The interplay of \textit{atoh1} genes in the lower rhombic lip during hindbrain morphogenesis

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Abstract

The Lower Rhombic Lip (LRL) is a transient neuroepithelial structure of the dorsal hindbrain, which expands from r2 to r7, and gives rise to deep nuclei of the brainstem, such as the vestibular and auditory nuclei and most posteriorly the precerebellar nuclei. Although there is information about the contribution of specific proneural-progenitor populations to specific deep nuclei, and the distinct rhombomeric contribution, little is known about how progenitor cells from the LRL behave during neurogenesis and how their transition into differentiation is regulated. In this work, we investigated the \textit{atoh1} gene regulatory network operating in the specification of LRL cells, and the kinetics of cell proliferation and behavior of \textit{atoh1a}-derivatives by using complementary strategies in the zebrafish embryo. We unveiled that \textit{atoh1a} is necessary and sufficient for specification of LRL cells by activating \textit{atoh1b}, which worked as a differentiation gene to transition progenitor cells towards neuron differentiation in a Notch-dependent manner. This cell state transition involved the release of \textit{atoh1a}-derivatives from the LRL: \textit{atoh1a} progenitors contributed first to \textit{atoh1b} cells, which are committed non-proliferative precursors, and to the \textit{lhx2b}-neuronal lineage as demonstrated by cell fate studies and functional analyses. Using \textit{in vivo} cell lineage approaches we revealed that the proliferative cell capacity, as well as the mode of division, relied on the position of the \textit{atoh1a} progenitors within the dorsoventral axis. We showed that \textit{atoh1a} may behave as the cell fate selector gene, whereas \textit{atoh1b} functions as a neuronal differentiation gene, contributing to the \textit{lhx2b} neuronal population. \textit{atoh1a}-progenitor cell dynamics (cell proliferation, cell differentiation, and neuronal migration) relies on their position, demonstrating the challenges that progenitor cells face in computing positional information from a dynamic two-dimensional grid in order to generate the stereotyped neuronal structures in the embryonic hindbrain.

Introduction

The assembly of functional neural circuits requires the specification of neuronal identities and the execution of developmental programs that establish precise neural network wiring. The generation of such cell diversity happens during embryogenesis, at the same time that the brain undergoes a dramatic transformation from a simple tubular structure, the neural tube, to
a highly convoluted structure—the brain—, resulting in changes in the position of neuronal progenitors and their derivatives upon time. Thus, the coordination of progenitor proliferation and cell fate specification is central to tissue growth and maintenance.

The comprehension of how neuronal heterogeneity is achieved implies the understanding of how the neurogenic capacity is acquired, how the number of progenitors vs. differentiated neurons is balanced, and how their relative spatial distribution changes upon morphogenesis. Neurogenesis is initiated by proneural genes, which trigger the specification of neuronal lineages and commit progenitors to neuronal differentiation by promoting cell cycle exit and activating a downstream cascade of differentiation genes [1]. Once neuronal progenitors are committed, the first step towards achieving the diversity observed in adults occurs early in development with the division of neuronal progenitor cells into distinct domains along dorsoventral (DV) axis, which will give rise to different types of neurons in response to morphogen signals emanating from local organizing centers [2]. The next level of complexity arises with the interpretation of the two-dimensional grid, along the DV and anteroposterior (AP) axes, of molecularly distinct progenitor regions that will control the final neuronal fate.

The hindbrain undergoes a segmentation process along the AP axis leading to the formation of seven metameres named rhombomeres (r1-r7) that constitute developmental units of gene expression and cell lineage compartments [3–5]. This compartmentalization involves the formation of a cellular interface between segments called the hindbrain boundary [6], which exhibit distinct features such as specific gene expression [7] and biological functions [8–11]. The hindbrain is the most conserved brain vesicle along evolution [12,13], and in all vertebrates the dorsal part of the hindbrain gives rise to a transient neuroepithelial structure, the rhombic lip (RL). RL progenitors will generate different neuronal lineages according to their position along the AP axis. The most anterior region of the RL, which coincides with the dorsal pole of r1, is known as Upper Rhombic Lip (URL) and produces all granule cells of the external and internal granular layers of the cerebellum [14,15]. The rest of the RL, which expands from r2 to r7, is known as Lower Rhombic Lip (LRL) and gives rise to deep nuclei of the brainstem, such as the vestibular and auditory nuclei and most posteriorly the precerebellar nuclei [16,17]. The genetic program for cerebellum development is largely conserved among vertebrates [16]; as an example, zebrafish and mouse use similar mechanisms to control cerebellar neurogenesis with a crucial role of atoh1 and ptf1 genes [17,18]. For the LRL, we know both the contribution of ptf1a/atoh1a proneural progenitor populations to specific deep nuclei [19], and the distinct rhombomeric identity [20]. However, little is known about how progenitor cells from the LRL behave during neurogenesis and how their transition into differentiation is regulated, in order to balance the rate of differentiation and proliferation to produce the proper neuronal numbers.

In this work, we sought to understand the role of atoh1 genes in the generation of the neuronal derivatives of LRL. We used complementary strategies in the zebrafish embryos to provide information about the gene regulatory network operating in the specification of LRL cells, and the kinetics of cell proliferation and behavior of atoh1a-derivatives. We unveiled that atoh1a is necessary and sufficient for specification of LRL cells by activating atoh1b, which worked as a differentiation gene to transition progenitor cells towards neuronal differentiation in a Notch-dependent manner. This cell state transition involved the release of atoh1a-derivatives from the LRL: atoh1a progenitors contributed first to atoh1b cells, which are committed non-proliferative precursors, and to the lhx2b-neuronal lineage as demonstrated by cell fate studies and functional analyses. Using in vivo cell lineage approaches we showed that the proliferative cell as well as their mode of division, relied on the position of the atoh1a progenitors within the dorsoventral axis.
Materials and methods

Zebrafish lines and genotyping

Zebrafish (*Dario rerio*) were treated according to the Spanish/European regulations for the handling of animals in research. All protocols were approved by the Institutional Animal Care and Use Ethic Committee (Comitè Etica en Experimentació Animal, PRBB) and the Generalitat of Catalonia (Departament de Territori i Sostenibilitat), and they were implemented according to European regulations. Experiments were carried out in accordance with the principles of the 3Rs.

Embryos were obtained by mating of adult fish using standard methods. All zebrafish strains were maintained individually as inbred lines. The transgenic line Mu4127 carries the KalTA4-UAS-mCherry cassette into the 1.5Kb region downstream of egr2a/krx20 gene, and was used for targeting UAS-constructs to rhombomeres 3 and 5, or as landmark of these regions [21]. Tg[ßactin:HRAS-EGFP] line, called Tg[CAAX:GFP] in the manuscript, displays GFP in the plasma membrane and was used to label the cell contours [22]. Tg,tp1:d2GFP] line is a readout of cells displaying Notch-activity [23] in which cells with active Notch express GFP. The Tg[HuC:GFP] line labels differentiated neurons [24]. Tg[atoh1a:Kalta4;UAS:H2A-mCherry] and Tg[atoh1a:Kalta4;UAS:GFP] fish lines label atoh1a-positive cells and their derivatives due to the stability of the fluorescent proteins. They were generated by crossing Tg [atoh1a:Gal4] [25] with Tg[UAS:H2A-mCherry] or Tg[UAS:GFP] lines, respectively, and accordingly were called Tg[atoh1a:H2A-mCherry] and Tg[atoh1a:GFP] all along the manuscript for simplification.

*atoh1a*fh282 mutant line in the Tg[atoh1a:GFP] background, which carried a missense mutation within the DNA-binding domain, was previously described in [18]. Embryos were phenotyped blind and later genotyped by PCR using the following primers: Fw primer 5′-ATGGATGGAATGAGCACGGA-3′ and Rv primer 5′-GTCGTTGTCAAAGGCTGGGA-3′. Amplified PCR products underwent digestion with AvaI (New England Biolabs), which generated two bands: 195 bp + 180 bp for the WT allele and 195 bp + 258 bp for the mutant allele. Since the *atoh1a*fh282 mutant allele only caused a deleterious phenotype in homozygosity, wild type and heterozygous conditions showed identical phenotypes and they were displayed in all our experiments as a single wild type condition.

Whole mount *in situ* hybridization and immunostainings

Zebrafish whole-mount *in situ* hybridization was adapted from [26]. The following riboprobes were generated by *in vitro* transcription from cloned cDNAs: *atoh1a* and *atoh1b* [27], *ptf1a*, *ascl1a*, *ascl1b* [28], *neurog1* [29], and *neurod4* [30]. lhxl and lhxb probes were generated by PCR amplification adding the T7 promoter sequence in the Rv primers (lhxl Fw primer, 5′-CAGAGAAGACAGGAA-3′; lhxb Rv primer, 5′-ATATTTTCTCAAAGGCTGGA-3′). Amplified PCR products underwent digestion with Aval (New England Biolabs), which generated two bands: 195 bp + 180 bp for the WT allele and 195 bp + 258 bp for the mutant allele. Since the *atoh1a*fh282 mutant allele only caused a deleterious phenotype in homozygosity, wild type and heterozygous conditions showed identical phenotypes and they were displayed in all our experiments as a single wild type condition.

For immunostaining, embryos were blocked in 5% goat serum in PBS-Tween20 (PBST) during 1h at room temperature and then incubated O/N at 4°C with the primary antibody. The primary antibodies were the following: anti-GFP (1:200; Torrey Pines), anti-pH3 (1:200; Upstate), anti-HuC (1:100, Abcam). After extensive washings with PBST, embryos were incubated with secondary antibodies conjugated with Alexa Fluor®594 or Alexa Fluor®633 (1:500,
Invitrogen). Either Draq5™ (1:2000; Biostatus, DR50200) or DAPI were used to label nuclei. After staining, embryos were either flat-mounted and imaged under a Leica DM6000B fluorescence microscope, or whole-mounted in agarose and imaged under a SP8 Leica confocal microscope.

**BrdU staining and TUNEL analysis**

Cells in S-phase were detected by BrdU-incorporation (Roche). Briefly, embryos were dechorionated and incubated in 10mM BrdU diluted in 5%DMSO 30min at RT. Embryos were washed with fresh water, fixed in 4%PFA at RT, and dehydrated in MetOH. After progressive dehydration, embryos were permeabilized with Proteinase K (Invitrogen) at 10μg/ml 15min at RT, fixed 20min in 4%PFA, and washed 3x10min in PBS before immunostaining with anti-
BrdU (1:50, Becton Dickinson).

Distribution of apoptotic cells was determined by TdT-mediated dUTP nick-end labeling of the fragmented DNA (TUNEL, Roche). Briefly, whole embryos at 30hpf were fixed in 4%
PFA and dehydrated in 100% MetOH were permeabilized with Proteinase K at 25μg/ml, and preincubated with TUNEL mixture during 60 min at 37˚C according to the manufacturer’s instructions. DAPI (1:500; Molecular Probes) was used to label nuclei.

**Quantification of the phenotypes**

For quantifying the number of differentiated neurons in atoh1a^WT and atoh1a^fh282 embryos, confocal MIP of ventral stacks were used and all cells present in the r4/r5 and r5/r6 domain were counted (see Table 1 for numbers and statistics).

In order to quantify the number of proliferating LRL-cells in atoh1a^WT and atoh1a^fh282 embryos in the Tg[atoh1a:GFP] background, the number of mitotic figures within the atoh1a:GFP progenitor domain was assessed (see Table 2 for numbers and statistics).

For the quantification of the total number of LRL atoh1a:cells in atoh1a^WT and atoh1a^fh282 embryos in the Tg[atoh1a:GFP] background, embryos at 24hpf were stained with Draq5 and the total number of nuclei of atoh1a:GFP cells was assessed in r5 (see Table 2 for numbers and statistics).

For the quantification of the delamination time of atoh1a:cells in atoh1a^WT and atoh1a^fh282 embryos in the Tg[atoh1a:GFP] background, we kept track of the time of division of a given cell (t0) and the time of delamination of the resulting cells (tf) and calculated the difference between tf and t0.

Table 1. Quantification of differentiated cells in atoh1a^WT and atoh1a^fh282 embryos at 24hpf and 36hpf with the t-test values (Fig 4M and 4N).

|        | atoh1a^WT | n | atoh1a^fh282 | n | p   |
|--------|-----------|---|--------------|---|-----|
| r4/r5-24hpf | 20.5 ± 4  | 14 | 1.4 ± 1.9    | 11 | < 0.001 |
| r5/r6-24hpf | 11.9 ± 3.3 | 14 | 0.25 ± 0.7   | 11 | < 0.001 |
| r4/r5-36hpf | 85.8 ±18.2 | 18 | 26.7 ± 9.5   | 7  | < 0.001 |
| r5/r6-36hpf | 75.6 ± 21.1 | 18 | 25.1 ± 11.9  | 7  | < 0.001 |

Table 2. Quantification of LRL cells and hallmarks of apoptosis in atoh1a^WT and atoh1a^fh282 embryos with the t-test values (Fig 5A–5D).

|                      | atoh1a^WT | n   | atoh1a^fh282 | n   | p  |
|----------------------|-----------|-----|--------------|-----|----|
| mitotic atoh1a:GFP LRL cells | 17.9 ± 3.6 | 15  | 15.9 ± 3.1   | 8   | ns |
| total LRL atoh1a:GFP cells   | 69.5 ± 6.4 | 15  | 68.4 ± 7.5   | 8   | ns |
| hindbrain apoptotic cells    | 10.5 ± 8.1 | 17  | 8.2 ± 4.7    | 5   | ns |

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3D+time imaging
Double transgenic Tg[atoh1a:H2A-mCherry]Tg[CAAX-GFP] embryos, or atoh1aWT
Tg[atoh1a:GFP] and atoh1afh282Tg[atoh1a:GFP] embryos were anesthetized and mounted dor-sally in 1% LMP-agarose. Time-lapse imaging was performed from 24hpf to 34hpf in a Leica
SP8 system using PMT detectors and a 20x objective. Experimental parameters for the videos were: voxel dimension (nm), x416.6 y416.6 z1200; time frame 8 min; total time 14 h; pinhole 1
Airy; zoom 1.3; objective 20x immersion; NA 0.70. The videos were processed and analyzed
using Fiji software (NIH). Cell tracking was performed using the MaMuT software (Fiji plug-in) [31].

Conditional overexpression
The full-length coding sequences of zebrafish atoh1a- and atoh1b [27] were cloned into the
MCS of a custom dual vector that expressed Citrine from one side of 5xUAS sequence and the
cDNA of interest from the opposite side [32]. Mu4127 embryos (expressing KalT4 in r3 and
r5) were injected either with H2B-citrine:UAS, H2B-citrine:UAS:atoh1a or H2B-citrine:UAS:
atoh1b constructs at the one-cell stage, grown at 28.5˚C and analyzed at 24hpf for atoh1a/b
and lhxb2 in situ hybridization and Citrine expression.

Pharmacological treatments
atoh1aWT Tg[atoh1a:GFP] and atoh1afh282 Tg[atoh1a:GFP] sibling embryos were treated either
with 10 μM of the gamma-secretase inhibitor LY411575 (Stemgent) or DMSO for control. The
treatment was applied into the swimming water at 28.5˚C from 24hpf to 30hpf. After treat-
ment, embryos were fixed in 4% PFA for further analysis.

Results
Expression of proneural genes within the zebrafish hindbrain
We first analyzed the formation of molecularly distinct neural progenitor domains, each of
them able to generate particular neuronal cell types, during hindbrain embryonic develop-
ment. We performed a comprehensive spatiotemporal analysis of the expression of distinct
proneural genes along the anteroposterior (AP) and dorsoventral (DV) axes within the hind-
brain and defined the DV order of proneural gene expression. The expression profiles of
atoh1a, ptf1a, ascl1a, ascl1b, and neurog1 indicated that their onset of expression differed
along the AP axis (S1 Fig). The dorsal most progenitor cells expressed atoh1a all along the AP
axis from 18hpf onwards, which remained expressed there until at least 48hpf (S1A–S1C Fig;
Fig 1A–1E). ptf1a expression started in rhombomere 3 (r3) at 18hpf and from 21hpf onwards
it expanded anteriorly towards r1 and r2 (S1D and S1E Fig), ending up expressed all along the
AP axis of the hindbrain with different intensities (S1F Fig; [17]). These two proneural genes
were the most dorsally expressed as shown by transverse sections (S1A’–S1F’ Fig). ascl1a and
ascl1b displayed overlapping expression profiles along the AP axis in a rhombomeric restricted
manner with slightly different intensities (S1G and S1J Fig). Nevertheless, their DV expression
differed: ascl1a expression was adjacenty dorsal to ascl1b and constituted a smaller territory
(S1G’–S1I’, S1J’–S1L’ and S1R Fig). Indeed, ascl1a and ptf1a mainly overlapped along the DV
axis occupying the region in between atoh1a and ascl1b (S1P–S1R Fig). Although by 24hpf
ascl1a-cells seemed to be more laterally located than ascl1b-cells (compare S1I with S1L Fig),
this just reflected the lateral displacement of the dorsal part of the neural tube upon hindbrain
ventricle opening: the hindbrain at early stages was a closed neural tube resembling the spinal
cord (S1 Fig, 18-21hpf stages), whereas at late stages all progenitor cells were in the ventricular
Fig 1. Spatiotemporal analysis of atoh1a and atoh1b within the hindbrain. A-E) Whole mount double in situ hybridization with atoh1a (green) and atoh1b (magenta) in wild type embryos from 14hpf to 42hpf. Dorsal views with anterior to the left. A'-E') Reconstructed transverse views of dorsal views in (A-E) at the level indicated by the white arrow depicted in (A-E). Note that the expression of atoh1b is more lateral than atoh1a-cells. Dotted line corresponded to the neural tube contour. F-H) Whole mount double in situ hybridization with atoh1a (green) and atoh1b (magenta) on Tg[HuC:GFP] embryos from 24hpf to 42hpf, where HuC expression was displayed in white. Dotted line corresponded to the neural tube and the HuC-expression contours (only half of it). I-J) Embryos at 30hpf were double in situ hybridized with atoh1a (green) and atoh1b (magenta) and cell proliferation was assessed by anti-PH3 staining (white). Dorsal views with anterior to the left. I'-J') Reconstructed transverse views of (I-J) at the level pointed by the white arrow in (I-J). Note atoh1a-cells underwent mitosis, whereas fewer atoh1b-cells did. Dotted line corresponded to the neural tube contour. K-K") Tg[ato1a:GFP] embryo after anti-PH3 (magenta) and DAPI (blue) staining. K'-K") Reconstructed transverse views of the region framed in (K), which is a dorsal view with anterior to the left. This is an example of an apical atoh1a:GFP cell undergoing division (black asterisk) and lateral atoh1a:GFP cell that did not (white asterisk), with (K') or without (K") the red-PH3 staining. Note that atoh1a:GFP cell nuclei expressing PH3 are located in the apical region (black asterisks), whereas atoh1a:GFP cell nuclei negative for PH3 (most probably atoh1b-positive, white asterisk) are in the most lateral domain. L-L") Tg[ato1a:GFP] embryo incubated for 30min with BrdU (blue) and assayed for atoh1b in situ hybridization (magenta). Reconstructed transverse views with (L-L") atoh1b-staining. White asterisks indicate atoh1b cells that did not incorporate BrdU. Dotted line corresponded to the neural tube contour. op, otic placode; ov, otic vesicle; r, rhombomere. Scale bars correspond to 50 μm.

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zone facing the brain ventricle after lumen expansion (S1C Fig, 24hpf; compare S2A', S2B', S2E' and S2F' with S2C', S2D', S2G' and S2H' Fig). At 24hpf, \textit{ascl1a/b} expression was restricted to rhombomeres, and by 42hpf their expression was clearly confined to the rhombomeric domains that flank the hindbrain boundaries (S2A–S2D Fig) as previously shown in [32,33]. Finally, \textit{neurog1} was expressed in a more ventral position (S1M–S1O and S1M'–S1O' Fig), just below \textit{ascl1a} (S1S Fig), and its expression restricted to the flanking boundary domains by 42hpf (S2E–S2H' Fig; [32]). Thus, by double \textit{in situ} hybridization experiments we could assess the organization of the different proneural progenitor pools along the DV axis as following: \textit{atoh1a}, \textit{ptf1a/ascl1a}, \textit{ascl1b}, \textit{neurog1}, being \textit{atoh1a}-cells the dorsal most progenitor cell population (S1P–S1S Fig). Interestingly, this was not the same order than proneural gene expression in the zebrafish spinal cord, where a second domain of \textit{neurog1} progenitors positioned just underneath the \textit{atoh1a} domain [34]. Proneural genes were expressed in non-differentiated progenitors, and accordingly, non-overlapping expression was observed with HuC-staining (S2A'–S2H' Fig; S3A', S3B and S3C Fig). Interestingly, progenitors located in the dorsal most domain, became placed more lateral upon morphogenesis (see \textit{atoh1a}-expressing cells in Fig 1E and 1E'; S3A' Fig); and progenitors in the ventral region such as \textit{neurog1}-cells, ended up in a more medial position (S2E'–S2H' Fig), showing the impact -and therefore the importance- of morphogenetic changes in the allocation of progenitor cells.

\textit{atoh1a} and \textit{atoh1b} were sequentially expressed in partially overlapping domains

The three \textit{atoh1} paralogs -\textit{atoh1a}, \textit{atoh1b} and \textit{atoh1c} - were shown to be expressed within the hindbrain and to contribute to the development of the cerebellum, with the expression of \textit{atoh1c} restricted to the upper rhombic lip [17,18]. Since our main interest was understanding the development of the lower rhombic lip (LRL), we focused on the study of \textit{atoh1a} and \textit{atoh1b} and compared their onset of expression. \textit{atoh1a} preceded the expression of \textit{atoh1b} in the most dorsal progenitor cells of the hindbrain at 14hpf (Fig 1A and 1A'). This was in contrast with the onset in the otic epithelium, where \textit{atoh1b} was expressed earlier than \textit{atoh1a} (see magenta in the otic placode in Fig 1A; [27]). At 18hpf, \textit{atoh1a} expression remained in the dorsal most cells, whereas \textit{atoh1b} expression domain was more lateral, overlapping with \textit{atoh1a}-cells and mostly contained within this expression domain (Fig 1B, 1B', 1C and 1C'). Upon the opening of the neural tube, the \textit{atoh1a/b} domains were laterally displaced and \textit{atoh1a} remained medial whereas \textit{atoh1b} positioned lateral (Fig 1D and 1D'), and by 42hpf -when the fourth ventricle was already formed- \textit{atoh1b} expression was completely lateral, and \textit{atoh1a} remained dorsal and medial (Fig 1E and 1E'). Thus, \textit{atoh1a} and \textit{atoh1b} were dorsally expressed but they differed in their mediodorsal (apicobasal) position. To demonstrate that they were kept as progenitor cells, we stained Tg[HuC:GFP] embryos with \textit{atoh1a/b} and observed that neither \textit{atoh1a} nor \textit{atoh1b} were expressed in differentiated neurons (Fig 1F–H and 1F'–1H'). Their differential apicobasal distribution and the fact that progenitor cell divisions always happened in the apical domains, suggested that \textit{atoh1b}-progenitor cells might have experienced a basal displacement of their cell body before undergoing differentiation. To demonstrate this, we stained embryos with \textit{atoh1a/b} and anti-pH3, a marker for mitotic figures, and observed that more \textit{atoh1a} than \textit{atoh1b} cells seemed to undergo mitosis (Fig 1I, 1I', 1J and 1J'). In this same line, analyses of single mitotic cells in the transgenic Tg[\textit{atoh1a}:GFP] fish line that labeled \textit{atoh1a}-expressing cells and their derivatives [18], showed that mitotic \textit{atoh1a}:GFP cells were always located in the ventricular domain (Fig 1K–1K"; see black asterisks in Fig 1K' and 1K") whereas the ones that did not divide were laterally displaced just above the neuronal differentiation domain (see white asterisks in Fig 1K' and 1K") as \textit{atoh1b} cells. To demonstrate
that indeed basal atoh1b did not proliferate, embryos were incubated with BrdU and assayed for atoh1b expression (Fig 1L–1L”). We observed that indeed atoh1b cells did not incorporate BrdU, and therefore did not undergo S-phase (see white asterisks in Fig 1L–1L”). Thus, atoh1b cells may derive from atoh1a progenitors that diminished their proliferative capacity and behaved as committed progenitors transitioning towards differentiation.

**ato1a progenitors gave rise to atoh1b cells and lhx2b neurons**

Next, we sought to unravel whether atoh1b cells derived from atoh1a progenitors and to which neuronal derivatives the atoh1a progenitors gave rise. For this we used the same Tg[atoh1a:GFP] fish line than before [18], which allows to label the cell derivatives of atoh1a progenitors due the stability of GFP, and combined in situ hybridization experiments with immunostaining, using atoh1 probes and specific neuronal differentiation genes such as lhx2b, lhx1a, and pan-neuronal differentiation markers such as HuC (Fig 2; S3 Fig). Although neuronal progenitors expressing atoh1a were restricted to the dorsal most region of the hindbrain, their derivatives were allocated in more ventral domains already at early stages of neuronal differentiation (Fig 2A and 2A’, compare magenta and green domains). atoh1b cells, located more laterally than atoh1a cells, expressed GFP (Fig 2B and 2B’, see white arrowhead in B’ pointing to magenta/white cells in the green territory) indicating that indeed, they derived from atoh1a progenitors and according to their position they were transitioning towards differentiation. At this stage in which neuronal differentiation just started, ventral atoh1a derivatives constituted a lateral subgroup of differentiated neurons expressing the terminal factor lhx2b (see white asterisks indicating magenta/white cells in Fig 2C and 2C’). Note that the more medial lhx2b neurons in r4 did not arise from atoh1a cells (Fig 2C, see white arrowhead, and compare it with D). This was expected because the lateral domain of lhx2b cells always fell below the atoh1a progenitors (S3A’ Fig), when compared to the more medial domain falling underneath ascl1b cells (S3A’ and S3B Fig). When the pan-neuronal differentiation marker HuC was analyzed (Fig 2E and 2F), we could clearly observe that at these early stages atoh1a derivatives contributed to a portion of differentiated cells (compare Fig 2E and 2E’, with 2F and 2F’). Thus, the Tg[atoh1a:GFP] line labeled several cell populations: i) two progenitor cell pools -the one expressing atoh1a, and another expressing atoh1b-, and ii) the lateral domain of differentiated lhx2b neurons. By 48hpf, most of the atoh1a progenitors have differentiated, and the remaining atoh1a/b progenitor pools were very small (Fig 2G, 2H, 2G’ and 2H’). Although lhx2b neurons occupied two territories, one lateral and one medial (see white asterisk and arrowhead in S3A and S3A’ Fig, respectively), the atoh1a derivatives specifically contributed to the most laterally located lhx2b neurons (see white asterisk pointing to magenta/white cells in Fig 2I and 2I’). Although lhx2b neurons occupied two territories, one lateral and one medial (see white asterisk and arrowhead in S3A and S3A’ Fig, respectively), the atoh1a derivatives specifically contributed to the most laterally located lhx2b neurons (see white asterisk pointing to magenta/white cells in Fig 2I and 2I’). As expected, cells organized properly along the DV axis according to their differentiation state: progenitor cells in the ventricular domain and cells transitioning towards differentiation more ventrally located (S3C–S3C” Fig). To better understand the dynamics of atoh1a-expressing progenitors, we in vivo monitored how the atoh1a-GFP cells populated the ventral domain of the hindbrain. We observed that the first-born atoh1a neurons occupied the rhombomeric edges or boundary regions (see white arrowhead in S4A–S4C Fig; Fig 2D). By 48hpf, atoh1a-derivatives already populated the basal domain of the hindbrain (which it is ventrally located at this morphogenetic stage), generating arched-like structures that coincided with rhombomeric boundaries (see yellow arrowhead in
Fig 2G–2L, see white arrowheads in S4 Fig), implying that once the dorsal progenitors commit, they undergo cellular migration during differentiation. In summary, \(\text{atoh1a}\) progenitors gave rise to \(\text{atoh1b}\) cells and to the lateral domain of \(\text{lhx2b}\) neurons. First differentiated \(\text{atoh1a}\) cells placed between rhombomeres to finally populate the basal hindbrain and generate arched-like structures.

**Reconstruction of the \(\text{atoh1a}\) lineage**

Next question was to address how the rate of differentiation and proliferation of \(\text{atoh1a}\) cells was balanced to achieve the needed cell diversity. For this, we used genetic lineages that allowed to delineate cell types arising from \(\text{atoh1a}\) subsets. To trace the \(\text{atoh1a}\) neuronal
lineages we used a transgenic line that expressed the H2A-mCherry fluorescent reporter protein under the control of enhancer elements of the atoh1a. Tg[atoh1a:H2A-mCherry] fish were crossed with Tg[CAAX:GFP] -to have the contour of the cells- and embryos at 24hpf were imaged over 14h. Information about plasma membrane, cell fate and position was simultaneously recorded every 7min (Fig 3A as an example). We monitored the atoh1a progenies and studied their behavior according to their position along the DV axis to (Fig 3B–3E). We tracked 40 atoh1a-cells, 22 dorsal most (see cells encircled in orange in Fig 3B) and 20 adjacent ventral (see cells encircled in white in Fig 3C), and analyzed their trajectories, when and how many times they divided during the 14h that they were imaged (Fig 3D), and by which mode of division they did so (Fig 3E) attending to their morphology and location: symmetrically giving rise to two progenitor cells (PP) or two neurons (NN), or asymmetrically generating one progenitor cell and one neuron (NP). Of the 22 tracked dorsal most cells (Fig 3B and 3D), only 59% of them divided, and they did so only once (Fig 3D, orange bars; n = 13/22). On the other hand, 82% of cells located just in the underneath domain underwent cell division either once or twice (Fig 3C and 3D, white bars; n = 14/17). Dorsal most atoh1a cells undergoing division gave rise always to two cells ending up as differentiated neurons (Fig 3E, dorsal cells NN n = 13/13), whereas the atoh1a cells located just below divided according to the three modes of division: 35% gave to two progenitor cells (Fig 3E, ventral cells PP n = 7/20) or two differentiated neurons (Fig 3E, ventral cells NN n = 7/20), and 30% displayed an asymmetric division (Fig 3E, ventral cells NP n = 6/20). These results demonstrated that the dorsal most domain allocated atoh1a cells already transitioning towards differentiation, whereas the proliferating atoh1a-progenitor pool occupied the region just underneath, generating a dorsoventral gradient of neuronal differentiation.

atoh1a is necessary and sufficient for neuronal specification

Our observations suggested that proliferating atoh1a progenitors gave rise to post-mitotic atoh1b precursors and lhx2b neurons in a sequential manner. However, in order to elucidate the hierarchy between these factors and cellular types, we analyzed the effect of atoh1a mutation on the neuronal differentiation domain (Fig 4). We made use of the available atoh1a^{fh282} mutant fish in the Tg[atoh1a:GFP] background, which carried a missense mutation within the DNA-binding domain [18]. First, we observed that mutation of atoh1a resulted in a complete loss of atoh1b expression within the hindbrain (Fig 4A, 4A', 4D, 4D', 4G, 4G', 4J and 4J'), suggesting that atoh1a was necessary for atoh1b expression and supporting the previous result that atoh1b cells derived from atoh1a progenitors. This phenotype was accompanied with the loss of the most lateral lhx2b-neuronal population (see white asterisk in Fig 4B, 4B', 4E, 4E', 4H, 4H', 4K and 4K'), but not of the lhx2b-medial column in r4 that remained unaffected (see white arrowhead in Fig 4B, 4B', 4E, 4E', 4H, 4H', 4K and 4K'), as it was anticipated since this specific population of lhx2b neurons did not derive from the atoh1a cells (Fig 2D). Although the overall pattern of neuronal atoh1a:GFP cells was not dramatically changed (Fig 4C, 4C', 4F, 4F', 4I, 4I', 4L and 4L'), when the number of neurons at different AP positions was assessed we could observe a clear decrease in the number of differentiated atoh1a neurons in the atoh1a^{fh282} mutant embryos at both the onset and progression of neuronal differentiation (Fig 4M and 4N, quantification of green dashed inserts in Fig 4C, 4F, 4I and 4L; Table 1).

To address the possibility that the decrease in the number of neurons in atoh1a^{fh282} mutants was the result of a smaller number of atoh1a progenitor cells, we quantified the number of LRL atoh1a:GFP cells undergoing mitosis (Fig 5A), and the overall number of atoh1a:GFP cells (Fig 5B), both in atoh1a^{WT} and atoh1a^{fh282} embryos. No significative differences were observed, suggesting that loss of atoh1a function did not affect the original number of LRL.
progenitors (Fig 5A; LRL atoh1a:GFP cells displaying PH3-staining: atoh1a\(^{WT}\) 17.9 ± 3.6 cells n = 15 vs. atoh1a\(^{fh282}\) 15.9 ± 3.1 cells, n = 8; Fig 5B; total atoh1a:GFP cells: atoh1a\(^{WT}\) 69.5 ± 6.4 cells n = 15 vs. atoh1a\(^{fh282}\) 68.4 ± 7.5 cells, n = 8; see Table 2). Next, we investigated whether atoh1a mutation resulted in an increase of apoptotic cells by TUNEL assay (Fig 5C and 5D). The pattern of cell death was the same sparse staining in the wild type and atoh1a\(^{fh282}\) sibling
embryos (Fig 5C and 5D; Table 2), suggesting that mutation of atoh1a did not result in a substantial increase of apoptosis. Since the domains of neural bHLH gene expression are established and/or maintained by cross-repression resulting in the control of specific neuronal populations [1], we sought whether this neuronal loss was due to a change in cell fate rather than to a reduction of the number of progenitor cells. Thus, we analyzed proneural gene expression changes both in wild type and mutant context (Fig 5E–5J; atoh1a<sup>WT</sup> n = 8, atoh1a<sup>fh282</sup> n = 10). We observed that upon atoh1a mutation, atoh1a expression dramatically increased as previously reported [18] (compare Fig 5E and 5E' with 5H and 5H'). In addition, these cells remained in an intermediate domain since they did not completely migrate towards their final ventral destination as they did in atoh1a<sup>WT</sup> embryos (compare Fig 5F' and 5I'; see white arrow in Fig 5H'–5J'). When we analyzed their possible cell fate switch, by assessing whether the GFP-expressing progenitor cells in the mutant context acquired the expression of the adjacent proneural gene ptf1a, atoh1a:GFP progenitors in the atoh1a<sup>fh282</sup> embryos did not display ptf1a expression (compare Fig 5G, 5G', 5J and 5J', see white arrow in J'). These observations indicated that in the absence of atoh1a function cells remained as post-mitotic but undifferentiated progenitors, and the LRL domain was properly specified since no changes in the number of cells was observed.
Loss of atoh1 function resulted in accumulation of atoh1:GFP progenitors unable to migrate and finally differentiate. In order to demonstrate that these committed precursors arrested, we performed high-resolution time-lapse imaging of both atoh1WT and atoh1fh282.
embryos from 24hpf onwards and followed the birth and migration of these atoh1a:GFP progenitors (Fig 5K and 5L). Before migrating, atoh1a progenitors in the wild type context, extended their apical and basal feet along the mediolateral axis of the neuroepithelium (dorsal stacks in Fig 5K; white asterisk indicating the tracked cell), and then moved away from the dorsal epithelium towards the mantle zone where they resided as differentiated neurons (see ventral stacks in Fig 5K; white asterisk indicating the tracked cell). This transition was accomplished in an average period of 4.5h (Fig 5K and 5M; t = 275min ± 102; n = 28 tracked cells).

In contrast, atoh1afh282 progenitors failed to transition and detach (see dorsal stacks in Fig 5L; white asterisk indicating the tracked cell) to barely migrate basally (see medial stacks in Fig 5L; white asterisk indicating the tracked cell). Indeed, after 9.5h of imaging most of atoh1afh282 cells still remained in the dorsomedial epithelial region (Fig 5L and 5M; t = 569min ± 180; n = 9/12 tracked cells). Thus, our observations revealed that atoh1a was necessary for initial steps of neuronal differentiation (apical abscission and migration).

To further demonstrate the requirement of atoh1a in atoh1b expression and lhx2b neuronal differentiation, and to better dissect the proneural gene hierarchy, we performed conditional gain of function experiments. We injected Mu4127 embryos expressing Gal4 in r3 and r5 with H2B-citrine:UAS vectors carrying either atoh1a or atoh1b genes, and analyzed the effects in atoh1 genes and lhx2b neurons (Fig 6, Table 3). The atoh1a transgene proved successful, as atoh1a expression was spread along the DV axis, where it induced the expression of atoh1b (compare Fig 6A’, 6B’, 6D’ and 6E’) as well as ectopic lhx2b neurons in r5 (compare Fig 6C’ and 6F’), a rhombomere usually devoid of these neurons at this stage. This was a cell autonomous effect, since all cells expressing atoh1b or lhx2b ectopically expressed Citrine, and therefore atoh1a (compare green cells in Fig 6E–6H with magenta cells in E’–H’). On the other hand, although atoh1b expression resulted in ectopic lhx2b induction (Fig 6H’ and 6I’) it did not activate atoh1a expression (Fig 5G’), demonstrating that atoh1b and atoh1a were not interchangeable, and atoh1a was upstream atoh1b. Overall, our results proved that atoh1a progenitors activated atoh1b, which allowed them to transition towards differentiation and contribute to the lhx2b neuronal population. Moreover, these experiments demonstrated the neurogenic potential of atoh1b, and importantly, its role in assigning a neuronal identity subtype.

**Notch-signaling regulates the transition of atoh1a cycling progenitors towards atoh1b committed cells**

We showed that atoh1a cycling cells gave rise to atoh1b post-mitotic committed precursors. Since this commitment is suspected to be irreversible and leading towards neuronal differentiation, we thought the Notch signaling pathway as a reasonable candidate to be regulating this transition. Thus, we explored the Notch activity within the LRL to understand how atoh1b expression was restricted to a given atoh1a-domain in the neural tube. First, we assessed Notch activity by the use of the Tg[tp1:d2GFP] transgenic line, which is a readout of Notch-active cells [23]. Indeed, Notch-activity was restricted to the most dorsomedial atoh1a cell population (Fig 7A and 7A’), whereas the more laterally located atoh1b cells were devoid of it (Fig 7B and 7B’). This suggested that Notch activity was responsible of preventing atoh1a progenitors to transition to atoh1b and therefore modulating neuronal differentiation. To demonstrate this, we conditionally inhibited Notch activity by incubating Tg[atoh1a:GFP] embryos with the gamma-secretase inhibitor LY411575, and asked whether atoh1a/b expression domains were altered. Upon inhibition of Notch activity, there was an increase of atoh1b-expression at expense of atoh1a (Fig 7C, 7D, 7F and 7G): atoh1b expression was expanded more medially, and atoh1a expression dramatically decreased (compare the border of the atoh1b expression in Fig 7D’ with 7G’). As expected, the atoh1b cells did not arise de novo but
derived from atoh1a:GFP progenitors (Fig 7E, 7E’, 7H and 7H’), supporting the hypothesis that Notch-pathway regulated either the transition from neural stem cells to neuronal progenitors, or the transition of atoh1a progenitors towards differentiation. To respond to this question, we conditionally inhibited the Notch-pathway in embryos where atoh1a was mutated, and therefore no cells could be transitioning towards differentiation. Upon LY-treatment, atoh1a\textsuperscript{fh282} embryos displayed a similar phenotype than non-treated mutant embryos (compare Fig 6. atoh1a is upstream of atoh1b and is necessary for lhxb2 neurons. Mu127 embryos expressing Gal4 in rhombomeres 3 and 5 were injected with H2B-citrine:UAS (A-C), H2B-citrine:UAS:atoh1a (D-F) or H2B-citrine:UAS:atoh1b (G-I) constructs in order to ectopically express the gene of interest in r3 and r5. Injected embryos were assayed for Citrine expression (green) and atoh1a (A-A’, D-D’, G-G’), atoh1b (B-B’, E-E’, H-H’) or lhxb2 (C-C’, F-F’, I-I’) expression (magenta). Reconstructed transverse views displaying the merge of the red and green channels (A-I), or only the red channel (A’-I’). Note that ectopic expression of atoh1a in more ventral domains induces atoh1b and lhxb2 expression (see white arrowheads in D-F, D’-F’), whereas ectopic atoh1b expression induces lhxb2 but not atoh1a (see white arrowheads in H-I, H’-I’). See Table 3 for numbers of analyzed embryos. r, rhombomere. Scale bars correspond to 50 μm.

Table 3. Analysis of the phenotypes in gain-of-function experiments (Fig 6).

|        | atoh1a | atoh1b | lhxb2 |
|--------|--------|--------|-------|
| H2B-citrine:UAS | 16/16  | 13/13  | 18/18 |
| H2B-citrine:UAS:atoh1a | 35/35  | 18/25  | 12/13 |
| H2B-citrine:UAS:atoh1b  | 16/16  | 28/28  | 10/14 |

Numbers indicate embryos displaying a phenotype as the one shown in Fig 6, over the total number of analyzed embryos (X/Y).
Fig 7. Notch-signaling regulates the transition of atoh1a cycling progenitors towards atoh1b committed cells. A-B) Whole mount double in situ hybridization with atoh1a (green) and atoh1b (magenta) in Tg[tp1:GFP] embryos (readout of Notch-activity in white). A’-B’) Reconstructed transverse views of embryos displayed as dorsal views in (A-B) through the point indicated by the white arrow. Note that Notch-activity is restricted to the most dorsomedial tip of the hindbrain, corresponding with atoh1a cells. C-K) atoh1a$^{+/+}$Tg[atoh1:GFP] (C-H) and atoh1a$^{fh282}$Tg[atoh1:GFP] (I-K) siblings were double in situ hybridized with atoh1a (green) and atoh1b (magenta) after treatment with DMSO (C-E, n = 10) or the gamma-secretase inhibitor LY411575 (F-H, n = 15; I-K n = 3). The atoh1a derivatives were followed by anti-GFP staining in white. C’-K’) Reconstructed transverse views of embryos displayed as dorsal views in (C-K) at the level indicated by the white arrow. Note how the atoh1b-domain expands at expense of atoh1a progenitors after blocking Notch-activity in wild type embryos, but not in atoh1a$^{fh282}$ mutants. A-D, F-G, I-J) Dorsal views of confocal MIP from dorsal stacks with anterior to the left. E, H, K) Dorsal views of confocal MIP from ventral hindbrain with anterior to the left. ov, otic vesicle. Scale bars correspond to 50 μm.

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Fig 7I–7K to Figs 4 and 5), namely; atoh1a expression increased (Fig 7I and 7I’; [18]), atoh1b expression was highly diminished (Fig 7J and 7J’), and GFP-expressing progenitor cells failed to reach the neuronal differentiation domain (Fig 7K and 7K’). Thus, even though inhibition of N-activity triggered the neurogenic program, lack of atoh1a function impeded the LRL-progenitors to proceed towards differentiation, supporting the hypothesis that the transition of atoh1a progenitors towards differentiation depends on atoh1a function and is regulated by Notch.
Discussion

Progenitor cell populations undergo important changes in their relative spatial distribution upon morphogenesis, which need to be precisely coordinated with the balance between progenitor cells vs. differentiated neurons. Here, we have defined the role of atoh1 genes along the development of the LRL population, and how this progenitor cell population behaves during the early neurogenic phase.

The spatiotemporal activation of proneural genes in the hindbrain shows that the neurogenic capacity is regionalized along the AP axis, such as that hindbrain boundaries and rhombomere centers remain devoid of neurogenesis [33]. This is valid for most of proneural genes except for atoh1 genes, because these are expressed all along the AP axis in the dorsal most hindbrain; however, RL derivatives delaminate from the dorsal epithelium, migrate and temporarily locate in the boundary regions. Interestingly, our results demonstrate that the function of different atoh1 genes depends on the context. In the inner ear, atoh1a and atoh1b cross-regulate each other but are differentially required during distinct developmental periods: atoh1b activates atoh1a early, whereas in a late phase atoh1a maintains atoh1b [27]. In the URL, atoh1a and atoh1c have equivalent function in the generation of granular cells progenitors [18], whereas we argue that in the LRL atoh1a and atoh1b are not interchangeable, since they work directionally and have distinct functions. Although in the URL atoh1a activates the expression of neurod1 in intermediate, non-proliferative precursors [35], neurod1 expression is not detected in the zebrafish LRL before the 48hpf, implying that atoh1b is the one defining LRL intermediate precursors rather than neurod1 during early LRL-derived neurogenesis.

Zebrafish has three atoh1 genes, atoh1a, atoh1b and atoh1c, which are expressed in overlapping but distinct progenitor domains within the rhombic lip [17,18]. Although atoh1a and atoh1c specify different, non-overlapping pools of progenitors within the URL, in the LRL while atoh1b largely overlaps with atoh1a it defines a cellular state rather than a progenitor lineage. atoh1b is expressed in a cell population that derives from atoh1a progenitors, and it has diminished its proliferative capacity; thus, atoh1b cells experienced a basal displacement of their cell body behaving as committed progenitors transitioning towards differentiation. This observation implies that atoh1 gene duplication in teleosts resulted in a gene sub-functionalization: atoh1a may behave as the cell fate selector gene, whereas atoh1b functions as a neuronal differentiation gene maintaining the transcriptional program initiated by atoh1a. In our conditional functional experiments, atoh1a ectopic expression was rapidly downregulated, whereas ectopic atoh1b remained active at later stages, highlighting the different roles of atoh1a and atoh1b in initiating vs. maintaining the differentiation program, and that atoh1a and atoh1b are not interchangeable. Interestingly, atoh1a/b/c proteins are conserved in the basic region, characterized by being arginine-rich, and in the two helixes but not in the loop, which is known to be variable. This conserved region, the core of bHLH proteins, is located in the center of the three proteins. The N- and C-terminal regions are highly divergent except for certain amino acids such as serine and threonine, predicted to be phosphorylation sites that may modulate the function of the distinct atoh1 proteins (S5 Fig).

Interestingly, first-born neurons from the LRL delaminate and migrate towards medio-ventral positions to allocate in rhombomeric boundaries. Later-born LRL neurons follow the same trajectory, pile up with them and settle more laterally generating what we call neuronal arch-like structures. We think that this pattern of neuronal organization responds to some kind of chemo-attractant signal derived from boundary cells, as first atoh1a derivatives have a tendency to allocate within rhombomeric boundaries independently from their AP position upon differentiation. Many of such signalling pathways have been described for LRL migrating cells in the mouse embryo [36]; however, signals participating in this particular context are
unknown. Nonetheless, boundary cells are signalling centres instructing the neuronal allocation in the neighbouring tissue [9]; thus, one plausible hypothesis is that boundary cells might dictate the allocation of newly-differentiated neurons.

Balancing the rate of differentiation and proliferation in developing neural tube is essential for the production of appropriate numbers and achieving the needed cell diversity to form a functional central nervous system (CNS). This requires a finely tuned balance between the different modes of division that neural progenitor cells undergo [37]. Three distinct modes of divisions occur during vertebrate CNS development: self-expanding (symmetric proliferative, PP) divisions ensure the expansion of the progenitor pool by generating two daughter cells with identical progenitor potential, self-renewing (asymmetric, PN) divisions generate one daughter cell with the same developmental potential than the parental cell and another with a more restricted potential, and self-consuming (symmetric terminal neurogenic, NN) divisions generate two cells committed to differentiation, thereby depleting the progenitor pool [37,38].

Our in vivo cell lineage studies shed light into this specific question in respect to the atoh1a cell population. We reveal the importance of the initial allocation of atoh1a progenitors: dorsal most atoh1a progenitors display more neurogenic capacity than ventral ones, since they give rise only to NN divisions upon the early neurogenic phase, whereas atoh1a progenitors located just underneath undergo the three distinct modes of division ensuring the expansion of the atoh1a-pool and providing committed progenitors. Most probably, the originally located dorsal progenitors will quickly become atoh1b and transition towards differentiation allocating more laterally. Interestingly, in the amniote spinal cord the modes of progenitor division are coordinated over time [39], instead of space. Why such a difference? One explanation is that in the LRL, where the position of progenitor cells changes dramatically over time, the most efficient way to provide fast neuronal production without exhausting the pool of progenitors could be regionalising the proliferative capacity. On the other hand, in vivo experiments in the chick spinal cord showed that an endogenous gradient of SMAD1/5 activity dictated the mode of division of spinal interneuron progenitors, in such a way that high levels of SMAD1/5 signalling promoted PP divisions, whereas a reduction in SMAD1/5 activity forced spinal progenitors to reduce self-expanding divisions in favour of self-consuming divisions [40]. This would suggest that dorsal most atoh1a cells would respond less to BMP signalling than ventral atoh1a cells. However, during hindbrain morphogenesis there is an important change in the position of atoh1a progenitors, and therefore their relative position in respect to the gradient sources. Since morphogen gradients quickly decrease with distance [41,42], it is difficult to apply the same rationale here than in the spinal cord. Still very little is known about how these gradients are established within the hindbrain [43], and how hindbrain progenitors interpret the quantitative information encoded by the concentration and duration of exposure to gradients. An alternative explanation is that different E proteins may control the ability of atoh1a to instruct dorsal or ventral neural progenitor cells to produce specific, specialized neurons, and thus ensure that the distinct types of neurons are produced in appropriate amounts as it happens in the chick spinal cord [44].

The loss of atoh1a function clearly affects the formation of the lateral column of lhx2b differentiated neurons and decreases the number of overall differentiated neurons. But what are the derivatives of these atoh1a-derived lhx2b cells? It has been described that the hindbrain displays a striking organization into transmitter stripes reflecting a broad patterning of neurons by cell type, morphology, age, projections, cellular properties, and activity patterns [45]. According to this pattern, the lateral lhx2b column would correspond to glutamatergic neurons expressing the barhl2 transcription factor [46], which in turn is an atoh1a target [46,47]. Moreover, our observations revealed that atoh1a was necessary for initial steps of neuronal differentiation, such as apical abscission and migration. Interestingly, this phenotype resembled
to the one of atoh1<sup>fl367</sup> mutants, in which the release of granule neuron progenitors from the URL required functional atoh1c [18], indicating that atoh1a replaced atoh1c function in this context.

Notch has been extensively studied as a regulator of proneural gene expression by a process called lateral inhibition, in which cells expressing higher levels of proneural genes are selected as “neuroblasts” for further commitment and differentiation, while concomitantly maintaining their neighbors as proliferating neural precursors available for a later round of neuroblast selection [48]. Indeed, in the LRL the transition atoh1a to atoh1b seems to be regulated by Notch-activity, since upon Notch-inhibition most of the atoh1a cells disappear and they become atoh1b, and therefore are ready to undergo differentiation. Thus, although atoh1a is the upstream factor in LRL cell specification, several mechanisms seem to be in place to precisely coordinate acquisition of the neurogenic capacity and progenitor vs. differentiation transitions.

Supporting information

S1 Fig. Proneural gene expression within the zebrafish embryonic hindbrain. Whole mount in situ hybridization at 18hpf, 21hpf and 24hpf using atoh1a (A-C, Q), ptf1a (D-F, P), ascl1a (G-I, P-S), ascl1b (J-L, R) and neurog1 (M-O, S) probes. Dorsal views with anterior to the left. A’-O’) Transverse views at the level pointed by the black arrowhead of embryos displayed in (A-O). P-S) Transverse views of double in situ hybridized embryos with the indicated probes. ov, otic vesicle; r, rhombomere. (TIF)

S2 Fig. Expression of ascl1b and neurog1 proneural genes along the dorsoventral axis in the context of the neuronal differentiation domain. Tg[HuC:GFP] embryos were in situ hybridized with ascl1b (A-D) or neurog1 (E-H) from 24hpf until 48hpf. A-H) Dorsal views with anterior to the left; A’-H’) Reconstructed transverse views at the level pointed by the white arrow in (A-H). Note that progenitor domain in magenta diminishes in size and constitutes the ventricular zone as neuronal differentiation increases over time. ov, otic vesicle. Scale bars correspond to 50 μm. (TIF)

S3 Fig. Comparison of the progenitor and differentiated domains upon morphogenesis. Tg [HuC:GFP] embryos were in situ hybridized either with atoh1a and lhx2b (A-A’), ascl1b and lhx1a (B), or ascl1b and neuroD4 (C-C”). Reconstructed transverse views except for (A), which is a dorsal view, showing the distinct position of progenitors (atoh1a or ascl1b in magenta) and differentiated neurons (lhx2b and lhx1a in green), and cells transitioning towards differentiation (neuroD4 in green) along the DV axis. ov, otic vesicle; r, rhombomere. Scale bars correspond to 50 μm. (TIF)

S4 Fig. First born atoh1a cells allocate within the rhombomeric boundaries. A-E) Double transgenic Tg[atoh1a:GFP]Mu4127 embryos were in vivo imaged at different developmental stages. Dorsal views of confocal MIP from ventral hindbrain with anterior to the left. Note that most of the first born atoh1a:GFP cells (green) at 21hpf position at the rhombomeric boundaries as indicated by the magenta staining in r3 and r5 (see white arrowheads indicating the most ventral atoh1a:GFP derivatives). Later, more atoh1a:GFP cells are generated and populate the whole AP axis (see white asterisks in (B-E)) piling up with the first-born atoh1a:GFP cells (see white asterisks). A’-E’, A”-E”) Reconstructed transverse views of (A-E) at the level of r4/r5 displaying either the two channels (A’-E’) or only the green one (A”-E”). See how the atoh1a:
GFP cells corresponding to atoh1a-derivatives end up generating a neuronal arch-like structure (see white arrowheads) as development proceeds. ov, otic vesicle; r, rhombomere. Scale bars correspond to 50 μm.

(TIF)

S5 Fig. Amino acid sequence comparison of zebrafish atoh1 proteins. Comparison of zebrafish atoh1a, atoh1b and atoh1hc proteins by Multiple Sequence Alignment CLUSTALW (MSA, EMBL-EBI). Sequence conservation (>70%) is displayed at the top as grey blocks with different hues. Amino acids highlighted in green correspond to those that match with the consensus sequence, which is displayed at the top in bold. Note how the three atoh1 proteins are conserved in the central regions and their sequence diverge in the N- and C-terminal domains.

(TIF)

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