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Ash1a and Neurogenin1 function downstream of Floating head to regulate epiphysial neurogenesis

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SUMMARY

The homeodomain transcription factor Floating head (Flh) is required for the generation of neurones in the zebrafish epiphysis. It regulates expression of two basic helix loop helix (bHLH) transcription factor encoding genes, *ash1a* (*achaete/scute homologue 1a*) and *neurogenin1* (*ngn1*), in epiphysial neural progenitors. We show that *ash1a* and *ngn1* function in parallel redundant pathways to regulate neurogenesis downstream of *flh*. Comparison of the epiphysial phenotypes of *flh* mutant and of *ash1a/ngn1* double morphants reveals that reduced expression of *ash1a* and *ngn1* can account for most of the neurogenesis defects in the *flh*-mutant epiphysis but also shows that Flh has additional activities. Furthermore, different cell populations show different requirements for *ash1a* and *ngn1* within the epiphysis. These populations do not simply correspond to the two described epiphysial cell types: photoreceptors and projection neurones. These results suggest that the genetic pathways that involve *ash1a* and *ngn1* are common to both neuronal types.

Key words: Neurogenesis, bHLH transcription factor, *floating head*, Prepattern, Epiphysis, Zebrafish

INTRODUCTION

In order to generate neurones with appropriate identities in the developing CNS, the acquisition of positional identity by neuroepithelial cells must be coupled to the genetic programmes by which neurones are produced. Considerable progress is being made in elucidating the genetic pathways underlying both of these aspects of neuronal development. For example, the establishment of anterior fates within the CNS appears to require the suppression of signals that promote posterior neural identities (Stern, 2001; Kudoh et al., 2002). Subsequent to the initial establishment of anteroposterior (AP) pattern, additional signals that include Wnt and Fgf proteins, act more locally within the CNS to refine AP regionalisation (Rhinn and Brand, 2001; Houart et al., 2002). Similarly, signals that include sonic hedgehog (Shh), and Nodal and bone morphogenetic proteins (Bmps) contribute to the establishment of positional identity along the dorsoventral (DV) axis of the CNS (reviewed by Wilson and Rubenstein, 2000; Jessell, 2000).

With respect to the genetic pathways underlying neurogenesis, the activity of members of a subclass of basic helix loop helix (bHLH) transcription factors is instrumental in most, and perhaps all, vertebrate neuronal lineages (for a review, see Bertrand et al., 2002). These transcription factors are vertebrate homologues of invertebrate proneural proteins, which in flies are both necessary and sufficient for the commitment of ectodermal cells to a neural progenitor fate (for reviews, see Campos-Ortega, 1993; Modolell, 1997). In vertebrates, neural bHLH transcription factor activity is required at several discrete stages during the formation of neurones, and both loss- and gain-of-function data support the notion that bHLH proteins can function both in networks and in cascades in various neuronal lineages (Ma et al., 1996; Kanekar et al., 1997; Fode et al., 1998; Ma et al., 1998; Perron et al., 1999; Cau et al., 2002).

Although both the mechanisms by which neural cells acquire positional identity and the genetic programmes underlying neurogenesis are beginning to be deciphered, it is less clear how these two events are connected. For example, how do patterning molecules that are expressed in discrete CNS areas influence the expression and activity of bHLH transcription factors that are common to many distinct areas of the brain? Furthermore, how is it that within the CNS compartments that are defined by regional cues, it is only a subset of cells that initiate expression of proneural genes? One current hypothesis is that proteins conferring positional identity regulate the expression of so called ‘prepattern genes’, which in turn spatially restrict expression of proneural bHLH transcription factors. Prepattern genes would thus be a link between genes specifying pattern and genes regulating neurogenesis (for reviews, see Ghysen and Dambly-Chaudiere, 1989; Skeath and Carol, 1994; Simpson, 1996). In at least some cases in flies, prepattern genes exhibit additional activities during the specification of neuronal phenotypes. For instance, it is the prepattern genes of the Iroquois complex, and not proneural genes, that are responsible for the acquisition of lateral versus medial identity by

mechanosensory bristles of the notum (Grillenzoni et al., 1998).

Several vertebrate homologues of *Drosophila* prepattern genes have been implicated in the regulation of neurogenesis (Ishibashi et al., 1995; Bellefroid, 1998; Gomez-Skarmeta et al., 1998; Saito et al., 1998; Cau et al., 2000). However, even in cases where such upstream regulators have been identified, it is not clear how much of their activity is mediated by downstream proneural gene targets. To explore the relationship between CNS patterning and neurogenesis, we studied the function of a potential vertebrate prepattern protein: the homeodomain-containing transcription factor Flh (Talbot et al., 1995; Masai et al., 1997). Within the CNS, *flh* expression is localised to the epithalamic region of the dorsal diencephalon. The major nucleus within this region is the epiphysis or pineal organ, a simple photoreceptive structure that has roles both in the detection of light (Foster and Roberts, 1982) and in the regulation of circadian rhythms (for a review, see Natesan et al., 2002). The spatial restriction of *flh* expression to the prospective epiphysis is tightly regulated by both Wnt and Bmp signals. For example, in the *masterblind* (*mbl*) mutant, enhanced Wnt activity in the neural plate leads to expansion of *flh* expression into regions of the anterior forebrain that should normally form telencephalon (Masai et al., 1997; Heisenberg et al., 2001). Similarly, reduced levels of Bmp activity in the *swirl* (*swr*) mutant lead to expansion of *flh* expression into more lateral ectodermal cells (Barth et al., 1999). Together, these studies have led to a simple model by which the anterior and posterior limits of *flh* expression are determined by thresholds of Wnt activity, and the dorsal and ventral limits are determined by thresholds of Bmp activity. With respect to function, genetic studies have shown that Flh is required to mediate epiphysial neurogenesis and to maintain expression of the bHLH transcription factor Ash1a (Asha – Zebrafish Information Network) (Masai et al., 1997). Flh thus has the hallmarks of a vertebrate prepattern gene.

In order to elucidate the pathways regulating epiphysial neurogenesis, we have investigated the regulation of three bHLH transcription factors, Ash1a, Ngn1 (Neurog1 – Zebrafish Information Network) and NeuroD (Neurod – Zebrafish Information Network), which are expressed in the epiphysis. We show that Flh is required to maintain the expression of *ash1a* and to initiate expression of *ngn1* and *neuroD*. Using morpholino antisense oligonucleotides (MOs) (Nasevicius and Ekker, 2001) to impair Ash1a and Ngn1 activity, we demonstrate that these two bHLH proteins are essential regulators of epiphysial neurogenesis. Ash1a and Ngn1 show some degree of redundancy and function downstream of Flh but upstream of *neuroD*. By comparing the epiphysial phenotypes of *flh* mutants and *ash1a*/*ngn1* morphants, we show that although the reduction in *ash1a* and *ngn1* expression can account for most of the neurogenesis defects in the *flh*-mutant epiphysis, Flh is unlikely to function solely as a regulator of *ash1a* and of *ngn1*. We also show that impairment of Ash1a or Ash1a and Ngn1 activity affects both epiphysial photoreceptors and projection neurones, suggesting that these genes are not involved in the fate choice between these two neuronal cell types. Our results confirm that Flh functions as a prepattern gene, linking patterning to neurogenesis, and reveal a crucial role for two bHLH proteins, acting downstream of Flh, in the control of epiphysial neurogenesis.

MATERIALS AND METHODS

Zebrafish lines

Fish heterozygous for the *flh*^{n1}-null allele (Talbot et al., 1995) were intercrossed to generate homozygous embryos, which were identified by the reduction of axial mesoderm. Fish heterozygous for the *ngn1* mutation (Golling et al., 2002) were raised in the laboratory of Dr Uwe Strähle (Strasbourg, France) and crossed to obtain homozygous embryos that were identified based on their reduced production of sensory neurones in the spinal cord.

Morpholino antisense oligonucleotides (MOs)

MOs (Gene Tools) were designed against *ash1a* (GenBank Accession Number U14587) (Allende and Weinberg, 1994) and *ngn1* (GenBank Accession Number AF017301) (Blader et al., 1997):

ash1a MO (complementing bases 121-145), 5′-ATCTTGGCGGT-GATGTCCATTTCGC-3′;

ash1a$^{5'UTR}$ MO (complementing bases 83-107), 5′-AAGGAGT-GAGTCAAAGCACTAAAGT-3′; and

ngn1 MO (complementing bases 222-246), 5′-TATACGATCTC-CATTGTTGATAACC-3′. [This MO has been used in previous studies (Cornell and Eisen, 2002; Andermann et al., 2002).]

MOs were diluted in Danieau's media (Nasevicius and Ekker, 2000) and were routinely injected at the one- to four-cell stages at concentrations of 2 mg/ml (*ash1a* MO) and 2.5 mg/ml (*ash1a*$^{5'UTR}$ MO, *ngn1* MO). The injection volume varied between 2 and 4 nl depending on the MO injected. Injection of two MOs were performed either sequentially or by using a mixture of the two MOs at their normal usage concentrations. Co-injection of *ash1a* MO and *ash1a*$^{5'UTR}$ MO was performed either with *ash1a* MO at 1 mg/ml and *ash1a*$^{5'UTR}$ MO at 1.25 mg/ml, or with *ash1a* MO at 2 mg/ml and *ash1a*$^{5'UTR}$ MO at 2.5 mg/ml. Similar results were obtained in these two sets of experiments.

To control the specificity of *ash1a* MO, we generated two different constructs: *ash1a::gfp* and *mutash1a::gfp*. The *ash1a::gfp* construct contained part of the *ash1a* gene (from base 115 to 393), which included the *ash1a* target sequence (see above), fused in frame with the *gfp* coding sequence. The *mutash1a::gfp* construct was identical to *ash1a::gfp* except for four single base mutations inside of the *ash1a* MO target sequence (**C**CGA**T**ATG**C**A**G**ATCACCGCCAAGAT). Embryos injected with RNA from either construct showed a bright green fluorescence owing to the expression of GFP (41 out of 45 embryos for *ash1a::gfp* and 38 out of 40 for *mutash1a::gfp*). The vast majority of embryos injected with both *ash1a::gfp* RNA and *ash1a* MO showed no fluorescence (41 out of 44 embryos). By contrast, most of the embryos injected with both *mutash1a::gfp* RNA and *ash1a* MO were fluorescent (37 out of 41).

RNA in situ hybridisation and immunohistochemistry

In situ hybridisation and immunohistochemistry were performed using standard procedures (Masai et al., 1997). Details of the probes are available upon request. The opsin antibody was a gift from P. Hargrave.

RESULTS

Flh regulates the expression of *ash1a*, *ngn1* and *neuroD* in different populations of epiphysial cells

The homeodomain transcription factor Flh is necessary for the production of neurones in the epiphysis. In *flh* mutants, the first epiphysial neurones are produced but after 18-somite stage (s) neuronal production stops. In the absence of Flh function, expression of *ash1a* is not maintained, raising the possibility that Flh is an activator of *ash1a* and that loss of Ash1a activity

might account for the defects in epiphysial neurogenesis (Masai et al., 1997). However, as bHLH proteins frequently operate in networks or cascades to promote production of neurones, we analysed if other bHLH protein encoding genes are also expressed in the epiphysis. Both *ngn1* (Blader et al., 1997) and *neuroD* (Korzh et al., 1998; Mueller and Wulliman, 2002a; Mueller and Wulliman, 2002b) are expressed in the epiphysis and could therefore contribute to the regulation of neurogenesis in this region.

ash1a is expressed in the diencephalic territory, that includes the presumptive epiphysis, as early as 6s (Masai et al., 1997) and intense localised epiphysial expression is observed from 8s onwards (Fig. 1A,D,G,J). Epiphysial expression of *ngn1* is detected from 12s onwards, with low levels of transcripts in a few cells in the posterior part of the epiphysis (Fig. 1B,E,H,K). *ngn1* is thus expressed later and in a more restricted posterior domain of the epiphysis than *ash1a*. *neuroD* is first expressed in a few epiphysial cells at around 18s (Fig. 1C,F,I), the same stage as the appearance of the first post-mitotic neurones (Masai et al., 1997) (data not shown). This is consistent with the observation that *neuroD* is usually expressed in newly born neurones (Lee et al., 1995; Cau et 1997; Korzh et al., 1998; Mueller and Wulliman, 2002a; Mueller and Wulliman, 2002b).

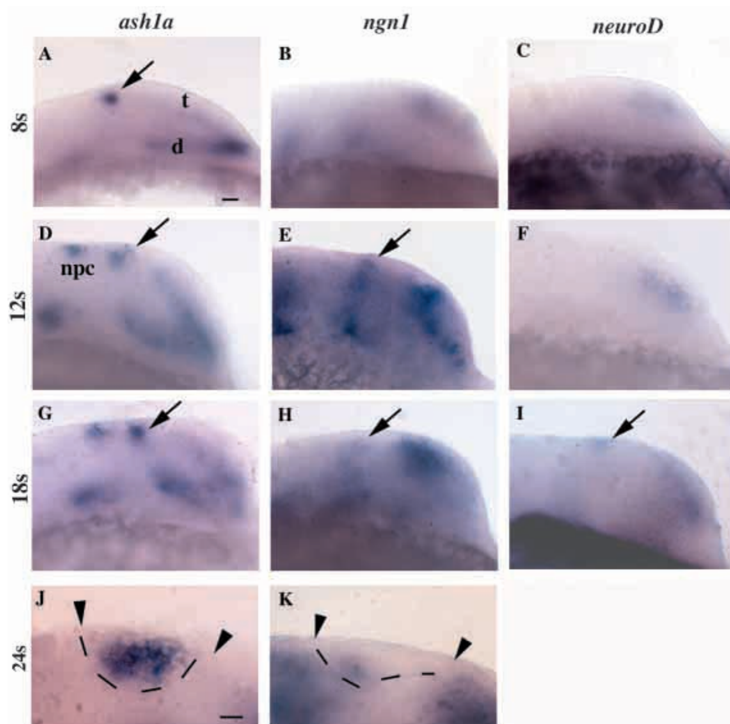

Fig. 1. *ash1a*, *ngn1* and *neuroD* are expressed in spatially and temporally different populations of cells in the epiphysis. Lateral views of whole brains with anterior to the right, showing expression of *ash1a*, *ngn1* and *neuroD* at 8-, 12-, 18- and 24-somite stages. Stage is indicated on the left and probe above. Arrows indicate the location of the epiphysis and arrowheads indicate the anterior and posterior limits of the epiphysis, which is delineated by the dashed lines (J,K). *ash1a* was expressed both earlier and more broadly than *ngn1* in the presumptive epiphysis, whereas *neuroD* was expressed later than both *ash1a* and *ngn1*. npc, nucleus of the posterior commissure; t, telencephalon; d, diencephalon. Scale bar: in A, 50 μm for A-I; in J, 10 μm for J,K.

To elucidate the role of Flh in the regulation of these bHLH transcription factors, we examined their expression in the epiphysis of *flh* mutants. Expression of *ash1a* was initially normal in the *flh*-mutant epiphysis (Fig. 2A,B) (Masai et al., 1997), but by 24s, the number of *ash1a*-expressing epiphysial cells was reduced to about 10 in mutants compared with 15-20 in wild type (Fig. 2C,D). Expression of *ash1a* continued to decrease such that by 24 hpf, transcripts were absent from the *flh*-mutant epiphysis (Masai et al., 1997) (data not shown). At 14s, a few *ngn1*-positive cells were detected in the wild-type epiphysis, whereas most *flh* mutants showed no expression (Fig. 2E-F). By 22-24s, 10-15 epiphysial cells expressed *ngn1* in wild-type embryos whereas only one to two ventrally located neuroepithelial cells expressed *ngn1* in *flh*-mutant embryos (Fig. 2G,H); by 26s epiphysial *ngn1* expression was absent (data not shown). *neuroD* expression was absent in the *flh*-mutant epiphysis at both 14 and 24s, although one or two *neuroD*-positive cells were usually detected by 30 hpf (Fig. 2I-L; data not shown).

These results suggest that *ash1a*, *ngn1* and *neuroD* are expressed in spatially and temporally different populations of neural progenitors, and that correct expression of all three genes depends upon Flh activity.

Ash1a and Ngn1 are required for the production of neurones in the epiphysis

As *ash1a* and *ngn1* are expressed early during epiphysial neurogenesis, we speculated that they could have an important role during the formation of neural progenitors in this structure. In order to test this hypothesis, we used MOs to abrogate the activity of Ash1a or Ngn1 proteins, or both.

Injection of an MO encompassing the start site of *ash1a*-coding sequence (*ash1a* MO) drastically impaired the expression of *islet1* (*isl1*) in the dorsal hypothalamus and adenohypophysis (in 84.6% embryos, *n*=91; Fig. 3G-H), which are both sites of strong *ash1a* expression (data not shown). Neuronal production in regions that do not express *ash1a*, for example in the cranial ganglia, appeared to be unaffected (Fig. 3G-H; see Materials and Methods for further controls). To determine whether *ash1a* is important for the production of epiphysial neurones, we compared the expression of *isl1* in the epiphysis of normal embryos and *ash1a* morphants, and counted the number of *isl1*-positive cells in a few representative embryos. Injection of *ash1a* MO led to a modest but reproducible reduction in the number of neurones produced in the epiphysis (Fig. 3A,B; Table 1). A second non-overlapping MO designed against the 5′UTR of *ash1a* ($ash1a^{5'UTR}$ MO) gave a similar phenotype (Fig. 3A-C), albeit at a lower frequency (54.7%, *n*=53). Co-injection of the two morpholinos (*ash1a* MO and $ash1a^{5'UTR}$ MO) gave a similar phenotype in 70% of the cases (*n*=77; Table 1; Fig. 3A-C,E).

Injection of an MO directed against the sequence at the *ngn1* start site (*ngn1* MO) impaired neurogenesis in olfactory, cranial and lateral line placodes, as well as impairing the formation of Rohon Beard and dorsal root ganglia neurones (Cornell and Eisen, 2002; Andermann et al., 2002) (E.C. and S.W.W., unpublished) in 82% of the embryos (*n*=39). However, this MO did not induce any

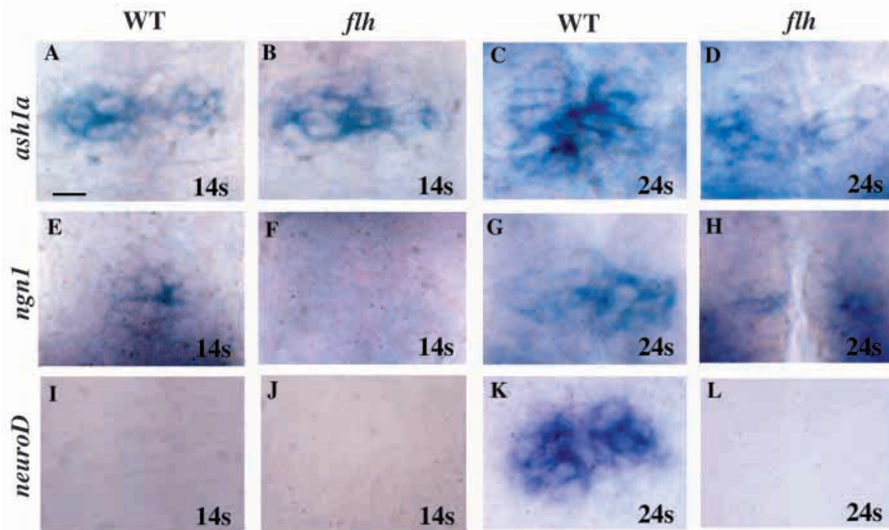

Fig. 2. *flh* regulates the expression of *ash1a*, *ngn1* and *neuroD* in the epiphysis. Dorsal views of whole brains with anterior at the top, showing expression of *ash1a*, *ngn1* and *neuroD* in the epiphysis of wild-type (WT) and *flh*-mutant embryos at 14- and 24-somite stages. Genotype is indicated above, genes analysed on the left of the panels, and stage in the bottom right of each image. Flh is required for the maintenance of *ash1a* expression (D) and for the activation of *ngn1* and of *neuroD* expression (F,J). Scale bar: 15 μm.

significant change in the numbers of epiphysial neurones (Fig. 3A,D; Table 1). To confirm that the loss of Ngn1 function does not alter epiphysial neurogenesis, we assessed embryos homozygous for an insertion in the *ngn1* gene that is likely to remove all Ngn1 activity (Golling et al., 2002). Similar to the morphants, $ngn1^{-/-}$ embryos exhibited a very strong reduction of sensory neurones and cranial ganglia neurones, but had the same number of epiphysial *isl1*-positive cells as their wild-type siblings (*n*=10; Table 1; data not shown). This confirms that loss of Ngn1 function alone does not significantly alter the production of neurones in the epiphysis.

The relatively mild phenotype observed in the epiphysis of *ash1a* morphants, and the absence of a detectable phenotype in the epiphysis of *ngn1* morphants and mutants, led us to analyse the possibility of genetic compensation occurring between *ash1a* and *ngn1*.

We first analysed possible cross-regulation between *ash1a* and *ngn1*. At 20s, *ngn1* was expressed in ~15 neuroepithelial epiphysial cells in both wild-type and *ash1a* MO-injected embryos (Fig. 3I,J). Likewise, at 17s, *ash1a* was expressed in ~10 neuroepithelial epiphysial cells in both wild-type and *ngn1* MO-injected embryos (Fig. 3K,L). Indeed, we did not detect any obvious difference in the expression of *ash1a* in *ngn1* MO-injected embryos at all stages examined (data not shown).

In contrast to the mild epiphysial phenotype in *ash1a* morphants, and to the absence of a detectable epiphysial phenotype in *ngn1* morphants, reducing the activity of both Ash1a and Ngn1 strongly impaired neuronal differentiation. Eighty percent of the double MO-injected embryos (*n*=30) showed both the '*ash1a* phenotype' (impairment in the production of *isl1*-positive cells in the hypothalamus

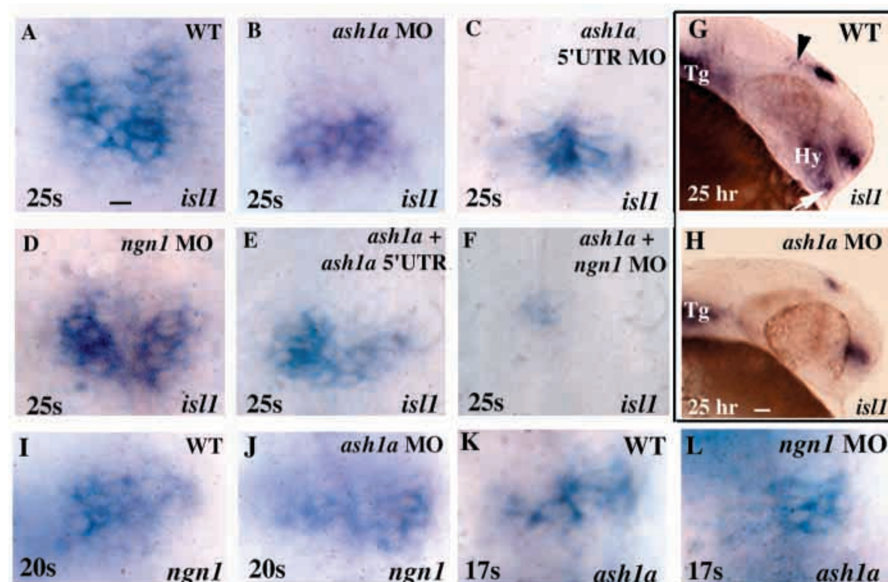

Fig. 3. *ash1a* and *ngn1* are important regulators of neurogenesis in the epiphysis. A-F and I-L are dorsal views of the epiphysis with anterior at the top. G and H are lateral views of the brain with anterior to the right. (A-F) Expression of *isl1* at the 25-somite stage in the epiphysis of wild-type (WT), *ash1a* MO-, $ash1a^{5'UTR}$ MO-, *ngn1*MO-, *ash1a* and $ash1a^{5'UTR}$MO-, or *ash1a* and *ngn1* MO-injected embryos. Neuronal production was reduced in *ash1a* morphants (B,C,E) but was normal in the *ngn1* morphant (D). A stronger effect was observed in the double *ash1a/ngn1* morphant (F) compared with single *ash1a* morphants (B,C,E). A combination of $ash1a^{5'UTR}$ MO and *ash1a* MO gave rise to a similar phenotype (E) to the single *ash1a* or $ash1a^{5'UTR}$ morphants (B,C). (G,H) Expression of *isl1* at 25 hours in the heads of wild-type and *ash1a*-morphant embryos. The black arrowhead indicates the nucleus of the posterior commissure and the white arrow indicates the adenohypophysis (G); both are sites where *isl1* expression is disrupted in the *ash1a* morphant (H). A reduction of the number of neurones was observed in the epiphysis of the *ash1a* morphant (H). By contrast, structures in which *ash1a* is not expressed, like the trigeminal ganglia, are not affected in the *ash1a* morphant. (I,J) Expression of *ngn1* in wild-type and *ash1a*-morphant embryos at the 20s stage. (K,L) Expression of *ash1a* in wild-type and *ngn1*-morphant embryos at the 17s stage. Hy, hypothalamus; Tg, trigeminal ganglia. Scale bars: in A, 10 μm for A- F,I-L; in H, 50 μm for G,H.

Table 1. Effects of *ash1a* and *ngn1* MOs on the expression of *flh, islet1* and *neuroD*

'Genotype'	*flh* (24 hours)	*neuroD* (27 hours)	*islet1* (27 hours)	*islet1* (25s)
	Number of cells positive for each probe			
WT	23.3±2.3 (*n*=3)	23.7±4.7 (*n*=3)	41.3±2.9 (*n*=6)	34.3±2.08 (*n*=3)
ash1a MO	29.0±6.1 (*n*=3)	16.7±1.7 (*n*=3)	24.1±8.9 (*n*=6)	19.3±3.78 (*n*=6)
ash1a$^{5'UTR}$ MO	nd	nd	24.6±5.4 (*n*=6)	19.0±1.0 (*n*=3)
ash1a and *ash1a*$^{5'UTR}$ MO	nd	nd	nd	18.83±5.03 (*n*=6)
ngn1 MO	26.7±2.1 (*n*=3)	21±3.5 (*n*=3)	36.7±7.5 (*n*=3)	29.7±1.53 (*n*=3)
ash1a and *ngn1* MOs	26.3±2.05 (*n*=3)	1.0±1.7 (*n*=3)	0.0±0.0 (*n*=3)	1.2±1.79 (*n*=6)
ngn1$^{-/-}$	nd	nd	nd	29.7±3.05 (*n*=3)
ngn1$^{-/-}$ and *ash1a* MO	nd	nd	nd	6.25±4.35 (*n*=3)

Numbers indicate mean number of cells±s.d. in preparations viewed at high magnification.
n, number of embryos scored.
nd, not determined.

and the adenohypophysis) and the '*ngn1* phenotype' (loss of cranial ganglia and primary sensory neurones). These embryos also exhibited severely reduced or absent epiphysial *isl1* expression at 25s (Fig. 3A,F; Table 1). However, some *isl1*-positive cells are still produced in the pancreas and ventral neural tube of double morphant embryos. The double morphant phenotype is thus specific to restricted domains of the nervous system. In addition, we observed a similar phenotype in *ngn1*$^{-/-}$ embryos injected with *ash1a* MO (six out of eight *ngn1*$^{-/-}$ embryos; Table 1; data not shown).

Altogether, these results suggest that *ash1a* and *ngn1* are expressed largely, or completely, independently of each other in the epiphysis and, together, play important and partially redundant functions during the production of neurones in this structure.

ash1a and *ngn1* function downstream of *flh* but upstream of *neuroD*

To determine if epiphysial cells are still present when Ash1a and Ngn1 activities are reduced, we analysed *flh* expression in *ash1a*, *ngn1* and *ash1a/ngn1* morphants. In all morphants, both the number and the organisation of *flh*-postitive cells were similar to non-injected embryos (Fig. 4A-D; Table 1).

As *ash1a* and *ngn1* are expressed before *neuroD* in the epiphysis, we analysed whether reducing Ash1a and Ngn1 function affects the expression of *neuroD*. Injection of *ash1a* MO led to a reduction in the number of *neuroD*-positive epiphysial cells (Fig. 4E-F; Table 1). Furthermore, co-injection of *ash1a* and *ngn1* MOs led to a severe reduction or absence of epiphysial *neuroD* expression (Fig. 4E-H; Table 1). Expression of *isl1* was similarly reduced/absent in the double morphant embryos (Table 1; data not shown).

Together, these data suggest that Ash1a and Ngn1 function downstream of *flh* but upstream of *neuroD*, which suggests that these bHLH proteins are not required for the establishment of an epiphysial territory but rather for the production of neurones within this territory.

Ash1a and Ngn1 are redundantly required for the expression of Delta and *otx5* genes

Ash and Ngn genes function as proneural (or neural determination) genes in a number of neuronal lineages (Cau et al., 1997; Fode et al., 1998; Ma et al., 1998; Casarosa et al., 1999). However, in the murine olfactory epithelium, *Mash1* (the closest known murine *ash1a* homologue; *Ascl1* – Mouse Genome Informatics) functions as a neural determination gene upstream of *Ngn1,* which functions as a differentiation gene (Cau et al., 1997; Cau et al., 2002). To investigate how *ash1a* and *ngn1* function during neural determination/differentiation in the epiphysis, we analysed how they regulate the expression of potential regulators of neurogenesis.

In both fly and vertebrates, neurogenesis involves the selection of neural progenitors through activation of the Notch signalling pathway. Neural determination genes initiate this process through the activation of expression of Delta genes that encode ligands for Notch receptors (Kunisch et al., 1994; Fode et al., 1998; Ma et al., 1998; Casarosa et al., 1999; Cornell and Eisen, 2002). We therefore analysed the expression of zebrafish *deltaA*, *deltaB* and *deltaD* genes (Haddon et al., 1998a) in the epiphysis of normal and MO-injected embryos.

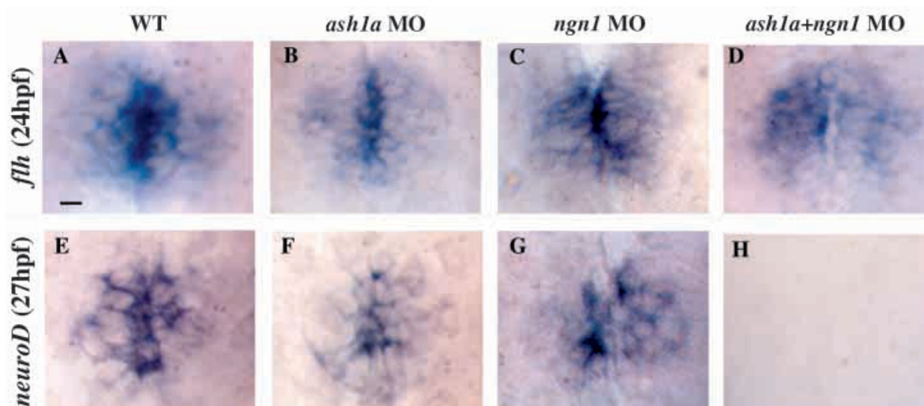

Fig. 4. Ash1a and Ngn1 function downstream of Flh and upstream of *neuroD*. Dorsal views of brains with anterior at the top, showing expression of *flh* and *neuroD* in wild-type (WT), *ash1a, ngn1* and *ash1a/ngn1*-double morphant embryos. Probe and stage are indicated on the left, 'genotype' above. Expression of *flh* was unaffected following the impairment of either or both Ash1a and Ngn1 function (B-D). By contrast, expression of *neuroD* was affected by the reduction of Ash1a (F) or of both Ash1a and Ngn1 (H). Scale bar: 10 μm.

In wild type, very weak expression of *deltaA* and *deltaD* was detected in ~20 epiphysial cells at 13-14s, with a few cells expressing the genes more strongly (Fig. 5A,G). *deltaA* and *deltaD* expression was absent at early stages in *ash1a* MO-injected embryos (Fig. 5C,I), but by 23-24s a few *deltaA*-positive and *deltaD*-positive cells were detectable (Fig. 5K,S). By contrast, expression of *deltaA* and *deltaD* remained absent in most of the *ash1a*-positive, *ngn1* MO-injected embryos (Fig. 5M,U). Similarly, the early expression of *deltaB* in bilateral clusters of one to two cells was severely reduced or absent in 13-14s *ash1a* morphants (Fig. 5D,F). By 23-24s, reduced *deltaB* expression was detected in *ash1a* morphants, but expression remained absent in *ash1a/ngn1* double morphants (Fig. 5N,O,Q).

In *ngn1* MO-injected embryos, expression of *deltaA*, *deltaB* and *deltaD* was normal in the epiphysis at 23-24s (Fig. 5J,L,N,P,R,T), whereas it was reduced/absent in other areas where Ngn1 is required for neurogenesis (cranial ganglia and dorsal spinal cord) (Cornell et al., 2002) (data not shown).

The homeodomain transcription factor Otx5 is required for the expression of several circadian genes by epiphysial cells (Gamse et al., 2002). To compare the functions of Ash1a and Ngn1 further, we analysed the expression of *otx5* in wild-type and MO-injected embryos. By 22-24s, epiphysial *otx5* expression was reduced in *ash1a* morphants but unaffected in *ngn1*-morphants (Fig. 6A-C). *otx5* expression was further reduced or absent in embryos injected with both *ash1a* and *ngn1* MOs (Fig. 6D).

These results demonstrate that *ash1a* and *ngn1* play partially redundant roles in the regulation of *otx5* and Delta gene expression. Together with the observation that *ash1a* and *ngn1* are expressed largely, or completely, independently of each other in the epiphysis, this analysis suggests that *ash1a* and *ngn1* function at the same level, rather than in a cascade, during epiphysial neurogenesis.

Flh regulates aspects of epiphysial development independent of Ash1a and Ngn1

Our data suggest that the defects in neurogenesis in *flh*$^{-/-}$ embryos can be explained by the loss of activity of Ash1a and Ngn1 in the mutants. To address whether Flh is likely to regulate other aspects of epiphysial development through pathways independent of Ash1a and Ngn1, we compared regulation of epiphysial gene expression in *flh* mutants and *ash1a/ngn1* double morphants.

At 13-14s, the expression of *deltaA* and *deltaD* was very severely reduced in the *flh*-mutant epiphysis, whereas the expression of *deltaB* was normal (Fig. 5A,B,D,E,G,H). In addition, expression of *otx5* was not detected in the *flh*-mutant epiphysis at 24s (Fig. 6A,E), nor before this stage (data not shown). By contrast, some expression of *otx5* was observed in the epiphysis of *flh* mutants at later stages (Gamse et al., 2002) (E.C. and S.W.W., unpublished).

ash1a is expressed normally in the *flh*-mutant epiphysis until the 14s stage, and continues to be expressed later, albeit at reduced levels (Fig. 2A-D) (Masai et al., 1997). If the only function of Flh was to maintain or activate the expression of *ash1a* and *ngn1*, we would have expected some initially normal expression of *otx5*, *deltaA* and *deltaD* in *flh* mutants. As this is not the case, it suggests that Flh could play a role in the regulation of Delta genes and *otx5*, in addition to its role in the maintenance of *ash1a* expression and the activation of *ngn1*.

There are further differences between *flh* mutants and *ash1a/ngn1* double morphants. *flh* expression is mainly

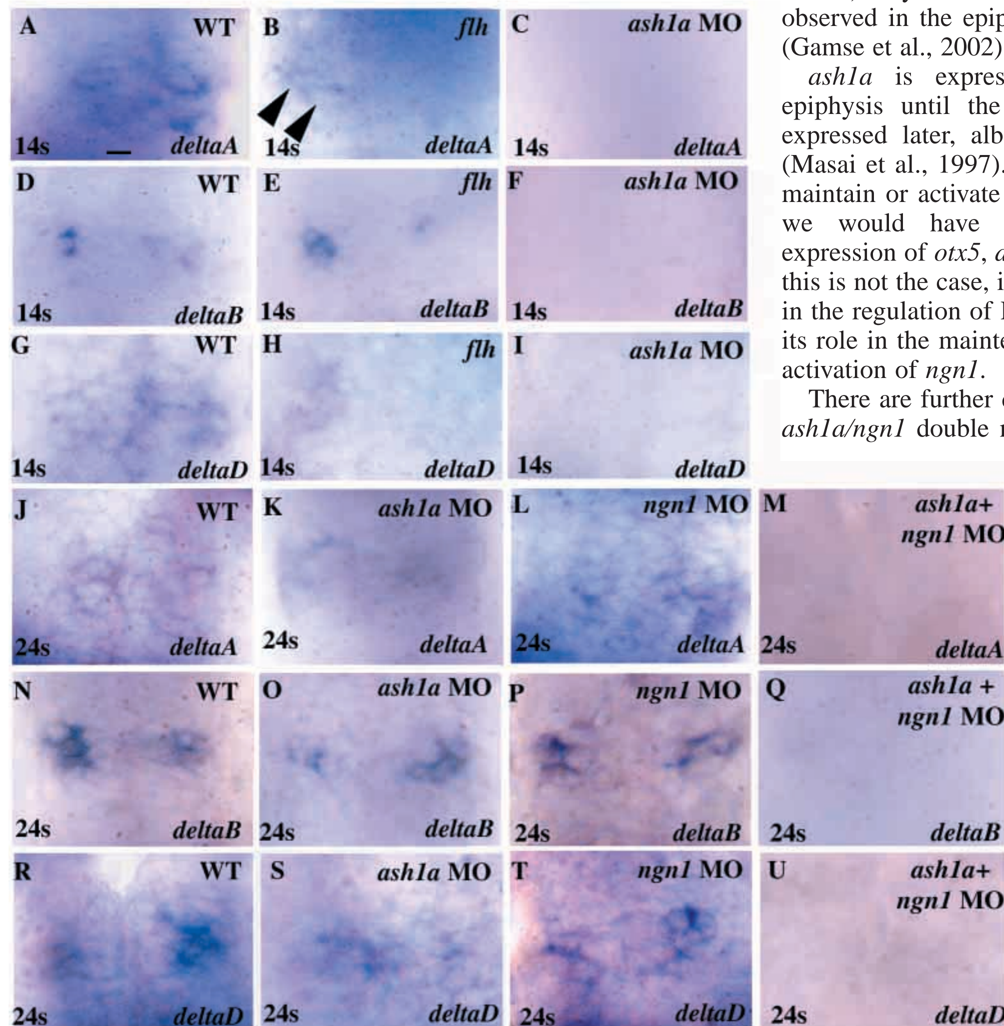

Fig. 5. Ash1a and Ngn1 regulate the expression of Notch ligands. Dorsal views of brains showing expression of *deltaA*, *deltaB* and *deltaD* in wild-type (WT), *flh*$^{-/-}$, and single *ash1a, ngn1* or double morphants. Probes used are indicated bottom right, stage bottom left, and 'genotype' at the top right of each panel. Arrowheads in B indicate two faint *deltaA*-positive cells. Expression of all three Delta genes was affected in the *ash1a* morphants (C,F,I,K,O,S), and more severely reduced or completely absent in the *ash1a/ngn1*-double morphants (M,Q,U). Scale bar: 10 μm.

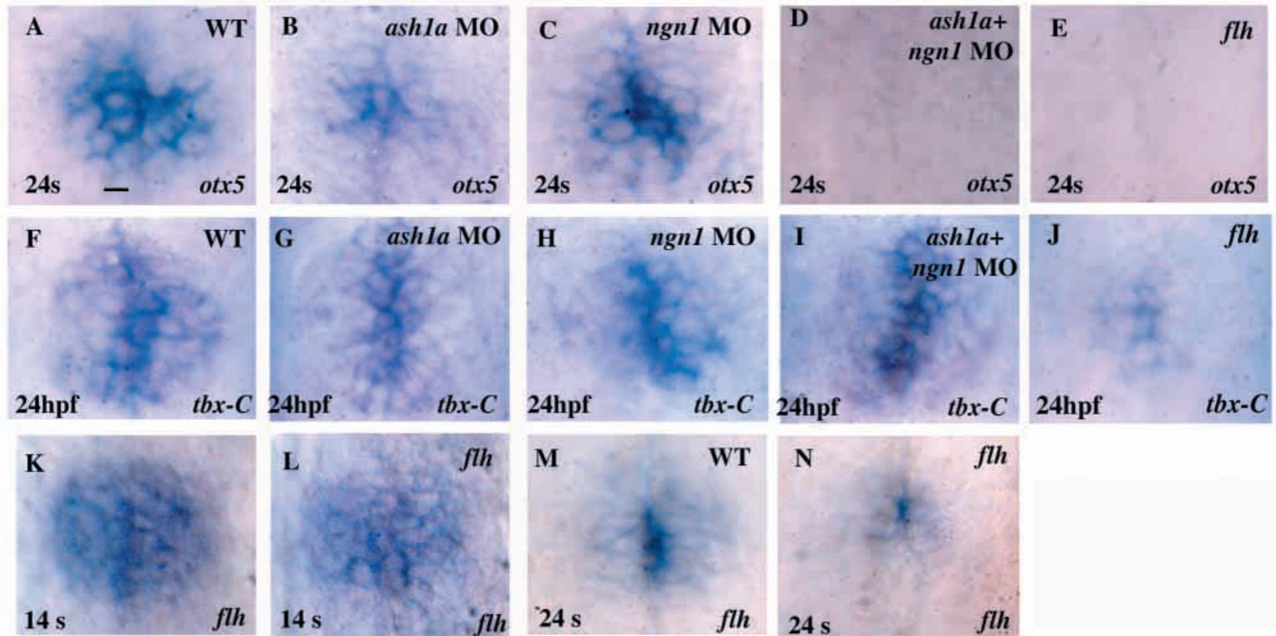

Fig. 6. Flh regulates aspects of epiphysial development independent of Ash1a and Ngn1. Dorsal views of brain showing expression of *otx5, tbxC* and *flh* in wild-type (WT), *flh,* and single *ash1a, ngn1* or double morphants. Probes used are indicated on the bottom right, stage on the bottom left and 'genotype' at the top right of each panel. Ash1a and Ngn1 are implicated in the regulation of *otx5* (B,D) but not of *tbxC* (G,I), whereas Flh is required for the expression of both genes (E,J). In addition, Flh is involved in the regulation of its own expression (N). Scale bar: 10 μm.

independent of *ash1a* and *ngn1* (Fig. 4A-D) whereas, by contrast, although *flh* expression is initiated normally in the *flh* mutant, it is greatly reduced by 24s (Fig. 6K-N) and absent by 24 hours (data not shown), which suggests that Flh regulates *flh* expression independently of Ash1a and Ngn1 activity.

The T-box transcription factor TbxC is proposed to be a potential effector of Flh during notochord development (Dheen et al., 1999). Flh also functions upstream of *tbxC* (*tbx2c* – Zebrafish Information Network) in the epiphysis. At 24 hpf, the *flh*-mutant epiphysis contains around five to eight *tbxC*-positive cells whereas the wild-type epiphysis contains ~40 *tbxC*-positive cells (Fig. 6F,J). In addition, no *tbxC* expression is observed in the *flh*-mutant epiphysis at the 14s stage (data not shown). By contrast, impairment of *ash1a* and *ngn1* function does not obviously affect the expression of *tbxC* (Fig. 6F-I). Altogether, these results suggest a role for Flh in the regulation of *otx5*, *deltaA*, *deltaD*, *flh* and *tbxC* that is independent of Ash1a and Ngn1 activity.

Reducing the activity of *ash1a* and *ngn1* affects both projection neurones and photoreceptors

Two different neuronal types have been described in the zebrafish epiphysis (Masai et al., 1997). Projection neurones are laterally located cells that appear to express the homeodomain transcription factor encoding gene *onecut* (Masai et al., 1997; Hong et al., 2002) (E.C. and S.W.W., unpublished). Photoreceptors are medially located cells that express the photoreceptive molecule Opsin (Masai et al.,

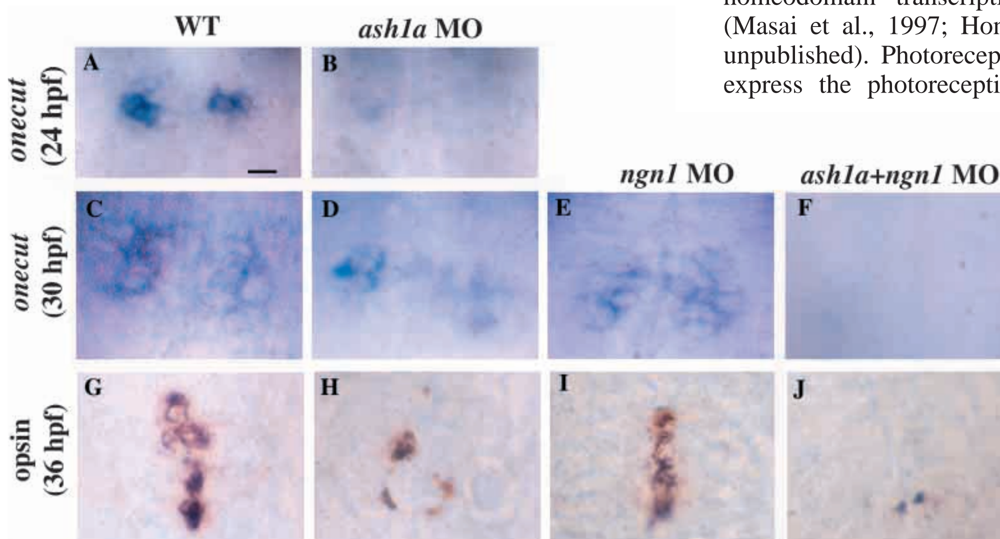

Fig. 7. Impairment of Ash1a and of Ngn1 activity affects both photoreceptors and projection neurones. Dorsal views of brains showing expression of *onecut* and opsin in wild-type (WT) and single *ash1a, ngn1* or double-morphant embryos. Probe and stage are indicated on the left and 'genotype' above. Both markers were affected following the impairment of Ash1a (B,D,H) or of both Ash1a and Ngn1 activity (F,J). Scale bar: 15 μm.

1997). In order to determine whether one or both cell types were affected by reduction of *ash1a* and *ngn1* activity, we analysed the expression of *opsin* and *onecut* in morphants.

At 24 hours, *onecut* transcripts were detected in around three to four cells on each side of the epiphysis. This expression was strongly reduced in *ash1a* MO-injected embryos (Fig. 7A,B). At 30 hours, the lateral clusters of *onecut* expression have reached a size of four to five cells in wild type, whereas in *ash1*a MO-injected embryos the clusters contained only one to two *onecut*-positive cells (Fig. 7C,D). Injection of *ngn1* MO impaired the expression of *onecut* in cranial ganglia but not in the epiphysis at all stages examined (Fig. 7C,E; data not shown). Double MO-injected embryos showed no *onecut* staining at 30 hours (Fig. 7F).

At 36 hours, expression of Opsin was detected in the outer segments of the photoreceptor cells (Fig. 7G). *ngn1* MO-injected embryos showed normal Opsin staining (Fig. 7G,I). By contrast, injection of *ash1a* MO reduced the quantity of Opsin-positive cells and disrupted their organisation (Fig. 7G,H). The expression of Opsin was even more severely reduced, or was completely absent, in double MO-injected embryos (Fig. 7G,J; data not shown).

Reducing Ash1a or both Ash1a and Ngn1 activity affected both photoreceptors and projection neurones, which suggests that *ash1a* and *ngn1* are not involved in the decision to make one versus the other cell type.

ash1a- and *ngn1*-dependent neurones have different locations along the AP axis of the epiphysis

The results described above demonstrate the existence of two distinct populations of neurones: one that depends only on the function of *ash1a*, and one that depends on the redundant functions of *ash1a* and *ngn1*. As these populations do not simply correspond to the two main neuronal types produced in the epiphysis, we looked at their distribution along the AP and DV axes of the epiphysial vesicle.

At 24 hours of development, the wild-type epiphysis contained 25-30 neurones, as judged by *isl1* expression. By contrast, in *ash1a* MO-injected embryos, only 15-20 neurones were produced in the epiphysis (Fig. 8A,B,E,F). These neurones will be referred to as the *ash1a*-independent lineage. Although less neurones were present, the density of expression of *isl1* was normal in *ash1a* MO-injected embryos, but the group of neurones was shorter along the AP axis of the vesicle. Moreover, the *ash1a*-independent neurones were always located posteriorly in the epiphysis, which is the domain in which *ngn1* is expressed (Fig. 8F and Fig. 1K). Similarly, by 24 hpf, about five to eight neurones were produced in the absence of *flh* function (referred to as *flh*-independent lineage; Fig. 8C,G) (Masai et al., 1997). To determine whether the *flh*-independent neurones require *ash1a*, we injected *ash1a* MO into the progeny of crosses between carriers of the *flh* mutation. *flh*-mutant embryos that have reduced *ash1a* activity showed no epiphysial neurones as judged by *isl1* expression (Fig. 8D). This suggests that the *flh*-independent lineage is dependent upon *ash1a*.

We can thus define three different populations in the epiphysis based on their requirement for *flh*, *ash1a* and *ngn1*: (1) a population of *flh*-independent, *ash1a*-dependent neurones; (2) a population of posteriorly positioned neurones that is *flh* dependent and depends on the redundant function of *ash1a* and *ngn1*; and (3) an anterior population that requires *flh* and *ash1a* but not *ngn1*.

DISCUSSION

In this paper, we compare the functions of Flh with two of its downstream targets, *ash1a* and *ngn1*, during epiphysial neurogenesis. In the absence of Flh function, expression of *ash1a* and *ngn1* is impaired, and the production of epiphysial neurones is severely compromised. We demonstrate that Ash1a and Ngn1 are essential regulators of neurogenesis that function downstream of Flh and upstream of *neuroD*. Although the reduced activity of Ash1a and Ngn1 is likely to be the primary cause of the neurogenesis defects in the *flh*-mutant epiphysis, Flh has activities in addition to the regulation of these genes.

Flh functions as a prepattern gene

Prepattern genes are defined by their ability to link positional identity to neurogenesis. Their expression is regulated by signals that establish positional identity and their targets include neural determination genes (Ghysen and Dambly-Chaudiere, 1989; Skeath and Caroll, 1994; Simpson, 1996). *flh* fulfils these criteria in that its expression is regulated by signalling pathways that mediate positional identity within the nervous system (Masai et al., 1997; Barth et al., 1999; Heisenberg et al., 2001), and its regulatory targets include genes encoding proneural bHLH proteins. Flh is an essential upstream regulator of the neural determination gene *ngn1,* and

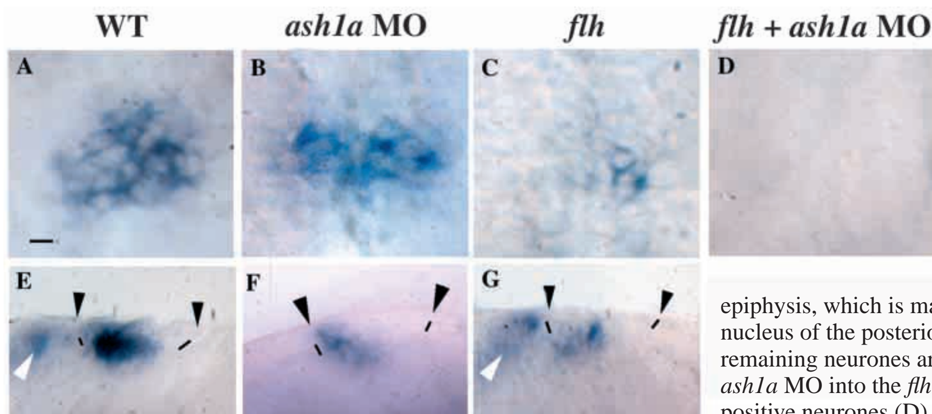

Fig. 8. Spatially distinct populations of epiphysial neurones show different requirements for *flh*, *ash1a* and *ngn1*. Dorsal (A-D) and lateral (E-G) views of brains with anterior at the top (A-D) or to the right (E-G), showing expression of *isl1* at 24 hours in wild-type (WT), *flh*-mutant and *ash1a*-morphant embryos, and a *flh* mutant injected with *ash1a* MO. In E-G, black arrowheads indicate the limits of the epiphysis, which is marked by a line; white arrowheads indicate the nucleus of the posterior commissure. In the *ash1a* morphant, remaining neurones are located posteriorly (F). The injection of *ash1a* MO into the *flh* mutant leads to the loss of the remaining *isl1*-positive neurones (D). Scale bar: 10 μm.

has a more complex role in the regulation of *ash1a*; it is required for maintenance but not early induction of expression.

In *Drosophila*, different prepattern genes regulate distinct domains of expression of neural determination genes within a given organ (for reviews, see Skeath and Caroll, 1994; Simpson, 1996) and can show genetic redundancy (see Gomez-Skarmeta et al., 1996; Sato et al., 1999). By analogy, one hypothesis is that an as yet unknown prepattern gene functions redundantly with *flh* to regulate early *ash1a* expression. Alternatively, Flh could have a role distinct to a prepattern function in the maintenance of *ash1a* expression. Biphasic regulation of neural determination genes has been reported previously. For example, initiation of expression of *Drosophila achaete* in early medial and lateral column neuroblasts requires a 3′ element, whereas a 5′ element mediates later expression (Skeath et al., 1994). It is not clear whether different proteins bind to these 3′ and 5′ elements; however, the HMG box transcription factor SoxNeuro is only required for the late expression and not the initiation of *achaete* expression (Buescher et al., 2002). Thus, the role of Flh may be to maintain *ash1a* expression while other proteins independently activate initial transcription of this gene.

Ash1a and Ngn1 regulate the production of neurones in the epiphysis

Our results demonstrate that Ash1a and Ngn1 regulate genes that are likely to be important for the development of neurones in the epiphysis. First, Ash1a and Ngn1 regulate the expression of three genes (*deltaA*, *deltaB* and *deltaD*) that encode Notch receptor ligands. The Notch signalling pathway mediates the selection of neural progenitors through the process of lateral inhibition, by which cells inhibit their neighbours from adopting a neuronal fate (see Lewis, 1998). A preliminary analysis of the epiphysis in the *mindbomb* mutant (*mib*^{ta52b}), in which lateral inhibition is impaired (Jiang et al., 1996; Schier et al., 1996; Haddon et al., 1998b; Itoh et al., 2003), suggests that epiphysial neurones are produced prematurely and in excess (E.C. and S.W.W., unpublished). These results suggest that the Notch signalling pathway controls neuronal production in the epiphysis.

We have also implicated Ash1a and Ngn1 in the regulation of a third bHLH protein encoding gene, *neuroD*. Our results corroborate observations showing that Ash and Ngn genes function upstream of *neuroD* in other species (Ma et al., 1996; Blader et al., 1997; Cau et al., 1997; Cau et al., 2002; Fode et al., 1998; Ma et al., 1998). As *neuroD* has been implicated in neuronal differentiation in a variety of neural lineages (Miyata et al., 1999; Liu et al., 2000; Schwab et al., 2000; Kim et al., 2001), its absence is likely to contribute to the neurogenesis defects observed in *ash1a*- and *ash1a*/*ngn1*-morphant embryos.

ash1a and *ngn1* are also required to activate *otx5* and *onecut*, genes that may function in the specification and/or differentiation of photoreceptors and projection neurones. Indeed, Otx5 is required to activate genes that show circadian expression in epiphysial cells (Gamse et al., 2002). *Drosophila onecut* functions as a differentiation gene during the formation of retinal photoreceptors (Nguyen et al., 2000); because zebrafish *onecut* appears to be expressed specifically by projection neurones, it may play a comparable role in the formation of these epiphysial neurones.

Overall, our study shows that Ash1a and Ngn1 function downstream of Flh, and upstream of genes that mediate production and differentiation of neurones. However, although Ash1a and Ngn1 are crucial effectors of epiphysial development, our data suggest that aspects of epiphysial development are independent of these genes.

Flh regulates aspects of epiphysial development independent of *ash1a* and *ngn1*

Although the neuronal deficits in *flh* mutants and *ash1a*/*ngn1*-double morphants are similar, several lines of evidence dispute a simple model in which Flh function is restricted to the regulation of *ash1a* and *ngn1* transcription (Fig. 9). First, we have demonstrated that *flh* is required for the induction of *tbxC* and the maintenance of its own transcription. This function does not appear to be shared with *ash1a* and *ngn1* because a reduction of both *ash1a* and *ngn1* function did not affect the expression of *tbxC*, whereas it did lead to a severe impairment of *isl1* expression in the epiphysis. This suggests that expression of *tbxC* is independent of Ash1a and Ngn1. An alternative interpretation is that residual activity of these transcription factors in morphants is sufficient to induce *tbxC.* We cannot completely exclude this a hypothesis but we think that it is unlikely given the seemingly high efficacy of the MOs, and given that it would imply that the levels of Ash1a and Ngn1 required to induce *tbxC* are considerably lower than the levels required to induce *isl1*.

TbxC is proposed to function downstream of Flh during notochord development, although it is unlikely to be the main effector of Flh function as *tbxC* overexpression does not rescue

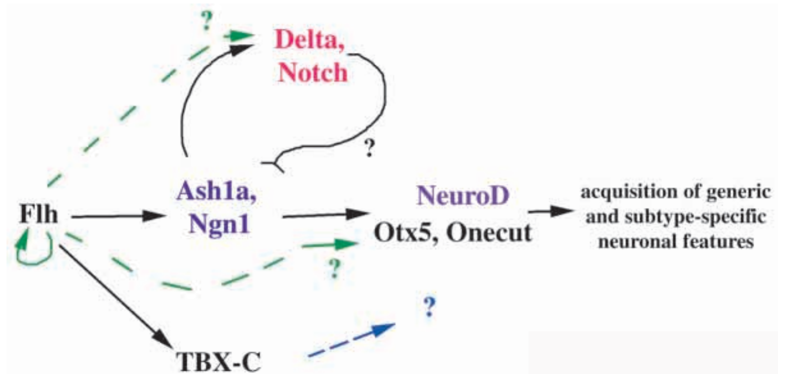

Fig. 9. Proposed interactions between Flh, Ash1a, Ngn1 and other regulators during epiphysial neurogenesis. Flh has several distinct activities, it regulates its own expression (green solid arrow), and the expression of the bHLH transcription factors Ash1a and Ngn1 (in purple), which in turn activate lateral inhibition genes (in red), and the potential differentiation factors NeuroD, Otx5 and Onecut. Flh also activates TbxC expression independently of Ash1a and Ngn1 activity; the function of TbxC in the epiphysis is currently unknown (blue dashed arrow with a question mark). In addition, roles for Flh in the transcriptional regulation of lateral inhibition and differentiation genes, independent of the regulation of *ash1a* and *ngn1* transcription, is inferred from analysis of the epiphysis in *flh* mutants (green dashed arrows with question marks, see text for details). Note that the early expression of *ash1a* is independent of Flh suggesting that another (as yet unknown) factor operates, possibly redundantly with Flh, to regulate bHLH gene expression at early stages (see Discussion).

the notochord phenotype of *flh*-mutant embryos (Dheen et al., 1999). In the epiphysis, TbxC could function either as an intermediate step between Flh and Ash1a/Ngn1 or in a pathway parallel to the one in which Ash1a and Ngn1 act. However, as the 14s stage *flh*-mutant epiphysis showed normal expression of *ash1a* but no expression of *tbxC*, it is unlikely that TbxC is the main regulator of *ash1a* downstream of Flh. We favour the hypothesis that TbxC participates in a pathway parallel to Ash1a/Ngn1.

Despite the fact that initial expression of *ash1a* was normal in the *flh*-mutant epiphysis, the activation of *neuroD*, *deltaA* and *deltaD* and *otx5* expression was impaired. Therefore, Flh might have roles during epiphysial neurogenesis, additional to, and distinct from, the regulation of *ash1a* and *ngn1* transcription. However, as Flh was not sufficient to initially activate *otx5*, *neuroD*, *deltaA* and *deltaD* expression in Ash1a/Ngn1 double morphants, it is unlikely that Flh and these bHLH proteins function in parallel pathways to regulate neurogenesis. Instead, Flh may regulate the activity of Ngn1 and/or Ash1a by other means, for example, by regulating the expression of a cofactor or an inhibitor of bHLH protein activity, or by influencing post-translational modification of the bHLH transcription factors.

Ash1a and Ngn1 function in parallel rather than in a cascade in the epiphysis

ash1a and *ngn1* function redundantly in the epiphysis to regulate targets that include the Delta genes, *otx5* and *onecut*. The most likely interpretation of our results is that Ash1a and Ngn1 function in parallel redundant pathways rather than in a cascade during the formation of epiphysial neurones. This situation is in contrast to that encountered in the murine olfactory epithelium. Indeed, in most olfactory progenitors, *Mash1* functions as a neural determination gene, upstream of *Ngn1*, in a genetic cascade. In these progenitors *Ngn1* bears the characteristics of a differentiation gene and is not involved in regulating the expression of Notch ligands (Cau et al., 1997; Cau et al., 2002). However, in a minority of olfactory progenitors *Mash1* and *Ngn1* do function redundantly as neural determination genes (Cau et al., 2002), which is similar to the situation encountered in epiphysial progenitors.

Distinct populations of neurones with different requirements for Ash1a and for Ngn1 coexist in the epiphysis

Our study has revealed some unexpected diversity within the zebrafish epiphysis as some early, anteriorly positioned neurones depend only on Ash1a activity, whereas, in posterior cells, Ngn1 activity is able to compensate for the lack of Ash1a activity. Although bHLH transcription factors can function in the specification of distinct neuronal subpopulations (for a review, see Bertrand et al., 2002), these two different populations of cells (Ash1a dependent, and Ash1a and Ngn1 dependent) do not correspond to the two neuronal populations described in the zebrafish epiphysis (photoreceptors and projection neurones). Furthermore, unpublished observations also suggest that *ash1a* and *ngn1* are not involved in specifying the expression of different opsins by epiphysial photoreceptors. Therefore, as yet, there is no indication that *ash1a* and *ngn1* have any involvement in the specification of neuronal phenotype in the epiphysis.

Loss of Flh or reduction of Ash1a and Ngn1 activity affects both photoreceptors and projection neurones

Absence of Flh, as well as impairment of Ash1a and Ngn1 function, affects production of both epiphysial photoreceptors and projection neurones. Several possibilities could explain these observations. First, *flh*, *ash1a* and *ngn1* could be required to specify a progenitor common to both photoreceptors and projection neurones. Second, the genetic programme involving *flh*, *ash1a* and *ngn1* could function independently in two distinct populations of progenitors, one for projection neurones and one for photoreceptors. A third possibility is that generation of one class of neurones is dependent upon the presence of the other. Such recruitment mechanisms are implicated in the development of the *Drosophila* eye (for a review, see Frankfort and Mardon, 2002), chordotonal organs (Lage et al., 1997; Okabe and Okano, 1997; zur Lage and Jarman, 1999) and olfactory sensillae (Reddy et al., 1997), and may also occur in the vertebrate eye (Masai et al., 2000; Neumann et al., 2001). Analysis of lineage relationships between the various epiphysial cell types should help resolve the nature of the cellular interactions and proliferation patterns that generate discrete epiphysial neurone classes.

In the vertebrate retina, removal of the function of specific bHLH transcription factors impairs the development of specific cell types (for reviews, see Vetter and Brown, 2001; Marquardt and Gruss, 2002). For instance, in both zebrafish and mouse, absence of Ath5 (Atoh7 – Zebrafish Information Network and Mouse Genome Informatics) specifically affects ganglion cells (Brown et al., 2001; Kay et al., 2001; Wang et al., 2001), whereas *neuroD* and *Math3* (*Neurod4* – Mouse Genome Informatics) are required for formation of amacrine cells (Morrow et al., 1999; Inoue et al., 2002), and *Mash1* and *Math3* promote bipolar cell development (Tomita et al., 2000). Thus, different populations of retinal neurones cells can be distinguished by their requirement for different bHLH proteins. By contrast, in the epiphysis, both projection neurones and photoreceptors are affected by reduction in the activity of Ash1a and Ngn1. Thus, the genetic mechanisms that govern neurogenesis in the two photoreceptive structures of the zebrafish embryo appear to be quite divergent.

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