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Partially redundant proneural function reveals the importance of timing during zebrafish olfactory neurogenesis

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SUMMARY

Little is known about proneural gene function during olfactory neurogenesis in zebrafish. Here, we show that the zebrafish Atonal genes *neurogenin1* (*neurog1*) and *neurod4* are redundantly required for development of both early-born olfactory neurons (EONs) and later-born olfactory sensory neurons (OSNs). We show that *neurod4* expression is initially absent in *neurog1* mutant embryos but recovers and is sufficient for the delayed development of OSN. By contrast, EON numbers are significantly reduced in *neurog1* mutant embryos despite the recovery of *neurod4* expression. Our results suggest that a shortened time window for EON development causes this reduction; the last S-phase of EON is delayed in *neurog1* mutant embryos but mutant EONs are all post-mitotic at the same stage as EONs in wild-type embryos. Finally, we show that expression of certain genes, such as *robo2*, is never detected in *neurog1* mutant EONs. Failure of *robo2* expression to recover correlates with defects in the fasciculation of *neurog1* mutant olfactory axonal projections and in the organisation of proto-glomeruli because projections arrive at the olfactory bulb that are reminiscent of those in *robo2* mutant embryos. We conclude that the duration of proneural expression in EON progenitors is crucial for correct development of the zebrafish olfactory system.

KEY WORDS: Zebrafish, Olfactory neurogenesis, Proneural gene, *neurog1*; *neurod4*, Developmental timing

INTRODUCTION

The nervous system is established via a tightly controlled series of events, known as neurogenesis, which assures that the appropriate subtypes of neurons are born in the correct places and numbers, and at the correct times during embryogenesis. Many of the molecular mechanisms that regulate neurogenesis have been unravelled in the fruit fly (Gomez-Skarmeta et al., 2003). A key step during the initial phase of this process is the establishment of so-called proneural clusters in the *Drosophila* neuroectoderm, which are characterised by expression of proneural genes that belong to the basic helix-loop-helix (bHLH) family of transcriptional activators (Dambly-Chaudiere and Vervoort, 1998). The conservation of genes homologous to *Drosophila* proneural genes, such as members of the neurogenin (neurog) and acheate-scute like (ascl) families, indicates that similar mechanisms are also at work in vertebrates (Bertrand et al., 2002).

The olfactory system has proven to be an attractive model for studying the role of proneural genes during vertebrate neurogenesis. In the mouse embryo, olfactory sensory neurons (OSN) are born in the sensory epithelium, which is itself derived from a pair of epidermal thickenings, or placodes, located on either side of the anterior neural plate (Cuschieri and Bannister, 1975a; Cuschieri and Bannister, 1975b; Smart, 1971). Loss-of-function studies in mice have shown that the sequential activity of members of the Ascl and Neurog families of proneural genes are required for the majority of OSN that develop. Indeed, the current model suggests that progenitor cells expressing *Ascl1* gives rise to intermediate precursors that require *Neurog1* for differentiation (Cau et al., 2002; Cau et al., 1997; Guillemot et al., 1993; Nicolay et al., 2006). More recently, it has been shown that the winged-helix transcription factor Foxg1 acts upstream of Ascl1 for correct olfactory neurogenesis (Duggan et al., 2008). Similarly, microRNAs of the miR-200 family have been implicated in the maintenance of olfactory neurogenesis (Choi et al., 2008).

As in the mouse, olfactory neurogenesis in the zebrafish embryo occurs in placodes flanking the anterior neural plate. It has been shown that olfactory placodes themselves fate-map to a horseshoe-shaped population of cells at the boundary between the anterior neural plate and the adjacent non-neural ectoderm (Whitlock and Westerfield, 2000). Although this region gives rise to both the olfactory placodes and the telencephalon, progenitors of olfactory neurons are generally located more laterally and telencephalic progenitors more medially (Whitlock and Westerfield, 2000). Progenitors of olfactory neurons coalesce to form the olfactory placodes in a process that requires the chemokine receptor Cxcr4b and its ligand Cxcl12a (previously known as Sdf1a) (Miyasaka et al., 2007). Subsequently, neurons are born in two waves with the development of a population of so-called pioneer neurons preceding the birth of mature OSNs (Whitlock and Westerfield, 1998). Pioneer neurons are required for the correct projection of OSN axons to the olfactory bulb and undergo apoptosis once the first OSN axons have correctly reached their targets (Whitlock and Westerfield, 1998). As for correct placode formation at earlier stages, the Cxcl12a-Cxcr4b interaction is required for the proper guidance of pioneer axons (Miyasaka et al., 2007; Yoshihara, 2009); the Robo2 receptor and Slit ligands have also been implicated in fasciculation and guidance of axons of olfactory neurons (Miyasaka et al., 2005; Yoshihara, 2009). Surprisingly, however, although a requirement for Foxg1 and microRNAs has apparently been conserved between zebrafish and mouse during the early steps of olfactory neurogenesis, nothing is

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known concerning proneural gene function during the development of either early or late-born olfactory neurons in zebrafish (Choi et al., 2008; Duggan et al., 2008).

Here, we describe the role of *neurogenin1* (*neurog1*) and a second proneural gene of the Atonal family, *neurod4*, during olfactory neurogenesis in the zebrafish embryo. Our results indicate that these genes have overlapping functions during the development of early-born olfactory neurons and later olfactory sensory neurons. We also show that although the initial phase of *neurod4* expression requires Neurog1, *neurod4* expression recovers in the *neurog1* mutant; the late recovery of *neurod4* expression is sufficient for a reduced number of early-born neurons to develop. Finally, we show that there is a significant delay in the formation of mature OSNs in *neurog1* mutants despite the recovery of Neurod4 activity. Taken together, these results show that zebrafish uses a strategy for olfactory neurogenesis that is distinct from that used in the mouse.

MATERIALS AND METHODS

Fish lines and developmental conditions

Embryos were raised and staged according to standard protocols (Kimmel et al., 1995). Embryos homozygous for the *neurog1*^{hi1059} mutation (Golling et al., 2002) were obtained by intercrossing heterozygous carriers; adults heterozygous for the *neurog1*^{hi1059} allele were identified by PCR genotyping of tail-clip genomic DNA. The previously described *Tg(8.4neurog1:gfp)* transgenic line was used to visualise early-born olfactory neurons (Blader et al., 2003). Embryos were fixed overnight at 4°C in 4% paraformaldehyde in PBS, after which they were dehydrated through an ethanol series and stored at –20°C until use.

In situ hybridisation and immunostaining

In situ hybridisation was performed as previously described (Oxtoby and Jowett, 1993). Antisense DIG-labelled probes for *neurog1* (Blader et al., 1997), *gfp* (Blader et al., 2003), *neurod4* (Park et al., 2003), *deltaA* (Haddon et al., 1998), *olfactory marker protein* (Celik et al., 2002), *robo2* (Lee et al., 2001), *gefiltin* (*inab* – Zebrafish Information Network) (Asch et al., 1998), *olfactomedin 1b* (Nakaya and Tomarev, 2007) and *insulinoma-associated 1b* (Lukowski et al., 2006) were generated using standard procedures. In situ hybridisations were visualised using either BCIP and NBT (Roche) or Fast Red (Roche) as substrates. Immunohistochemical staining was performed as previously described (Masai et al., 1997) using either anti-GFP (1/1000, Torrey Pines Biolabs), zns-2 (1/250) (Trevarrow et al., 1990), anti-HuC/D (1/500, Molecular Probes), anti-Myc (1/10) (Evan et al., 1985), PCAM (1/500) (Mizuno et al., 2001) and SV2 [1/20; Developmental Studies Hybridoma Bank (DSHB) at University of Iowa]; secondary antibodies used were Alexa 488- or Alexa 555-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1/1000, Molecular Probes).

Antisense morpholino injection and mis-expression constructs

For morpholino knockdowns, embryos were injected at the one-cell stage with either of two morpholinos specific for *neurod4*, neurod4-MO1 and neurod4-MO2, using the previously described concentrations (Park et al., 2003). For Ngn1 and Neurod4 mis-expression studies, the pI-*Sce*1-*hsp70:myc-neurod4* vector was construct using the strategy previously described for the generation of pI-*Sce*1-*hsp70:myc-ngn1* (Halloran et al., 2000; Madelaine and Blader, 2011; Thermes et al., 2002); for reintroducing Neurod4 activity in *neurog1* mutant embryos, a myc-tagged coding region of the gene was fused to 8.4 kb of genomic DNA upstream of the *Neurog1* initiation codon using the previously described Tol2kit (Blader et al., 2003; Kwan et al., 2007). The resulting constructs were injected as previously described (Madelaine and Blader, 2011).

BrdU labelling, image acquisition and cell counting

For birthdating, embryos were incubated on ice in fish water supplemented with 10 mM 5-bromo-2-deoxyuridine (BrdU) and 8% DMSO for 20 minutes. Subsequently, embryos were incubated at 28.5°C in the continued presence of 10 mM BrdU until the desired stage was reached; embryos incubated in BrdU beginning at 8, 12, 16 and 20 hours post-fertilisation (hpf) were fixed at 24 hpf and embryos incubated from 24 hpf were fixed

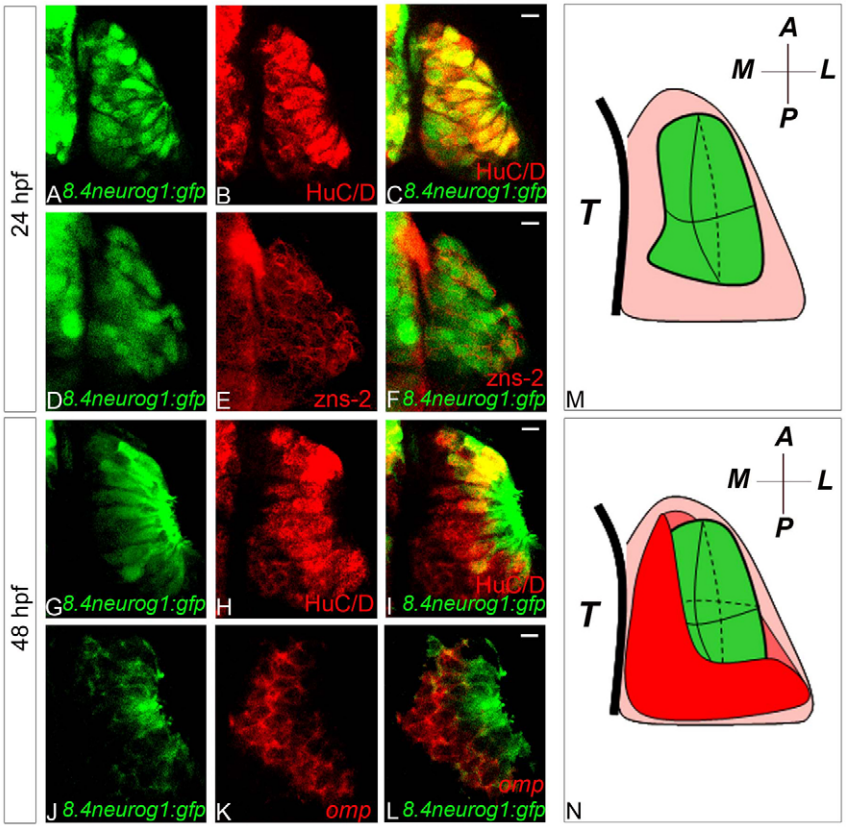

Fig. 1. *Tg(8.4neurog1:gfp)* is a marker of zebrafish early-born olfactory neurons. (**A-F**) Single confocal sections of olfactory placodes showing co-expression of HuC/D (A-C) or the previously described pioneer neuron marker zns-2 (D-F) with GFP from the *Tg(8.4neurog1:GFP)* transgene at 24 hpf. (**G-L**) Single confocal sections of olfactory placodes showing immunolabelling of HuC/D (G-I) or in situ hybridisation and immunolabelling of *omp* (J-L) and GFP from the *Tg(8.4neurog1:GFP)* transgene at 48 hpf. Whereas all GFP-positive cells in the placode are HuC/D- and zns-2-positive at 24 hpf, at 48 hpf HuC/D+/GFP– cells are present; little, if any, overlap is detected between the expression of *omp* transcripts and GFP at 48 hpf. (**M**,**N**) Schematics of olfactory placodes at 24 hpf (M) or 48 hpf (N). Early-born olfactory neurons are shown in green and olfactory sensory neurons in red. A, anterior; L, lateral; M, medial; P, posterior; T, telencephalon. Scale bars: 10 μm.

at 28 hpf. BrdU incorporation was detected by immunohistochemistry using an anti-BrdU antibody (G3G4, 1/500, DSHB). Confocal acquisitions were carried out using a Leica SP5 confocal microscope. Images were manipulated using Photoshop (Adobe) software. For cell counting, confocal stacks were analysed using ImageJ software.

RESULTS

Tg(8.4neurog1:gfp) is a marker of early-born olfactory neurons in the zebrafish olfactory placode

The expression of the proneural gene *neurogenin1* (*neurog1*) at the border between the neural and non-neural ectoderm in the anterior part of the neural plate suggests that this basic helix-loop-helix (bHLH) transcription factor is involved in the development of early-born olfactory neurons and/or olfactory sensory neurons (Blader et al., 1997). To address this possibility, we first mapped GFP expression driven from a transgene carrying a subset of *neurog1* regulatory elements, *Tg(8.4neurog1:gfp)*, relative to these two neural populations (Blader et al., 2003). Although this transgene only recapitulates a part of the overall *neurog1* expression pattern, the expression of *neurog1* transcripts overlaps with the GFP driven by the transgene in the olfactory system at early stages (see Fig. S1D,D′ in the supplementary material). Furthermore, the persistence of GFP at later stages allows us to use the transgene as a short-term lineage label after endogenous *neurog1* expression is no longer detected in the developing olfactory placode (see Fig. S1E-G′ in the supplementary material). At 24 hpf, all GFP+ cells in the olfactory placode express the early marker of post-mitotic neurons HuC/D (Elavl3/4 – Zebrafish Information Network) (Fig. 1A-C,M). Similarly, all transgene positive cells are recognised by the zns-2 antibody, previously described as a marker of pioneer neurons in the zebrafish olfactory placode (Fig. 1D-F,M) (Trevarrow et al., 1990; Whitlock and Westerfield, 1998). Thus, at 24 hpf *Tg(8.4neurog1:gfp)*+ neurons are the only post-mitotic cells in the olfactory placode and all appear to be pioneer neurons. By 48 hpf, the size of the olfactory placode has increased. Nonetheless, the number of *Tg(8.4neurog1:gfp)*-expressing cells remains unchanged after the first day of development; 95.15±11.77 *Tg(8.4neurog1:gfp)*-positive cells at 24 hpf versus 94.40±13.32 at 36 hpf. Also, whereas at 24 hpf all HuC/D-positive cells are GFP positive, at 48 hpf a significant proportion of the HuC/D-positive cells is negative for transgene expression (Fig. 1G-I). In contrast to GFP+ neurons, which are located superficially in the olfactory placode at 48 hpf, the HuC/D+;*Tg(8.4neurog1:gfp)*– cells are located closer to the telencephalon. Olfactory sensory neurons can be visualised at 48 hpf by the expression of the *olfactory marker protein* (*omp*) gene (Sato et al., 2005). Similar to the HuC/D+;*Tg(8.4neurog1:gfp)*– cells, cells expressing *omp* are located more deeply in the olfactory placode at this stage (Fig. 1J-L). Thus, at 48 hpf the olfactory placode can be schematically represented as an inner HuC/D+;*Tg(8.4neurog1:gfp)*+ population of early-born olfactory neurons (EON) surrounded by an outer cup of *omp*+;*Tg(8.4neurog1:gfp)*– olfactory sensory neurons (OSN; Fig. 1N).

The development of early-born olfactory neurons is affected but not eliminated in *neurog1* mutant embryos

The restricted expression of the *Tg(8.4neurog1:gfp)* reporter line suggests a role for *neurog1* in the generation of EON. Thus, we assessed the development of these neurons in a *neurog1* mutant background at 24 hpf by following the expression of the *Tg(8.4neurog1:gfp)* transgene and/or HuC/D (Golling et al., 2002); expression of the transgene is not regulated by *neurog1* itself and, thus, can be used to count the number of EON in the mutant context (see Fig. S1A-C in the supplementary material). We found that the development of early-born olfactory neurons is affected in *neurog1* mutant embryos with mutant embryos displaying approximately half the number of EONs as their wild-type siblings (Fig. 2A,C and Fig. 5A,B,E); the deficit in neurons is variable between embryos (Fig. 2B-D).

Development of early-born olfactory neurons is delayed in *neurog1* mutant embryos

The reduction in the number of early-born olfactory neurons in *neurog1* mutant embryos at 24 hpf could reflect either that EON are born at the correct time but at a reduced rate or that they are born later. To distinguish between these possibilities, we assessed the deficit at earlier stages in the *neurog1* mutant. At 22 hpf, a clear reduction in the number of HuC/D+ cells is already apparent in *neurog1* mutant embryos relative to wild-type siblings (Fig. 2E,F). More strikingly, at 19 hpf, when the dorsal telencephalic and olfactory lineages have become visibly segregated, a complete absence of HuC/D neurons is apparent in the olfactory anlagen in *neurog1* mutant embryos (Fig. 2G,H). These results suggest that the genesis of EON is delayed in the absence of *neurog1* function.

HuC/D is a marker of post-mitotic neurons. Thus, the delay in the generation of HuC/D+ neurons in the olfactory anlage of *neurog1* mutant embryos suggests that the last cell cycle of EON progenitors is also delayed in the mutant. However, the percentage of post-mitotic EON cannot be assessed accurately at stages prior to the segregation of the telencephalic and olfactory lineages using HuC/D. To address the question of cell-cycle delay differently, we

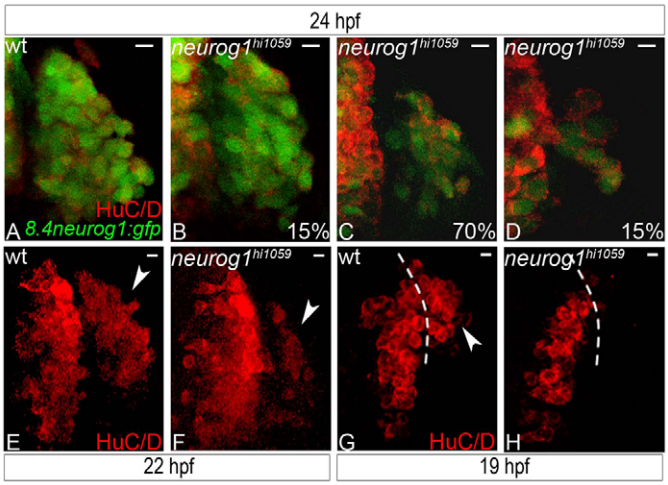

Fig. 2. Zebrafish early-born olfactory neuron development is delayed in the absence of *neurog1* function. (**A-D**) Confocal projections of wild-type (A) or *neurog1* mutant (B-D) olfactory placodes at 24 hpf labelled for HuC/D and GFP from the *Tg(8.4neurog1:GFP)* transgene; mutant embryos show a variable but reduced number (shown as percentage of wild type) of early-born olfactory neurons. (**E**,**F**) Confocal projections of wild-type (E) or *neurog1* mutant (F) olfactory placodes at 22 hpf labelled for HuC/D; a reduced number of HuC/D-positive neurons is detected in the olfactory placode of mutant embryos at this stage. (**G**,**H**) Confocal projections of wild-type (G) or *neurog1* mutant (H) olfactory placodes at 19 hpf labelled for HuC/D; a complete absence of HuC/D neurons is apparent in the olfactory anlagen in *neurog1* mutant embryos. Dashed white line indicates the boundary of the telencephalon and the olfactory placode; arrowheads indicate HuC/D expression in olfactory placode. Scale bars: 10 µm.

performed experiments using BrdU in *Tg(8.4neurog1:gfp)* transgenic embryos. When embryos were treated with BrdU continuously from 8 or 12 hpf to 24 hpf, virtually all GFP+ cells are BrdU+, indicating that EON progenitors have yet to undergo their last S-phase at these stages in either wild-type or mutant embryos (Fig. 3G). By contrast, whereas ~90% of *Tg(8.4neurog1:gfp)*+ are BrdU+ when mutant embryos are treated from 16 hpf, only 56% of EONs are labelled in wild-type embryos incubated in BrdU from the same stage (Fig. 3A,D,G). Labelling from 20 hpf leads to an overall reduction in the number of *Tg(8.4neurog1:gfp)*+ that are BrdU+ in either mutant or wild-type embryos. Nonetheless, over twice as many EON progenitors still incorporate BrdU in the mutant relative to wild type at this stage (75±14% in mutants versus 35±15% in wild-type siblings; Fig. 3B,E,G). Finally, very few early-born olfactory neurons incorporate BrdU at 24 hpf in either mutant or wild-type embryos (Fig. 3C,F,G). The differences between the incorporation curves obtained for mutant and wild-type embryos suggests that the last cell cycle of early-born olfactory neurons is delayed in the absence of Neurog1 function. Furthermore, our BrdU experiments support our counts of *Tg(8.4neurog1:gfp)*+ neurons at 24 and 36 hpf that suggested that few EON progenitors are cycling after 24 hpf in either genetic context.

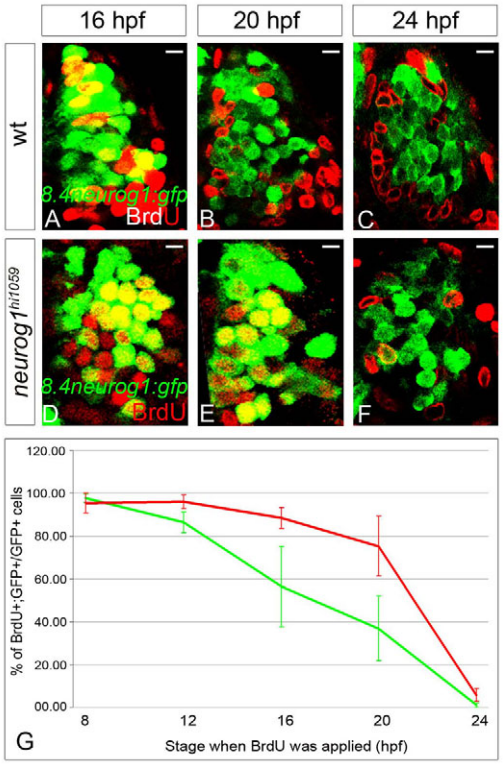

Fig. 3. The last S-phase of early-born olfactory neurons is delayed in the *neurog1* mutant zebrafish. (**A-F**) Confocal sections of olfactory placodes immunolabelled for BrdU (red) and GFP (green) from the *Tg(8.4neurog1:GFP)* transgene in wild-type (A-C) or *neurog1* mutant (D-F) embryos at 24 hpf (A,B,D,E) or 28 hpf (C,F). The stages at which BrdU incubation were started is indicated. Scale bars: 10 μM. (**G**) The proportion BrdU+;GFP+ cells over total number of GFP+ cells in the olfactory placode after BrdU treatment in wild-type (green line) or *neurog1* mutant (red line) embryos. Although early-born olfactory neurons generally leave the cell cycle later in mutant than in wild-type embryos, at 24 hpf few, if any, early-born olfactory neurons are still cycling in either genetic context. Error bars represent s.d.

Neurod4 acts redundantly with Neurog1 during development of early-born olfactory neurons

Although *neurog1* plays an apparently important role for the development of early-born olfactory neurons in the zebrafish, the incomplete penetrance of the EON deficit in *neurog1* mutant embryos suggests that there are other proneural factors with at least partially overlapping functions. The *neurod4* gene encodes a transcription factor with a bHLH domain highly similar to that of Neurog1 (Park et al., 2003; Wang et al., 2003). Furthermore, Neurog1 and Neurod4 have redundant functions during the development of some cranial ganglia in zebrafish (Park et al., 2003); correspondingly, a variety of potential target genes behave identically after mis-expression of the two proneural factors (see Fig. S2E-G′ in the supplementary material; data not shown). Thus, we analysed the expression of *neurod4* to address the possibility of redundancy with *neurog1* during neurogenesis in the zebrafish olfactory placode.

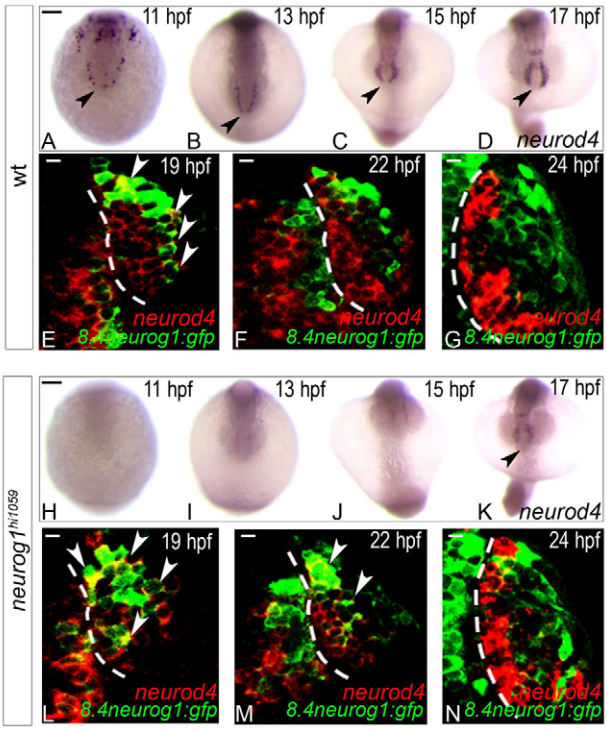

Fig. 4. Expression of *neurod4* in the zebrafish olfactory anlage is dependent on *neurog1* at early but not late stages. (**A-D**,**H-K**) Whole-mount in situ hybridisation against *neurod4* in wild-type (A-D) and *neurog1* mutant (H-K) embryos at 11 (A,H), 13 (B,I), 15 (C,J) and 17 (D,K) hpf. Although *neurod4* expression is absent in mutant embryos up to 15 hpf, expression recovers to wild-type levels at 17 hpf. Embryos are viewed dorsally with anterior down; black arrowheads indicate the mixed telencephalo-olfactory expression domain. (**E-G**,**L-N**) Single confocal sections of in situ hybridisation and immunolabelling against *neurod4* and GFP from the *Tg(8.4neurog1:GFP)* transgene in the olfactory placode of wild-type (E-G) or *neurog1* mutant (L-N) embryos at 19 (E,L), 22 (F,M) and 24 (G,N) hpf. At 24 hpf, *neurod4* is restricted to cells of the olfactory placode immediately adjacent to the telencephalon and is excluded from *Tg(8.4neurog1:gfp)*-positive early-born olfactory neurons. White arrowheads indicate double-labelled cells. Dashed white line indicates the boundary of the telencephalon and the olfactory placode. Scale bars: in A, 100 μM for A-D; in H, 100 μM for H-K; in E-G,L-N, 10 μm.

The expression pattern of *neurod4* resembles that of *neurog1* (Blader et al., 1997; Korzh et al., 1998; Park et al., 2003; Wang et al., 2003). In the developing olfactory system, *neurod4* expression is first detected at 11 hpf in cells at the boundary of the anterior neural plate, shortly after the onset of expression of *neurog1* (Fig. 4A) (Blader et al., 1997; Korzh et al., 1998). Increasing levels of *neurod4* expression in this region accompany the convergence of the anterior neural plate such that by 17 hpf strong expression is detected in the mixed telencephalo-olfactory lineage (Fig. 4B-D); during this period *neurod4* expression overlaps broadly with *neurog1* and the proneural target gene *deltaA* (see Fig. S3A-I in the supplementary material) (Haddon et al., 1998). At 19 hpf, when the telencephalic and olfactory lineages have segregated, robust *neurod4* expression is detected in both territories (Fig. 4E). Expression of *neurod4* continues in the developing olfactory placode at 22 hpf where it is largely excluded from *Tg(8.4neurog1:gfp)+* cells (Fig. 4F). Finally, at 24 hpf the expression of *neurod4* is detected as an outer cup of cells surrounding the population of *Tg(8.4neurog1:gfp)+* neurons (Fig. 4G). Surprisingly, the expression of *neurod4* is absent in *neurog1* mutant embryos up to 17 hpf (Fig. 4H-J). Furthermore, mis-expression of Neurog1 rapidly induces the expression of *neurod4*, suggesting that it is a direct target gene at stages up to 17 hpf (see Fig. S2C-D′ in the supplementary material); mis-expression of Neurod4 does not induce *neurog1* expression (see Fig. S2A-B′ in the supplementary material). At later stages, however, the expression of *neurod4* in the olfactory placode is reminiscent of that in wild-type embryos (Fig. 4D-G versus K-N). Thus, the recovery of *neurod4* expression at 17 hpf in *neurog1* mutant embryos is in agreement with a role for the gene in the generation of residual EONs in absence of *neurog1* function.

Next, we used a morpholino knockdown approach alone or in the *neurog1* mutant background to confirm a role for *neurod4* in the development of early-born olfactory neurons. As described above, there is a 57% reduction in the number of *Tg(8.4neurog1:gfp)+* neurons in *neurog1* mutant embryos (Fig. 5A,B,E). There is also a significant, although mild, reduction in the number of EONs in *neurod4* morphants alone (Fig. 5C,E); co-injection with a *p53* morpholino has no effect on this reduction (data not shown). Simultaneous abrogation of both *neurod4* and *neurog1* function, however, reduces the number of EONs by 80% relative to wild type (Fig. 5D,E); this reduction is significantly greater when compared with counts in *neurog1* mutant embryos alone (Fig. 5E). Finally, re-introduction of Neurod4 partially rescues the EON deficit in *neurog1* mutant embryos, suggesting that the proneural function of the two genes is interchangeable (Fig. 5E). We conclude from our expression studies and functional analyses that the generation of early-born olfactory neurons is controlled by the partially redundant activities of Neurog1 and Neurod4.

Neurog1 and Neurod4 redundantly control olfactory sensory neurons development

The expression of *neurod4* in the olfactory placode at 24 hpf is excluded from *Tg(8.4neurog1:gfp)*-positive neurons (Fig. 4G). Furthermore, our birthdating studies indicate that the cells at this position incorporate BrdU at this stage (Fig. 3C). These results suggest that *neurod4*-expressing cells at 24 hpf are cycling neural progenitors. As few, if any, early-born neurons remain in the cell cycle at this stage, we conclude that these *neurod4*-expressing cells are progenitors of OSNs.

To investigate the role of *neurod4* in the development of OSNs, we analysed the expression of markers of neural progenitors in *neurod4* morphant embryos. The transcription of *deltaA* is directly regulated by proneural genes in neural progenitors (Madelaine and Blader, 2011). Likewise, a second target of proneural genes, *insulinoma-associated 1b* (*insm1b*) is expressed in the olfactory placode during development (see Fig. S4A-C in the supplementary material) (Castro et al., 2006; Lukowski et al., 2006); *deltaA* and *insm1b* expression are comparable to that of *neurod4* at 24 hpf (Fig. 6A,A′; see Fig. S4G in the supplementary material). A similar pattern of *deltaA* or *insm1b* expression is detected in embryos injected with the *neurod4* morpholino, suggesting that, as for EONs, redundancy between proneural factors exists during the

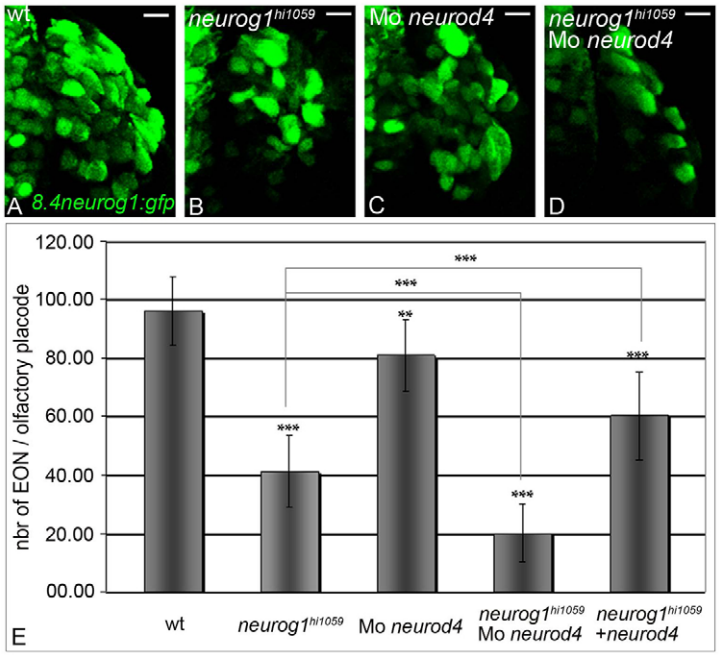

Fig. 5. Neurog1 and Neurod4 act redundantly during development of zebrafish early-born olfactory neurons. (**A-D**) Confocal projections of GFP from the *Tg(8.4neurog1:GFP)* transgene in the olfactory placode of wild-type (A), *neurog1* mutant (B), *neurod4* morphant (C) and *neurog1*/*neurod4* double loss-of-function (D) embryos at 24 hpf. Abrogation of both Neurod4 and Neurog1 function leads to a severe deficit in the differentiation of early-born olfactory neurons. Placodes are oriented with anterior up. Scale bars: 10 μM. (**E**) Counts of early-born olfactory neurons per placode at 24 hpf in wild-type, *neurog1* mutant, *neurod4* morphant, *neurog1*/*neurod4* double loss-of-function embryos and *neurog1* mutant embryos in which Neurod4 has been mis-expressed. A minimum of twelve olfactory placode was analysed for each context. Error bars represent s.d. **P*<0.05, ***P*<0.001, ****P*<0.0005, determined by *t*-test.

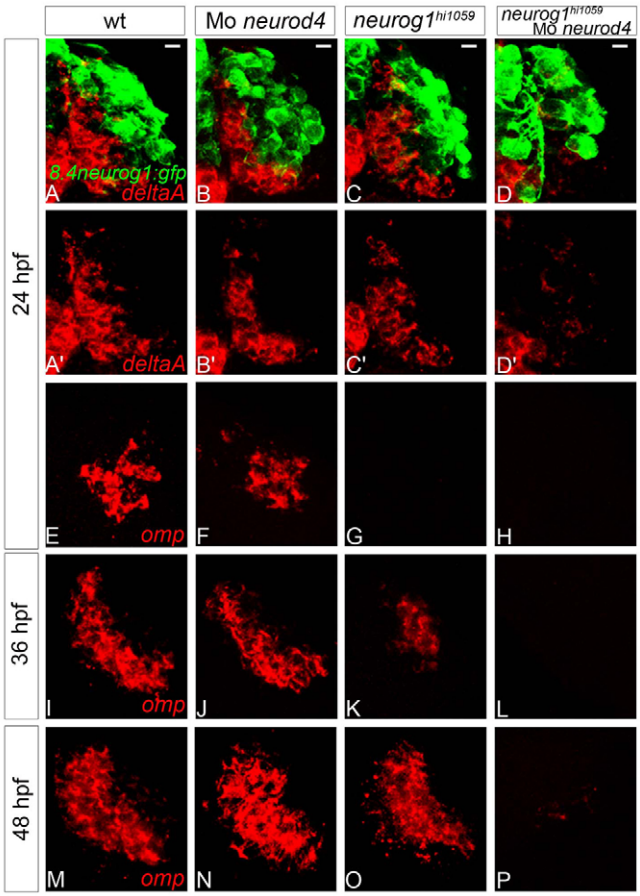

Fig. 6. Neurod4 and Neurog1 act redundantly during OSN development in zebrafish. (**A-D′**) Confocal projections of in situ hybridisation and immunolabelling against *deltaA* and GFP in wild-type (A,A′), *neurod4* morphant (B,B′), *neurog1* mutant (C,C′) and *neurog1/neurod4* double loss-of-function (D,D′) olfactory placodes of *Tg(8.4neurog1:GFP)* embryos at 24 hpf. Although no difference is apparent in the expression of *deltaA* in single loss-of-function embryos relative to wild-type siblings, *deltaA* expression is absent when both *neurog1* and *neurod4* function is abolished. (**E-P**) Confocal sections of in situ hybridisation labelling against *olfactory marker protein* (*omp*) in wild-type (E,I,M), *neurod4* morphant (F,J,N), *neurog1* mutant (G,K,O) and *neurog1/neurod4* double loss-of-function (H,L,P) olfactory placodes at 24 (E-H), 36 (I-L) and 48 (M-P) hpf. Although *omp* expression resembles the wild-type pattern in the single loss-of-function context at 48 hpf, there is a delay in the initiation of *omp* expression in the absence of Neurog1 function. The expression of *omp* is never detected when both *neurog1* and *neurod4* function is abolished. Scale bars: 10 μm.

development of OSNs (Fig. 6B,B′; see Fig. S4H in the supplementary material). Although *neurog1* is no longer expressed in the olfactory placode at 22 hpf, residual expression can be detected up to 20 hpf leaving open the possibility that *neurog1* also acts together with *neurod4* during the early phase of OSN development (see Fig. S1E-G′ in the supplementary material). Consistent with this idea, whereas *deltaA* and *insm1b* expression is unaffected in *neurog1* mutant embryos at 24 hpf (Fig. 6C,C′; see Fig. S4D-F,I in the supplementary material), no *deltaA* or *insm1b* expression is detected in embryos lacking both *neurod4* and *neurog1* function (Fig. 6D,D′; see Fig. S4J in the supplementary material).

Next, we analysed the effect of abrogating *neurod4* and/or *neurog1* function on the expression of a marker of specified OSNs, *omp* (Sato et al., 2005). As for *deltaA*, *omp* expression in *neurod4* morphants is indistinguishable from wild type at all stages analysed (Fig. 6E,I,M versus F,J,N). On the contrary, whereas *omp* expression in *neurog1* mutant embryos resembles that in wild-type siblings at 48 hpf, expression is severely reduced in the mutant at 36 hpf and cannot be detected at 24 hpf (Fig. 6G,K,O); in the absence of both *neurod4* and *neurog1* activity the expression of *omp* is missing at all stages up to 48 hpf (Fig. 6H,L,P). We conclude that the redundant function of *neurod4* and *neurog1* is necessary for the formation of OSNs.

Neurog1 controls aspects of early-born olfactory neuron behaviour

Our results suggest that the majority of the residual EONs present in *neurog1* mutant embryos finish their last S-phase after 20 hpf. This correlates with the late onset of *neurod4* expression, which becomes independent of Neurog1 a few hours earlier. Thus, future EONs appear to express bHLH proneural factors for a significantly shorter time in the absence of Neurog1 function, from 16-24 hpf rather than 10-24 hpf as in wild type. We, thus, asked whether this shortened time window of proneural expression is nonetheless sufficient for EONs that are born in *neurog1* mutant embryos to acquire all aspects of their genetic programme.

The zebrafish *robo2* gene encodes a transmembrane receptor protein of the immunoglobulin superfamily. The expression of *robo2* in the olfactory system begins at 20 hpf, is robust between 24 and 36 hpf, after which its expression quickly diminishes (Miyasaka et al., 2005). We find that *robo2* expression overlaps completely with the *Tg(8.4neurog1:gfp)* transgene at 24 and 36 hpf in wild-type embryos, suggesting that it is specific to EONs (Fig. 7A-C,J-L). In contrast to the wild-type situation, *robo2* expression is not detected in *Tg(8.4neurog1:gfp)*+ cells in *neurog1* mutant embryos at 24 hpf and never significantly recovers in the mutants at later stages (Fig. 7D-F,M-O; data not shown). Similarly, the expression of *olfactomedin 1b* (*olfm1b*) and *gefiltin* (*gef*) is restricted to EONs at 24 hpf in wild-type embryos and is absent from *neurog1* mutant olfactory placodes (see Fig. S5A-L in the supplementary material) (Asch et al., 1998; Nakaya and Tomarev, 2007). Thus, although neurons with certain characteristics of EONs can develop in the absence of Neurog1 function, not all aspects of EON specification are apparent. Importantly, re-introduction of Neurod4 expression under the control of *neurog1* regulatory elements leads to a partial rescue of *robo2* expression at 24 hpf (Fig. 7G-I); *robo2* does not behave as a direct target of either Neurog1 or Neurod4 (see Fig. S2H-J′ in the supplementary material). We conclude, therefore, that the failure of late EON markers, such as *robo2*, to recover in *neurog1* mutant embryos reflects the change in the time window of exposure to proneural activity and not to differences in the intrinsic activities of Neurog1 and Neurod4.

The activity of Robo2 is required for fasciculation of axons as they project to the olfactory bulb (Miyasaka et al., 2005). Likewise, the organisation of proto-glomeruli in the olfactory bulb is also disturbed in the *robo2* mutant, *astray* (Miyasaka et al., 2005). The failure of *robo2* expression to recover in EONs in *neurog1* mutant embryos suggests that these processes might be compromised in the early-born olfactory neurons that develop in the absence of Neurog1 function. Thus, we analysed the fasciculation of olfactory axons and the formation of proto-glomeruli in the *neurog1* mutant background by performing immunostaining against PCAM

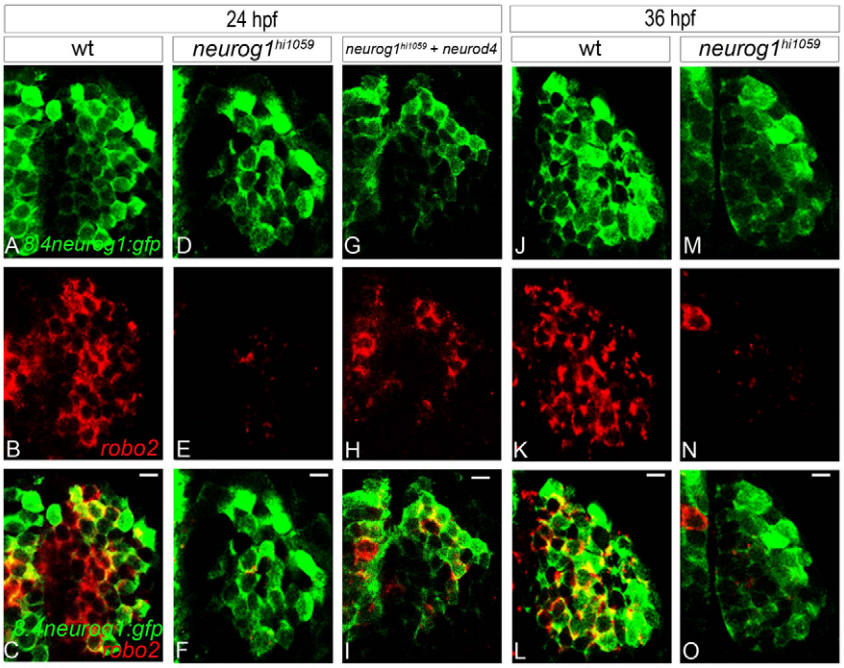

Fig. 7. In *neurog1* mutant zebrafish embryos, residual early-born olfactory neurons fail to express certain EON markers. (**A-I**) Confocal sections of in situ hybridisation and immunolabelling against *robo2* and GFP from the *Tg(8.4neurog1:GFP)* transgene at 24 hpf in wild type (A-C), *neurog1* mutant embryos (D-F) and *neurog1* mutant embryos in which Neurod4 has been mis-expressed (G-I). Whereas early-born olfactory neurons in wild-type embryos robustly express *robo2*, expression is absent in *neurog1* mutant embryos; mis-expression of Neurod4 partially rescues *robo2* expression. (**J-O**) Confocal sections of in situ hybridisation and immunolabelling against *robo2* and GFP from the *Tg(8.4neurog1:GFP)* transgene in wild-type (J-L) and *neurog1* mutant (M-O) embryos at 36 hpf. The expression of *robo2* never recovers in the few early-born olfactory neurons that develop in *neurog1* mutant embryos. Scale bars: 10 μm.

(Ncam1b – Zebrafish Information Network) and SV2; PCAM labels olfactory axons and their termini and, together with the synaptic vesicle protein SV2, highlights proto-glomeruli in the developing olfactory bulb (Miyasaka et al., 2005; Mizuno et al., 2001). In wild-type embryos, PCAM+ olfactory axons form a single fasciculated tract until they enter the telencephalon where they disperse to form distinct PCAM+/SV2+ proto-glomeruli (Fig. 8A-C,J-L). By contrast, in *neurog1* embryos, olfactory axons are often defasciculated in a manner similar to *ast* mutants (Fig. 8D-F). In several cases, we detected aberrant projections of olfactory axons as soon as they leave the olfactory placode or subsets of projections that are apparently incapable of leaving the placode (Fig. 8G-I; see Fig. S6A-L in the supplementary material). Finally, in the *neurog1* mutant embryos, PCAM+/SV+ proto-glomeruli appear less well organised (Fig. 8M-O) Together, these results suggest that failure of the expression of *robo2* to recover in EONs of *neurog1* mutant embryos has effects on the organisation of the projections of mature olfactory neurons. Furthermore, we propose that the combined failure of the expression of *robo2* and other genes, such as *gef* and *olfm1b*, to recover in *neurog1* mutant EONs underlies the fact that the *neurog1* phenotype is generally stronger than that of *robo2* mutants.

DISCUSSION

In the present study, we describe the role of two proneural genes, *neurog1* and *neurod4*, during olfactory neurogenesis in the early zebrafish embryo. Our results suggest that these genes have largely redundant functions during the development of olfactory neurons. Interestingly, the early phase of *neurod4* expression requires Neurog1. As a consequence of this partial redundancy, there is a delay in the development of olfactory neurons in the *neurog1* mutant (schematically represented in Fig. 9). Here, we discuss these results relative to previous studies in the zebrafish and mouse.

Our interest in identifying proneural genes required for the development of olfactory neurons in the zebrafish led us to characterise the expression of the *Tg(8.4neurog1:gfp)* transgene in the olfactory system (Blader et al., 2003). We found that at 24 hpf, GFP expression is restricted to post-mitotic neurons in the olfactory placode. As all GFP+ cells are recognised by the antibody zns-2, we initially concluded that the *Tg(8.4neurog1:gfp)* transgene is a novel marker of pioneer neurons; zns-2 was originally described as a marker of pioneer neurons (Whitlock and Westerfield, 1998). However, whereas our counts suggest that there are ~95 GFP+/zns-2+ cells per placode, only 11 zns-2+ neurons/placode were reported in the initial characterisation of zns-2 expression at the same stages (Whitlock and Westerfield, 1998). We subsequently performed birthdating studies on *Tg(8.4neurog1:gfp)*+ cells. Again our results differ from those of earlier work; based on single cell fate mapping and morphological criteria it was previously shown that pioneer neurons are post-mitotic by 12 hpf (Whitlock and Westerfield, 1998; Whitlock and Westerfield, 2000), but our results indicate that *Tg(8.4neurog1:gfp)*+ cells can incorporate BrdU up to 20-24 hpf. An explanation for these discrepancies is that only a subset of GFP+ cells are in fact pioneer neurons; the corollary being that zns-2 is not a specific marker of pioneer neurons, an idea supported by the limited co-expression of the olfactory sensory neuron marker *Tg(omp:YFP)* and zns-2 (Miyasaka et al., 2007). However, it does not appear that the majority of *Tg(8.4neurog1:gfp)*+ cells are olfactory sensory neurons either as only a few cells co-express *omp* and the *Tg(8.4neurog1:gfp)* transgene (R.M. and P.B., unpublished observations). It is apparent, therefore, that there is more heterogeneity in olfactory neurons subtypes than previously appreciated. To avoid confusion with the previous 'pioneer/OSN' nomenclature, we have chosen to refer to the *Tg(8.4neurog1;gfp)*+ neurons described in our study as early-born olfactory neurons (EONs) rather than pioneer neurons. The characterisation of novel molecular markers should help refine our understanding of the complexity of early neural subtypes in this system.

The conserved role of Foxg1 and members of the miR-200 microRNA family during early olfactory development in mice and zebrafish suggests an overall conservation of developmental strategy for the olfactory system in these two species (Choi et al., 2008;

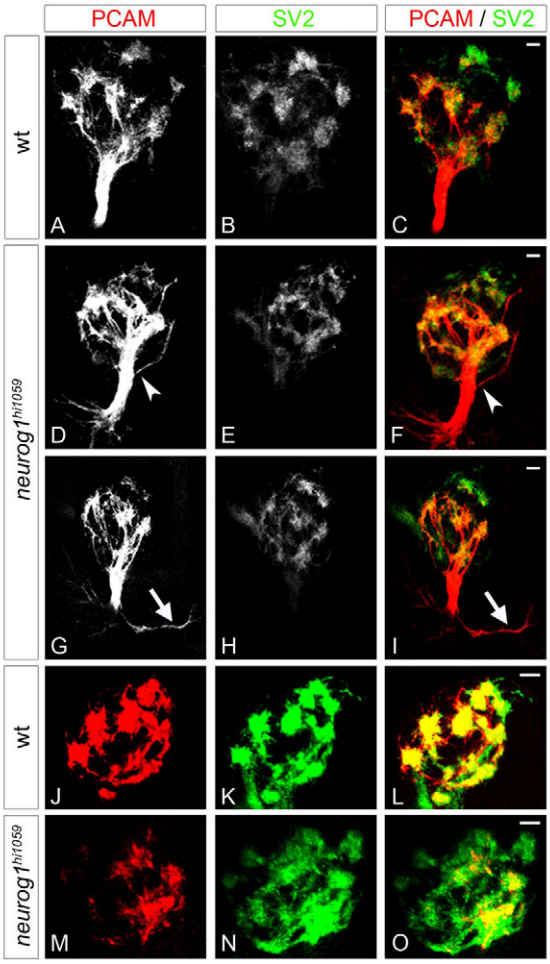

Fig. 8. The fasciculation of olfactory axons and organisation of proto-glomeruli are flawed in *neurog1* mutant zebrafish embryos. (**A-I**) Confocal projections of immunolabelling against PCAM and SV2 of olfactory projections in wild-type (A-C) and *neurog1* mutant (D-I) embryos at 72 hpf. Whereas projections in wild-type embryos form a single fasciculated tract from the olfactory placode to the olfactory bulb, fasciculation (arrowheads in D,F) is aberrant in *neurog1* mutant embryos; certain mutant embryos display abnormal guidance of projections immediately after they leave the placode (arrows in G,I). (**J-O**) Confocal projections of immunolabelling against PCAM and SV2 in the olfactory bulb in wild-type (J-L) and *neurog1* mutant (M-O) embryos at 72 hpf. Proto-glomeruli appear less well organised in *neurog1* mutant embryos relative to wild-type siblings. Embryos are viewed frontally (A-I) or dorsally with anterior up (J-O). Scale bars: 10 μM.

Duggan et al., 2008); the conserved role of Robo2 would seem to confirm this idea (Cho et al., 2007). Our study, however, shows that this is not completely the case as although in the mouse olfactory sensory epithelium *Ascl1* acts as the dominant proneural factor, in the zebrafish a redundant pair of Atonal family members, *neurog1* and *neurod4*, play this role (this study) (Cau et al., 2002; Cau et al., 1997; Guillemot et al., 1993). The significance of this difference is not clear. Knock-in experiments in the mouse have shown that Ngn2 can effectively replace Ascl1a in the development of the mouse olfactory epithelium, suggesting that there is no apparent 'specification role' for the proneural gene used after olfactory neurons are determined (Parras et al., 2002). However, the deficit in early-born olfactory neurons in the *neurog1* mutant in zebrafish can be rescued by the mis-expression of Neurog1 but not Ascl1a (R.M. and P.B., unpublished observations). We conclude that the choice of proneural gene is, at least in the case of EONs, crucial for correct specification of neural sub-type identity in the zebrafish olfactory system. It is possible that there is no equivalent to zebrafish early-born olfactory neurons in the mouse. Furthermore, *ascl1a* is expressed in a restricted domain of OSN progenitors in the zebrafish olfactory placode at 24 hpf, suggesting that it might have a role to play in the development of OSN in the zebrafish, as it does in the mouse (R.M. and P.B., unpublished observations). However, in the *ascl1a* mutation, *pituitary absent* (*pia*), we have not detected defects in olfactory neurogenesis (R.M. and P.B., unpublished observations) (Pogoda et al., 2006). Moreover, the abrogation of both *neurog1* and *neurod4* activity is sufficient to eliminate OSN development up to 48 hpf, indicating that at best *ascl1a* acts downstream of the combined activity of these two genes. The question of whether there is a later role for Ascl1a during olfactory neurogenesis in the zebrafish remains open.

The correct development of early-born and mature olfactory sensory neurons in the zebrafish olfactory placode requires the redundant activity of Neurog1 and Neurod4. Our data also suggest that *neurod4* is a transcriptional target of Neurog1 up to 17 hpf, after which its expression becomes independent of Neurog1 activity. The timing of the recovery of cell-cycle exit of early-born olfactory neurons in the olfactory placode of *neurog1* mutant zebrafish embryos coincides with the recovery of *neurod4* expression in the mutant and our rescue experiments suggest that the expression of either gene is sufficient to drive the progenitors of early-born olfactory neurons out of the cell cycle. In the mouse, it has been suggested that Neurod4 (also known as Math3) synergises with Neurog2 during cortical development (Mattar et al., 2008); physical interaction between these bHLH proteins has been shown to lead to an increased efficiency in target gene induction. Furthermore, Neurod4 behaves as a Neurog2 target gene in the mouse cortex highlighting similarities between the two systems. However, it is not clear whether Neurog2 and Neurod4 have truly redundant functions in the mouse as, to our knowledge, double Neurog2-Neurod4 loss-of-function studies have not been reported for the cortex. It is also not clear whether Neurog1 and Neurod4 synergise to regulate the transcription of targets in the zebrafish olfactory system. A slight reduction in the number of early-born olfactory neurons is detected in *neurod4* morphant embryos. However, it remains unclear whether this reflects that Neurog1 functions less well in the absence of Neurod4 or that the last early-born neurons cannot develop in the *neurod4* morphant once *neurog1* expression stops in the olfactory placode at 20 hpf.

Our BrdU experiments suggest that early-born olfactory neurons begin to exit the cell cycle between 10 and 12 hpf in wild-type embryos, shortly after the expression of *neurog1* is initiated in the anlage of the zebrafish olfactory placodes. Furthermore, we show that early-born olfactory neurons in *neurog1* mutant embryos initiate their last S-phase after 16 hpf, concomitant with the recovery of expression of *neurod4*. However, virtually all early-born olfactory neurons in both wild-type and *neurog1* mutant embryos are post-mitotic at 24 hpf; the stable number of *Tg(8.4neurog1:gfp)+* neurons in both wild type or mutants after 24 hpf is consistent with this idea. Thus, although the development of these neurons is apparently initiated by the expression of *neurog1* or *neurod4*, even with the sustained expression of *neurod4* in cycling progenitors after 24 hpf early-born olfactory neurons can

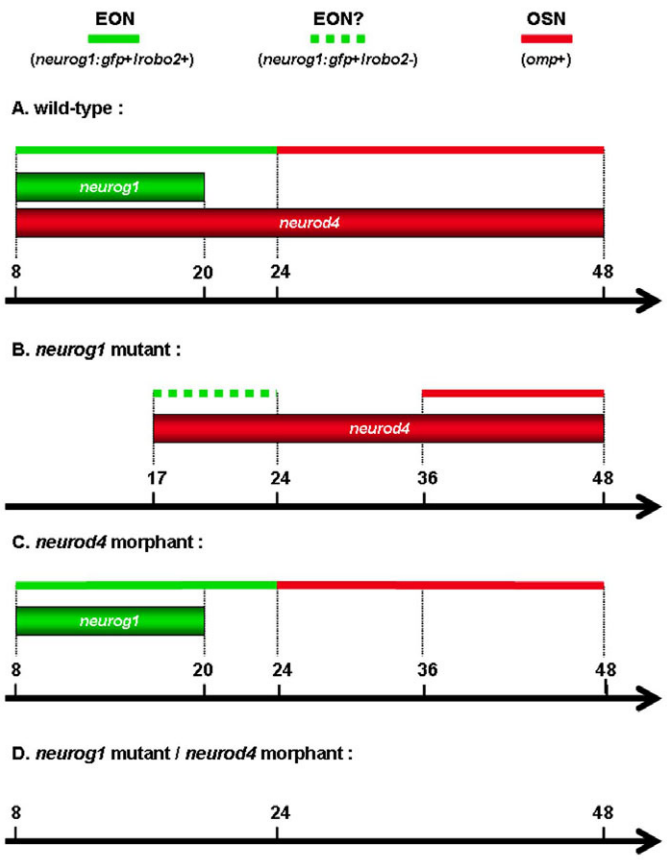

Fig. 9. Schematic of neurogenesis in the zebrafish olfactory placode. (**A-D**) A schematic recapitulation of olfactory neurogenesis in wild-type (A), *neurog1* mutant (B), *neurod4* morphant (C) and *neurog1/neurod4* double loss-of-function (D) embryos. The period of expression of *neurog1* and *neurod4* are shown as thick green and red bars, respectively, and the birth of the different types of olfactory neurons is shown as thin bars. Neurog1 and Neurod4 share redundant activity during the development of early-born olfactory neurons (EON) and olfactory sensory neurons (OSN). Furthermore, in the absence of Neurog1 the complete specification of early-born olfactory neurons is never achieved and the early phase of OSN differentiation is delayed. In double loss of function of *neurog1* and *neurod4*, virtually all early-born olfactory neurons and olfactory sensory neurons are lost in the developing olfactory placode.

no longer be generated. The mechanism that controls the end of the determination phase of early-born olfactory neurons development is not known. One possibility is that from the earliest stages of olfactory neurogenesis there are separate progenitor lineages for early and late neuronal sub-types and the progenitor pool for the early-born neurons is exhausted at 24 hpf. A previous study has shown that distinct progenitors exist for pioneer neurons and olfactory sensory neurons (Whitlock and Westerfield, 2000). However, it is not clear whether this is also the case for the *Tg(8.4neurog1;gfp)*+ neurons and OSNs; although limited, the overlap of zns-2 and *Tg(omp:YFP)* argues against this idea (Miyasaka et al., 2005). Furthermore, even though the development of early-born neurons is delayed in *neurog1* mutants, these neurons also stop being generated at 24 hpf in the mutant, suggesting either that the pool of progenitors is smaller to start with in the absence of Neurog1 function or that parallel mechanisms actively repress the birth of the early-born population after 24 hpf.

Redundancy between transcription factors of the same families is not particularly novel. Indeed, the overlapping roles of the Neurog1 and Neurog2 genes during dorsal root ganglion (DRG) development in the mouse is similar to that of Neurog1 and Neurod4 during olfactory neurogenesis in the zebrafish (Ma et al., 1999) (this study). Indeed, in mouse the early (TrkB+ and TrkC+; Ntrk2 and Ntrk3, respectively – Mouse Genome Informatics) and late (TrkA+; Ntrk1 – Mouse Genome Informatics) waves of DRG neurons resemble the early EON and late OSN waves we describe in the zebrafish olfactory placode. Moreover, the early phase of *Neurog1* expression depends on Neurog2 function in mouse DRGs as does *neurod4* on Neurog1 in the fish olfactory placode. Nonetheless, there are several differences between the two systems. First, during the late wave of DRG neurogenesis only Neurog1 is required for TrkA+ neurons to develop. By contrast, both Neurog1 and Neurod4 are necessary for the correct development of late OSN neurons in the fish. Secondly, whereas the delay in the onset of expression of Neurog1 in Neurog2 mutant mice ultimately has no effect on the number of early TrkB+ and TrkC+ neurons that are born in DRGs (apparently complete redundancy with a delay), development of early olfactory neurons is significantly perturbed in *neurog1* mutants in the fish despite the recovery of *neurod4* expression (only partial redundancy with a delay). A 'delayed neurogenesis' phenotype similar to that highlighted in our study has also been reported during the development of the nucleus of the solitary tract (nTS) and sympathetic ganglia in the *Ascl1* knockout mouse (Pattyn et al., 2006); in these structures, neurons appear with a 24 hour delay in the mutant and accumulate at a slower pace. However, although the authors suggested that the sequential expression of two proneural factors could regulate development of the nTS and sympathetic ganglia, the second proneural factor in this system is unknown (Pattyn et al., 2006).

Although there is a delay in the development of the sympathetic ganglia in the *Ascl1* knockout and TrkB+ and TrkC+ neurons in Neurog2 mutant DRGs in the mouse, this delay is eventually overcome in both systems (Ma et al., 1999; Pattyn et al., 2006). Although this also appears to be the case for the development of *omp*+ OSNs in the zebrafish, we show that in the absence of Neurog1 function, markers such as *robo2* are never expressed by the residual neurons that develop before 24 hpf in the *neurog1* mutant. Furthermore, the failure of the expression of *robo2* to recover correlates with abnormal fasciculation of olfactory axons and their correct organisation into proto-glomeruli in the olfactory bulb, phenotypes previously reported for the *robo2* mutant (Miyasaka et al., 2005). The aberrant projection of olfactory axons in some *neurog1* mutant embryos suggests that the expression of other, as yet unidentified, genes also fails to recover in time to permit correct development; our results with *gef* and *olfm1b* show that *robo2* is not the only gene for which expression fails to recover in residual EONs in the *neurog1* mutant. Thus, we propose that the length of developmental time available to early-born olfactory neurons after proneural gene activity drives them to differentiate is a crucial parameter for the correct acquisition of EON identity and for the development of the zebrafish olfactory system. Understanding whether this reflects a general principle during neurogenesis will require further study.

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Competing interests statement
The authors declare no competing financial interests.

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