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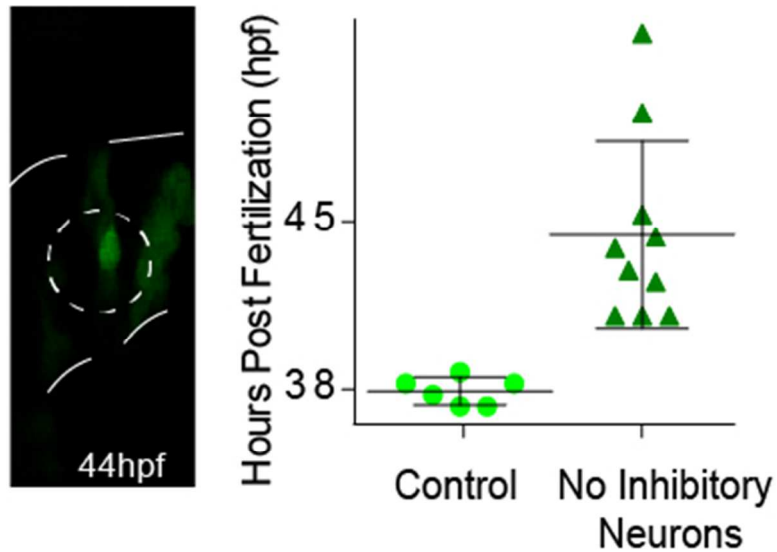
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Accepted Article

**Feedback from each retinal neuron population drives expression of subsequent fate determinant genes without influencing the cell cycle exit timing**

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## **Abstract**

During neurogenesis progenitors balance proliferation and cell cycle exit together with expression of fate determinant genes to ensure that the correct number of each of these neuron types is generated. While intrinsic gene expression acting cell-autonomously within each progenitor drives these processes, the final number of neurons generated is also influenced by extrinsic cues, which represents a potential avenue to direct neurogenesis in developmental disorders or regenerative settings without the need to change intrinsic gene expression. Thus it is important to understand which of these stages of neurogenesis are amenable to such extrinsic influences. Additionally, all types of neurons are specified in a highly conserved histogenic order, though its significance is unknown. Here, we make use of conserved patterns of neurogenesis in the relatively simple, yet highly organised zebrafish retina model, in which such histogenic birthorder is well characterised. We directly visualised and quantified birthdates and cell fate determinant expression in wild type vs environments lacking different neuronal populations. Our work shows that extrinsic feedback from developing retinal neurons is important for the temporal expression of intrinsic fate determinants but not the timing of birthdates. We found no changes in cell cycle exit timing, but a significant delay in the expression of genes driving the generation only of later, but not earlier born cells, suggesting that the robustness of this process depends on continuous feedback from earlier formed cell types. Thus, extrinsic cues selectively influence cell fate determinant progression which may explain the function of the observed retinal histogenic order.

## Introduction

During neurogenesis multipotent neural progenitors generate a fully functioning central nervous system (CNS) through distinct stages including proliferation, cell cycle exit, cell migration, differentiation and synaptogenesis. The genes acting within these progenitors to control these stages exhibit a high level of conservation both among CNS regions as well as among all vertebrate species. The neural retina has become an important CNS model to study the process of neurogenesis due to its relatively simple, yet highly organised neuronal architecture, and accessibility. In particular, the zebrafish retina model has for the first time allowed us to achieve the milestone of performing *in vivo* analysis of the entire development of a vertebrate CNS structure, which has opened up the possibility of directly assessing the timing and gene interactions.

The different stages of neurogenesis including proliferation vs. cell cycle exit and fate specification are regulated by highly co-ordinated intrinsic gene expression (Fujitani et al., 2006; Jusuf et al., 2011; Liu et al., 2001; Ma et al., 2004; Poggi et al., 2005; Vitorino et al., 2009), as well as secreted signalling factors (Hashimoto et al., 2006; Jusuf et al., 2011; Lillien and Cepko, 1992; Van Raay and Vetter, 2004; Wallace, 2008) and gradients (Del Bene et al., 2008). How these signals interact to give rise to the final robustly organised neural tissue remains unclear, although common observations across species suggest that conserved developmental mechanisms evolved across all vertebrates.

A key developmental observation across all studied vertebrate species has been the distinct histogenic order in which the five main types of neurons of the retina are generated (Barbosa-Sabanero et al., 2012; Karl and Reh, 2010). The high

conservation of this histogenic order suggests an important developmental mechanisms, though its significance and to what extent it might be important for the proper progression of neurogenesis and what signals control this progression remain unknown.

Since the different cell types are born at distinct developmental times, both the timing of cell cycle exit as well as timing of fate determinant gene expression together will influence the number of each neuron type generated. Whether or which of these key aspects is responsible for the observed difference in the proportion of different types generated in differing environments is unknown.

The switch between proliferative to differentiation phase within individual progenitors and the timing of this will determine both the final number of cells within the entire neural retina, but also the relative numbers of different cell types. Gene manipulations of intrinsic cell-autonomously acting factors such as p27<sup>Kip1</sup> and p57<sup>Kip2</sup> cyclin kinase inhibitors (Dyer and Cepko, 2000; 2001) and dynactin-1 (Del Bene et al., 2008), as well as general extrinsic factors including Wnt/Frizzled, Hedgehog, vascular endothelial growth factor, transforming growth factor $\alpha$ , epidermal growth factor and fibroblast growth factor (FGF) signalling (Hashimoto et al., 2006; Karl et al., 2008; Lillien and Cepko, 1992; Moshiri et al., 2005; Takeda et al., 2008; Van Raay and Vetter, 2004; Wallace, 2008) can affect this proliferative to differentiation switch, which in turn affects cell fate specification, as early cell cycle exit will drive generation of early born cell types, such as ganglion cells (Ohnuma et al., 2002).

Fate determination is directly controlled by basic helix loop helix (bHLH) and homeobox transcription factors are expressed within progenitors to bias them towards specific fates (Levine et al., 1997; Liu et al., 2001), for example, cone-rod

homeobox, visual homeobox transcription factor 1 (Vsx1), and pancreas transcription factor 1a (Ptf1a) bHLH proteins are required for photoreceptor, bipolar, and horizontal / amacrine fates respectively (Hayashi et al., 2000; Jusuf et al., 2011; Poggi et al., 2005; Vitorino et al., 2009). Other factors can also simultaneously affect multiple stages, such as visual homeobox transcription factor 2 (Vsx2), which maintains progenitors in the proliferative phase as well as driving bipolar fate determination (Vitorino et al., 2009). Similarly, the bHLH Atonal homolog 7 (Atoh7) drives cell cycle exit as well as specifies ganglion cell fate (Brown et al., 2001; He et al., 2012; Ohnuma et al., 2002; Vitorino et al., 2009; Wang et al., 2001). In the Atoh7 zebrafish mutant *lakritz* cell cycle exit is delayed resulting in larger clones with more of the remaining retinal neuron types generated instead of the first born ganglion cells (He et al., 2012; Kay et al., 2001). Fate specification is also influenced by extrinsic cues including FGF signalling, which biases the precursor pool of photoreceptor progenitors towards amacrine cell fate (Frohns et al., 2009). Extrinsic cues directly from surrounding developing neurons also affect fate biases, as loss of specific retinal neurons during development can bias progenitors in the growing ciliary margin zone or transplanted wild type progenitors to generate a higher proportion of the missing cell type (Jusuf et al., 2011; Poggi et al., 2005; Reh and Tully, 1986).

At the same time, some aspects of neurogenesis are also driven stochastically. Thus, individual progenitors do not undergo the same predefined program, but rather produce a whole variety of clone sizes composed of different combinations of neuronal cell types (He et al., 2012; Holt et al., 1988).

Given the strong genetic control, yet some degree of stochasticity, we asked whether the function of the highly conserved histogenic birth order observed may lie in

overseeing the process of cell cycle exit and gene expression timing to ensure the final accurate neural structure that is so robustly generated. Using the imaging, molecular and chimera advantages of the zebrafish vertebrate retina, we thus investigated whether the loss of a neuronal populations within the developing retina affects cell cycle progression and timing of fate determinant gene expression to understand which developmental processes are either linked or independently controlled and the significance of the highly conserve birth order. Here we show that the timing of fate determination factors is directly influenced by the absence or presence of earlier born cell types in the environment, while the cell cycle progression and exit is timed independently.

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## **Materials and Methods**

### ***Zebrafish husbandry***

Zebrafish were housed, bred and raised at FishCore facility at Monash University in accordance with local animal guidelines. WIK (RRID:ZIRC\_ZL84), Tg(*vsx1a*:GFP), Tg(*atoh7*:GAP-RFP), Tg(*ptf1a*:GFP) embryos of either gender were maintained according to standard protocol, staged as previously described (Kimmel et al., 1995), and used exclusively before free feeding stages. Relevant numbers are described separately for each result.

### ***Morpholino injections***

Translation blocking and standard morpholino oligonucleotides (MO) obtained from Gene Tools were reconstituted as 1 mM stock solutions in water and injected into the yolk embryos during the 1-2 cell stage. A well established, previously characterised Ptf1a MO targeted against 44 bp upstream of the translational start site with sequences 5'-TTGCCAGTAACAACAATCGCCTAC-3' was used to inhibit generation of inhibitory horizontal and amacrine cells at 10-12 ng / embryo (Jusuf et al., 2011). Standard MO with sequences 5'-CCTCTTACCTCAGTTACAATTTATA-3' injected up to 12 ng / embryo had no significant biological activity in zebrafish.

### ***Transplantation***

To follow transgene expression of wild type progenitor cells in different host environments, Tg(*atoh7*:RFP) or Tg(*vsx1*:RFP) all cells in donor embryos were

labelled with *H2A-GFP* or *H2B-RFP* RNA injected into the yolk of single cell stage embryos. Hosts were wildtype embryos injected with either standard or Ptf1a MO as described above. At the blastula stage 10 – 50 cells were transplanted from donor embryos into the animal poles of unlabelled host embryos (Jusuf et al., 2013). For this embryos were dechorionated using 0.5 µg/µl pronase and placed into 2% agarose moulds covered in E3. Embryos were subsequently transferred to agarose coated petri dishes in E3.

### ***Bromodeoxyuridine (BrdU) injections***

For injections, BrdU (B5002, Sigma) was diluted in 5 mM sodium citrate, pH 6.0 to a 10 mM stock solution and of 3 nl was injected embryos starting at different developmental time points: 30, 36, 42, 48, 54, 60 and 66 hours postfertilisation (hpf) allowing for immediate availability and more accurate temporal control. Zebrafish were anaesthetised with 0.0006% tricaine in embryonic medium (E3) and placed onto petri dish coated with 2% agarose. The BrdU was injected into the third brain ventricle using a needle pulled from a 1.0 mm O.D x 0.78 mm I.D glass capillary (Harvard Apparatus) and a Narishige IM 300 microinjector. After the first injection zebrafish were maintained in 2.5 mM BrdU diluted in E3, and all embryos were fixed at 75 hpf when central retinal development is complete. All images were taken at 75 hpf.

### **Antibody Characterization**

Detection of BrdU labelling was performed using immunohistochemistry with anti-

BrdU antibody (Roche cat. no. 11170366001, BMC 9318 clone, RRID: AB\_233622, Table 1). This antibody was generated by immunizing BALB/c mice with a BrdU-bovine serum albumin conjugate. The BrdU-BSA conjugate recognizes and binds to bromodeoxyuridine and crossreacts with iodouridine, but not fluorodeoxy-uridine, nor with any endogenous cellular components such as thymidine or uridine. The antibody raised against BrdU recognizes cells that have incorporated BrdU in vivo and in vitro, but does not show any nonspecific antibody binding in brain sections of mice that did not receive BrdU injections (Silvestroff et al., 2010) nor in zebrafish retinal sections that we did not expose to BrdU.

### ***Immunohistochemistry***

Embryos were fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich) in phosphate buffered saline (PBS, pH 7.4), cryoprotected in 7.5% gelatine / 15% sucrose in PBS solution and cryostat sectioned at 14  $\mu\text{m}$  thickness using a Leica CM3050S Cryostat. Antibody staining was performed using standard protocols with all steps were performed at room temperature. Sections were blocked in 5% fetal bovine serum (FBS) / 0.5% Triton x-100 in PBS, incubated in mouse anti-BrdU primary antibody (1:500) diluted in the same block solution overnight at 4°C. Secondary antibody used was anti-mouse Alexa Fluor-488 fluorophores (1:400, Life Technology diluted in the same block). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1:10,000, Life Technology). Sections were mounted with Mowiol (Sigma-Aldrich).

### ***Image acquisition***

Images of fixed sections were obtained on a Zeiss Z1 (20x objective) using an AxioCam (HRm 13-megapixel, monochrome) and Axiovision software. Brightness and contrast were adjusted with Photoshop (Adobe).

### ***Time-lapse imaging***

*Live in vivo* time-lapse imaging was used to quantify the timing of transgene expression indicating cell fate determinant expression, specifically of transgenes such as the Tg(*vsx1:GFP*) which is weakly expressed in progenitors and then upregulated to drive differentiation of the last born bipolar populations. Imaging was conducted using previously described methods (Jusuf et al., 2011). Briefly, embryos were anaesthetised in 0.0006% tricaine and mounted in 1% low melt agarose in E3 medium. Time-lapses were imaged on an inverted widefield fluorescent microscope (Olympus CellR Widefield Microscope) or Zeiss LSM710 confocal microscope. Using the 488 nm (GFP or Alexa 488 fluorophore) and 560 nm (RFP or Alexa 546 fluorophore) lasers images taken with a W Plan-Apochromat 20x/1.0 DIC M27 70mm objective at 1.5 zoom. Stacks of optical sections at 2  $\mu\text{m}$  step intervals for a total of 110 - 170  $\mu\text{m}$  stacks were taken every 24 mins at 32<sup>0</sup>C between 25 - 60 hpf. Montages were generated using Fiji software, and brightness and contrast were adjusted with Photoshop (Adobe).

### ***Analysis***

For quantification of cell cycle exit (250 – 300 cells / section from n = 20 embryos per time point), all analysis was conducted of the same retinal region because

differentiation proceeds in central to peripheral as well as ventral to dorsal waves. Thus, sections surrounding the optic nerve were subdivided into four equal sectors drawn from the centre of the lens and the central two sectors, which contain the central most differentiated cells were quantified as done in similar studies (Jusuf et al., 2011).

- For quantification of timing of Tg(*ptf1a:GFP*) expression onset in *lakritz* mutants (*lak*), embryos (n = 93 mutants and 438 siblings) that showed the very obvious start of expression in the eye were sorted every 0.5 hpf and kept in individual wells in embryo mix until 5 dpf, when homozygous mutants could be identified by the black body pigmentation. For similar phenotype identification of Tg(*vsx1:GFP*) embryos (n = 21 mutants and 61 siblings), individual embryos were released from the agarose after time-lapse imaging and similarly kept in individual wells in embryo mix until 5 dpf.

Statistical analyses were conducted using a non-parametric Mann-Whitney test (GraphPad) for pairwise comparison and Kruskal Wallis (GraphPad) for multiple groups.

## **Results**

### **Timing of cell cycle exit occurs independently of earlier generated cell types**

Different types of neurons are specified at distinct developmental times (Wong and Godinho, 2003) following a histogenic order, which is conserved across all vertebrates (Fujita and Horii, 1963; La Vail et al., 1991; Nawrocki et al., 1985; Rapaport et al., 2004; Stiemke and Hollyfield, 1995). Thus, the balance between the timing of proliferation and differentiation within the progenitor populations plays an important role in determining the final number of different neuron types generated.

Since feedback from existing cells in the developing tissue has been shown to influence the number of different neuron fates generated, we studied, whether such feedback might act through altering cell cycle progression, in particular the timing of cell cycle exit. In order to test this, we examined whether the absence of the two main types of neurons generated in the middle of the stereotypical birth order progression was sufficient to change the cell cycle exit timing of both earlier and later born neuron types, or whether progenitors had sufficient cues / gene expression programs to control this process completely independently.

Thus, the birth times of the main types of neurons were quantified using prolonged BrdU birthdating experiments established previously (Jusuf et al., 2011). The birthdates of excitatory photoreceptors, bipolar cells and ganglion cells was quantified in control retinas and compared to sibling retinas that lacked the two main inhibitory neuron types horizontal and amacrine cells. Inhibitory neurons are generated in the middle of the histogenic birthorder of neuron types in the retina. Their absence has been shown to positively influence transplanted wild type progenitors to be biased towards generating more of the missing inhibitory neuron

types (Jusuf et al., 2011). If they also influence the cell cycle progression and cell cycle exit, then later born neurons (namely photoreceptors and bipolar cells) may have altered birthdates. Retinas without inhibitory neurons were generated as previously described using the Ptf1a morpholino (Jusuf et al., 2011), which is cell-autonomously necessary and sufficient to specify inhibitory horizontal and amacrine cell fates in the retina. Unlike the *Atoh7* transcription factor, which is expressed prior to the final mitosis and drives cell cycle exit and specifies ganglion cells (Kay et al., 2001; Poggi et al., 2005), Ptf1a expression is only switched on after the last progenitor division (Jusuf and Harris, 2009) playing a role in progenitors that have already decided to exit the cell cycle. The normal developmental timing of morphant embryos was verified by comparing the timing of transgene expression in an unrelated tissue that does not express Ptf1a. For this, the timing of expression of reporter protein in *Tg(acta1:mCherry)* transgenic fish line was compared in uninjected, standard MO control and Ptf1a MO injected embryos. The mCherry reporter is driven by the alpha actin (*acta1*) promoter, which drives a major component of the contractile apparatus of skeletal muscle. Analysis of somite development timing at 31 hpf and 42 hpf showed comparable onset of transgene in uninjected, standard and Ptf1a MO injected embryos ( $p > 0.05$ , Kruskal-Wallis test, Figure 1, A-H).

To follow the timing of cell cycle exit (BrdU negative cells) for all of the different retinal cell fates BrdU injections followed by BrdU immersion was conducted at distinct developmental time points in age matched control standard MO and Ptf1a MO injected siblings.

Comparison of the birthdates as quantified by the proportion of BrdU negative cells (i.e. those that had undergone terminal division prior to the time of BrdU injection)

revealed robust developmental timing of ganglion (earlier born) as well as bipolar and photoreceptors (later born) cells. There is no significant difference between the timing when progenitors first exit the cell cycle and start as well as complete generating these neural populations, although the peak rate of differentiation can differ. Thus, the different types of excitatory retinal neurons are born and generated at the same developmental time independent of the presence or absence of inhibitory neurons (Figure 2, A-J). Temporal co-ordination of genes that control the timing of progenitor proliferation vs. cell cycle exit and differentiation are therefore independently controlled and are not driven by feedback from the previously born cell type as reflected by the comparable time at which half of each neuron populations is born (Table 2).

#### **Fate determinant expression is timed by cues from previous neuron types**

The second key process, which ultimately affects the final number of different neuron types generate fate specification independent of cell cycle exit is the expression timing of cell-autonomous fate determinants within progenitors. In fact the conserved histogenic order and progression of such gene expression observed across all vertebrates may be the common mechanism to ensure sufficient numbers of each neuron type are generated, before progenitors change their competence to start generating the next neuron type. Whether the switch in gene expression from one to the next type of fate determinant is timed intrinsically within each progenitor, or whether this progression is driven by accumulating extrinsic feedback signals when sufficient earlier born cell types have been generated, remains unknown.

Thus, we compared the timing of expression of key fate determinant factors that specify ganglion cells (first born), horizontal and amacrine cells (intermediate born) or bipolar cells (last born) populations in the retina. Two distinct “environments” were generated to compare with the wild type control.

In the first set of experiments, standard MO injected embryos were compared to Ptf1a MO injecting sibling embryos, in which the intermediate born inhibitory horizontal and amacrine neurons are lost in the retina (Figure 1 I, J) (Jusuf et al., 2011). Here we quantified how the loss of such inhibitory neurons affected the timing of the earlier born *Atoh7* expressing ganglion cells and later born *Vsx1* expressing bipolar cells (Hayashi et al., 2000; Vitorino et al., 2009).

In the second set of experiments, homozygous *lakritz* mutants lacking functional *Atoh7* (*lak*) (Kay et al., 2001) were compared with siblings with wild type phenotype. In *lak* mutants, the absence of *Atoh7* causes the complete loss of the first born ganglion cells in the retina. Here, we quantified how the loss of ganglion cells affected the timing of gene expression of *Ptf1a*, which drives middle born horizontal and amacrine cell specification, or *Vsx1*, which drives late born bipolar specification.

As previously shown development and neurogenesis is first observed in the anterior ventral area and progresses around the retina as development progresses (Kay et al., 2005). Thus the timing of first differentiation of ganglion, horizontal, amacrine and bipolar cells was analysed in this region by quantifying the onset of transgene expression in *Tg(atoh7:GAP-RFP)*, *Tg(ptf1a:GFP)*, *Tg(vsx1:GFP)* or appropriate double transgenic embryos. *Ptf1a:GFP* expression onset could clearly be visualised in living embryos, which were sorted for expression every 0.5 hpf. The *Vsx1:GFP* transgene is expressed at low levels in progenitors initially and upregulated strongly

specifically as bipolar cells are specified and start differentiating. Thus, we quantified the onset of strong GFP expression in late born bipolar cells, which was visualised using time-lapse imaging at the confocal microscope. *Atoh7*:GAP-RFP was simultaneously imaged using double transgenics in these time-lapse movies.

In the *Ptf1a* morphants, we find no change in the timing of *Atoh7*:GAP-RFP transgene (around 28 hpf) and thus ganglion cell specification ( $p = 0.0998$ , Mann-Whitney test, Figure 3 A&C, Figure 4). Because ganglion cells are born prior to any inhibitory neurons, these results are consistent with timing of ganglion cell differentiation occurring independently of the presence of the later born inhibitory neurons.

The developmental timing of *Vsx1* expression in the time-lapse imaging series showed that bipolar cells are generated at a significantly later time point ( $40 \text{ hpf} \pm 0.240 \text{ SEM}$ ) compared to the wild types ( $38 \text{ hpf} \pm 0.217 \text{ SEM}$ ) ( $p < 0.0001$ , Mann-Whitney test, Figure 3 B&D, Figure 4). While *Vsx1* expression does turn on eventually and bipolar cells are indeed generated, the robust timing of this process is dependent on feedback from the intermediate born horizontal and amacrine cells.

Similarly, the loss of the firstborn ganglion cell population in the *lak* mutant also causes significant delays in the timing of expression of *Ptf1a*:GFP ( $p < 0.0001$ , Mann-Whitney test, Figure 4) and *Vsx1*:GFP ( $p < 0.0346$ , Mann-Whitney test, Figure 4) transgenes, showing again that usually signals from the earlier born cells generated do in fact control the switch and timing of relevant genes to generate the later born neuron types (Table 3).

### **Fate gene progression delayed in chimeras: Wildtype progenitors in different environments**

The later born neuron types themselves do not usually express the genes that were knocked out to generate the different extrinsic environments and progenitors giving rise to these should not have been directly affected by the knock out conditions. We tested this directly by generating chimeras in which genetically wild type progenitors were transplanted into the different environment to assess, if the delay in fate determinant gene expression was still observed. For these experiments, we used the Ptf1a vs Standard MO comparison, in which we can obtain sufficient embryos of each condition. Progenitors in wild type embryos were labelled either using *H2B-RFP* or *H2A-GFP* RNA injections into the yolk of one-cell stage embryos. At the blastula stage a handful of labelled progenitors were transplanted into unlabelled host embryos at the same stage that had been injected either with standard (control) or Ptf1a MO to generate different environments for the wild type progenitors to develop in. Time-lapse imaging was performed as above to assess the onset of *Vsx1:GFP* and *Atoh7:GAP-RFP* transgenes in the transplanted wild type progenitors. These chimeras confirmed that the onset of *Atoh7:GAP-RFP* in these wild type progenitors is unaffected by the loss of the later born inhibitory neurons. In the Ptf1a morphant hosts, we find no significant change in the timing of *Atoh7:GAP-RFP* transgene onset ( $n = 6, 35 \text{ hpf} \pm 2.96 \text{ SEM}$ ) compared to that in donors transplanted into control host background ( $n = 8, 40 \text{ hpf} \pm 3.83 \text{ SEM}$ ) ( $p < 0.3623$  Mann-Whitney test, Figure 5). Thus the *Atoh7* fate determinant transcription factor is expressed normally to generate ganglion cells, which are born prior to any inhibitory neurons. These results are consistent with timing of ganglion cell differentiation occurring independently of the presence of the later born inhibitory neurons. Additionally this

result acts as a control to show that transplantation of cells into this host does not cause any delays in itself.

In these chimera experiments, we do however find a significant delay in the onset of Vsx1:GFP expression which signals bipolar differentiation. Thus, wildtype donor cells in a Ptf1a morphant host turned on Vsx1:GFP to generate bipolar cells at a significantly later time point ( $n = 6$ , 38 hpf  $\pm$  0.247 SEM) compared to control donor cells into a host background ( $n = 9$ , 45 hpf  $\pm$  1.70 SEM,  $p < 0.0002$  Mann-Whitney test, Figure 5). Thus the robust timing of progression of fate determinant factor expression is directly dependent on the generation of and feedback from the previously born cell types (in this case horizontal and amacrine cells, Table 3).

The larger variation in the onset of transgenes in the chimeric experiments compared to that in the previous time-lapse experiments are likely due to the random integration of donor cells into different areas of the forming retina, including differing depth and differing circumferential location, which would increase variation due to the developmental waves in which retinogenesis occurs.

This confirms that our observed affect on fate determinant gene timing is due to the differences in cell composition in the environment, even when the progenitors we are following and analysing have completely wild type gene expression. Specifically feedback from the relatively earlier born cell types are needed to control the precise switch of gene expression to start generating the later born cell types at the appropriate developmental time. Thus, signals from earlier born neurons drive the very robust timing the onset of fate determinant expression within developing progenitors independently of cell cycle exit timing.

## Discussion

It has long been described that different types of retinal neurons are born in a specific histogenic birthorder conserved across all vertebrates (Livesey et al., 2004).

In this context the generation of a fully functioning retina thus depends on the appropriately timed cell cycle exit and fate determination. While it is known that progenitors can be biased towards different proportions of cell types by the absence or presence of neurons in the environment (Jusuf et al., 2011; Poggi et al., 2005; Reh and Tully, 1986), it remains unclear whether such feedback and the role of the histogenic order may in fact control the timing of these key stages during development.

The fate determination of retinal neural progenitors can be biased towards generating more of the missing cell type demonstrating that feedback signalling from neurons as they are generated (Jusuf et al., 2011; Poggi et al., 2005; Reh and Tully, 1986). Since the different cell types are born at distinct developmental times, both the timing of cell cycle exit as well as timing of fate determinant gene expression together will influence the number of each neuron type generated. Whether or which of these key aspects is responsible for the observed difference in the proportion of different types generated in differing environments was unknown.

Here, we directly examined the timing of retinal progenitor cell cycle exit and fate specification during differentiation in a wild type environment, where all cell types are generated normally compared to one that is missing either earliest born ganglion cells (*lak*) or intermediate born inhibitory neuron types (*Ptf1a* morphant). In these changed environments we were able to quantify any changes in earlier born or later born cell populations. While the specificity of the *Ptf1a* MO has been well

characterised (Jusuf et al., 2011; Lin et al., 2004), the use of the MO for this study is not to directly study gene function, but as a tool to generate a retina lacking inhibitory neurons (Jusuf et al., 2011) regardless of its mode of action. In these changed environments we were able to quantify any changes in earlier born and later born cells populations.

- In both experimental conditions, the lack of earliest born ganglion cells or intermediate born inhibitory cells did not affect the timing of cell cycle exit, suggesting this aspect is controlled robustly independent of such feedback. Thus, cell cycle exit progression and ultimately the size of clones and final number of neurons seems to be independent of feedback from earlier born cell types (Figure 6)

However, in both changed environments (loss of first born ganglion cells in *lak* or loss of intermediate born inhibitory neurons in *Ptf1a* morphants), the timing of key fate determinants of later born, but not earlier born cell types was delayed. The onset of gene expression in different body organs (*Acta1:mCherry* expression in muscle) and gene expression of earlier born retinal neuron type (in this case *Atoh7* expression in earlier born ganglion cells in the *Ptf1a* morphant) within the same embryo act as an internal experimental control. These showed no delay revealing that the morpholino injection and embryo manipulation itself does not result in any developmental delay. as the same experimental procedure is performed, but the environment in which these progenitors find themselves at this earlier time point is equivalent, while the later born neuron types are generated in an environment that is missing key feedback from neuron types that should have been generated previously (Figure 6). While there was only a few hours delay, the highly significant difference indicates that the timing is fine-tuned by such feedback. While the later born neurons do not express and do not require the genes that we knocked out to generate the

different environments, we performed transplantation studies. In combination with *in vivo* imaging to look at the timing of transgene expression in truly “wild type” progenitors in these changed environments we found the same pattern showing no delay in the Atoh7 expression and a delay in Vsx1 expression. Again the Atoh7 expression acts as an internal control showing that expression of earlier genes are not affected by the presence or absence of later born neurons, and furthermore that the experimental procedure itself does not cause any gene expression delays.

The Vsx1 expression was also observed to be significantly delayed, though to a larger extent (7 hours). While this could be due in part to the actual setup (following wild type progenitors in a morphant host environment), transplantation itself has caveats that need to be carefully considered. We cannot accurately control the retinal position into which such transplanted cells integrate. Since gene expression starts in the anterior-ventral patch and spreads three dimensionally as a wave both towards posterior regions as well as deeper eye regions, transplanted cells even in the control condition will show vastly different timing of gene expression (> 12 hours for a single label, see Figure 2, (Almeida et al., 2014)(Poggi et al., 2005). This is reflected by the increase in the standard deviation of this data even within the Atoh7 control expression. While we could track the circumferential location and show a similar spread of control vs. morphant cells (Figure 5), the depth of cells could not be easily determined due to potential mounting angle differences.

Given that later born neurons are in fact generated (rather than arresting retinogenesis completely) suggests that intrinsic factors within progenitors are sufficient to progress retinogenesis with a recent study also showing that probabilistic gene expression can account largely for the resulting neural fate composition (Boije et al., 2015). We believe that the feedback and changes we

observe here may act to fine-tune this process. As the development of the zebrafish retina occurs rather rapidly and the difference between the first born (starting around 28 hpf) and last born (starting around 38 hpf) is only about 10 hpf at any given retinal location, these observed delays of a few hours can significantly influence the total number of each neuron type being generated.

Therefore, we believe that the highly conserved birthorder of neuron fates observed across vertebrate retinas acts specifically to ensure that the correct number of each neuron types are generated, before changing the gene expression of fate determinant genes to drive neurogenesis of the next cell type. While cell cycle progression and exit during zebrafish retinal development are independently controlled to ensure the correct overall number of neurons, the histogenic order and feedback from each generated cell type ensures that this overall number is correctly subdivided to specify the right proportion of each cell type.

### **Other acknowledgements**

We thank Dr Lucia Poggi (Centre for Organismal Studies, Heidelberg, Germany), and Professors William A. Harris (Cambridge University, UK), Shin-ichi Higashijima (National Institute for Physiological Sciences, Okazaki, Japan) and Steven D. Leach (Johns Hopkins Medical Institute, Baltimore, MD) for the transgenic lines used in this study. We thank FishCore staff for taking care of our animal stocks and Monash Micro Imaging facility and Drs Ashley Siegel, Wouter Masselink and Phong Nguyen for technical assistance.

### **Conflict of interest statement**

We have no conflict of interest.

### **Role of authors**

All authors had full access to all the data in the study and take responsibility for the integrity for the data and the accuracy of the data analysis. JN carried out the experiments and wrote the first draft of the manuscript. SD carried out transplantations experiments. PC and PJ conceived the study, supervised and reviewed the manuscript.

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## Figure Legends

### **Figure 1. Timing of embryonic development is unaltered when pancreas transcription factor is knocked down**

General developmental progression of embryos was assessed by comparing the developmental timing of embryonic structures that do not express the pancreas transcription factor 1a (Ptf1a) and should not be affected by the knockdown. (A – H) Micrographs show the trunk region of Tg(*acta1:mCherry*) embryos, in which a fluorescent protein marker is expressed in developing muscles. Comparison at 31 hours postfertilisation (hpf) (A – D) and 42 hpf (E – H) showing micrographs of uninjected (A, E), standard morpholino (MO) injected (B, F) or Ptf1a MO injected (C, G) embryos. The number of embryos that had turned on the Acta1a:RFP transgene expression compared to the total number of embryos is indicated in each micrograph. Bar graphs (D, H) show proportion of larvae ( $n > 30$  larvae per group) turning on Acta1:mCherry in the embryonic musculature of the trunk is comparable at 31 hpf (D, 40 – 50%) and 42 hpf (H,  $> 90\%$ ) in all groups ( $p > 0.05$ , Kruskal-Wallis test, error bars are SEM). (I-J) Micrographs of DAPI labelled cross sections through the central retina of 75 hpf standard (I) and Ptf1a MO injected embryos (J). Micrographs show loss of inhibitory neural retinal layers (100% loss of horizontal cells: arrows, 80% loss of amacrine cells: brackets) caused by efficient Ptf1a knockdown most evident in high power inserts (white boxes, I2 and J2). ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; n.s.: not significant. Scale bar G (for A – G) = 50  $\mu\text{m}$ , scale bar J (for I, J) = 20  $\mu\text{m}$ , scale bar J2 (for I2, J2) = 10  $\mu\text{m}$ .

**Figure 2. Timing of neural birthdates is controlled independent from signalling from neighbouring neurons**

(A - H) Micrographs of retinal sections of wild type embryos at 75 hpf (hours postfertilisation) injected either with standard (A - D) or Ptf1a (E - H) morpholino (MO) showing DAPI in blue and BrdU in magenta. Embryos were subsequently injected with BrdU followed by continuous BrdU incubation until 75 hpf starting at different ages (as indicated above each panel). (I) High power images of Ptf1a MO injected embryo with BrdU incubation starting at 30 hpf. Single channel BrdU (I, magenta), DAPI (I2, blue) and merged channels (I3) show examples of BrdU positive (asterisks) and BrdU negative (circles) nuclei (identified by DAPI labelling). (J) Graph shows comparison of the birthdates of ganglion cells (blue), bipolar cells (red) and photoreceptors (green) in standard MO (dark) vs. Ptf1a (lighter) MO injected embryos. Birthdates show no significant difference between standard and ptf1a MO injected embryos at all-time points for all cell types ( $p > 0.05$ ), except for 48 hpf ganglion cells ( $p < 0.05$ ), 48 hpf-bipolar cells ( $p < 0.01$ ) and 54 hpf-photoreceptors ( $p < 0.05$ , Mann-Whitney test). Error bars are SEM. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer. Scale bar A (for A - H) = 20  $\mu\text{m}$ , scale bar I3 (for I - I3) = 10  $\mu\text{m}$ .

**Figure 3. Timing of expression of later Vsx1, but not earlier Atoh7 factors is influenced by signals from inhibitory neurons**

(A - D) Micrographs from time-lapse movies of developing retinas in *Tg(vsx1a:GFP / atoh7:GAP-RFP)* double transgenic embryos starting at 27 hpf (hours postfertilisation). (A, C) Atoh7:RFP (magenta) which marks the earliest born neurons

(ganglion cells) is first expressed in the developing retina at 28 hpf, in both standard and Ptf1a morpholino (MO) injected embryos. The Ptf1a MO induced loss of the later born inhibitory neurons (horizontal and amacrine cells) does not affect the timing of the fate determinant Atoh7. (B, D) Vsx1:GFP (green) which marks progenitors and is upregulated in the later born bipolar cells is first expressed at 38 hpf in Ptf1a MO and at 40 hpf in standard MO injected embryos. Ptf1a MO induced loss of inhibitory neurons affects the robust timing of upregulation of the late born bipolar cell fate determinant Vsx1. Dashed circle indicates the first onset of Atoh7:RFP transgene or first upregulation of Vsx1:GFP transgene. Scale bar D (for A – D) = 20  $\mu$ m.

**Figure 4. Transgene onset for later cell fates are dependent on generation of earlier born cell types.**

Box and Whisker plots shows the timing (hours postfertilisation – hpf) of Atoh7:RFP, Ptf1a:GFP and Vsx1:GFP transgenes onset for each embryo imaged. (A) Comparison of timing of transgene expression in standard or Ptf1a morpholino (MO) injected embryos. Atoh7:RFP expression (open symbols) onset in the first born ganglion cell neurons is comparable in standard vs Ptf1a injected MO (n.s.  $p = 0.10$ ), whereas timing of Vsx1:GFP expression (closed symbols) onset in developing later born bipolar cell neurons in Ptf1a injected MO is significantly delayed compared to the standard injected MO. (B) Comparison of timing of transgene expression in *lak* (Atoh7 mutant) or sibling embryos. The timing of expression of Ptf1a:GFP (open symbols) and Vsx1:GFP (closed symbols), both driving generation of later born neuron types are significantly delayed in the *lak* mutants. \*  $p = 0.035$ , \*\*\*  $p < 0.0001$ . Error bars are SEM.

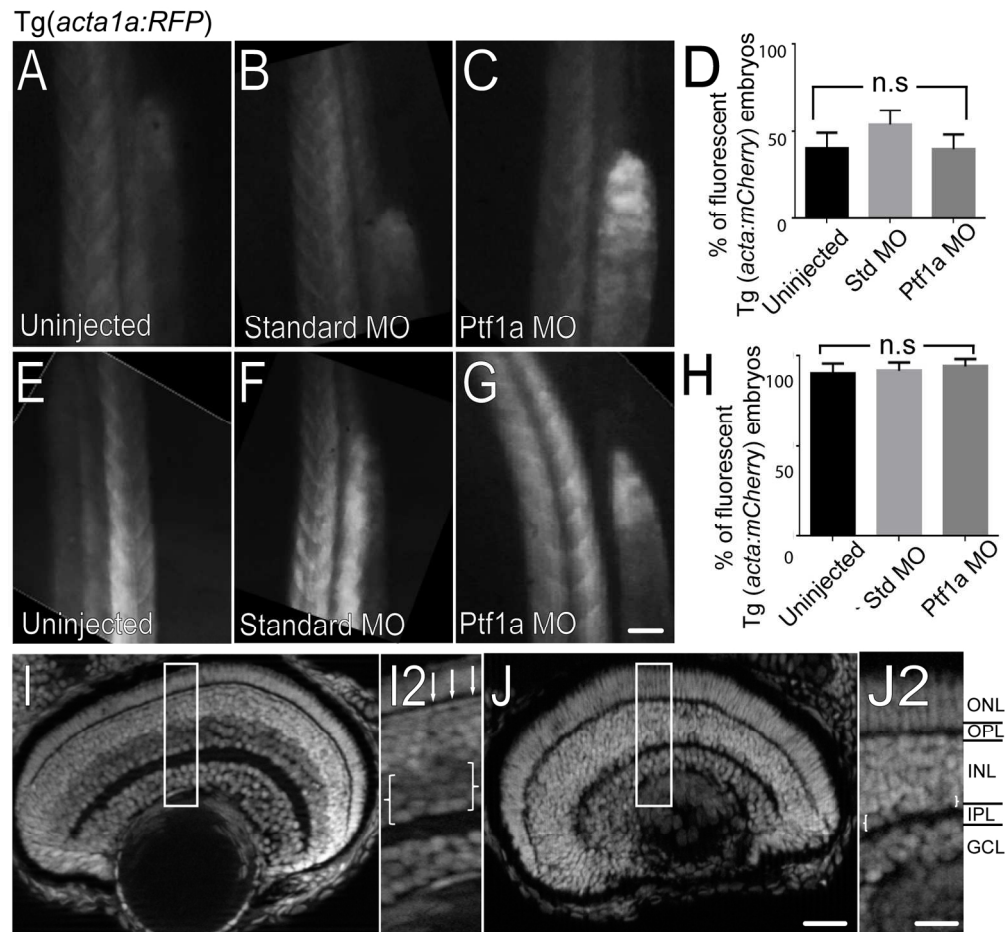
**Figure 5. Wild type donor cells transplanted into different environment show a delay in timing of expression of fate determinant genes of relatively later born neurons.**

(A, B) Micrographs from time-lapse movies of developing wild type donor cells from Tg(*vsx1:GFP*) transgenic embryos in standard or Ptf1a morpholino (MO) injected host background starting at 36 hpf (hours postfertilisation). *Vsx1:GFP* marks progenitors and is upregulated in the latest born bipolar cells at 38 and 44 hpf in Ptf1a MO and standard MO injected embryos respectively. (C) Box and Whisker plot showing the timing of transgene (either *Atoh7* – open symbols or *Vsx1* – solid symbols) onset for each embryo imaged. Retinal schematics above each experimental data shows the spatial distribution of individual transplanted donor cells in standard MO (circles) and Ptf1a MO (triangles) injected host environments (A = Anterior, P = Posterior). Retinogenesis begins in the ventral anterior quadrant (near A) and progresses as a wave towards posterior regions (P). Timing of *Atoh7:RFP* expression onset in developing first born ganglion cell neurons is comparable in standard vs Ptf1a morphant hosts (n.s.  $p = 0.3623$ , Mann-Whitney test). Timing of *Vsx1:GFP* expression onset in developing first born bipolar cell neurons is significantly delayed in Ptf1a vs standard morphant hosts (\*\*\*)  $p < 0.0002$ , Mann-Whitney test). Error bars are SEM. Scale bars A & B = 10  $\mu\text{m}$ .

**Figure 6. Summary schematic of changes in cell birth and fate gene expression timing after loss of different neuronal population**

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During retinal development in a wild type environment, different types of neurons are born at distinct times and the expression of differential fate genes for each neuronal types similarly occurs in a stereotypical manner at distinct developmental times. In environments where a neuron population is missing during development, such as ganglion cells in the *lak* mutant or inhibitory cells (i.e. horizontal and amacrine cells) in the *Ptf1a* morphant, the birthdates are unchanged (vertical arrows still at the same developmental time), however, the timing of gene expression for later born neuron populations are delayed (such as *Ptf1a* and *Vsx1*), while the expression timing of earlier staged neuron fate genes (*Atoh7*) remains unaffected.



**Figure 1.** Timing of embryonic development is unaltered when pancreas transcription factor is knocked down. General developmental progression of embryos was assessed by comparing the developmental timing of embryonic structures that do not express the pancreas transcription factor 1a (Ptf1a) and should not be affected by the knockdown. (A – H) Micrographs show the trunk region of Tg(*acta1*:mCherry) embryos, in which a fluorescent protein marker is expressed in developing muscles. Comparison at 31 hours postfertilisation (hpf) (A – D) and 42 hpf (E – H) showing micrographs of uninjected (A, E), standard morpholino (MO) injected (B, F) or Ptf1a MO injected (C, G) embryos. The number of embryos that had turned on the *Acta1a*:RFP transgene expression compared to the total number of embryos is indicated in each micrograph. Bar graphs (D, H) show proportion of larvae ( $n > 30$  larvae per group) turning on *Acta1*:mCherry in the embryonic musculature of the trunk which is comparable at 31 hpf (D, 40 – 50%) and 42 hpf (H, > 90%) in all groups ( $p > 0.05$ , Kruskal-Wallis test, error bars are SEM). (I–J) Micrographs of DAPI labelled cross sections through the central retina of 75 hpf standard (I) and Ptf1a MO injected embryos (J). Micrographs show loss of inhibitory neural retinal layers (100% loss of horizontal cells: arrows, 80% loss of amacrine cells: brackets) caused by efficient Ptf1a knockdown most evident in high power inserts (white boxes, J2 and J2). ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; n.s.: not significant. Scale bar G (for A – G) = 50  $\mu$ m, scale bar J (for I, J) = 20  $\mu$ m, scale bar J2 (for J2, J2) = 10  $\mu$ m.

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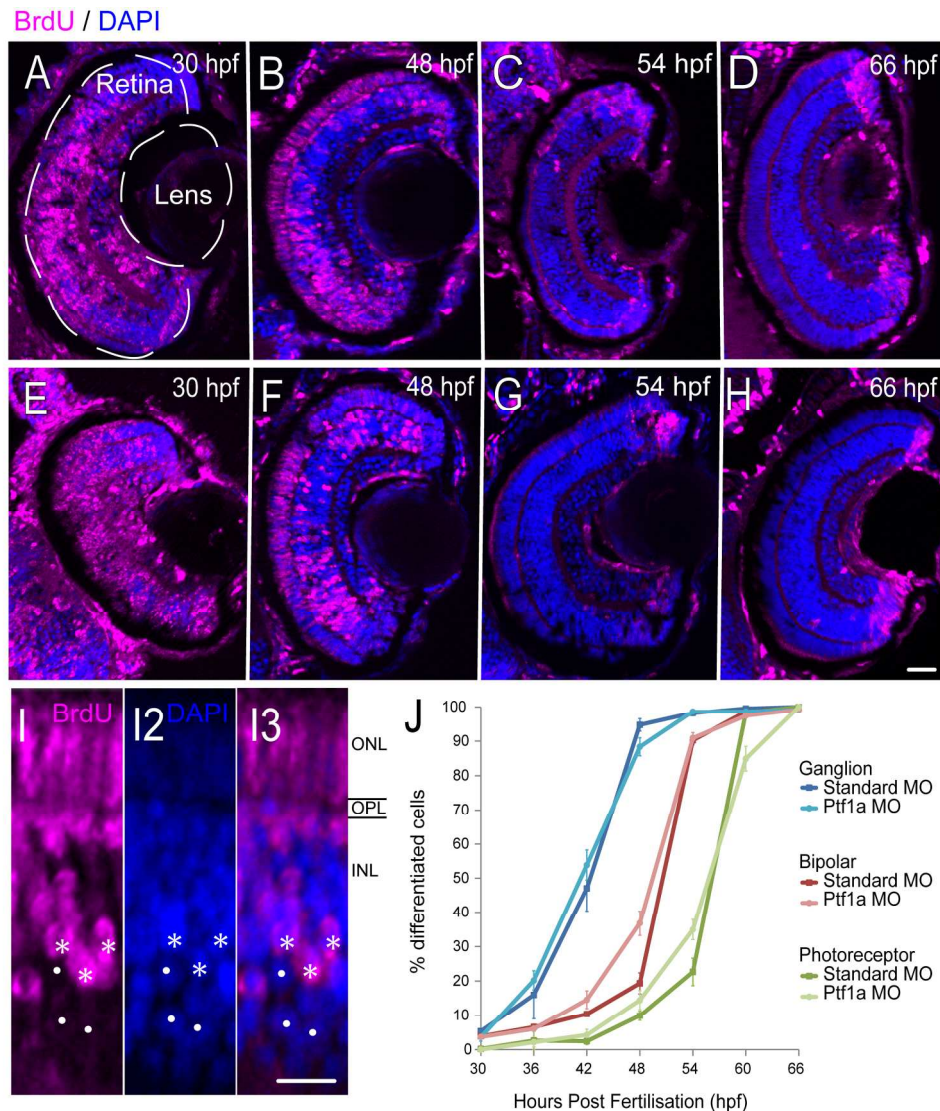


Figure 2. Timing of neural birthdates is controlled independent from signalling from neighbouring neurons (A - H) Micrographs of retinal sections of wild type embryos at 75 hpf (hours postfertilisation) injected either with standard (A - D) or Ptf1a (E - H) morpholino (MO) showing DAPI in blue and BrdU in magenta. Embryos were subsequently injected with BrdU followed by continuous BrdU incubation until 75 hpf starting at different ages (as indicated above each panel). (I) High power images of Ptf1a MO injected embryo with BrdU incubation starting at 30 hpf. Single channel BrdU (I, magenta), DAPI (I2, blue) and merged channels (I3) show examples of BrdU positive (asterisks) and BrdU negative (circles) nuclei (identified by DAPI labelling). (J) Graph shows comparison of the birthdates of ganglion cells (blue), bipolar cells (red) and photoreceptors (green) in standard MO (dark) vs. Ptf1a (lighter) MO injected embryos. Birthdates show no significant difference between standard and ptf1a MO injected embryos at all-time points for all cell types ( $p > 0.05$ ), except for 48 hpf ganglion cells ( $p < 0.05$ ), 48 hpf-bipolar cells ( $p < 0.01$ ) and 54 hpf-photoreceptors ( $p < 0.05$ , Mann-Whitney test). Error bars are SEM. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer. Scale bar A (for A - H) = 20  $\mu\text{m}$ , scale bar I3 (for I - I3) = 10  $\mu\text{m}$ .

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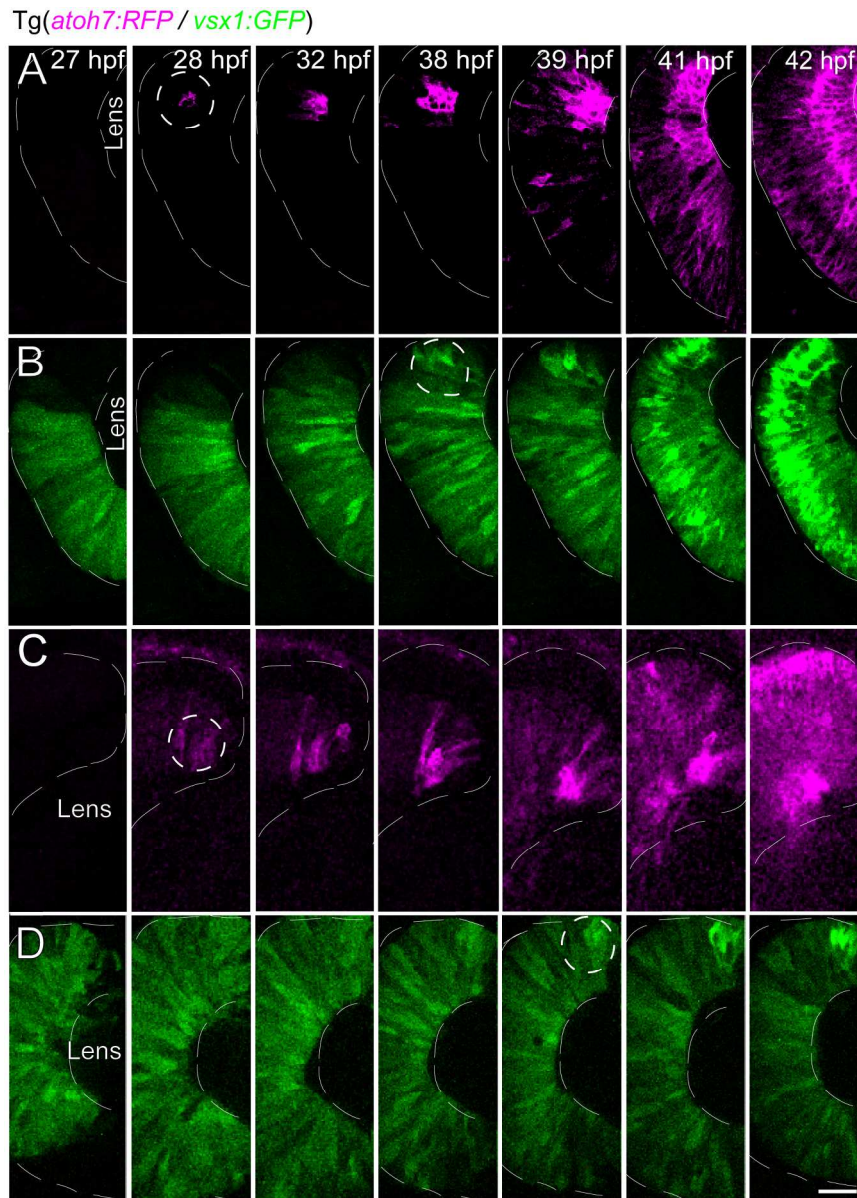


Figure 3. Timing of expression of later *Vsx1*, but not earlier *Atoh7* factors is influenced by signals from inhibitory neurons

(A - D) Micrographs from time-lapse movies of developing retinas in Tg(*vsx1a:GFP* / *atoh7:GAP-RFP*) double transgenic embryos starting at 27 hpf (hours postfertilisation). (A, C) *Atoh7:RFP* (magenta) which marks the earliest born neurons (ganglion cells) is first expressed in the developing retina at 28 hpf, in both standard and *Ptf1a* morpholino (MO) injected embryos. The *Ptf1a* MO induced loss of the later born inhibitory neurons (horizontal and amacrine cells) does not affect the timing of the fate determinant *Atoh7*. (B, D) *Vsx1:GFP* (green) which marks progenitors and is upregulated in the later born bipolar cells is first expressed at 38 hpf in *Ptf1a* MO and at 40 hpf in standard MO injected embryos. *Ptf1a* MO induced loss of inhibitory neurons affects the robust timing of upregulation of the late born bipolar cell fate determinant *Vsx1*. Dashed circle indicates the first onset of *Atoh7:RFP* transgene or first upregulation of *Vsx1:GFP* transgene. Scale bar D (for A - D) = 20  $\mu$ m.

170x232mm (300 x 300 DPI)

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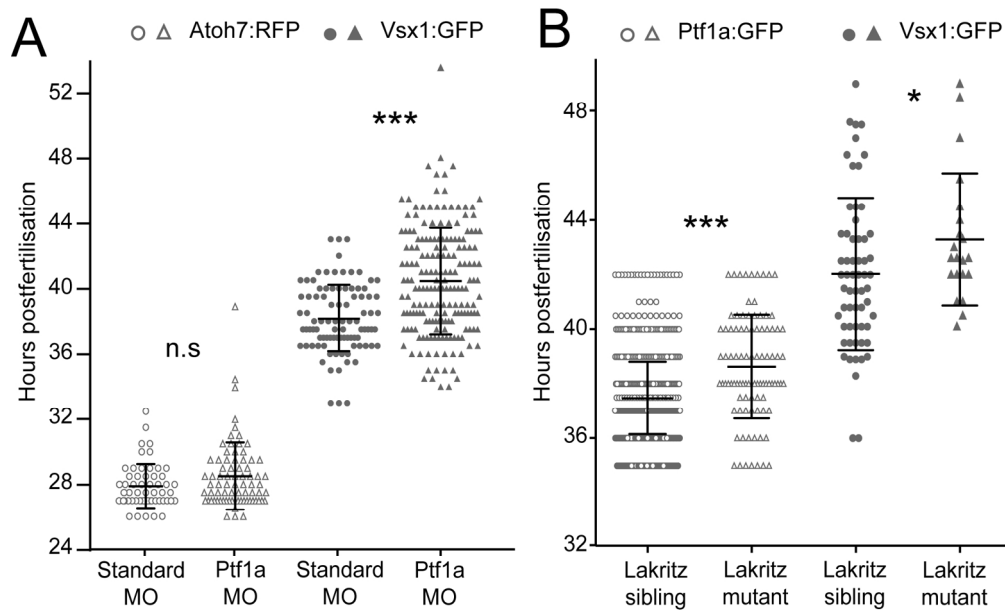


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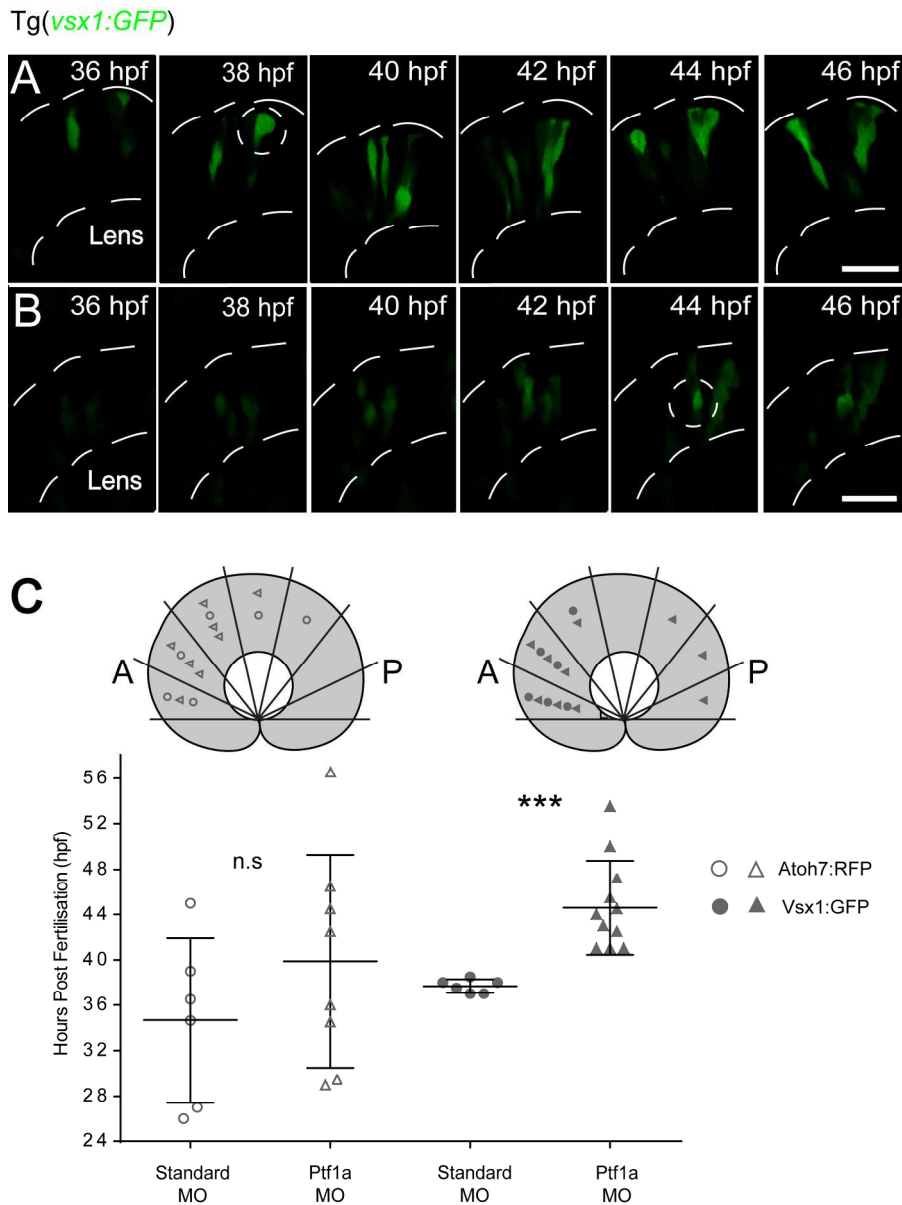


Figure 5. Wild type donor cells transplanted into different environment show a delay in timing of expression of fate determinant genes of relatively later born neurons.

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morphant hosts (\*\**p* < 0.0002, Mann-Whitney test). Error bars are SEM. Scale bars A & B = 10  $\mu$ m.

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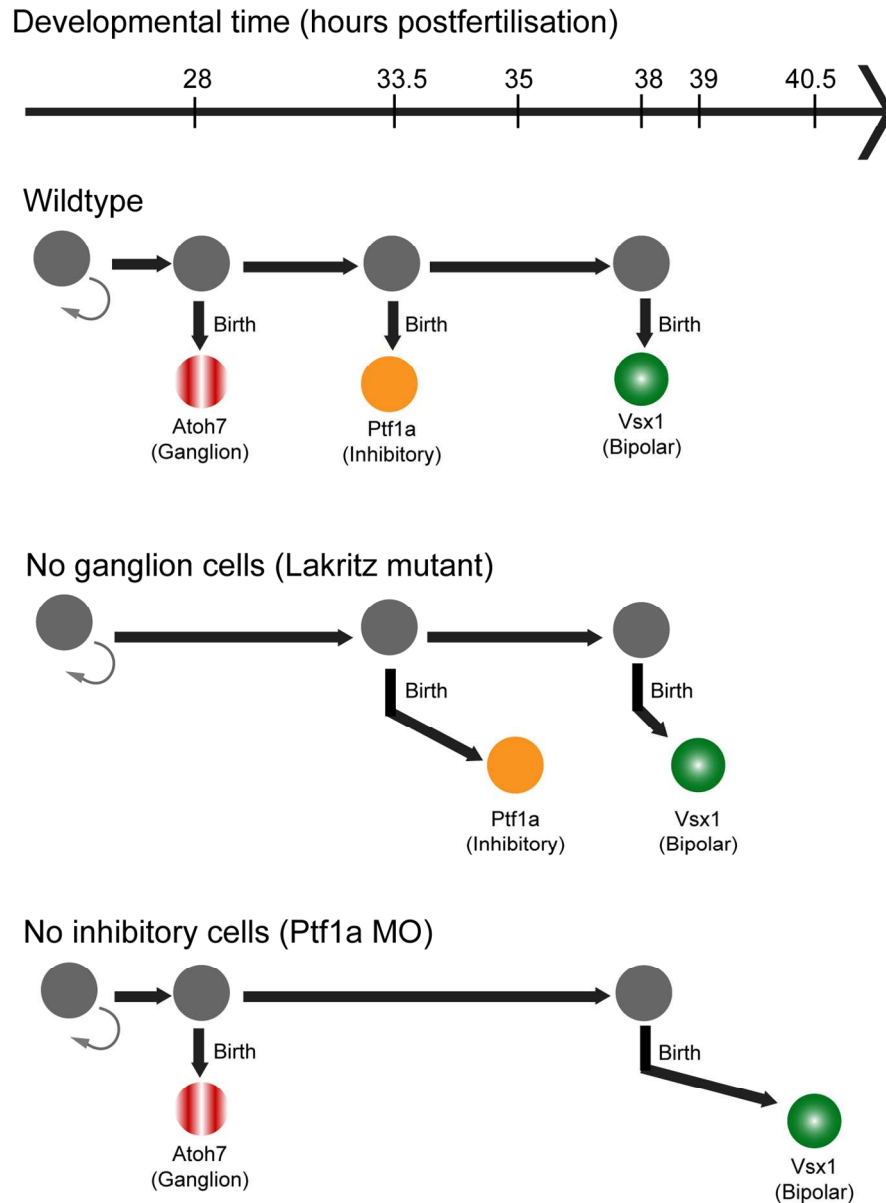


Figure 6. Summary schematic of changes in cell birth and fate gene expression timing after loss of different neuronal population

During retinal development in a wild type environment, different types of neurons are born at distinct times and the expression of differential fate genes for each neuronal types similarly occurs in a stereotypical manner at distinct developmental times. In environments where a neuron population is missing during development, such as ganglion cells in the lak mutant or inhibitory cells (i.e. horizontal and amacrine cells) in the Ptf1a morphant, the birthdates are unchanged (vertical arrows still at the same developmental time), however, the timing of gene expression for later born neuron populations are delayed (such as Ptf1a and Vsx1), while the expression timing of earlier staged neuron fate genes (Atoh7) remains unaffected.

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Table 1. Table of Primary Antibody Used

<b>Antigen</b>	<b>Description of Immunogen</b>	<b>Source, Host Species, Cat. #, Clone or Lot#, RRID</b>	<b>Concentration Used</b>
BrdU	Bromodeoxyuridine-bovine serum albumin conjugate	Roche, mouse IgG1 monoclonal, Cat# 11170366001, Clone BMC 9318, RRID:AB_233622	0.25 µg/ml (IF)

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Table 2. Developmental time in hours postfertilisation (hpf) at which 50% of each cell type is born in central retina (intrapolated)

Background	Ganglion cells (hpf)	Bipolar cells (hpf)	Photoreceptor cells (hpf)
Wildtype	42	50	56
Standard MO	42	50	56
Ptf1a MO (no inhibitory neurons)	42	51	56

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Table 3. Developmental time in hours postfertilisation (hpf) at which fate determinant master gene expression first starts in different backgrounds

Background	Ganglion fate Onset of Atoh7 (hpf $\pm$ SEM)	Inhibitory cell fate Onset of Ptf1a (hpf $\pm$ SEM)	Bipolar cell fate Onset of Vsx1 (hpf $\pm$ SEM)
Lakritz sibling (control)	-	33.48 $\pm$ 0.09	38.030 $\pm$ 0.36
Lakritz mutants (no ganglion cells)	-	34.63 $\pm$ 0.20	39.30 $\pm$ 0.53
Standard MO (control)	28 $\pm$ 0.18	-	38 $\pm$ 0.22
Ptf1a MO (no inhibitory neurons)	28.5 $\pm$ 0.24	-	40.5 $\pm$ 0.24
WT in standard MO (control chimera)	35 $\pm$ 2.96	-	38 $\pm$ 0.25
WT in Ptf1a MO (no inhibitory neuron chimera)	40 $\pm$ 3.83	-	45 $\pm$ 1.70