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 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).

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 epiphiyal neural progenitors. We show that ash1a and ngn1 function in parallel redundant pathways to regulate neurogenesis downstream of flh. Comparison of the epiphiyal 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 epiphiyal 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

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

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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; Bellenfroid, 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 (mbi) 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 epiphyseal 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 epiphyseal 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 epithysis. We show that Flh is required to maintain the expression of ashl and to initiate expression of ngnl 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 epiphyseal neurogenesis. Ash1a and Ngn1 show some degree of redundancy and function downstream of Flh but upstream of neuroD. By comparing the morphogenetic phenotypes of flh mutants and ashlngn1 morphants, we show that although the reduction in ashl and ngn1 expression can account for most of the neurogenesis defects in the flh-mutant epithysis, Flh is unlikely to function solely as a regulator of ashl and of ngn1. We also show that impairment of Ash1a or Ash1a and Ngn1 activity affects both epithelial 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 epiphyseal neurogenesis.

**MATERIALS AND METHODS**

**Zebrafish lines**

Fish heterozygous for the flh<sup>flh</sup>-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 ashl (GenBank Accession Number U14587) (Allende and Weinberg, 1994) and ngn1 (GenBank Accession Number AF017301) (Blader et al., 1997): ashl <sup>MO</sup> (complementing bases 121-145), 5′-ATCTTGGCGGT-GATGTCCATTTCGCG-3′; ashl<sup>UTR</sup> MO (complementing bases 83-107), 5′-AAGGAGT-GAGTCAAAAGCACTAAAGT-3′; and ngn1 MO (complementing bases 222-246), 5′-TATACGACTC-CATTTGTTGATAACC-3′. [This MO has been used in previous studies (Cornell and Eisen, 2002; Andermann et al., 2002).]

MOs were diluted in Danieu’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<sup>UTR</sup> 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 ashl MO and ashl<sup>UTR</sup> MO was performed either with ashl MO at 1 mg/ml and ashl<sup>UTR</sup> MO at 1.25 mg/ml, or with ashl MO at 2 mg/ml and ashl<sup>UTR</sup> MO at 2.5 mg/ml. Similar results were obtained in these two sets of experiments.

To control the specificity of ashl MO, we generated two different constructs: ashl::gfp and mutash1a::gfp. The ashl::gfp construct contained part of the ashl gene (from base 115 to 393), which included the ashl target sequence (see above), fused in frame with the gfp coding sequence. The mutash1a::gfp construct was identical to ashl::gfp except for four single base mutations inside of the ashl<sup>UTR</sup> MO target sequence (CGATATGCGAGATACCGCCAAGAT). Embryos injected with RNA from either construct showed a bright green fluorescence owing to the expression of GFP (41 out of 45 embryos for ashl::gfp and 38 out of 40 for mutash1a::gfp). The vast majority of embryos injected with both ashl::gfp RNA and ashl MO showed no fluorescence (41 out of 44 embryos). By contrast, most of the embryos injected with both mutash1a::gfp RNA and ashl 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 opsins antibody was a gift from P. Hargrave.

**RESULTS**

Flh regulates the expression of ashl, ngn1 and neuroD in different populations of epiphyseal cells

The homeodomain transcription factor Flh is necessary for the production of neurones in the epithysis. In flh mutants, the first epiphyseal neurones are produced but after 18-somite stage (s) neuronal production stops. In the absence of Flh function, expression of ashl is not maintained, raising the possibility that Flh is an activator of ashl 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 al., 1997; Korzh et al., 1998; Mueller and Wulliman, 2002a; Mueller and Wulliman, 2002b).

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 Ng1n 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 Ng1n 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 (ash1a5′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 ash1a5′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
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. 3B,D)) and the activation of ngn1 (Fig. 3G,J). Scale bar: 15 μm.

**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.

**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, J-L; in H, 50 μm for G,H.
and the adenohypophysis) and the 'ngn1 phenotype' (loss of cranial ganglia and primary sensory neurones). These embryos also exhibited severely reduced or absent epiphysial islet1 expression at 25s (Fig. 3A,F; Table 1). However, some islet1-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-positive 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. 4G-H; Table 1).

Expression of islet1 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.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>flh (24 hours)</th>
<th>neuroD (27 hours)</th>
<th>islet1 (27 hours)</th>
<th>islet1 (25s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>23.3±2.3 (n=3)</td>
<td>23.7±4.7 (n=3)</td>
<td>41.3±2.9 (n=6)</td>
<td>34.3±2.08 (n=3)</td>
</tr>
<tr>
<td>ash1a MO</td>
<td>29.0±6.1 (n=3)</td>
<td>16.7±1.7 (n=3)</td>
<td>24.1±8.9 (n=6)</td>
<td>19.3±3.78 (n=6)</td>
</tr>
<tr>
<td>ash1a UTR MO</td>
<td>nd</td>
<td>nd</td>
<td>24±5.4 (n=6)</td>
<td>19.0±1.0 (n=3)</td>
</tr>
<tr>
<td>ngn1 MO</td>
<td>26.7±2.1 (n=3)</td>
<td>21.6±3.5 (n=3)</td>
<td>36.7±7.5 (n=3)</td>
<td>29.7±1.53 (n=3)</td>
</tr>
<tr>
<td>ash1a and ngn1 MOs</td>
<td>26.3±2.05 (n=3)</td>
<td>1.0±1.7 (n=3)</td>
<td>0.0±0.0 (n=3)</td>
<td>1.2±1.79 (n=6)</td>
</tr>
<tr>
<td>ngn1−/− and ash1a MO</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>29.7±3.05 (n=3)</td>
</tr>
</tbody>
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| Numbers indicate mean number of cells±s.d. in preparations viewed at high magnification. n, number of embryos scored. nd, not determined.

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/engn1-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 expression remained absent in most of the ngn1-positive, MO-injected embryos (Fig. 5H,N).

In ngn1 MO-injected embryos, expression of deltaA, deltaB and deltaD was normal in the epiphysis at 23-24s (Fig. 5J,L,N,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 flh 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...
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., 1997).
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 ash1a 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. 7J; 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 prepatterning 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-Chauphier, 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. Spatial distribution 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.](image-url)
has a more complex role in the regulation of ash1a; it is required for maintenance but not early induction of expression.

In Drosophila, different prepatterning 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 prepatterning gene functions redundantly with flh to regulate early ash1a expression. Alternatively, Flh could have a role distinct to a prepatterning 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 (miha526), 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 ash1alnrg1-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 ashlalnrg1-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

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**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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