Ascl1 as a Novel Player in the Ptf1a Transcriptional Network for GABAergic Cell Specification in the Retina

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Abstract

In contrast with the wealth of data involving bHLH and homeodomain transcription factors in retinal cell type determination, the molecular bases underlying neurotransmitter subtype specification is far less understood. Using both gain and loss of function analyses in Xenopus, we investigated the putative implication of the bHLH factor Ascl1 in this process. We found that in addition to its previously characterized proneural function, Ascl1 also contributes to the specification of the GABAergic phenotype. We showed that it is necessary for retinal GABAergic cell genesis and sufficient in overexpression experiments to bias a subset of retinal precursor cells towards a GABAergic fate. We also analysed the relationships between Ascl1 and a set of other bHLH factors using an in vivo ectopic neurogenic assay. We demonstrated that Ascl1 has unique features as a GABAergic inducer and is epistatic over factors endowed with glutamatergic potentialities such as Neurog2, NeuroD1 or Atoh7. This functional specificity is conferred by the basic DNA binding domain of Ascl1 and involves a specific genetic network, distinct from that underlying its previously demonstrated effects on catecholaminergic differentiation. Our data show that GABAergic inducing activity of Ascl1 requires the direct transcriptional regulation of Ptf1a, providing therefore a new piece of the network governing neurotransmitter subtype specification during retinogenesis.

Introduction

During development, neural specification leads to the emergence of a large diversity of neuronal subtypes that will serve distinct functions in the adult nervous system. A key issue concerns the nature and action of the molecular cues underlying the acquisition of both generic and specific characteristics of neurons. A small number of basic helix-loop-helix (bHLH) transcription factors are necessary and sufficient for progenitor cell commitment towards a neuronal lineage at the expense of a glial fate and have consequently been qualified as “proneural genes” [1]. Beside their generic function, several studies have shown that proneural genes also display context-dependent effects contributing to the differentiation of particular neuronal subtypes [1,2]. This is the case for the atonal-related neurogenin genes (Neurog1 and Neurog2) and the achaete-scute gene Ascl1 (achaete-scute complex homolog 1; also called Mash1 in mouse or Xash1 in Xenopus). These genes are mostly expressed in complementary patterns in the murine central and peripheral nervous system where they drive the production of distinct neuronal populations [3,4,5,6]; reviewed in [1,7,8]. In particular, Neurog2 and Ascl1 appear respectively as key regulators of glutamatergic (excitatory) versus GABAergic (inhibitory) neuronal fates during telencephalic development [4,6,9,10,11,12]. The implication and requirement of Ascl1 in GABAergic cell specification has however proved to be highly time and space dependent [10,13,14]. In addition, Ascl1 is also involved in the development of other neuronal subtypes such as hindbrain serotoninergic neurons [15,16], central and peripheral noradrenergic neurons [17,18,19] or mesencephalic dopaminergic neurons [20]. Exploring its involvement in other nervous system regions, such as the retina, and unravelling how it integrates within distinct genetic networks are now required to better understand its divergent properties.

In the retina, beside its proneural function [21,22,23], Ascl1 may also have a more specialized role in the commitment of particular cell subtypes since its expression is restricted to subsets of neuronal progenitors, mostly distinct here again from the Neurog2-expressing ones [3,24,25,26]. If numerous studies have addressed the intrinsic mechanisms responsible for the generation of the diverse classes of retinal neurons such as photoreceptor or ganglion cells [27,28,29], studies reporting on the molecular cues governing the specification of retinal subtypes with a specific neurotransmitter phenotype remain sparse [29,30,31,32,33,34]. As in the brain, GABAergic and glutamatergic neurons represent the most abundant inhibitory and excitatory neurons of the retina, respectively. They are...
showed that distinct Ascl1-dependent transcriptional networks sustain the production of GABAergic and catecholaminergic neurons. Together, our data suggest that the GABAergic activity of Ascl1 requires Ptf1a, this latter being a direct transcriptional target of Ascl1.

Materials and Methods

Con structs

pCS2-Ascl1 (also called Xash1 [44]), pCS2-Ptf1a, pCS2-Ptf1a-GR [45], pCS2-XNeurog2 (also called XNgnr-1), pCS2-Neurog2-GR [46], pCS2-Atoh7 (also called Xath5 [47]), pCS2-NeuroD1 (also called NeuroD [48]), pCS2-GFP [49] and all glucocorticoid-inducible chimeric Ascl1:Neurog2 constructs [50] have previously been described. pCS2-flag-Ascl1-GR was generated by subcloning the Ascl1 coding sequence from pCS2-NLSMT plasmid into EcoRI and XhoI sites of pCS2-flag-GR [51]. Protein activity of GR constructs was induced by addition of 4 μg/ml dexamethasone (dex, Sigma).

Embryos, in vitro RNA synthesis and microinjection

Xenopus laevis embryos (up to stage 41) were obtained by conventional procedures of in vitro fertilization and staged according to Nieuwkoop and Faber [52]. At the desired stage, embryos were fixed in 4% paraformaldehyde. Capped sense mRNAs were prepared from CS2 plasmids after NotI digestion and transcribed using the mMessage mMachine SP6 kit (Ambion). mRNAs were then purified with Sephadex Column (Roche). 100–150 pg of mRNAs were injected into one or two blastomeres at the two- or four-cell stage. GFP mRNA was co-injected as a tracer. Loss of function experiments were performed using already described and validated antisense oligonucleotides morpholinos: Ptf1a-Mo [39], Ascl1-Mo (a mix of two morpholinos, Ascl1-Mo1 and Ascl1-Mo2, that target the two Xenopus laevis Ascl1 alloleles; [53]), Phox2a-Mo and Hand2-Mo [53]. As a control, standard morpholinos purchased from GeneTools (LLC) were used. 8 ng of morpholinos were injected into one or two blastomeres at the two-cell stage. All embryos were co-injected with GFP mRNA as a tracer and only embryos exhibiting GFP fluorescence in the eye were selected for further analysis.

In vivo lipofection

pCS2-Ascl1, pCS2-Neurog2, pCS2-Atoh7, pCS2-NeuroD1 were transfected in stage 18 neurula into the presumptive region of the retina as previously described [54,55]. pCS2-GFP was co-lipofected and used as a tracer to follow transfected cells. Of note, co-lipofection efficiency was previously shown to be extremely high (85%–100%) [54]. Embryos were fixed at stage 41 in 4% paraformaldehyde plus 0.3% glutaraldehyde and cryostat sectioned (12 μm). Transfected cells were counted and cell types were identified based upon their laminar position and morphology.

Immunohistochemistry

Immunohistochemistry was performed using rabbit polyclonal anti-GABA (1/1000; Immunostar), mouse monoclonal anti-GFP (1/200; Molecular Probes) and anti-mouse or anti-rabbit fluorescent secondary antibodies (1/1000; Alexa, Molecular Probes). Cell nuclei were counterstained with Hoechst (Sigma).

In situ hybridization

Digoxigenin-labeled antisense RNA probes for gad1, VGlut1 [56], TH [53], tubb2b [48], Bru [57], dopsL [58], Ptf1a [45] and Ascl1 [50] were generated according to the manufacturer’s instruction (Roche). Whole mount in situ hybridization [59] and double

Figure 1. Ascl1 is required for retinal GABAergic cell genesis. Whole mount in situ hybridization on stage 35 embryos following Ascl1 or control morpholino (Mo) injection in one blastomere at the two-cell stage. Shown for each indicated probe are representative pictures of the observed labelling in the head region (lateral views, anterior on the left) and on retinal cross sections (dorsal side up). The expression of the pan-neuronal marker Brü (A, B) is not affected by Ascl1 knockdown while gad1 and Ptf1a stainings are severely reduced (C-F). VGlut1 expression (G, H) is unequally affected in the different cell layers: decreased in the photoreceptor layer (white arrow) and extended in the inner nuclear layer (white arrowhead). L: Lens. Scale bar represents 300 μm (heads) or 50 μm (sections).

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distributed among the 6 major classes of retinal neurons: photoreceptor, bipolar and ganglion cells are mainly glutamatergic, whereas most GABAergic retinal neurons are found within amacrine and horizontal cells. Only a few factors, such as the bHLH transcription factor BHLHB5 [35], the cofactor LMO4 [36], the orphan nuclear receptor Nr4a2 [37] or the PACAP pan-neuronal marker Ascl1 activity as an inducer of neurotransmitter phenotypes. We
Ascl1 overexpression in the retina favours GABAergic cell genesis. Cell fate analysis following overexpression of the indicated construct by either mRNA injection in one blastomere at the four-cell stage and dex treatment at stage 16 (A, B; analysis at stage 38) or in vivo lipofection at the neurula stage (C, D; analysis at stage 41). In both cases, GFP was used as a tracer to visualize injected/transfected cells. Note that Ascl1, Neurog2 and Atoh7 lipofections all result in an increased percentage of cells in the ganglion cell layer but only Ascl1-overexpressing cells are biased towards a GABAergic destiny. Total number of analyzed retinas and counted cells per condition is indicated in each bar. Values are given as mean ± s.e.m. p < 0.001 (**), p < 0.01 (***), p < 0.05 (*) (Student’s t-test). (E) shows a typical section of stage 41 retinas lipofected with GFP plus Ascl1 and immunostained with anti-GABA (red) and anti-GFP (green) antibodies. Panels on the right are higher magnifications of the dotted square delineated region. The dotted line indicates the inner plexiform layer, with the ganglion cell layer on the left and the inner nuclear layer on the right. White arrow points to a transfected GABA-positive cell within the ganglion cell layer. L: Lens. Scale bar represents 300 μm (heads) or 50 μm (sections). doi:10.1371/journal.pone.0092113.g002
A

|                | Control | Ptf1a | Neurog2 | NeuroD1 | Atoh7 |
|----------------|---------|-------|---------|---------|-------|
| tubb2b         | ![Image](tubb2b) | ![Image](tubb2b) | ![Image](tubb2b) | ![Image](tubb2b) | ![Image](tubb2b) |
| VGlut1         | ![Image](VGlut1) | ![Image](VGlut1) | ![Image](VGlut1) | ![Image](VGlut1) | ![Image](VGlut1) |
| gad1           | ![Image](gad1) | ![Image](gad1) | ![Image](gad1) | ![Image](gad1) | ![Image](gad1) |
| Ascl1          | ![Image](Ascl1) | ![Image](Ascl1) | ![Image](Ascl1) | ![Image](Ascl1) | ![Image](Ascl1) |
| Ptf1a          | ![Image](Ptf1a) | ![Image](Ptf1a) | ![Image](Ptf1a) | ![Image](Ptf1a) | ![Image](Ptf1a) |
| Neurog2        | ![Image](Neurog2) | ![Image](Neurog2) | ![Image](Neurog2) | ![Image](Neurog2) | ![Image](Neurog2) |
| NeuroD1        | ![Image](NeuroD1) | ![Image](NeuroD1) | ![Image](NeuroD1) | ![Image](NeuroD1) | ![Image](NeuroD1) |
| Atoh7          | ![Image](Atoh7) | ![Image](Atoh7) | ![Image](Atoh7) | ![Image](Atoh7) | ![Image](Atoh7) |

B

% of embryos with ectopic VGlut1 expression

| Condition   | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
|-------------|----|----|----|----|----|----|----|----|----|----|
| Control     | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Ascl1       | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Ptf1a       | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Neurog2     | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| NeuroD1     | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Atoh7       | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Ascl1+Ptf1a | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Ascl1+Neurog2 | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Ascl1+NeuroD1 | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Ascl1+Atoh7 | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |

C

% of embryos with ectopic gad1 expression

| Condition   | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
|-------------|----|----|----|----|----|----|----|----|----|----|
| Control     | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Ascl1       | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Ptf1a       | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Neurog2     | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| NeuroD1     | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Atoh7       | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Ascl1+Ptf1a | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Ascl1+Neurog2 | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Ascl1+NeuroD1 | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |
| Ascl1+Atoh7 | 33 | 37 | 24 | 22 | 25 | 26 | 16 | 23 | 30 | 25 |

D

% of embryos with strong or weak ectopic gad1 expression

| Condition   | Strong | Weak |
|-------------|--------|------|
| Ascl1       | 12     | 11   |
| Ascl1+Neurog2 | 12 | 11   |
fluorescent in situ hybridizations on cryosections [60] were carried out as previously described.

RT-qPCR
Stage 19 embryos were treated with cycloheximide (CHX; 10 μg/ml) during 2.5 hours. Total RNA from 6 embryos was then isolated using the Nucleospin RNA XS kit (Macherey Nagel). Reverse transcription was performed using Iscript cDNA Synthesis Kit (BioRad). qPCR reactions were performed in triplicate using SsoFast Eva Green Supermix (BioRad) on a C1000 Thermal Cycler (CFX96 Real-Time System, BioRad). All values were normalized to the level of the reference gene ovaltine decarboxylase (ODC) using Biorad CFX Manager Software. Primers sequences are: tubb2b forward 5’GGACAGTGGTAGGGGCAAGCTCA3’; tubb2b reverse 5’GCCCACTTTATGCCACAGCCACTT3’, Ptf1a forward 5’GCCGCTCAGGAACCCCAAC3’, Ptf1a reverse 5’GGACGGCTAGCTTGAGGTC3’, ODC forward 5’CATGGCATTTCCCTGAAGTACAA3’ and reverse 5’GGACAGTGGTAGGGGCAAGCTCA3’.

Western-blot analyses
Total protein lysates were prepared from stage 14 embryos and submitted to western blot analysis using an anti-Myc antibody (Sigma) as previously described [61].

Image analysis and quantification
Shown in figures are representative data from one experiment that has been performed at least in duplicate. Fluorescent staining was visualized with a M2 Zeiss microscope. Images were captured with a digital camera AxioCam MRc and AxioVision Rel. 7.8 software. The quantification of in situ hybridization signal intensity in the eyes of whole embryos was quantified using Adobe Photoshop CS4.

Ethics statement
All animal procedures were conducted under the supervision of several licensed personnel, including the director of research of the CNRS and professor at the university Paris-Sud, with licenses to perform Xenopus experimentation (authorization 91-29 and 91-28), in accordance with French government policies. The study was conducted under an institutional license (number B 91-471-102 up to 2012 and C 91-471-102 since 2013). The study protocol was approved by the institutional animal care committee, the Direction Départementale de la Protection des Populations (license B/C 91-471-102).

Results
Ascl1 knockdown impairs retinal GABAergic neuron genesis
To address the potential requirement of Ascl1 in GABAergic and glutamatergic phenotype acquisition, we performed morphological analysis of whole mount in situ hybridization analyses of tubb2b, VGlut1 or gad1 expression on stage 24 embryos injected with the indicated mRNAs in one blastomere at the two-cell stage. (A) Arrows indicate ectopic expression in the epidermis (anterior on the left, dorsal side up). All the tested BHLH factors exhibit neurogenic activity, as inferred from tubb2b ectopic expression. However, only Ascl1 specifically induces gad1+ neurons without inducing VGlut1+ ones. (B, C) Quantification of embryos displaying VGlut1 (B) or gad1 (C) ectopic expression. Ascl1 interferes with Ptf1a, Neurog2, NeuroD1 and Atoh7-dependent production of VGlut1+ neurons, while none of these factors affect Ascl1 GABAergic inducing activity. (D) Quantification of embryos with weak or strong ectopic gad1 staining following mRNA injection of Ascl1 alone or together with Neurog2. Note that Neurog2 enhances Ascl1 GABAergic inducing activity. Total number of analyzed embryos per condition is indicated in each bar. Error bars represent 95% confidence intervals. p<0.001 (***) p<0.01 (**), p<0.05 (*) (Binomial test). Scale bar represents 300 μm.

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Figure 4. *Ascl1* overexpression together with *Neurog2* still promotes production of GABAergic cells in the retina. (A) Stage 41 retinal sections immunostained with anti-GABA (red) and anti-GFP (green) antibodies. Embryos were injected with the indicated mRNAs in one blastomere.
confirms that Ascl1 exerts a cell-autonomous function in GABAergic cell type determination.

In order to figure out whether this GABAergic inducing activity is specific to Ascl1 compared to other bHLH proteins endowed as well with neurogenic properties, we performed similar in vivo lipofection experiments with the atonal-related genes Neurog2 and Atoh7. As expected from previous studies [46,47,63], overexpression of these genes, as for Ascl1, resulted in an imbalanced production of early- versus late-born cell types (Fig. 2C and data not shown). However, in contrast to the Ascl1 gain of function phenotype, the ratio of GABAergic neurons among Neurog2 or Atoh7 transfected cells in the ganglion cell layer did not differ from the control situation (Fig. 2D).

Altogether, our data highlight that, although Ascl1 shares with Neurog2 or Atoh7 a neurogenic activity impacting on retinal cell type distribution, it has the specific ability to bias a subset of progenitors towards a GABAergic fate.

Ascl1 has a specific GABAergic inducing activity and is epistatic to glutamatergic factors

It has previously been shown that Ascl1, Neurog2, NeuroD1, Atoh7 and Ptf1a are all sufficient to induce ectopic neurogenesis when overexpressed in the epidermis of Xenopus embryos [47,50,64,66,67]. To further assess the functional specificity of Ascl1 as a GABAergic inducing factor, we injected the corresponding mRNA as well as those encoding the aforemen-

Figure 5. Comparison of the catecholaminergic inducing activities of five different bHLH factors. (A) Whole mount in situ hybridization analysis of Tyrosine Hydroxylase (TH) expression on stage 24 embryos injected with the indicated mRNAs in one blastomere at the two-cell stage (anterior on the left, dorsal side up). Arrowheads indicate the position of previously described TH-positive antero-ventral neurons [53], while arrows point to ectopic TH staining. Note that, among all tested bHLH factors, only Ascl1 induces ectopic TH expression. Sibling embryos were also hybridized with the tubb2b probe as a positive control (see Figure 3A). (B) Quantification of embryos displaying ectopic TH+ neurons, showing that Neurog2 and Ptf1a significantly reduce the catecholaminergic inducing activity of Ascl1. Total number of analyzed embryos per condition is indicated in each bar. Error bars represent 95% confidence intervals. p<0.05 (*) (binomial test). Scale bar represents 500 µm.

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tioned bHLH factors and compared the neurotransmitter phenotypes they induced in ectopic neurons formed within the epidermis. As expected, each of them induced ectopic tubb2b (previously called N-tubulin) expression in stage 24 embryos (Fig. 3A). Except for Ascl1, this was associated with a robust VGlut1 staining (Fig. 3A, B). Only Ptf1a and Ascl1 were able to promote ectopic gad1 expression (Fig. 3A, C). We therefore conclude that Ascl1 has the unique property among these factors, to convert presumptive epidermal cells into GABAergic neurons without simultaneously inducing glutamatergic ones.

We next wondered whether Ascl1 might act as an inhibitor of glutamatergic neuron genesis and thus investigated the genetic relationships between Ascl1 and the other bHLH genes, in co-injection experiments. The glutamatergic inducing activities of Neurog2, NeuroD1, Atoh7 and Ptf1a were all dramatically reduced upon Ascl1 misexpression (Fig. 3A, B), indicating that Ascl1 indeed actively represses the glutamatergic fate of ectopically produced neurons. In contrast, neither Neurog2, NeuroD1 or Atoh7 were able to interfere with the Ascl1-dependent induction of GABAergic neurons (Fig. 3A, C). Surprisingly, we found that embryos...
overexpressing both Ascl1 and Neurog2 displayed more ectopic gad1-positive cells compared to embryos injected with Ascl1 alone (Fig. 3D), suggesting an unexpected synergistic effect. Altogether, these data demonstrate that, in this ectopic context, Ascl1 is epitastic to these various bHLH genes. In the retina as well, overexpression of Ascl1 together with Neurog2 still resulted in an
increased production of GABAergic cells within the ganglion cell layer (Fig. 4A, B). However, no synergy was observed in this context.

Ascl1 catecholaminergic inducing activity is impaired by glutamatergic factors

As recently shown, Ascl1 misexpression in the epidermis also leads to the ectopic generation of catecholaminergic neurons [53], authenticated by the expression of tyrosine hydroxylase (TH). We thus decided to pursue our comparative study by monitoring TH labelling after Ascl1, Neurog2, NeuroD1, Atoh7 or Ptf1a mRNA injection. In contrast to Ascl1, none of these genes were able to promote the production of TH-positive neurons, emphasizing again the specific role of Ascl1 in neuronal subtype determination (Fig. 5A). Epistatic analyses revealed that the proportion of embryos displaying ectopic TH expression was slightly decreased upon co-injection of Ascl1 with either Atoh7 or NeuroD1, although this was not statistically significant. It was however significantly reduced when Ascl1 was misexpressed together with Neurog2 or Ptf1a, suggesting that these two genes could interfere with the Ascl1 catecholaminergic inducing activity (Fig. 5A, B). Such a result contrasts with the interaction previously demonstrated for the induction of GABAergic neurons and reflects that the same set of transcription factors establishes variable genetic interactions within distinct differentiation pathways.

The GABAergic and catecholaminergic inducing activities of Ascl1 are conferred by its basic domain

We next examined which Ascl1 domains account for the protein activity in neuronal subtype specification. We used a series of
whole mount (Fig. 6A, C; compare AbN to Ascl1 and NHA to Neurog2). This demonstrates that the functional specificity of these two proteins resides in their respective bHLH domains. In addition, we found that the presence of the Ascl1 basic domain in chimeric constructs was necessary and sufficient to trigger TH and gad1 ectopic expression (Fig. 6A, C; compare AbN and NHA to NtA for gad1 and TH staining). Conversely, only the chimeric proteins containing the Neurog2 bHLH domain had the ability to induce glutamatergic neurons (Fig. 6A, C; see VGlut1 labelling for AbN and NHA versus NtA). In line with this observation, the two chimeric proteins containing both the basic domain of Ascl1 and the bHLH of Neurog2 (AbN and NHA proteins) were able to simultaneously promote gad1, TH and VGlut1 expression. Altogether, these results suggest that the functional specificities of Ascl1 and Neurog2 in neurotransmitter subtype specification do not reside in the same protein domain. GABAAergic and catecholaminergic inducing activity of Ascl1 primarily relies on its basic domain while glutamatergic inducing activity of Neurog2 is imparted by its bHLH domain.

To assess whether this also holds true within the retina, we overexpressed the chimeric construct AbN by in vivo lipofection (Fig. 7A). Similarly to wild-type Ascl1, AbN transfection increased the proportion of cells within the ganglion cell layer (Fig. 7B) and biased these additional neurons towards a GABAAergic destiny (Fig. 7C). This indicates that Ascl1 basic domain is sufficient to drive a subset of retinal progenitor cells towards a GABAAergic fate in the retina.

**Phox2a and Hand2 are essential downstream components of Ascl1 catecholaminergic pathway but are dispensable for its GABAAergic inducing activity**

The DNA binding basic domain of Ascl1 being central to its activity, we next sought to identify potential transcriptional targets involved in its GABAAergic versus catecholaminergic inducing activity. The homeodomain Phox2a and bHLH Hand2 proteins act downstream Ascl1 for the determination and differentiation of noradrenergic neurons [17,19,68,69]. To examine whether these genes may also be involved in Ascl1 GABAAergic inducing activity, we simultaneously misexpressed Ascl1 while knocking down both Phox2a and Hand2 using specific morpholinos. As previously described, this resulted in a significantly decreased proportion of embryos exhibiting ectopic TH staining. In contrast, the ability of Ascl1 to induce gad1-positive neurons appeared largely unaffected (Fig. 8A, B). This demonstrates that Phox2a and Hand2, although required for Ascl1-dependent production of catecholaminergic neurons, are dispensable for its GABAAergic inducing activity.

**Ptf1a is required for Ascl1 GABAAergic inducing activity as a direct transcriptional target**

Since both Ascl1 and Ptf1a share the ability to induce ectopic GABAAergic neurons, in contrast to the other tested bHLH genes (Fig. 3), we next investigated whether they could act in the same transcriptional network. We first examined Ptf1a requirement for Ascl1 GABAAergic and catecholaminergic inducing activities by simultaneously overexpressing Ascl1 and knocking down Ptf1a (Fig. 8C, D). The Ascl1-dependent ectopic TH expression was unaffected by Ptf1a inhibition, showing that this gene is dispensable for Ascl1 catecholaminergic inducing activity. In contrast, the percentage of embryos displaying ectopic gad1
staining was significantly decreased by the concomitant blockade of Ptf1a. These results indicate that Ptf1a acts downstream of Ascl1 for the generation of ectopic GABAergic neurons.

We then tested whether this Ptf1a dependency might rely on a transcriptional interaction. We found that Ascl1 mRNA injection promoted Ptf1a ectopic expression in the epidermis (Fig. 9A, B). In contrast, Neurog2 misexpression was unable to do so. Together, these data suggest the existence of a positive transcriptional regulation exerted by Ascl1 on Ptf1a. We thus investigated whether Ptf1a could constitute a direct target of Ascl1. In this purpose, we performed gene induction assays on Ascl1-GR injected embryos in the absence of protein synthesis, using the translation-blocking drug cycloheximide (CHX). In the absence of CHX, Ascl1 overexpression lead to a strong increase of both tubb2b and Ptf1a expression level, as assayed by RT-qPCR analysis (Fig. 9C). Only Ptf1a up-regulation persisted upon CHX treatment, consistent with Ptf1a being a direct Ascl1 target gene (Fig. 9D).

In order to know whether such transcriptional interaction could also hold true in the retina, we first carefully compared their expression pattern during retinogenesis (Fig. 10). Consistent with a Ptf1a-independent proneural function, we found that Ascl1 was broadly expressed in early retinal precursors prior to the onset of Ptf1a expression. However, from stage 30 onwards, regions of overlapping expression were clearly observed in the neural retina of the optic cup and then within the neurogenic zone of the mature retina called the ciliary marginal zone (CMZ) [70]. We next asked whether Ptf1a overexpression could rescue Ascl1 knockdown. We found indeed that gad1 expression was restored to a control level in the retina of Ascl1 morphant embryos overexpressing Ptf1a (Fig. 11). Altogether, these results are
consistent with Ascl1 acting upstream Ptf1a in the transcriptional network controlling GABAergic neuron genesis in the retina.

**Discussion**

The molecular bases underlying neurotransmitter subtype determination in the retina remain largely unexplored. As previously shown in the brain and peripheral nervous system [6,15,18,68], our data suggest that during retinogenesis, Ascl1 does not simply act as a proneural factor committing progenitor cells to a generic neuronal fate, but also contributes to the specification of their neurotransmitter identity. We showed indeed that Ascl1 is required for retinal GABAergic cell type genesis and sufficient to redirect a subpopulation of progenitors towards this inhibitory neuronal destiny. Besides, taking advantage of an in vivo neurogenic assay in the *Xenopus* epidermis, we found that Ascl1 GABAergic determining activity is instructive, as inferred by its ability to counteract glutamatergic differentiation programs. Finally, we gained insights into downstream genetic networks underlying Ascl1 functions in neurotransmitter subtype specification by showing that its GABAergic activity involves the direct transcriptional regulation of Ptf1a (Fig. 12).

Ascl1 GABAergic determining activity in the retina

Most Ascl1 gain and loss of function analyses in the retina have so far uncovered phenotypes predominantly related to its
pronounce activity. Ascl1 has indeed been shown to be required for neuronal versus glial fate decisions during late neurogenesis [21,22,23,71] and to participate, with other pronuclear genes, to the spatiotemporal progression of the neurogenic wave in the retina [72]. In line with this, recent lineage experiments in mouse revealed that Ascl1-expressing progenitors contribute to all major cell types of the retina with the exception of ganglion cells [3]. Such a primary function in determining a generic neuronal program has thus hindered the identification of its potential specific activity in retinal cell subtype specification. In line with this, overexpression of Ascl1 or other bHLH genes such as Neurog2 or Atoh7 results in apparently similar cell type distribution defects within the Xenopus retina [46,47,55,63]. In the present manuscript, we revealed that Ascl1 is actually endowed with a GABAergic determining function that superimposes to its pronuclear activity. Of note, neither Neurog2 nor Atoh7 exhibit this property. Importantly, our results are consistent with a previous study in chick reporting that, unlike Neurog2, NeuroD1 or Atoh7, Ascl1 misexpression promotes an overproduction of amacrine cells, the major retinal GABAergic cell type [73]. Therefore, we propose that Ascl1 not only promotes neurogenesis but also biases, in combination with other bHLH factors (see below), a subset of precursor cells towards particular neurotransmitter subtypes, thereby contributing to neuronal diversity during retinogenesis.

Epistatic relationships of bHLH genes in neuronal subtype specification

The strong context-dependency of Ascl1 function in neurotransmitter subtype specification relies on the presence of other regionally expressed transcription factors including bHLH ones [2]. In line with this, our data show that Ascl1 ability to induce ectopic catecholaminergic neurons is impaired in the presence of Neurog2, NeuroD1 or Atoh7. In contrast, its GABAergic inducing activity is not counteracted by these factors, both ectopically and in retinal progenitors. Thus Ascl1 likely exerts an instructive role in promoting GABAergic cell fate as previously proposed in the ventral telencephalon [4,6].

Unexpectedly, Ascl1-dependent ectopic GABAergic neuron production was found to be enhanced by Neurog2 co-expression. This suggests that, in the few locations where both factors are co-expressed in the same cells [3,74,75,76], they might act synergistically or at least cooperate within neuronal subtype specification programs. This could be the case in the retina where, although the Ascl1 and Neurog2 lineages are largely distinct, such progenitors expressing both genes have recently been described [3]. Interestingly, Neurog2 was identified as a direct transcriptional target of Ptf1a in the chick dorsal spinal cord and cerebellum [77], two regions where Ptf1a, Ascl1 and Neurog2 expressions partially overlap [78]. Neurog2 might thus participate within these domains in the Ascl1/Ptf1a-dependent GABAergic program. Additionally, Ascl1 and Neurog2 were shown to heterodimerize and function in common transcriptional complexes [77,79,80]. Investigating Ascl1 interactions with other expressed bHLH genes in the retina will surely help understand the combinatorial codes governing neurotransmitter subtype determination.

Functional domain and transcriptional network of Ascl1 involved in GABAergic cell determination

Previous reports proposed that the HLH domain of murine Ascl1 contains the information underlying its specific activity in neuronal subtype specification [20,81]. In particular, a study using chimeric and mutated constructs concluded that Ascl1-mediated acquisition of GABAergic identity in forebrain precursor cultures does not occur through DNA binding, but rather through HLH domain protein-protein interactions [10]. The authors however raised the possibility that, in their assays, alterations of endogenous Ascl1 expression may have interfered with proper GABAergic differentiation. Our domain swapping experiments led to an opposite conclusion, highlighting the essential role of Ascl1 basic functional [82]. Additional Ascl1 binding sites may however lie in enhancer regions but have not proved to be functional [82]. We found in particular that Ascl1 ability to promote gad1-positive neurons requires Ptf1a. Additionally, Ascl1 was able to activate Ptf1a transcription and to be necessary for its retinal expression. In line with such a positive transcriptional regulation, previous data showed that Ascl1 is required for the induction and/or maintenance of Ptf1a during the late phase of dorsal sensory interneuron development [14,82]. Moreover, we provide some evidence that Ptf1a might constitute a direct transcriptional target of Ascl1. Consensus binding sites for Ascl1 have previously been described in two conserved Ptf1a enhancer regions but have not proved to be functional [82]. Additional Ascl1 binding sites may however lie in other Ptf1a regulatory regions.

Altogether, our work demonstrated for the first time a role for Ascl1 in the generation of GABAergic interneurons in the retina and provided insights into the regulatory circuits responsible for its activity in neurotransmitter subtype specification.
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Author Contributions

Conceived and designed the experiments: MP EB NM DP. Performed the experiments: NM KP DP SP JH. Analyzed the data: NM KP DP SP ML EB MP. Wrote the paper: NM KP PV ML EB MP.

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