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Expanded expression of Sonic Hedgehog in *Astyanax* cavefish: multiple consequences on forebrain development and evolution

Arnaud Menuet^{1,*}, Alessandro Alunni^{2,*}, Jean-Stéphane Joly², William R. Jeffery³ and Sylvie Rétaux^{1,†}

Ventral midline Sonic Hedgehog (Shh) signalling is crucial for growth and patterning of the embryonic forebrain. Here, we report how enhanced Shh midline signalling affects the evolution of telencephalic and diencephalic neuronal patterning in the blind cavefish *Astyanax mexicanus*, a teleost fish closely related to zebrafish. A comparison between cave- and surface-dwelling forms of *Astyanax* shows that cavefish display larger *Shh* expression in all anterior midline domains throughout development. This does not affect global forebrain regional patterning, but has several important consequences on specific regions and neuronal populations. First, we show expanded *Nkx2.1a* expression and higher levels of cell proliferation in the cavefish basal diencephalon and hypothalamus. Second, we uncover an *Nkx2.1b-Lhx6*-GABA-positive migratory pathway from the subpallium to the olfactory bulb, which is increased in size in cavefish. Finally, we observe heterochrony and enlarged *Lhx7* expression in the cavefish basal forebrain. These specific increases in olfactory and hypothalamic forebrain components are Shh-dependent and therefore place the telencephalic midline organisers in a crucial position to modulate forebrain evolution through developmental events, and to generate diversity in forebrain neuronal patterning.

KEY WORDS: Teleost, Telencephalon, Subpallium, Olfactory bulbs, LIM-homeodomain, Nkx, Dlx, GABA, Proliferation, Hypothalamus, Cavefish, *Astyanax mexicanus*

INTRODUCTION

The vertebrate forebrain is a sophisticated structure, which contains the neural regions controlling complex behaviours, such as sensorimotor, cognitive and limbic responses. The embryonic development of this structure, particularly the telencephalon and diencephalon, is orchestrated through a series of complex patterning, morphogenetic, migration and wiring events that are not completely understood. The genetic mechanisms controlling these developmental steps appear largely shared among vertebrates (Bachy et al., 2002; Murakami et al., 2005; Puelles et al., 2000; Rubenstein et al., 1994; Striedter, 1997; Wullmann and Mueller, 2004), even though the morphology and connectivity between forebrain areas vary considerably among different vertebrate species (Butler and Hodson, 1996).

Early in embryogenesis, the forebrain develops under the influence of signalling centres, called 'secondary organisers' in mammals, which secrete morphogen molecules such as Wnt (Wingless-Int), Bmp (Bone morphogenetic protein), Fgf (Fibroblast growth factor), or Shh (Sonic hedgehog) (Marin and Rubenstein, 2001; Ohkubo et al., 2002; Rubenstein and Beachy, 1998; Rubenstein et al., 1998; Wilson and Rubenstein, 2000). These signalling centres are located at the dorsal (Wnt, Bmp), anterior (Fgf8) and ventral (Shh) midline of the telencephalon, respectively. Likewise, diencephalon development is largely controlled by the floor or basal plate and by the secondary organiser of the zona limitans intrathalamica (*zli*), which both produce Shh (Kiecker and Lumsden, 2004; Vieira et al., 2005; Zeltser, 2005). In amniotes, these midline signalling centres and interactions between them

(Ohkubo et al., 2002; Shimogori et al., 2004; Tole et al., 2000) create a field of organisation in their zone of influence, and regulate the growth, patterning and regionalisation of forebrain areas. Thus, Shh midline signalling is well placed to have a major impact, not only on the development but also on the evolution of the forebrain, through subtle changes in signal intensity and/or position. For example, although the global developmental organisation of the forebrain is highly conserved in lampreys (jawless vertebrates) compared with gnathostomes (Murakami et al., 2001; Murakami et al., 2005), their ventral telencephalon 'lacks' a pallidum (Weigle and Northcutt, 1999). Strikingly, there is no Shh signalling at the lamprey telencephalic ventral midline (Osorio et al., 2005), suggesting that *Shh* expression in the anterior ventral midline was responsible for the appearance of a novel forebrain subdivision, and further that midline signalling may be a powerful motor for forebrain evolution in jawed vertebrates.

The surface-dwelling and cave-living forms of *Astyanax mexicanus* have been used as an advantageous model system in evolutionary developmental biology (Jeffery, 2001). The two forms of this single species split from a common ancestor about 10,000 years ago, a relatively short period of time during which the cave animals have evolved both regressive and constructive features. These include the loss of eyes, pigmentation and aggressive behaviour, and the increase in feeding apparatus (jaws, teeth, taste buds) and body fat content. Among these evolutionary changes, the loss of eyes in cavefish has drawn much attention. Recently, Yamamoto et al. (Yamamoto et al., 2004) have shown that eye regression is a consequence of early overexpression of hedgehog genes (*Hh*, including *Shh*) at the ventral midline of cavefish embryos. This expansion of midline Hh signalling causes hyperactivation of downstream genes, lens apoptosis and eye degeneration. Thus, a gain of function in Hh signalling is at the origin of the regressive eye phenotype. In fact, eyes may have been lost by default as a consequence of natural selection for constructive traits, such as feeding structures, that are positively regulated by Hh signalling (Jeffery, 2005).

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As shown in various model species, the Hedgehog family of secreted proteins regulates pattern formation, proliferation and differentiation. Here we have taken advantage of enlarged *Shh* expression in the embryonic midline of cavefish relative to surface fish embryos to test the idea of the impact of the ventral midline on the development and evolution of forebrain patterning, neuronal organisation and wiring. Through the analysis of *Shh* downstream genes of the *Nkx* and LIM-hd (LIM-homeodomain) families, cell proliferation patterns and GABAergic neuron differentiation, we show that *Shh* overexpression has multiple and region-specific effects on neuronal development in the hypothalamus, subpallium and olfactory bulbs. Moreover, comparison between cave and surface embryos allows the identification of new developmental mechanisms for neuronal specification in the fish telencephalon.

MATERIALS AND METHODS

Animals

Laboratory stocks of *A. mexicanus* surface fish and cavefish (Pachón population) were obtained from the Jeffery laboratory at the University of Maryland, College Park, MD. Fish were maintained at 23–26°C on a 12:12 hours light:dark cycle. Embryos were collected after spawning and fixed at various stages in 4% paraformaldehyde (PFA). After progressive dehydration in methanol, they were stored at –20°C. *A. mexicanus* development is almost identical to zebrafish in the first 48 hours post-fertilisation (hpf), so the zebrafish staging table can be used (Kimmel et al., 1995).

cDNA cloning

Total RNA from surface fish brains was reverse transcribed with random primers using AMV reverse transcriptase (Promega). Partial cDNA sequences for *Nkx2.1b* (278 bp, GenBank accession no. DQ431669), *Lhx6* (408 bp, DQ431667), *Lhx9* (1.2 kb, EF175738) and *GAD65* (445 bp, DQ431668) were amplified by PCR using degenerated primers (sequences on request) designed after alignments of several teleost sequences including zebrafish. PCR products were subcloned in TOPO-PCR II vector (Invitrogen) and sequenced. *Nkx2.1a* (AY661435), *Shh* (AY661431) and *Pax6* (AY651762) cDNAs were previously isolated by the Jeffery laboratory. The *Lhx7* and *Dlx2* plasmids were kindly provided by David Stock.

Phylogenetic analysis

Sequences of *Lhx6* and *Lhx7* genes (accession numbers on request) were aligned with Clustal W (Thompson et al., 1994). Phylogenetic tree (Neighbour-Joining) and bootstrap values were generated using the MEGA3.1 program (Kumar et al., 2004). During this work, an *Astyanax* *Lhx6* sequence was isolated by David Stock (DQ822512). The two *Lhx6* sequences shared 100% identity in the overlapping fragment.

Whole-mount in situ hybridisation

cDNAs were amplified by PCR, and digoxigenin- or fluorescein-labeled riboprobes were synthesised from PCR templates (Nguyen et al., 2001). A protocol for automated whole-mount in situ hybridisation (Intavis) was performed (Deyts et al., 2005). For probe penetration, embryos were treated with proteinase K for 30 minutes at 37°C (10 µg/ml for 36 hpf, 20 µg/ml for 48 hpf, 30 µg/ml for 60 hpf, 40 µg/ml for 72 hpf). Embryos were paraffin-sectioned at 8 µm, or their brains were dissected out and mounted in glycerol. Depending on the gene marker and the stage considered, five to 20 embryos from each population were analysed (except for cyclopamine treatments, where $n=4-5$), and three to four were sectioned. Quantification of the expansion of *Nkx2.1* and *Dlx2* domains was performed using ImageJ, on in toto pictures of embryos at 24, 36 and 48 hpf coming from at least two independent experiments.

PCNA and phosphohistone H3 immunohistochemistry

For Proliferating cell nuclear antigen (PCNA) immunohistochemistry embryos were fixed in Clark's solution (3:1 ethanol 100: acetic acid) overnight at 4°C. They were processed as described (Candal et al., 2005). Quantification was performed on the same serial section in cavefish and

surface fish embryos. Classical morphometric measurement was done, using a grid superimposed on the sections, and counting the arbitrary surface covered by PCNA-positive cells.

For phospho-H3 immunohistochemistry, embryos were fixed in 4% PFA. A primary rabbit polyclonal anti-phosphoH3 antibody (1/100^o, Euromedex) was used. Quantification in the hypothalamus was performed by counting the total number of phospho-H3 positive cells in the hypothalamus of each embryo through all serial transverse sections obtained. For control quantification in the tectum, the first five sections at tectal level were counted. Results were expressed as phospho-H3 positive cell density after measurement of the surface area of the hypothalamus or the tectum for each section using ImageJ. Statistical analysis was carried out using Student's *t*-test.

Cyclopamine treatment

At 15 hpf embryos were treated for 9 hours in 200 µmol/l or 20 µmol/l cyclopamine (Sigma) in water. Control embryos were exposed to 0.1% ethanol, as the cyclopamine stock solution was diluted in 100% ethanol. After treatment, they were washed twice, maintained in fresh water until 48 hpf and fixed.

RESULTS

Yamamoto et al. (Yamamoto et al., 2004) have recently shown that midline *Shh* expression is expanded at early embryonic stages (neural plate though 1–4 somites, or 10 hpf), and indirectly causes eye degeneration in cavefish. Here, we have investigated the impact of *Shh* modification later in forebrain embryogenesis, from 24 hpf to 1 week of development.

Shh expansion is maintained during cavefish development

First, we asked whether enlarged *Shh* expression in cavefish was maintained throughout development. At 20, 36 and 48 hpf, *Shh* was still more largely expressed in cavefish compared with surface fish (Fig. 1). This expanded expression included all anterior *Shh* expression domains, including the floor plate, the *zli*, the hypothalamus (Fig. 1A–H) and the subpallium (Fig. 1G,H). These data indicate that *Shh* expansion was not transient in cavefish, but rather was maintained and even spread to new locations during late embryogenesis, opening the possibility of multiple consequences on forebrain development.

The expression of *Nkx* family factors is enlarged in cavefish in a region-specific manner

As a next step, we investigated the expression of patterning and regionalisation genes of the *Nkx* family. *Nkx2.1* and *Nkx2.2* homeodomain factors are downstream in the *Shh* signalling pathway (Briscoe et al., 1999; Rallu et al., 2002; Xu et al., 2005). In other well-studied teleost species (zebrafish, medaka), there are two *Nkx2.1* members (Alunni et al., 2004; Rohr et al., 2001). In *Astyanax*, we also found that *Nkx2.1a* (Yamamoto et al., 2004) is accompanied by a duplicated gene, which we named *Nkx2.1b*. Consistent with expression patterns in other fishes, *Nkx2.1a* was found exclusively in the presumptive hypothalamus, encompassing almost the entire structure (Fig. 2I). By contrast, *Nkx2.1b* was expressed both in restricted nuclei of the hypothalamus, in the subpallial telencephalon (SP, subpallium), including the future basal ganglia and septum (see Wullmann and Mueller, 2004; Alunni et al., 2004), and in the preoptic region (po; Fig. 2E–G). Finally, *Nkx2.2* showed a typical 'alar-basal boundary' pattern, adjacent to and following the *Shh* pattern (Fig. 2C).

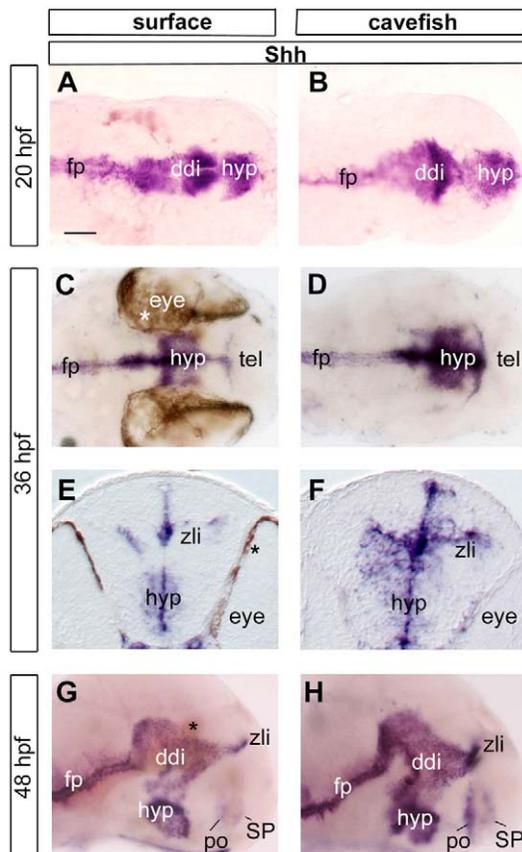


Fig. 1. Enlarged *Shh* expression is maintained during cavefish development. Brain distribution of *Shh* mRNA in surface fish (left) and cavefish (right) embryos at indicated stages. Transverse sections through the hypothalamus (E,F), or in toto ventral (A-D) and lateral views (G,H) show expansion of *Shh* signal in cavefish at all stages. In all panels and following figures, anterior is right and dorsal is up. In G,H, the eyes were removed to facilitate observation. In this and subsequent figures, note that pigment cells (asterisks) are only present in surface fish embryos, most of them corresponding to the retina pigmented epithelium. Scale bar: 50 μ m. ddi, dorsal diencephalon; fp, floor plate; hyp, hypothalamus; po, preoptic region; SP, subpallium; tel, telencephalon; zli, zona limitans.

There was no significant difference in *Nkx2.2* or *Dlx2* (subpallial marker) expression between surface and cave embryos at any stage (Fig. 2A-D). Quantification showed a non-significant +4.8% increase in *Dlx2* territory in cavefish, suggesting that global pallial and subpallial patterning was identical in both populations. *Nkx2.1b* expression was similar in the presumptive hypothalamus of cavefish and surface fish embryos (Fig. 2E-H), but was significantly expanded in the cavefish SP and preoptic region relative to surface fish embryos (Fig. 2E-H; +35% after quantification). This result was found reproducibly from 24 to 72 hpf. To investigate whether the *Nkx2.1b* expansion was at the expense of the rest of the subpallium, double labelling for *Dlx2* and *Nkx2.1b* were performed. They showed that the domain expressing only *Dlx2* was unchanged, and that *Nkx2.1b* territory enlargement was due to an anteroventral expansion of the *Nkx2.1b*

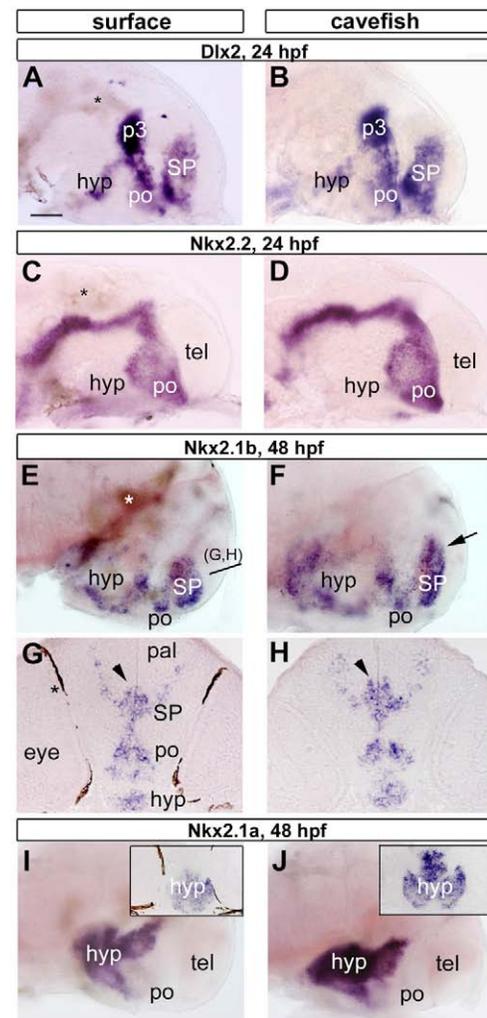


Fig. 2. Differential modulation of *Nkx* and *Dlx2* expression domains in cavefish embryos. *Dlx2* and *Nkx* gene expression in surface fish (left) and cavefish (right) embryos at indicated stages. (A-D) *Dlx2* and *Nkx2.2* on in toto lateral views. (E-H) *Nkx2.1b* in toto (E,F) and on horizontal sections (G,H, orientation indicated in E). The arrow in F and arrowheads in G,H point to larger expression in cavefish SP. (I,J) *Nkx2.1a* distribution in toto and in transverse sections through the hypothalamus (insets in I and J). Scale bar: 50 μ m. hyp, hypothalamus; p3, prosomere 3 (ventral thalamus); po, preoptic region; pal, pallium; SP, subpallium; tel, telencephalon.

domain (see Fig. S1A,B in the supplementary material). Finally, between 24 and 72 hpf, *Nkx2.1a* expression was enlarged in the developing hypothalamus of cavefish compared with their surface counterparts (Fig. 2I,J; +38%), possibly defining a larger hypothalamic territory.

Cavefish have a larger presumptive hypothalamus region

The above data, using regionalisation factors as comparative indexes of brain patterning between cave and surface embryos, suggested that specific presumptive regions are larger in cavefish embryos. To analyse the origin of these differences, particularly in po and hypothalamus, we compared cell proliferation in cavefish and surface fish embryos from 36 hpf to 5 days, by PCNA immunostaining (Fig. 3). Until 48 hpf, almost the entire

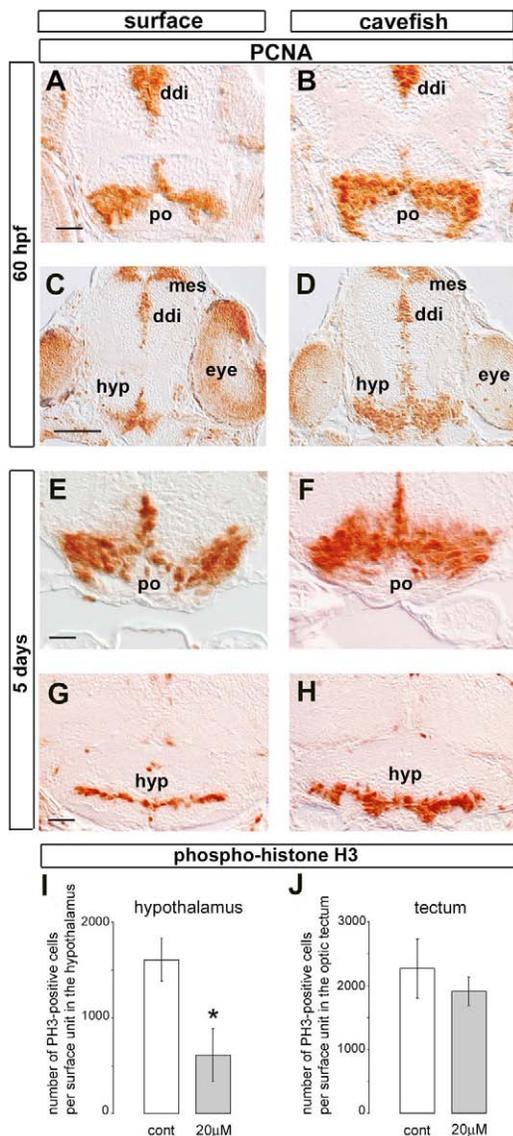


Fig. 3. Increased proliferation in the preoptic region and hypothalamus of cavefish embryos is Shh-dependent.

(A-H) PCNA immunoreactivity in transverse sections (surface fish, left; cavefish, right) at 60 hpf (A-D) and 5 days (E-H, high magnification) through the preoptic region (A,B,E,F) and the hypothalamus (C,D,G,H). Note that proliferation in the dorsal diencephalon and mesencephalon is identical in both populations. (I,J) Cavefish embryos were treated with 20 $\mu\text{mol/l}$ cyclopamine from 15 to 24 hpf, allowed to develop until 48 hpf, and proliferation was assessed with anti-phosphohistone H3 immunostaining (see Fig. S1 in the supplementary material for pictures). The results are expressed as the density of phospho-H3-positive cells (number of cells per surface area in arbitrary units). See methods for details. * indicates a significant effect ($P < 0.05$, $n = 4$ embryos; Student's t -test) when compared with the ethanol-treated controls. Scale bars: 50 μm . ddi, diencephalon; hyp, hypothalamus; mes, mesencephalon; po, preoptic region.

brain of both populations was proliferating (not shown), preventing any comparison. Slightly later, when proliferation was less extensive, an increased number of PCNA-positive cells was observed in the cavefish basal diencephalon (Fig. 3A-H). This increase was maintained for up to 5 days of development and was

specific to the developing hypothalamus and preoptic region. Quantification showed that the surface area covered by proliferating cells was 31 and 22% higher in the po of cavefish at 60 hpf and 5 days, respectively, and 75 and 60% higher in the hypothalamus of cavefish at the same stages. By contrast, the dorsal diencephalic and mesencephalic proliferative zones showed identical PCNA immunoreactivity in both populations (Fig. 3A-D).

To further demonstrate the causal role of enlarged Shh signalling in the increased proliferation and expansion of the basal diencephalon in cavefish embryos, we performed cyclopamine treatments. Cavefish embryos were treated with cyclopamine between 15 and 24 hpf, were allowed to develop until 48 hpf and were assessed for proliferation pattern using phospho-histone H3 (phosphoH3), which labels cells in M phase of the cell cycle and gives readily quantifiable number of immuno-positive cells. Cyclopamine treatment induced a reduction in cell proliferation both in the preoptic region and in the hypothalamus (see Fig. S2A-D in the supplementary material). Quantification of this effect in the hypothalamus showed a 38% reduction in the surface area of the hypothalamus, a 77% reduction in the number of phospho-H3 cells, and a significant decrease in the density of immuno-positive cells in this region in cyclopamine-treated embryos when compared with their controls (Fig. 3I). This suggested an Shh-dependent control of proliferation and size during early development of the basal diencephalon. The specificity of these effects were confirmed by parallel analysis in the optic tectum (Fig. 3J and see Fig. S2E,F in the supplementary material): there, a slight reduction of tectal surface (-11%) paralleled a slight reduction in the number of phospho-H3-positive cells (-18%), and resulted in a similar density of proliferating cells in control and treated embryos.

Downstream of *Shh* and *Nkx*: *Lhx6* and *Lhx7* LIM-homeodomain factors

We next sought to estimate the impact of enlarged *Nkx2.1a* and *Nkx2.1b* expression on neuronal specification processes in the hypothalamus and basal telencephalon. In other vertebrates, *Nkx2.1* governs the expression of *Lhx6* and *Lhx7*, neuronal specification factors of the LIM-hd family expressed in the basal forebrain (Sussel et al., 1999; Grigoriou et al., 1998). In mouse, *Lhx6* governs the tangential migration of GABAergic neurons generated in the subpallium towards the pallium (Alifragis et al., 2004; Lavdas et al., 1999), whereas *Lhx7* governs the specification of cholinergic neurons (Fragkouli et al., 2005; Mori et al., 2004; Zhao et al., 2003). The *Astyanax* *Lhx6* and *Lhx7* genes were identified as orthologs of *Lhx6* and *Lhx7* in other vertebrates, including zebrafish (Fig. 4A), and their expression was localised with respect to *Shh* expression using double-colour in situ hybridisation (Fig. 4B,C). In *Astyanax*, *Lhx6* was expressed in the SP and preoptic region, and in a complex hypothalamic pattern partly similar to that of *Nkx2.1b* (Fig. 4B, see also Figs 6 and 9). *Lhx7* transcripts were similarly detected in the SP and po (Fig. 4C, see also Figs 5 and 9), and also in the pituitary (see Fig. 4C-E and Fig. 5A-D). This pituitary expression has never been reported for *Lhx7* in other species (Alunni et al., 2004; Bachy et al., 2001; Grigoriou et al., 1998), and might be *Astyanax*-specific. Double-colour in situ hybridisation further showed that *Lhx6* and *Lhx7* were expressed in the same subpallial domain but had different expression territories in the preoptic region, *Lhx6* expression being restricted to the anterior po and included in a larger *Lhx7* domain (Fig. 4D,E).

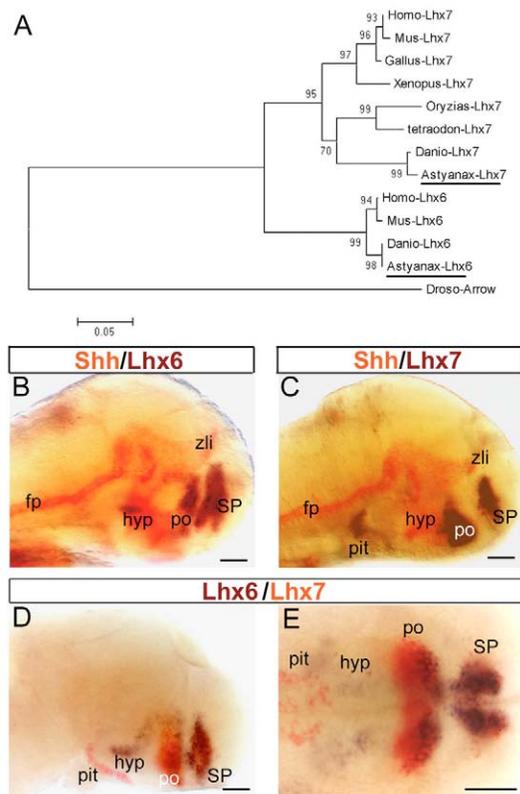


Fig. 4. Phylogenetic analysis and expression domains of *Lhx6* and *Lhx7* in *Astyanax*. (A) *Lhx6* and *Lhx7* sequences from different species were aligned and a phylogenetic tree was constructed. (B,C) Forty-eight hpf in toto lateral views of *Lhx6* (B) and *Lhx7* (C) expression patterns (purple) in cavefish brains that have been double-hybridised with *Shh* (red). (D,E) At 36 hpf, in toto lateral (D) and ventral (E, high power) views of cavefish brains double-hybridised for *Lhx6* (purple) and *Lhx7* (red). Scale bars: 50 μ m. fp, floor plate; hyp, hypothalamus; pit, pituitary; po, preoptic region; SP, subpallium; zli, zona limitans.

***Lhx7*: heterochrony and broader expression in the po of cavefish embryos**

Lhx7 spatiotemporal expression patterns were compared between surface fish and cavefish embryos (Fig. 5). First, we observed a shift in the onset of *Lhx7* expression in the SP: whereas the cavefish basal telencephalon already contained numerous *Lhx7*-expressing cells at 24 hpf, only two or three cells were reproducibly detected in their surface fish counterparts at this stage (Fig. 5A,B). This heterochrony in the onset of *Lhx7* in the telencephalon was rapidly compensated, as we could not detect significant differences in SP expression of *Lhx7* in later embryos, ranging from stage 36 to 60 hpf (Fig. 5C-H). Of note, and with respect to the role of *Lhx7* in cholinergic specification in mammals, the *Astyanax* ortholog was found in a few cells of the lateral subpallium at 48 and 60 hpf (Fig. 5G,H and inset). This region is called VI in fish (for area Ventralis lateralis) and contains cholinergic neurons (Mueller et al., 2004). *Lhx7* expression at this level also appears identical in cavefish and surface fish embryos (Fig. 5G,H). By contrast, *Lhx7* was continuously expressed as a

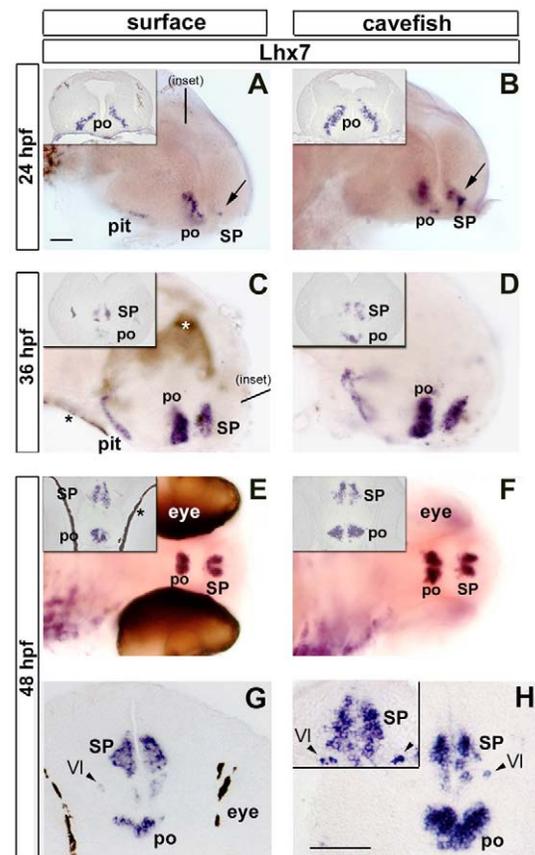


Fig. 5. *Lhx7* is expressed earlier in the subpallium, and as a larger domain in the preoptic region of cavefish embryos. Surface fish (left) and cavefish embryos (right) were hybridised for *Lhx7* at indicated stages (inset in H, 60 hpf). In each panel, insets show transverse (A,B, orientation given by black line in A) or horizontal sections (C-H, orientation given in C) at the level of the SP or po. In A,B, arrows points to the fewer than three cells reproducibly found in the surface fish subpallium, to be compared with the already large expression in cavefish at this stage. In G,H, arrowheads point to *Lhx7* cells in the lateral subpallium. pit, pituitary; po, preoptic region; SP, subpallium; VI area ventralis lateralis. Scale bars: 50 μ m.

larger domain in the po region of cavefish relative to surface fish embryos (Fig. 5C-H). Finally, hypothalamic and pituitary expression were identical in both populations (Fig. 5C,D).

In sum, *Lhx7* was expressed earlier in the SP and larger in the po of cavefish embryos, bringing up the possibility that *Shh* and *Nkx2.1b* expansion in these two regions could have effects on downstream neuronal specification events regulated by *Lhx7*.

***Lhx6*: expanded expression in the cavefish telencephalon and hypothalamus**

At all studied stages from 24 to 72 hpf, *Lhx6* expression domains were significantly broader in cavefish embryos than in their surface fish counterparts. This included the SP and po regions (shown in Fig. 6A,B at 24 hpf), and the hypothalamus (shown in Fig. 6C,D at 36 hpf). Moreover, starting at 48 hpf, an additional area of *Lhx6* expression was detected in the telencephalon of both *Astyanax* populations (Fig. 6E-H). At 48, 60 and 72 hpf, two bilateral columns of *Lhx6*-expressing cells extended from the dorsal aspect of the *Lhx6*-positive SP domain (dSP, Fig. 7A,B),

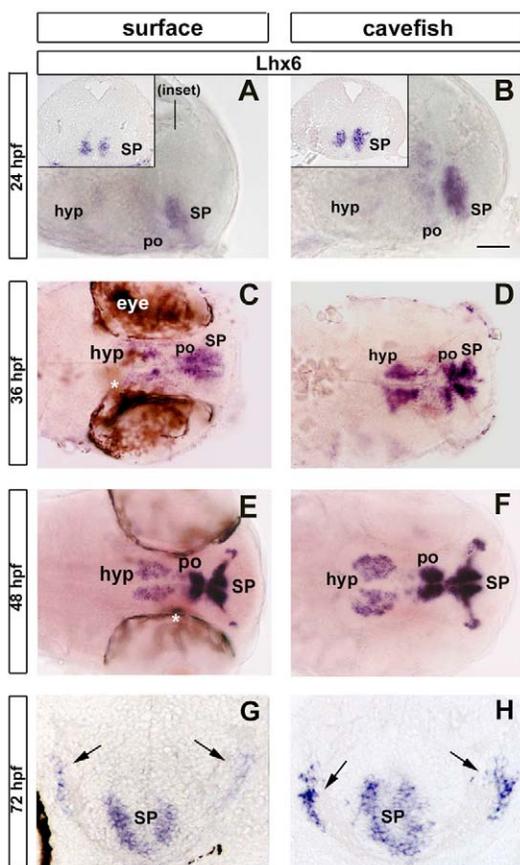


Fig. 6. *Lhx6* expression is enlarged in cavefish, especially in columns of cells extending from the SP. (A-H) Comparison of *Lhx6* pattern between surface fish (left) and cavefish embryos (right) at indicated stages on lateral (A,B) and ventral (C-F) in toto views. G and H show frontal sections through the forebrain of 72 hpf embryos, where arrows point to anteroventral located *Lhx6* cells. In A and B, an inset shows a transverse section at SP level (orientation given in A). Scale bar: 50 μ m.

ending as a pack of strong *Lhx6*-expressing cells located in the anterolateral part of the pallium (Fig. 6E-H and Fig. 7A,B). After analysis in sections, this *Lhx6* expression pattern is suggestive of cells migrating out of the SP towards pallial areas, and may therefore represent the fish counterpart of the *Lhx6*-positive, ganglionic-emergence-originated, tangentially migrating GABAergic neurons in mammals. Importantly, *Lhx6* expression was much more intense in the columns of cells emanating from the dorsal SP in cavefish than in surface fish embryos (Fig. 6E-H). Moreover, at 72 hpf, *Lhx6* expression in the SP and associated bilateral columns of putative migratory cells continued to be stronger in cavefish embryos (Fig. 6G,H).

To further support the idea that *Lhx6*-positive cells move outside the subpallium towards the pallium, we analysed how *Lhx6* expression relates to *Pax6* and *Lhx9*. In fish, *Pax6* marks the pallio-subpallial border (Wulliman and Mueller, 2004), and *Lhx9* is a pallial marker (Alunni et al., 2004). We could confirm that *Lhx6* cells cross the pallio-subpallial border, represented by the *Pax6*-expressing band in the telencephalon (Fig. 7J,K), and reach their target in a pallial region that also expresses weak levels of

Pax6 (Fig. 7J-M). To further explore the characteristics of the columns of *Lhx6*-expressing cells, we showed that at 60 hpf, when the columns are well developed, they also expressed *Nkx2.1b* (Fig. 7E,F, see also Fig. 2G,H at 48 hpf). Moreover, the columns of *Lhx6*-positive cells expressed *Astyanax* GAD65 (Glutamic acid decarboxylase), demonstrating that they are GABAergic (Fig. 7C,D). Finally, we found that the columns ended in a pallial area corresponding to the developing olfactory bulbs, as demonstrated both by the positions of their termini in the forebrain and by fluorescent dye (DiI) labelling of the olfactory bulbs through the primary olfactory projection (Fig. 7G-I). As the *Lhx6*-positive columns are more important in cavefish than in surface fish, these data suggest that the expansion of the ‘ventralising’ *Shh* morphogen has an impact on the establishment of neuronal circuits in various parts of the brain, including regions that are not directly in the range of *Shh* signalling activity.

Forebrain patterning modifications in cavefish are due to *Shh* expansion

Finally, we sought to demonstrate that the modifications of forebrain neuronal patterning in cavefish were indeed *Shh*-dependent. To this end, we performed cyclopamine treatments on cavefish embryos, trying to phenocopy the surface-type patterns by reduction of *Shh* signalling. Because we are studying late events in neurodevelopment, cavefish embryos were treated with cyclopamine between 15 and 24 hpf, and fixed at 48 hpf (Fig. 8). After cyclopamine treatment, *Dlx2* expression was affected in the SP and thalamic prosomere p3 but not in the hypothalamus (Fig. 8A-D), and *Nkx2.2* expression was strongly affected (Fig. 8E-H). The *Nkx2.1a* expression domain in the hypothalamus was also reduced by cyclopamine, phenocopying the difference between surface fish and cavefish embryos (Fig. 8I-L). Although *Nkx2.1b* expression was not strongly modified in the hypothalamus, it was reduced in size in the po and SP of treated cavefish (Fig. 8J-L). Finally, *Lhx6* and *Lhx7* expression were affected in the telencephalon (SP and po) of cyclopamine-treated embryos (Fig. 8Q-X), but not in the pituitary (*Lhx7*) nor in the hypothalamus (*Lhx6*). Of note, the columns of *Lhx6*-positive cells were affected similarly to the difference observed between cavefish and surface fish (Fig. 8U-X). In sum, cyclopamine treatments showed dose-dependent, gene-specific and region-specific effects, which demonstrate the *Shh*-dependence of the above described modifications in regional and neuronal patterning in cavefish (Fig. 9).

DISCUSSION

We have taken advantage of subtle, viable and probably adaptive expansion of *Shh* expression at the ventral midline of the cave-living form of *Astyanax mexicanus* to investigate the impact of midline signalling on forebrain neuronal patterning during development and evolution. The main results are as follows: (1) *Shh* expansion in cavefish is maintained throughout development; (2) transcription factors of the *Nkx2.1* (but not *Nkx2.2* or *Dlx2*) families are expressed in larger domains in the basal forebrain of cavefish embryos; (3) the LIM-hd cell-specification genes *Lhx6* and *Lhx7* also show broader expression in a region-specific manner in the subpallium and hypothalamus; (4) GABAergic interneuron migration towards the olfactory bulbs is increased in cavefish; (5) the hypothalamus of cavefish is larger, probably due to increased cell proliferation; and (6) all these effects are *Shh*-dependent. These findings indicate that a slight modification of midline *Shh* signalling can affect the specification, size and presumably the migration and

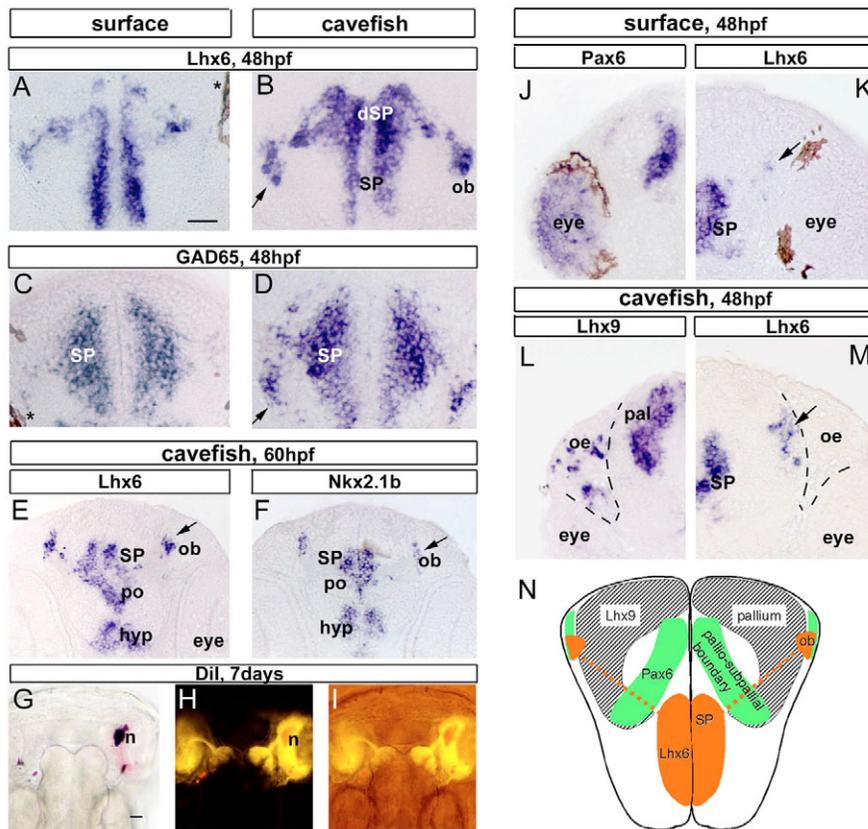


Fig. 7. Characterisation of *Lhx6*-positive columns of cells in the telencephalon.

(A-D) Frontal sections through the telencephalon of 48 hpf surface fish (left) and cavefish embryos (right) showing *Lhx6* and corresponding *GAD65* expression in the putative migratory stream. Arrows point to the increased *Lhx6* or *GAD65* component in cavefish. (E,F) Horizontal sections showing that *Lhx6* (E) and *Nkx2.1* (F) patterns are indistinguishable at 60 hpf in the columns of cells. (G-I) Dil labelling of the olfactory projection onto the olfactory bulbs of a 7-day-old cavefish, in dorsal views, where anterior is up. G (bright field), H (fluorescence) and I (combined) photomicrographs indicate that the olfactory bulbs are located in the anterolateral part of *Astyanax* telencephalon, i.e. where the *Lhx6*- and *GAD65*-positive cells terminate their migration. (J-M) Hemi-sections through the telencephalon of surface (J,K) or cavefish (L,M) embryos, to illustrate *Lhx6* pattern with regard to *Pax6* and *Lhx9*. In L,M, dashed lines delineate the brain and the adjacent olfactory epithelium (oe), which also expresses *Lhx9*. (N) Schema, in which gene expression is indicated on the left, with corresponding anatomical regions on the right. dSP, dorsal part of subpallium; hyp, hypothalamus; n, nostril; ob, olfactory bulb; po, preoptic region; SP, subpallium; tel, telencephalon. Scale bars: 50 μ m.

connectivity of specific neuronal populations, resulting in a global increase of the olfactory and neuroendocrine components of the brain in cave-living animals.

Increased Shh signalling in cavefish induces downstream cascades in a highly specific manner

We have shown that expanded *Shh* expression is maintained throughout in every region of the anterior central nervous system (ventral midline, zli, subpallium) up to 72 hpf in cavefish embryos. Such a pleiotropic 'upregulation' of *Shh* strongly suggests that a quantitative element, as opposed to spatio-temporal regulatory elements (Jeong et al., 2006), in the transcriptional regulation of *Shh* has been changed in cavefish. Alternatively, a global increase of an upstream regulator that would quantitatively control *Shh* expression independently of spatial regulatory elements is possible. In any case, as increased *Shh* signalling has been found in several independently evolved *Astyanax* cavefish populations (Yamamoto et al., 2004), it will be extremely interesting to identify this viable regulatory mutation.

Continuous expanded *Shh* signalling would be expected to induce major perturbations in brain development. However, the global patterning of forebrain development is not modified in cavefish relative to surface fish embryos, and only some of the specific gene cascades downstream of *Shh* signalling are increased in these embryos (summarised in Fig. 9). Hence, the expression of *Nkx2.2* is identical in surface and cavefish, and so is the expression of *Dlx2* (see also Xu et al., 2005), reinforcing the specificity of the effects discussed below, and suggesting that at least some natural compensatory mechanisms must be at work in cavefish to avoid a massive perturbation in brain development after *Shh* expansion. In

fact, when cavefish are treated with cyclopamine, *Dlx2* and *Nkx2.2* expressions are diminished, demonstrating the functional regulatory link between *Shh* and *Dlx2* or *Nkx2.2*, and unmasking a probable compensatory mechanism that has evolved in cavefish. Moreover and globally, the cyclopamine treatments also show that the observed effects on regionalisation and neuronal specification genes are indeed due to increased *Shh* signalling.

As schematised in Fig. 9, *Nkx2.1a* and *Nkx2.1b* expressions are enlarged in the cavefish hypothalamus and SP or po, respectively. However, *Nkx2.1b* is unchanged in the hypothalamus. This differential regulation can be viewed in terms of subfunctionalisation of the two *Nkx2.1* genes, which may constitute a feature characteristic of teleosts. Concerning LIM-hd genes, the enlarged expression of *Lhx6* in the hypothalamus can be interpreted as a consequence of *Nkx2.1a* expansion, and the enlarged expression of *Lhx7* and *Lhx6* in the SP or po as a consequence of *Nkx2.1b* expansion. Thus, we uncover specificity of the increased 'cascades', where each *Nkx2.1* paralog has an effect on the expression of one or the two LIM-hd paralogs, and where *Shh* signalling eventually regulates the neuronal composition of the forebrain. These results fit nicely with recent findings in mouse showing that *Shh* signalling during neurogenesis maintains cortical interneuron identity through regulation of *Nkx2.1* (Xu et al., 2005).

Shh and cell proliferation: a bigger hypothalamus in cavefish

Our findings suggest that the expanded *Nkx2.1a* and *Nkx2.1b*, *Lhx6* and *Lhx7* expression domains in the hypothalamus and po of cavefish embryos reflects a higher level of cell proliferation in these regions relative to surface fish embryos, eventually resulting in

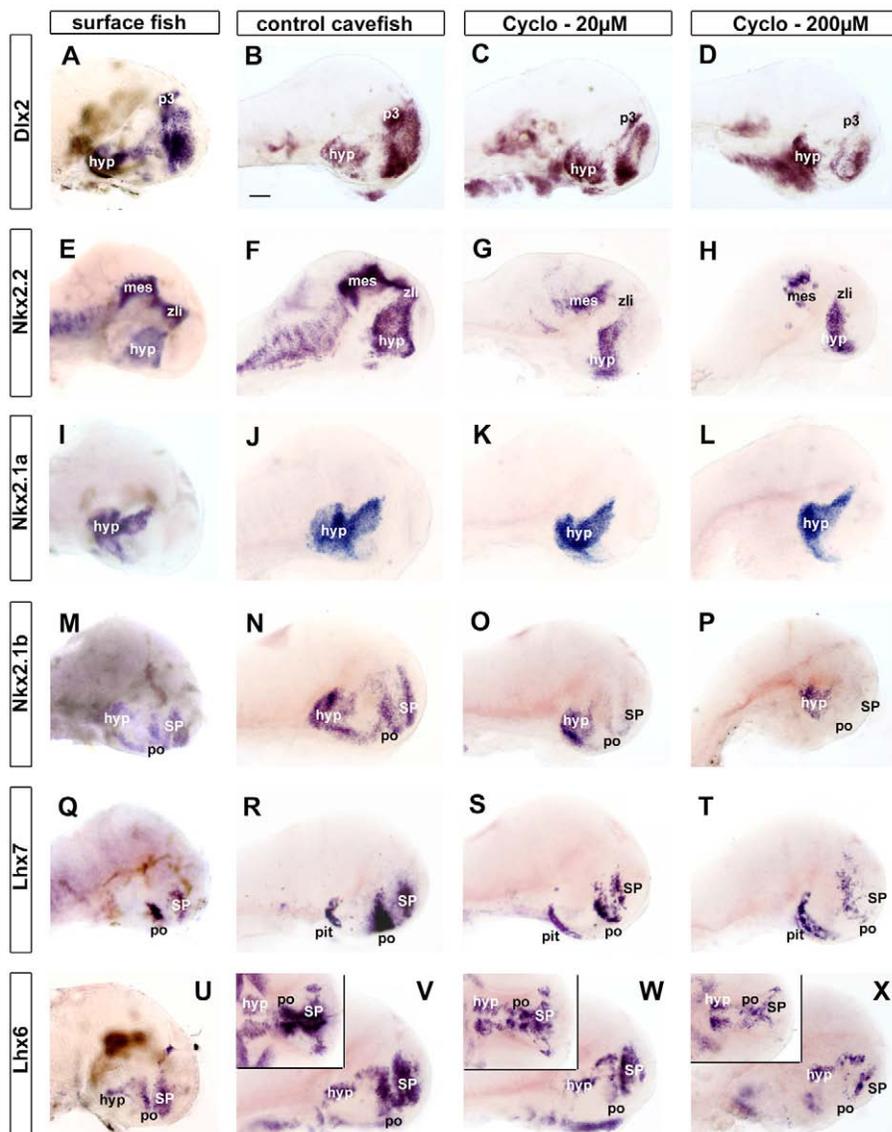


Fig. 8. Forebrain neuronal patterning differences observed in cavefish are Shh-dependent. Cavefish embryos were treated between 15 and 24 hpf with 0.1% ethanol (control), 20 $\mu\text{mol/l}$ or 200 $\mu\text{mol/l}$ cyclopamine and compared to surface fish embryos. In toto lateral views (**A-U**) and ventral views (insets **V-X**) of 48 hpf brains hybridised with the indicated probes. Note the dose-dependence and region-specificity of the effects. For example, the pituitary *Lhx7* or the dorsal hypothalamic *Lhx6* signals are not affected by cyclopamine. Scale bar: 50 μm . hyp, hypothalamus; mes, mesencephalon; p3, prosomere 3 (ventral thalamus); pit, pituitary; po, preoptic region; SP, subpallium; zli, zona limitans.

enlargement of this part of the basal forebrain. Consistent with this possibility, the mature hypothalamus is larger in cavefish than in surface fish adults (D. Soares and W. R. Jeffery, unpublished). The implication of Shh in the control of proliferation in the brain (and other organs) is documented (Britto et al., 2000; Fu et al., 2004; Ishibashi and McMahon, 2002; Lewis et al., 2004; Moshiri et al., 2005; Wechsler-Reya and Scott, 1999). However, despite the fact that Shh controls the proliferation of both ventral and dorsal brain structures (Dahmane et al., 2001), we observed a specific effect only in the hypothalamus and preoptic region. By contrast to the case of the telencephalon, where other organising centres secreting Bmps or Fgfs might naturally compensate for *Shh* overexpression (Ohkubo et al., 2002; Shimogori et al., 2004; Tole et al., 2000), the hypothalamus develops under the direct influence of the ventral midline and alar-basal boundary and in the vicinity of the zli diencephalic organiser, all of which secrete Shh. Thus, there is probably no possible compensation by neighbouring centres in this part of the forebrain, and this might explain why *Shh* expansion results in a bigger hypothalamus in cavefish. Does this have any physiological and/or adaptive consequences? Cave animals are fat and have lost aggressive behaviours compared with surface fish

(Jeffery, 2001). These traits might be related to the neuroendocrine controls exerted by the hypothalamus. However, several nuclei of the hypothalamus and preoptic region serve as tertiary gustatory centres (Folgueira et al., 2003; Lamb and Finger, 1996), and the increase in these nuclei might correspond to the increase in taste bud number in cavefish (Jeffery et al., 2000). Further investigations are clearly needed to functionally interpret this hypothalamic difference between cavefish and surface fish.

***Lhx7*: a rapidly compensated heterochrony of expression in the subpallium of cavefish embryos**

In mammals, the function of *Lhx7* is well studied: *Lhx7* specifies the cholinergic phenotype in the basal forebrain (Mori et al., 2004; Zhao et al., 2003), and mice lacking *Lhx7* show impaired spatial learning and memory (Fragkouli et al., 2005). More recently, *Lhx7* was also involved in the control of GABAergic fate in the basal forebrain (Manabe et al., 2005; Bachy and Rétaux, 2006). There, *Lhx7* inhibits GABAergic differentiation at early stages of embryogenesis to preserve a pool of future cholinergic neurons, the number of which is tightly controlled and which will undergo terminal differentiation much later in development (Bachy and Rétaux, 2006). Thus, our

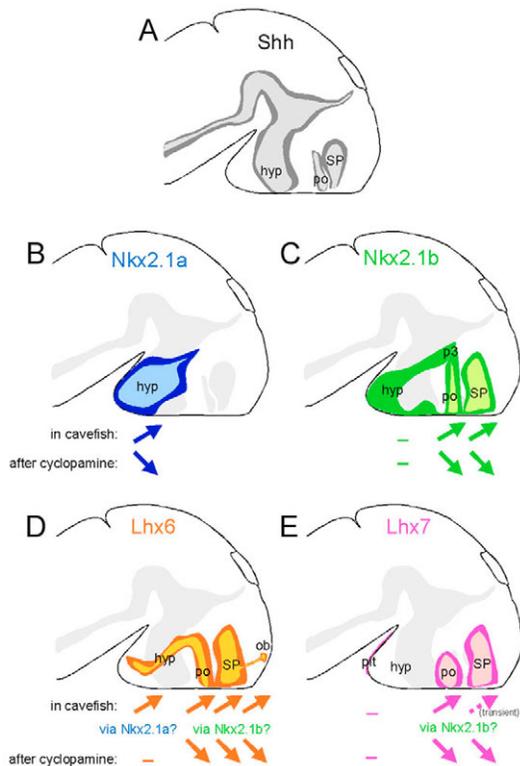


Fig. 9. A summary of gene expression differences in the developing forebrain of cavefish and surface fish embryos.

Schematic representations of lateral views of embryonic brains. The expression patterns of *Shh* (A), *Nkx2.1a* (B), *Nkx2.1b* (C), *Lhx6* (D) and *Lhx7* (E) are drawn in grey, blue, green, yellow and pink, respectively. For each gene, the dark and light colour schematises the enlarged expression domains in cavefish versus surface fish, respectively. In B to E, the *Shh* expression pattern, which is larger in cavefish, is also shown in grey. For each gene, the region-specific changes in expression observed in cavefish relative to surface fish embryos or after cyclopamine treatment in cavefish are indicated. The effect on *Lhx6* in the hypothalamus is probably mediated through the increase of *Nkx2.1a* in this region, whereas the effects on *Lhx6* and *Lhx7* in the po and telencephalon are probably mediated through the increase of *Nkx2.1b* in the respective regions.

observation of earlier *Lhx7* expression in cavefish embryos might simply reflect the fact that upstream *Shh* and *Nkx2.1b* are increased and reach a ‘threshold’ level earlier than in surface fish to induce *Lhx7* expression. Then, when the correct pool of cholinergic neurons is obtained, progenitors stop dividing, and the expression of *Lhx7* in surface fish and cavefish becomes similar after 36 hpf. In this case, and contrary to the case of *Lhx6* regulation, the (unknown) mechanisms that control the number of cholinergic neurons in the forebrain would naturally compensate for the primary effects of Shh overexpression in cavefish, and would not be expected to have any obvious consequence on SP cholinergic neuronal patterning.

By striking contrast to the case of the SP, *Lhx7* expansion is maintained in the po of cavefish relative to surface fish. The po is another cholinergic region in mammals and also contains cholinergic neurons in fish (Mueller et al., 2004) and other non-mammalian vertebrates (Marin et al., 1997; Pombal et al., 2001; Rodriguez-Moldes et al., 2002). Moreover, in fish, the po projects

to the olfactory bulbs (Folgueira et al., 2004), and these projections might represent the fish counterpart of the pathway from basal forebrain cholinergic nuclei of the diagonal band onto the olfactory bulbs of mammals (Shipley, 1985). Importantly, this cholinergic innervation of the bulbs actively participates in olfactory functions, such as early olfactory learning and discrimination (Fletcher and Wilson, 2002; Linster and Cleland, 2002; Wilson et al., 2004). Further analysis will be needed to investigate whether cavefish indeed have enhanced cholinergic projections to the olfactory bulbs, and whether this has functional implications (see also below).

***Lhx6*: a marker for a subpallium to pallium migratory stream in fish?**

We interpret the *Lhx6* expression domain in *Astyanax* telencephalon as a stream of migrating cells originating from the SP and populating the pallial region forming the olfactory bulbs. These cells fulfil criteria for migrating cells: (1) they express *Nkx2.1b*, a subpallial marker, expressed in the ventricular zone of the SP; (2) their pallial region of destination never expresses *Nkx2.1* or *Lhx6* in the ventricular or subventricular zone, suggesting that they are not derived from the pallial progenitor zone; (3) they follow a tangential trajectory in the neuroepithelium, where they end up in a subpial, marginal zone location; and (4) they cross the pallio-subpallial border defined by *Pax6* domain. These cells are GABAergic, as they express *GAD65*, and thus probably constitute a population of interneurons. We therefore propose that *Lhx6* labels the equivalent of the mammalian rostral migratory stream (RMS), which contributes granule and periglomerular GABAergic interneurons to the olfactory bulbs (Lois and Alvarez-Buylla, 1994; Luskin, 1993; Wichterle et al., 2001). In mammals, the RMS begins to migrate during the late embryonic and perinatal periods and continues throughout adulthood (Wichterle et al., 2001). The timecourse observed here in fish is similar, as the peak of *Lhx6* expression in the putative migratory cells is observed in 48 hpf embryos and continues at later stages. We did not observe at any stage *Lhx6*-positive cells that could constitute the fish counterpart of tangentially migrating GABAergic cortical interneurons (Anderson et al., 1999; Anderson et al., 1997; Anderson et al., 2001; Marin and Rubenstein, 2001; Marin and Rubenstein, 2003). In fish, these GABAergic pallial interneurons constitute a relatively scarce neuronal population (Anglade et al., 1999), and the absence of pallial *Lhx6* expression, together with the absence of *Dlx* gene expression in the fish pallium (Akimenko et al., 1994; Alunni et al., 2004; Zerucha and Ekker, 2000) (present data), suggest that these interneurons do not share common genetic specification mechanisms with their mammalian counterparts. In fact, an *Lhx6.1* isoform of *Lhx6* in mouse was reported to be expressed both in the cortex and the olfactory bulbs (Kimura et al., 1999). Altogether, this may suggest that an *Lhx6*-positive migratory pathway from the SP to the olfactory bulbs is ancestral in vertebrates, and that the dorsal pallium-directed *Lhx6* migrations are an innovation of mammals.

Strikingly, lampreys do not express *Shh* and *Nkx2.1* in their embryonic ventral forebrain (Murakami et al., 2001; Osorio et al., 2005), they do not have a pallidal division in their adult subpallium (Weigle and Northcutt, 1999), and they do not possess GABAergic interneurons in their pallium (Melendez-Ferro et al., 2002). Thus, the presence of regulatory cascades controlling cell migrations and generating diversity in neuronal patterning in the forebrain seems to be a novelty that emerged at the transition from agnathans to fish, and for which the impact of Shh midline signalling is crucial.

Cavefish have increased *Lhx6*-positive migrations to the olfactory bulbs

We have observed an increased *Lhx6*-positive migratory stream in the forebrain of cavefish relative to surface fish. From an adaptive point of view, what kind of advantage does having more olfactory bulb interneurons confer to cavefish for their life in perpetual darkness? In the mammalian bulbs, periglomerular GABA interneurons increase the contrast between activation levels in different glomeruli, and augment odour discrimination (Aungst et al., 2003; Lledo et al., 2004), whereas granular cells increase contrast and specificity of olfactory information by synchronising the discharges of mitral projection neurons (Lagier et al., 2004; Laurent, 2002; MacLeod and Laurent, 1996; Saghatelian et al., 2005). In sum, bulbar interneurons are crucial in olfactory circuitry, and a positive correlation between the abundance of newly generated bulbar interneurons and olfactory performances was reported (Scotto-Lomassese et al., 2003). An increase in olfactory modality through an augmentation or more rapid turnover of the RMS might therefore be advantageous for cavefish. Indeed, *Shh* was shown to be required for the production of new olfactory neurons from the stem cell niche of the forebrain subventricular zone in vivo (Palma et al., 2005).

Conclusions

Using the two forms of *Astyanax* as an evolutionary developmental model, we have shown that changes in *Shh* midline signalling can have various effects on brain development and evolution. Because *Shh* expansion in cavefish is physiological and probably adaptive, it could be compensated by other signalling pathways to prevent deleterious effects on brain development. Thus, *Shh* expansion in cavefish has highly specific consequences on the expression of downstream genes of the *Nkx2.1* and LIM-hd families, resulting either in expression heterochrony of *Lhx7*, which is rapidly compensated and has no consequences on telencephalic patterning, or in an increase of *Lhx6* expression, which might confer a selective advantage to the cavefish, or in an increase of the size of the hypothalamus, which is more difficult to interpret in terms of adaptation. The finding that cavefish have enhanced olfactory and neuroendocrine structures when compared to surface fish illustrates how midline signalling centres can have a major impact on forebrain development, evolution and diversification.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/5/845/DC1>

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