheless, the same experiments revealed that *atoh1a* is able to activate the expression of *atoh1b* but not the other way around, strongly suggesting that in the LRL progenitor population *atoh1a* functions as the cell fate selector gene, being crucial for neurogenesis initiation and promotion, whereas *atoh1b* is expressed downstream as a neuronal differentiation gene, such as *neurod1* and *neurod4* in other contexts, carrying out the neurogenic program.

Regarding *atoh1a* function, *atoh1a*^{fh282} mutants show a deficient phenotype in neurogenic production as well as a defect in progenitor delamination. These observations suggest that *atoh1a* in the LRL has the same function than *atoh1c* in the generation of URL-derived granule cells. Interestingly, *atoh1c* is required for the activation of *atoh1b* in URL progenitors, which suggests that the role of *atoh1b* as a downstream neuronal differentiation gene is conserved in all RL cells (Kidwell *et al*., 2018).

The impairment of neurogenesis observed in the LRL of *atoh1a*^{fh282} mutants is partially restored as development progresses, with “escaper” progenitors that are able to differentiate even in the absence of *atoh1a* function. We can explain the neurogenic recovery in *atoh1a*^{fh282} mutants by the features of the mutant allele itself: the mutation generated by TILLING technology results in a single nucleotide substitution that leads to an aminoacidic change in the DNA-binding domain of the Atoh1 protein, impairing its functionality and mainly generating hypomorph mutants (Kidwell *et al*., 2018). On the other hand, downregulation of Atoh1 protein by translation-blocking morpholino leads to a more severe phenotype, impairing completely the formation of the LRL-derived neuronal arches, even at late stages (Figure 5.1). Thus, the milder phenotype in the *atoh1a*^{fh282} homozygous mutants is most likely a consequence of behaving as a hypomorph mutation as already reported (Kidwell *et al*., 2018). Intriguingly, *atoh1b* expression never recovered in *atoh1a*^{fh282} mutants. Thus, the restored neurogenic phenotype observed in *atoh1a*^{fh282} homozygous mutants cannot be simply explained by the role of *atoh1b* compensating for the loss of *atoh1a*. This conclusion leads to two hypothetic scenarios: *atoh1b* induction and/or

maintenance needs efficient and sustained expression of functional *atoh1a*; and/or *atoh1a* might still be able to activate neurogenesis, even if non-efficiently, by interacting with dimerization partners such as E proteins. On behalf of this possibility, recent studies in the chick spinal cord suggest that the role of E proteins, often regarded as passive partners of proneural function, play an active role in modulating the neurogenic activity of proneural factors (Le Dréau *et al*., 2018). More on the matter, in the specific case of LRL progenitors the TCF4 E protein, Atoh1 dimerization partner, is required for the acquisition of pontine neuron identity in the mouse embryonic hindbrain (Flora *et al*., 2007). However, whether these dimerization proteins are able to rescue the loss of DNA-binding ability of *atoh1a* in the *atoh1a*^{fh282} mutant background remains to be elucidated.

Unlike amniotes, the zebrafish genome contains three *atoh1* genes: *atoh1a*, *atoh1b* and *atoh1c*. While the three of them are expressed in cerebellar progenitors, only *atoh1a* and *atoh1b* are expressed in the LRL. One of the main conclusions of our work within this progenitor population is that *atoh1a* behaves as the cell fate selector gene, whereas *atoh1b* functions as a neuronal differentiation factor maintaining the transcriptional program initiated by *atoh1a*. This implies that *atoh1* gene duplication in teleosts resulted in a sub-functionalization. In this scenario, while *atoh1a* is essential for neuronal differentiation in the LRL, the *atoh1b* loss-of-function experiments are less conclusive. The *atoh1b*-MO is less efficient, resulting in a gradient of phenotypes that go from mild to non-existent (Figure 5.1). One possible explanation for the lack of phenotype in *atoh1b* morphants could be a hypothetic compensatory role of *atoh1a* for the loss of *atoh1b,* carrying on with the neurogenic program by itself. Indeed, zebrafish embryos bearing the *atoh1b* ^{fh473} mutant allele did not show defects in neuronal migration and differentiation in cerebellar progenitors (Kidwell *et al*., 2018); reinforcing the idea that, in the absence of the downstream effector *atoh1b*, the upstream initiators *atoh1a* and *atoh1c* (in LRL and URL progenitors, respectively) can promote neurogenesis up to completion.

In the zebrafish hindbrain, such compensatory relationships between bHLH neurogenic factors would not be exclusive of the LRL. For example, in the ventral hindbrain, *neurog1* functions as the fate selector gene and *neurod4* as the

neuronal differentiation gene, even though *neurod4* loss-of-function does not impair neurogenesis in the absence of *neurog1* (Park *et al*., 2003). In this same line, it has been proposed that in some contexts proneural genes and neuronal differentiation genes engage in a cooperative relationship rather than in a transcriptional hierarchy in order to promote neurogenesis, according to the work done in the zebrafish olfactory bulb and habenula (Madelaine *et al*., 2011; Halluin *et al*., 2016). Specifically, neural progenitors of the olfactory bulb lacking *neurog1* function do not express *neurod4* at early stages. However, *neurod4* expression recovers with time and compensates for the loss of *neurog1*, enabling neuronal production and indicating that *neurod4* might be independently expressed from its proneural gene. Nonetheless, this does not seem to be the case in the zebrafish LRL, as *atoh1b* expression is never recovered in the *atoh1a* mutant context at least up to 48hpf. In our conditional experiments, *atoh1a* ectopic expression was rapidly downregulated, whereas ectopic *atoh1b* remained active at later stages, highlighting the different temporal windows in which proneural and neuronal differentiation genes are expressed in the classical transcriptional hierarchy.

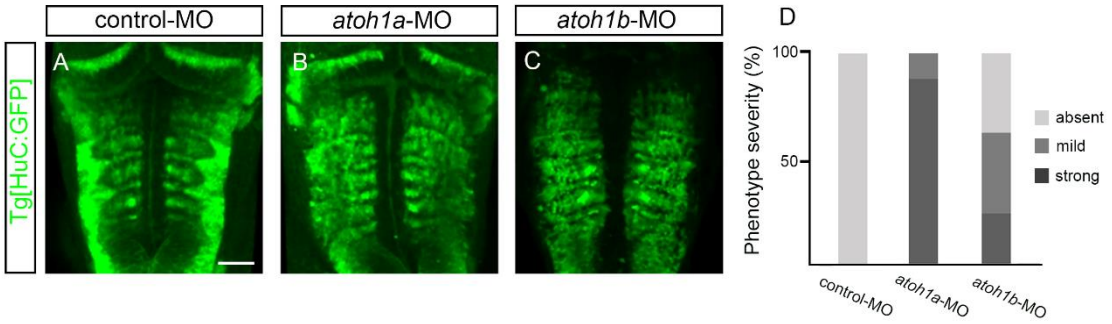

Figure 5.1. *atoh1a* and *atoh1b* differential requirement for neurogenesis. Tg[HuC:GFP] embryos were injected with control-MO (n=24) (A), and translation-blocking MOs for *atoh1a* (n=21) (B), and *atoh1b*-MO (n=20) (C) and analysed at 42hpf. Differentiated neurons are observed in green. **D**) Graphical representation of the phenotype proportion obtained for every morphant condition. None of embryos injected with the control MO showed aberrant phenotype (n=24/24). In the case of *atoh1a* morphants, 85% of the embryos had no LRL-derived neuronal arches (n=18/21), and 15% showed partial defects (n=3/21). *atoh1b*-MO injected embryos result in a disperse distribution of phenotype severity, being 40% unaffected (n=8/20), 40% with a mild phenotype (n=8/20) and 20% with a strong phenotype similar to *atoh1a* morphants (n=4/20). **C**). All MOs were injected at the amount of 5ng. The *atoh1a* and *atoh1b* MOs used were described in Millimaki *et al*., 2007. Images show dorsal views with anterior to the top. Scale bar corresponds to 50µm.

5.2. Notch signalling as the gatekeeper of LRL neurogenesis

One of the functions of Notch signalling is to single out precursors from a field of equipotent progenitors. In the context of neural cells, Notch signalling regulates the binary decision whether to engage into neurogenesis or to remain as a cycling progenitor. Therefore, Notch signalling works as a gatekeeper controlling the number of cells that undergo differentiation. Cells with active Notch signalling will activate the expression of *hes/her* genes, which will keep cells as undifferentiated proliferative progenitors. On the other hand, neighbouring cells without Notch activity will allow proneural gene expression, engaging progenitor cells into differentiation that will become post-mitotic and will leave the VZ. In our work, we show that Notch signalling regulates the transition from *atoh1a*-proliferative progenitors to *atoh1b*-neuronal precursors, as Notch blockade increases the number of *atoh1b* cells at the expense of *atoh1a*, confirming that the LRL behaves as a proneural cluster. Hence, cells with active Notch signalling will remain in the ventricular zone as proliferating progenitors as shown in the analysis of Tg[tp1:GFP] embryos, which express GFP in the Notch-active cells.

We do not know much about the main players of the Notch pathway in LRL neurogenesis. In the mammalian RL, *hes5* is the Notch target gene responsible for keeping Notch-responsive cells in a progenitor state (Gazit *et al*., 2004). Interestingly, the zebrafish *hes5* homologue, *her4*, is also expressed in zebrafish LRL progenitor cells, suggesting a conserved role of *her4*-related genes in LRL neurogenesis (Figure 5.2). Interestingly, *her4* is expressed in all the proneural domains of the hindbrain, not only the LRL. This highlights that, even if proneural function in the hindbrain has diverged to generate distinct neuronal lineages, the selection of neuronal precursors via Notch targets is conserved in all hindbrain proneural domains.

Taking into account the Notch oscillation model and assuming that it might apply in our context, LRL-proliferative progenitors would express *atoh1a* in an oscillatory fashion. Such oscillation would be complementary to the expression of Notch targets of the *hes/her* family, such as *her4*. While the expression of these antagonistic factors remained symmetric and oscillating, LRL-progenitor cells

would be able to progress through the cell cycle and proliferate. Upon high and sustained expression of *atoh1a*, the progenitor cell would activate the expression of *atoh1b* and exit the cell cycle, engaging into neuronal differentiation (Figure 5.3). Nonetheless, whether *atoh1a* expression oscillates in the LRL is still unknown, as well as the signals driving the symmetry break between *atoh1a* and Notch targets.

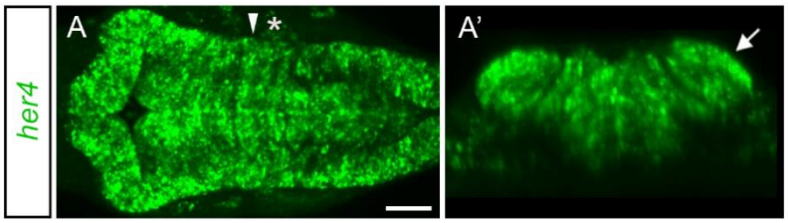

Figure 5.2. *her4* expression in hindbrain's proneural domains. Whole-mount *in situ* hybridization of *her4* in a 24hpf embryo. **A**) Dorsal view with anterior to the left. **A'**) Transverse view at the level indicated by the arrowhead in (A). Arrow in (A') points to the LRL, which is positive for *her4* expression. Asterisk indicates the position of the otic vesicle. Scale bar corresponds to 50µm.

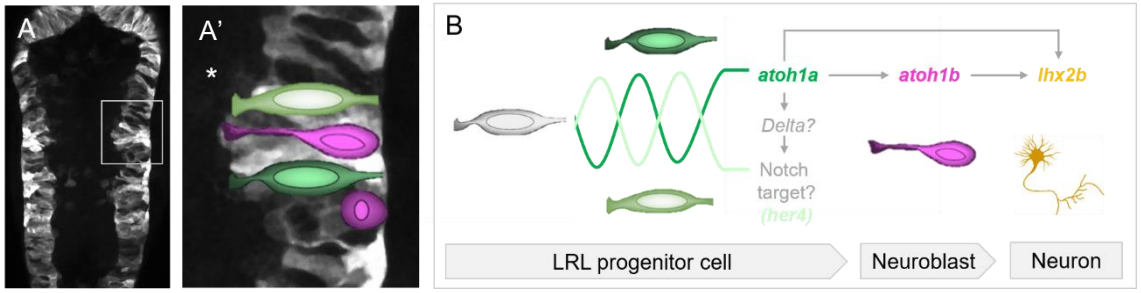

Figure 5.3. Oscillation model for zebrafish LRL neurogenesis. **A**) Tg[atoh1a:GFP] embryo at 24hpf, GFP signal is coloured in grey scale. **A'**) Magnification of the squared area in (A), with a schematic representation of the different cellular states found in the LRL: *atoh1a*-expressing cells (dark green), *her4*-expressing cells (light green) and *atoh1b*-expressing cells (magenta). Asterisk indicates the hindbrain's lumen. **B**) Schematic representation of LRL cells according to *atoh1a* proneural gene expression. Hypothetically, the expression of *atoh1a* (dark green) and the Notch target *her4* (light green) follow an oscillating and complementary expression pattern. High and sustained expression of *atoh1a* would activate the expression of *atoh1b* (magenta cells), which would induce the transition from proliferating neural progenitors to post-mitotic neuronal precursors and would be regulated by Notch signalling. In turn, both *atoh1a* and *atoh1b* would be able to activate the expression of *lhx2b* in LRL-derived neurons (orange cell).

5.3. On the spatiotemporal distribution of LRL neuronal derivatives

The proneural code provided by *atoh1a* and *atoh1b* ensures the production of *lhx2b* neurons by LRL progenitors. However, neuronal differentiation is only the initial step in the construction of a mature brain. After a neuron is born and has acquired a given identity, it has to extend its axons and integrate into the proper neuronal circuit. In some cases, this integration process involves extensive neuronal migration. LRL neuronal derivatives present a very interesting example of how neuronal organization after differentiation shapes the overall architecture of the hindbrain's mantle zone (MZ).

One of the distinctive features of the LRL when compared to the rest of the hindbrain proneural gene expression is that the expression of *atoh1a* is homogenous along the AP axis, with the corresponding neuronal production. However, we show that LRL-derived neurogenesis is mainly concentrated in interhombomeric boundaries and our *atoh1a*-lineage tracing experiments show that first-born *lhx2b* neurons, after migrating from dorsal positions, are located within the interface between rhombomeres at a ventral level. Later-born *lhx2b* neurons are located within the MZ following the first-born neurons, organizing in structures that we call neuronal arches.

The individual tracking of *atoh1a*-progenitor cells revealed an interesting observation: regardless their birthplace, first-born *atoh1a* neurons are located within rhombomeric boundaries, a region devoid of proneural gene expression and, as a consequence, of neurogenic activity during the initial steps of hindbrain neurogenesis (Voltes *et al*., 2019). This observation strongly suggests that there must be some kind of chemoattractant signal derived from the boundary cells that allow the migration of LRL cells towards ventral positions. Indeed, the boundary cell population has been proposed as a signalling centre that instructs the positioning of *fgf20*-expressing neurons within rhombomeric centres by expressing repellent cues such as semaphorines (Terriente *et al*., 2012). To date, no chemoattractant signals have been described to be secreted from boundary cells. However, there is a signalling pathway that is tempting to propose as a

candidate signal driving LRL migration towards rhombomeric boundaries in their ventral half: the netrin/DCC pathway, where netrin is the ligand and DCC is the receptor. This pathway is necessary for the proper migratory route of pontine neurons from the posterior RL to ventral r3 in the murine hindbrain (Kratochwil *et al*., 2017). In the zebrafish hindbrain, the netrin/DCC pathway has been implicated in the establishment of contralateral projections in cranial and reticulospinal motoneurons and the wiring of functional neuronal circuits (Suli *et al*., 2006; Jain *et al*., 2014). More specifically, *netrin1a* is expressed in the ventral midline all along the AP axis of the hindbrain and enriched in rhombomeric boundaries. Interestingly, DCC has been described to be expressed in LRL cells (Vanderlaan *et al*., 2005; Fricke *et al*., 2005). This information highlights the netrin/DCC pathway as a candidate molecular mechanism by which hindbrain neurons, LRL-derived in this case, would preferentially locate in the MZ of the boundaries.

Up to now, we have discussed the spatial distribution of LRL progenitors and their neuronal derivatives and the impact in the architecture of the MZ. However, we have to take into account another factor, time, since the cerebellar RL generates different neuronal types in a temporal sequence that is conserved along vertebrates (Hibi *et al*., 2017; Kidwell *et al*., 2018). Our birth-dating and cell-tracking experiments show that the time of neuronal differentiation determines the mediolateral position of LRL-derived neurons in which early-born neurons position ventromedially and late-born neurons position dorsolaterally (Figure 5.4). Although this may indicate that the generation of distinct neuronal types from the same progenitor population relies on time, the known markers for LRL-derived neurons (*lhx2b*, *lhx9*, *bahrl1*, *zn5, vglut2*) are expressed in all neurons of the LRL-derived neuronal arches (Sassa *et al*., 2007; Kinkhabwala *et al*., 2010), suggesting that in the LRL time does not confer a specific neuronal fate.

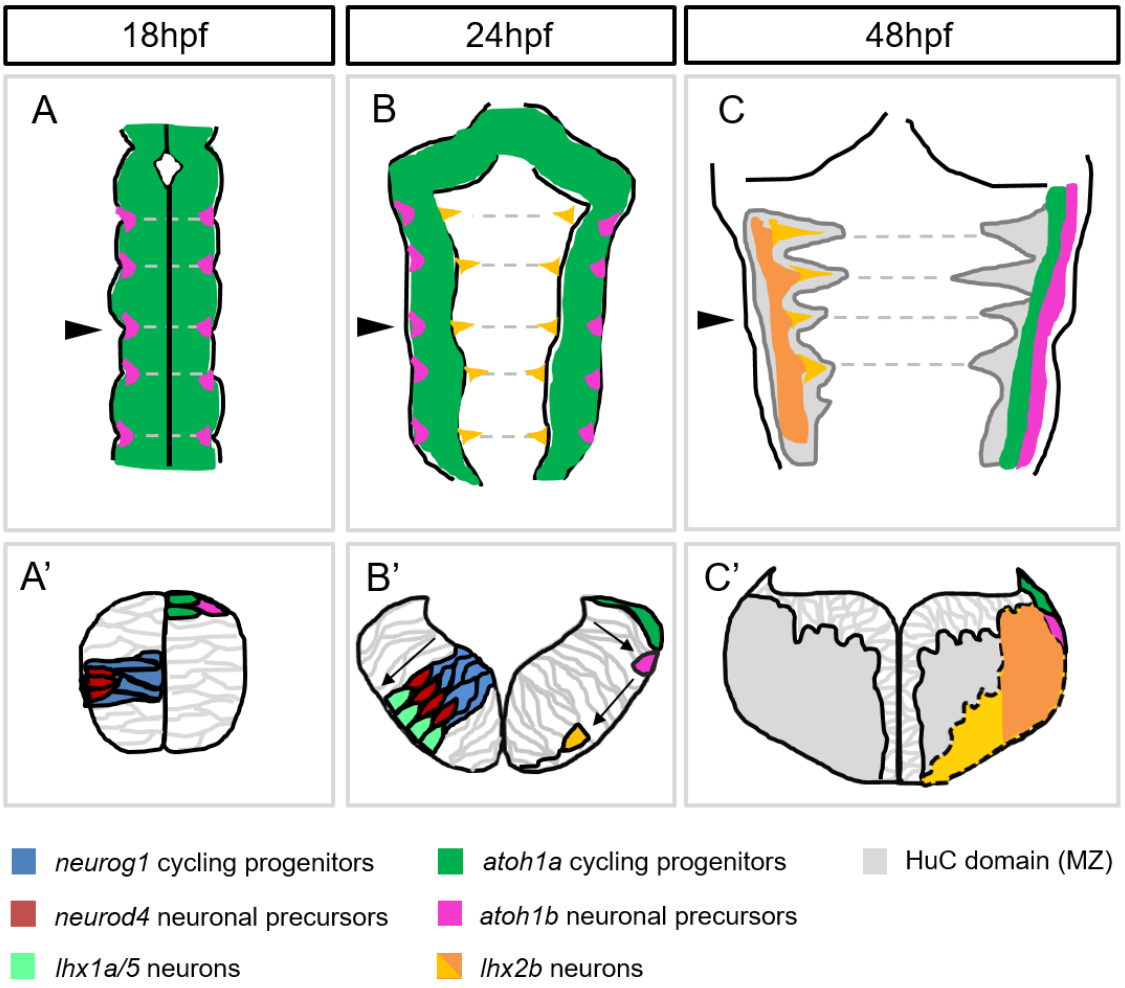

Figure 5.4. Sequence of events in hindbrain and LRL neurogenesis. A-A') At early embryonic stages (18hpf), the zebrafish hindbrain expresses *atoh1a* (dark green) all along the AP axis, and regionalizes along the DV axis in different proneural gene expression domains. Note that the *atoh1a*-population is specified at the most dorsal pole of the hindbrain (dark green), whereas other proneural domains are specified more ventrally, such as *neurog1* (blue). **B-B'**) The onset of LRL-derived neurogenesis is at 24hpf. *atoh1a*-proliferative progenitors transition into basally-located *atoh1b* cells (magenta), which give rise to *lhx2b* neurons (yellow) that upon tangential migration will locate in the interhombomeric boundaries within the hindbrain MZ. This process occurs alongside the dorsal opening of the neural tube, which generates the fouth ventricle. The rest of the hindbrain's proneural domains engage into neurogenesis approximately at the same time. For example, proliferative progenitors that express *neurog1* (blue cells) will give rise to *neurod4* neuronal precursors (red). In turn, these cells will differentiate into *lhx1a/5* neurons (light green). Black arrows indicate the directionality of cellular migration upon differentiation. **C-C'**) At 48hpf, first-born LRL-derived neurons are located medially, at the tip of the neuronal arches (yellow), which coincide with interhombomeric boundaries (dashed lines). Late-born neurons will accumulate in more lateral positions, at the base of the arches (orange). Note that the dorsal view in (C) depicts the progenitor populations of the right side and the neuronal populations on the left side. In the transverse view (C'), both progenitor and neuronal populations are represented in the right side. **A-C**) Dorsal views with anterior to the top with dashed lines depicting the interhombomeric boundaries. **A'-C'**) Transverse views of (A-C) at the level of the black arrowheads.

5.4. Balancing distinct modes of division within the developing LRL

During the development of the CNS, the balance between the production of differentiated neurons and self-renewing progenitor proliferation is of key importance to ensure the production of all the necessary neuronal cells to generate a fully functional brain, as well as keeping a reservoir of proliferative progenitors that account for the growth of the tissue. Therefore, the proportion of the different modes of division within domains of proliferative progenitors is a crucial event.

Progenitor cells within the LRL are actively engaged into mostly neurogenic divisions at the onset of neurogenesis. Interestingly, lineage tracing of *atoh1a* progenitors reveals that they display two different growth patterns depending on their position along the DV axis: symmetric neurogenic divisions (NN) occur in the most dorsal layer of progenitor cells; whereas more ventral *atoh1a* cells undergo the three modes of division: NN divisions, asymmetric neurogenic divisions (PN) and symmetric proliferative divisions (PP). Specifically, dorsal *atoh1a* progenitor cells are more prone to transition towards *atoh1b*-precursor cells. In contrast, *atoh1a* cells located just underneath the dorsal ones hold self-renewing capacity and contribute both to the *lhx2b*-neuronal population and to the LRL progenitor population in order to maintain the progenitor pool. Thus, the regionalization of different modes of division within the same progenitor population may be useful to ensure the rapid and continuous production of neurons while preventing the exhaustion of the progenitor pool. This regionalization may be due to dorsally-derived signals that favour neuronal differentiation and do not reach deeper cell layers. Upon differentiation, dorsal cells will migrate away from the LRL, leaving up vacant space that will be filled up by the proliferating cells underneath, which in turn will be exposed to the differentiation signal. However, how this dorsoventral gradient of neuronal differentiation is orchestrated is still unknown.

Nevertheless, one of the well characterised dorsally-derived signals in the developing neural tube is BMP, which is known for its critical role in the

specification of the *atoh1* cells in the spinal cord as demonstrated in mouse and chick embryos (Tong *et al*., 2015). More specifically, the mediators of BMP-signalling Msx1 and Msx2 act as transcriptional activators of the *atoh1* gene through the 3' enhancer in the murine spinal cord (Duval *et al*., 2014). In this sense, high exposure to BMP signalling in dorsal LRL cells could lead to high and sustained expression of *atoh1a* and the subsequent neuronal differentiation according to the oscillation model. In turn, ventral LRL cells would receive less BMP resulting in lower levels of *atoh1a* expression. However, this hypothesis is contradictory with the data on SMAD1/5 activity in the chick spinal cord, where high SMAD1/5 activity promotes self-renewing divisions in progenitors of spinal interneurons (Le Dreáu *et al*., 2014). Nonetheless, we still know little about the signalling gradients that govern progenitor specification in the developing zebrafish hindbrain. With some exceptions, the neuronal populations that arise within the embryonic hindbrain are equivalent to the ones found in the spinal cord (Hernández-Miranda *et al*., 2017a), suggesting that the role of patterning cues such as BMP should be quite similar. Nevertheless, we have to take into account that, unlike in the spinal cord, hindbrain morphogenesis impacts the position of progenitor cells, shifting the orientation of the apico-basal axis and positioning dorsal domains to lateral positions. How the dorsal-bound hindbrain lumen formation affects the establishment of signalling gradients is an issue that has not been addressed yet, but we believe that it is the first step in order to understand how cellular specification and progenitor dynamics are coupled with extensive brain morphogenesis.

CHAPTER 6: CONCLUSIONS

[1] The neurogenic capacity in the zebrafish embryonic hindbrain is regionalized along the dorsoventral (DV) axis in domains defined by the expression of the proneural genes *atoh1a*, *ptf1a*, *ascl1a, ascl1b* and *neurog1.* With time, neurogenesis becomes regionalized along the anteroposterior (AP) axis, confining proneural gene expression to boundary-flanking regions.

[2] The most dorsal region of the posterior hindbrain, the Lower Rhombic Lip (LRL), is defined by the expression of *atoh1a* and *atoh1b*. In contrast with the rest of proneural genes expressed in the hindbrain, *atoh1a* and *atoh1b* expression is not regionalized along the AP axis.

[3] *atoh1a* is expressed in proliferative progenitors of the LRL, whereas *atoh1b* is expressed in post-mitotic neuronal precursors derived from *atoh1a* progenitors.

[4] *atoh1a* progenitors give rise to neurons that preferentially locate within interhombomeric boundaries independently of progenitor position in the AP axis. *atoh1a* progenitors of the LRL give rise to *lhx2b* neurons.

[5] The mode of division of *atoh1a* progenitors relies on their DV position. Dorsal *atoh1a* progenitors undergo symmetric neurogenic (NN) divisions exclusively, whereas *atoh1a* progenitors located underneath undergo three types of division: symmetric proliferative (PP), asymmetric (PN) and symmetric neurogenic (NN) divisions.

[6] *atoh1a* is necessary for promoting delamination and neuronal differentiation in LRL progenitors.

[7] *atoh1a* is necessary and sufficient to activate the expression of *atoh1b* in LRL-derived neuronal precursors.

[8] *atoh1a* and *atoh1b* are both sufficient for promoting neuronal differentiation and activating the expression of *lhx2b* in hindbrain progenitor cells.

[9] Notch signalling regulates the transition of *atoh1a*-proliferative progenitors to *atoh1b* post-mitotic precursors.

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APPENDIX. OTHER CONTRIBUTIONS

Letelier, J., Terriente, J., Belzunce, I., Voltes, A., Undurraga, C.A., Polvillo, R., Devos, L., Tena, J.J., Maeso, I., Retaux, S., Gomez-Skarmeta, J.L., Martínez-Morales, J.R., Pujades, C. Evolutionary emergence of the *rac3b*/*rfng*/*sgca* regulatory cluster refined mechanisms for hindbrain boundaries formation.

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