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Role of *hlx1* in zebrafish brain morphogenesis

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ABSTRACT ***hlx1*** **is a related homeobox gene expressed in a dynamic spatiotemporal expression pattern during development of the zebrafish brain. The homologues of** ***hlx1*****, mouse** ***dbx1*** **and** ***Xenopus Xdbx*****, are known to play a role in the specification of neurons in the spinal cord. However, the role of these molecules in the brain is less well known. We have used two different approaches to elucidate a putative function for** ***hlx1*** **in the developing zebrafish brain. Blastomeres were injected with either synthetic** ***hlx1*** **mRNA in gain-of-function experiments or with antisense morpholino oligonucleotides directed against** ***hlx1*** **in loss-of-function experiments. Mis-expression of** ***hlx1*** **produced severe defects in brain morphogenesis as a result of abnormal ventricle formation, a phenotype we referred to as "*fused-brain*". These animals also showed a reduction in the size of forebrain neuronal clusters as well as abnormal axon pathfinding.** ***hlx1*** **antisense morpholinos specifically perturbed hindbrain morphogenesis leading to defects in the integrity of the neuroepithelium. While hindbrain patterning was in the most part unaffected there were select disruptions to the expression pattern of the neurogenic gene** ***Zash1B*** **in specific rhombomeres. Our results indicate multiple roles for** ***hlx1*** **during zebrafish brain morphogenesis.**

KEY WORDS: *hox, development, hindbrain, axon, tract, morpholinos, neurogenesis*

Introduction

Hox genes contain a highly conserved DNA binding motif known as the homeobox. In vertebrates these genes map to one of four *hox* gene clusters which exhibit both spatial and temporal colinearity (Duboule and Dolle, 1989; Duboule, 1994; Duboule and Morata, 1994). In the vertebrate central nervous system (CNS) the most anterior limit of *hox* gene expression is in the hindbrain. In this region *hox* genes exhibit remarkable expression patterns, with sharp borders present at rhombomere boundaries (McGinnis and Krumlauf, 1992; Krumlauf, 1994). There is also evidence to indicate that rhombomeres exhibit a two segment periodicity with respect to some gene expression patterns and features (Lumsden and Keynes, 1989; Lumsden, 1990). Rhombomeres are considered lineage restricted, segmental units which are characterised by their differential expression of *hox* genes and the presence of segmental cranial neurons (Fraser *et al.*, 1990). Alterations in the pattern of *hox* gene expression either through genetic mutation or ectopic expression have revealed that these genes are responsible for patterning the anterio-posterior axis of the hindbrain.

While genes mapping to the *hox* gene clusters are not expressed in the forebrain, related *hox* genes exhibit discrete expression domains in all regions of the CNS. Related *hox* genes contain a homeobox but do not map to any of the *hox* gene clusters. Examples of related *hox* genes included members of the *pax*, *six*, *emx* and *otx* gene families. The *pax* genes are expressed widely throughout the embryo during development. Mice carrying a homozygous mutation in the *pax6* gene exhibit small eyes and forebrain patterning defects (Roberts, 1967; Hogan *et al.*, 1986; Stoykova *et al.*, 1996). Members of the *pax* gene family are involved in the specification of interneurons and motor neurons in the ventral spinal cord as well as of neurons in the diencephalon (Burrill *et al.*, 1997; Ericson *et al.*, 1997; Mastick and Andrews, 2001). In zebrafish, *six3* is involved in forebrain development (Kobayashi *et al.*, 1998). *otx* genes are expressed throughout the rostral brain and are thought to be required for brain morphogenesis and regionalisation. Recent analysis of *otx2* and *emx2* double knockout mice has revealed that they cooperate in the

Abbreviations used in this paper: AC, anterior commissure; CNS, central nervous system; Di, diencephalon; drc, dorsal rostral cluster; DVDT, dorsoventral diencephalic tract; E, epiphysis; FB, forebrain; gfp, green fluorescent protein; HB, hindbrain; MB, midbrain; MHB, midbrain-hindbrain boundary; MLF, medial longitudinal fascicle; MO, morpholino; nTPC, nucleus of tract of posterior commissure; OV, otic vesicle; POC, postoptic commissure; sMO, standard morpholino; SOT, supraoptic tract; Te, telencephalon; TPC, tract of the posterior commissure; TPOC, tract of the postoptic commissure; vcc, ventrocaudal cluster; vrc, ventral rostral cluster; WT, wild type.

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formation of the diencephalon (Suda *et al.*, 2001). Other related *hox* genes such as *vax1* in mice are involved in axon guidance (Bertuzzi *et al.*, 1999). Thus, many different related homeobox genes clearly have very important roles in brain development.

hlx1 is a related homeobox gene expressed in the developing brain during embryogenesis in zebrafish (Fjose *et al.*, 1994). It is first detected at approximately 9 hpf during the early stages of neurulation in the anterior region of the developing neural plate. Later in development it is expressed in discrete domains throughout the neural axis. The dynamic spatiotemporal expression pattern of *hlx1* suggests that it has a unique role in brain morphogenetic events. Its expression in a longitudinal stripe in the ventral midline of the rostral brain is unusual for homeobox containing genes. More often these genes are restricted in expression to small patches or transverse bands (Hauptmann and Gerster, 2000). Comparison of the amino acid sequence of the homeodomains of *hlx1* and other related *hox* genes has revealed that *dbx1* in mice and *Xdbx* in *Xenopus* are homologues of *hlx1* (Gershon *et al.*, 2000; Lu *et al.*, 1994). Both *dbx1* and *Xdbx* have been implicated in neurogenesis in the spinal cord (Gershon *et al.*, 2000; Pierani *et al.,* 1999). In *Xenopus*, overexpression of *Xdbx* leads to inhibition of the generation of neurons in the early neural plate (Gershon *et al.*, 2000). This mechanism is possibly due to an inhibitory action of *Xdbx* on the expression of *xash3*. *xash3* is a neurogenic gene homologous to the *Drosophila melanogaster achaete-scute* (*ash*) gene complex. Overexpression of *xash3* in *Xenopus* causes ectopic generation of neurons (Ferreiro *et al.*, 1994). However, when both *xdbx* and *xash3* are co-expressed the ectopic generation of neurons is blocked (Gershon *et al.*, 2000). Thus, *Xdbx* acts to inhibit neurogenesis by limiting the expression of *xash3*. To date the roles of *dbx1* and *Xdbx* in the developing brain are yet to be investigated, while the function of *hlx1* remains unknown.

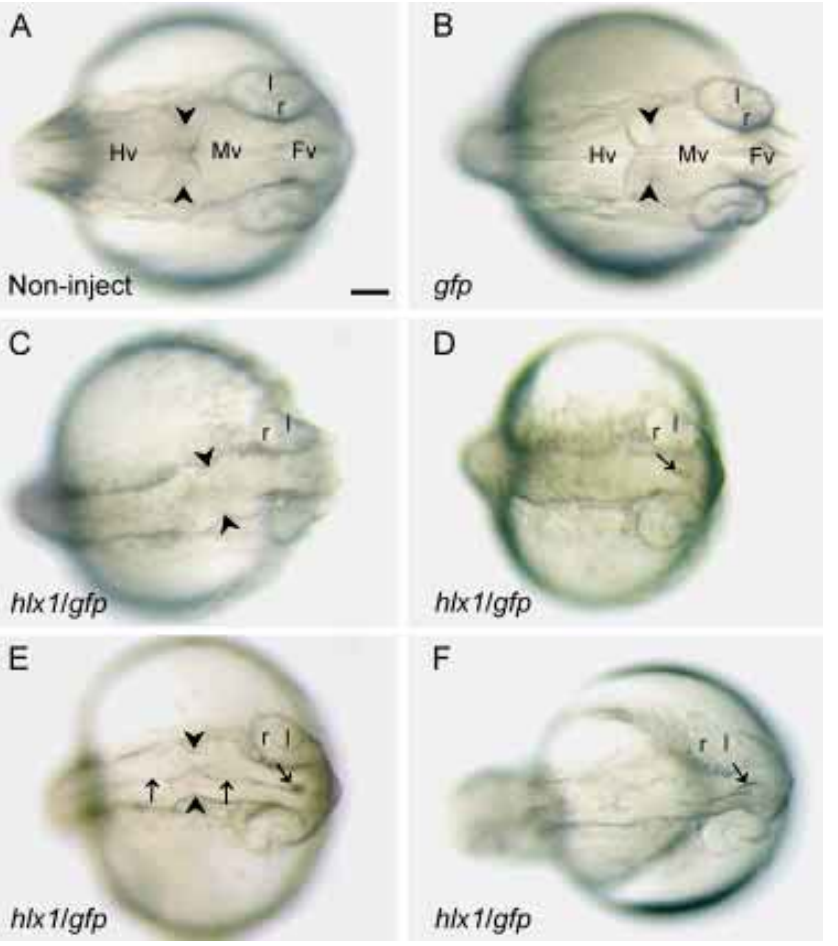

Fig. 1. Mis-expression of *hlx1* produces a fused-brain phenotype. *Dorsal views of the brain in live 24 hpf zebrafish embryos; rostral is to the right in all panels. In non-injected* **(A)** *and control gfp injected* **(B)** *animals, three prominent ventricles are present in the brain: the forebrain ventricle (Fv), the midbrain ventricle (Mv) and the hindbrain ventricle (Hv). The close apposition of the neuroepithelium is also evident at the midbrain/hindbrain boundary (arrowheads).* **(C-F)** *Embryos injected with hlx1 and gfp exhibit a fused-brain phenotype. In some cases, remnants of ventricles (arrows) and the midbrain/hindbrain boundary (arrowheads) are apparent. l, lens; r, retina. Bar, 400 μm.*

In the present study we have adopted two approaches to begin to understand the role of *hlx1* in the zebrafish brain. First, we mis-expressed *hlx1* by microinjecting synthetically generated *hlx1* RNA into 1-2 cell stage embryos. Second, we generated *hlx1* loss-of-function embryos by microinjection of antisense morpholino oligonucleotides directed against *hlx1* mRNA. Embryos mis-expressing *hlx1* exhibited a specific "*fused-brain*" phenotype involving abnormal ventricle formation. Anterio-posterior patterning in these embryos was normal despite disruptions in the template of axon tracts and a reduction in the size of forebrain neuronal clusters. Hlx1 loss-of-function affected hindbrain morphogenesis and the patterning of *zash1B* expression in specific rhombomeres. Our data indicate that *hlx1* has multiple functional roles in brain morphogenesis in zebrafish.

Results

hlx1 is a homeobox gene which exhibits a dynamic spatiotemporal expression pattern during embryonic development of the zebrafish brain (Fjose *et al.*, 1994; Hjorth and Key, 2001). *dbx1*, the mouse homologue of *hlx1*, plays an important role in the development of subpopulations of neurons in the developing spinal cord (Pierani *et al.*, 1999), while its *Xenopus* homologue, *Xdbx,* is also an essential part of the neurogenic pathway (Gershon *et al.*, 2000). To date these reports have only dealt with understanding the role that these genes play in the developing spinal cord. Here we examine the function of *hlx1* during development of the zebrafish brain. Our strategy involves two different approaches. First, we microinject the full length coding mRNA of *hlx1* to overexpress this gene in the developing embryo and, second, we microinject specific antisense morpholino oligonucleotides to reduce its expression.

Cloning of Zebrafish hlx1

To begin to understand the role of *hlx1* in zebrafish brain development we first isolated a full length cDNA for this gene. A segmentation period zebrafish cDNA library was screened using a

partial clone of *hlx1* (Fjose *et al.*, 1994). Three cDNA fragments of approximately the same size were isolated. DNA sequencing and analysis confirmed that all three fragments contained the full length coding region of *hlx1* and differed only in the lengths of 5' untranslated regions. During this time Seo *et al.* (1999) published the full coding sequence of this gene. Sequence analysis confirmed that our isolated cDNAs were identical to the published sequence.

Mis-Expression of hlx1 *leads to a* Fused-Brain *Phenotype*

We mis-expressed *hlx1* by microinjecting synthetic capped RNA into 1-2 cell stage zebrafish embryos. RNA encoding green fluorescence protein (*gfp*) was co-injected as a reporter molecule. By 24 hours post-fertilization (hpf) in both control non-injected and control *gfp* injected animals, the brain is well developed and three ventricles are clearly evident: the forebrain ventricle (Fv, Fig. 1 A,B), the midbrain ventricle (Mv, Fig. 1 A,B) and the hindbrain ventricle (Hv, Fig. 1 A,B). In addition, the eyes are well developed with the lens (l, Fig. 1 A,B) and retina (r, Fig. 1 A,B) easily distinguishable. At this age the close apposition of the two sides of the brain is obvious at the midbrain/hindbrain boundary (arrowheads, Fig. 1 A,B). In control *gfp* injected embryos, approximately 10% exhibited non-specific developmental defects at 24 hpf (Table 1). When *hlx1* RNA was injected a highly penetrant mutant phenotype was observed (Table 1; Fig. 1 C-F). The brain ventricles clearly failed to develop in embryos mis-expressing *hlx1*. This led to the appearance of the two sides of the brain being fused along the midline with an obvious loss of the midbrain/hindbrain constriction, a phenotype we refer to as "*fused-brain*" (Fig. 1 C-F). Despite these abnormalities, the eyes still appeared to form normally. Although remnants of the ventricles were sometimes present, these were dramatically reduced in size (arrows, Fig. 1 D-F). Some animals also displayed a rudimentary constriction at the putative midbrain/hindbrain boundary (arrowheads, Fig. 1 C,E). When 230 pg of *hlx1* RNA was injected into 1-2 cell stage embryos 44% exhibited this "*fused-brain*" phenotype at 24 hpf (Table 1). This penetrance increased to 70% when up to 1000 pg of *hlx1* RNA was injected (Table 1). Approximately 10% of animals exhibited non-specific defects similar to those observed in *gfp* injected animals (Table 1). These results clearly demonstrate that mis-expression of *hlx1* leads to a specific morphogenic defect involving malformation of the ventricular system of the brain.

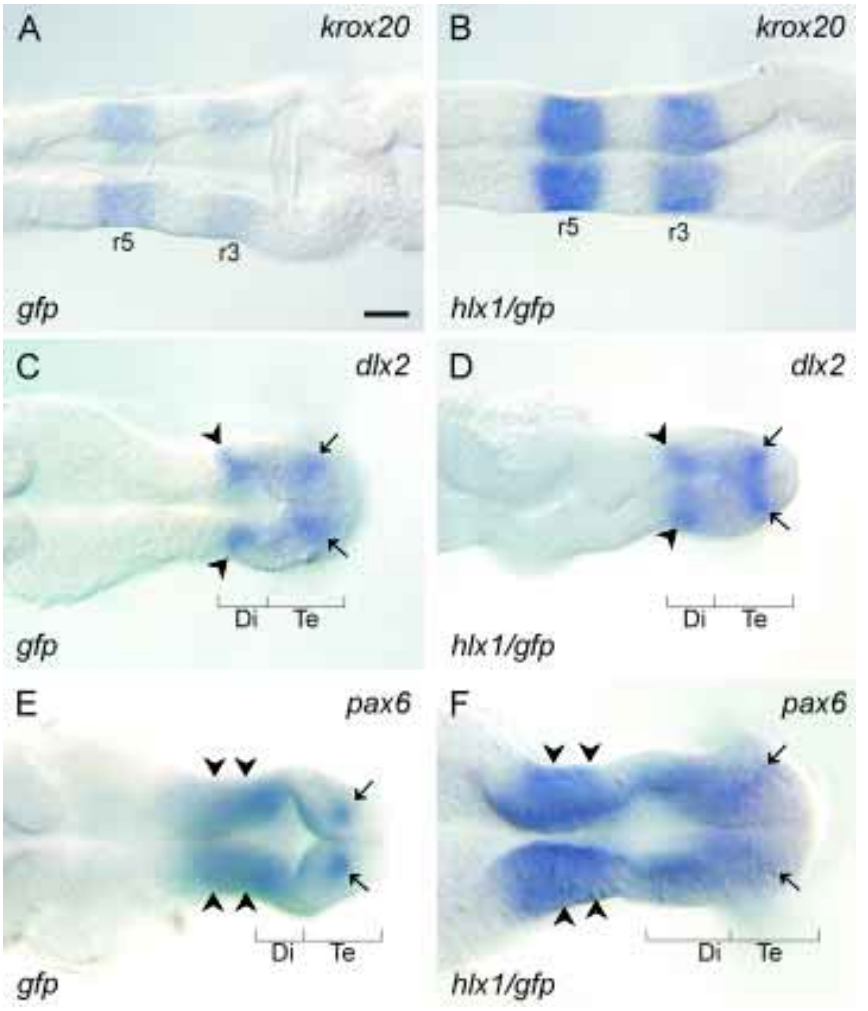

Fig. 2. Expression of anterio-posterior markers is unaffected by *hlx1* mis-expression. *Dorsal views of dissected 24 hpf zebrafish hindbrain (A,B) or rostral brain (C-F). Rostral is to the right in all panels.* **(A)** *gfp and* **(B)** *hlx1/gfp injected animals exhibit normal expression of krox20 in rhombomeres 3 (r3) and 5 (r5).* **(C)** *gfp and* **(D)** *hlx1/gfp injected animals exhibit normal expression of dlx2 in the telencephalon (arrows) and diencephalon (arrowheads).* **(E)** *gfp and* **(F)** *hlx1/gfp injected animals exhibit normal expression of pax6 in the telencephalon (arrows) and diencephalon (arrowheads). Di, diencephalon; Te, telencephalon; r3, rhombomere 3; r5, rhombomere 5. Bar, 70 μm.*

TABLE 1

MIS-EXPRESSION OF *HLX1* LEADS TO A *FUSED-BRAIN* PHENOTYPE IN ZEBRAFISH EMBRYOS

RNA injected	Amount (pg)	Survived to 24 hpf	Normal Morphology	General Defects	Fused-brain
gfp	230[a]	93	85 (91%)	8 (9%)	0 (0%)
	230 - 1000	150	135 (90%)	15 (10%)	0 (0%)
hlx1[b]	230	125	57 (46%)	13 (10%)	55 (44%)
	230 – 1000[c]	332	76 (23%)	22 (7%)	234 (70%)

[a] we routinely inject animals with up to 230pg of RNA. In latter experiments we injected up to 1000 pg without non-specific defects.
[b] 100 pg of *gfp* RNA was coinjected with *hlx1* as a marker of successful injections
[c] data for all injections greater than 230pg were pooled. While the phenotype remained constant, the penetrance did increase.

Brain Patterning is unaffected by Mis-Expression of hlx1

The *fused-brain* phenotype observed in *hlx1* injected animals led us to ask whether the patterning of the three major subdivisions of the brain was perturbed. The presence of normal eyes in *hlx1* injected animals suggested that forebrain regions continued to develop, while the rudimentary constriction at the midbrain/hindbrain boundary in some embryos suggested that the posterior brain was perhaps patterned correctly. To examine patterning in more detail we analysed zebrafish wholemounts by in situ hybridisation for the expression of the region-specific markers *krox20* (hindbrain), *pax6* (midbrain) and *dlx2* (forebrain; Fig. 2).

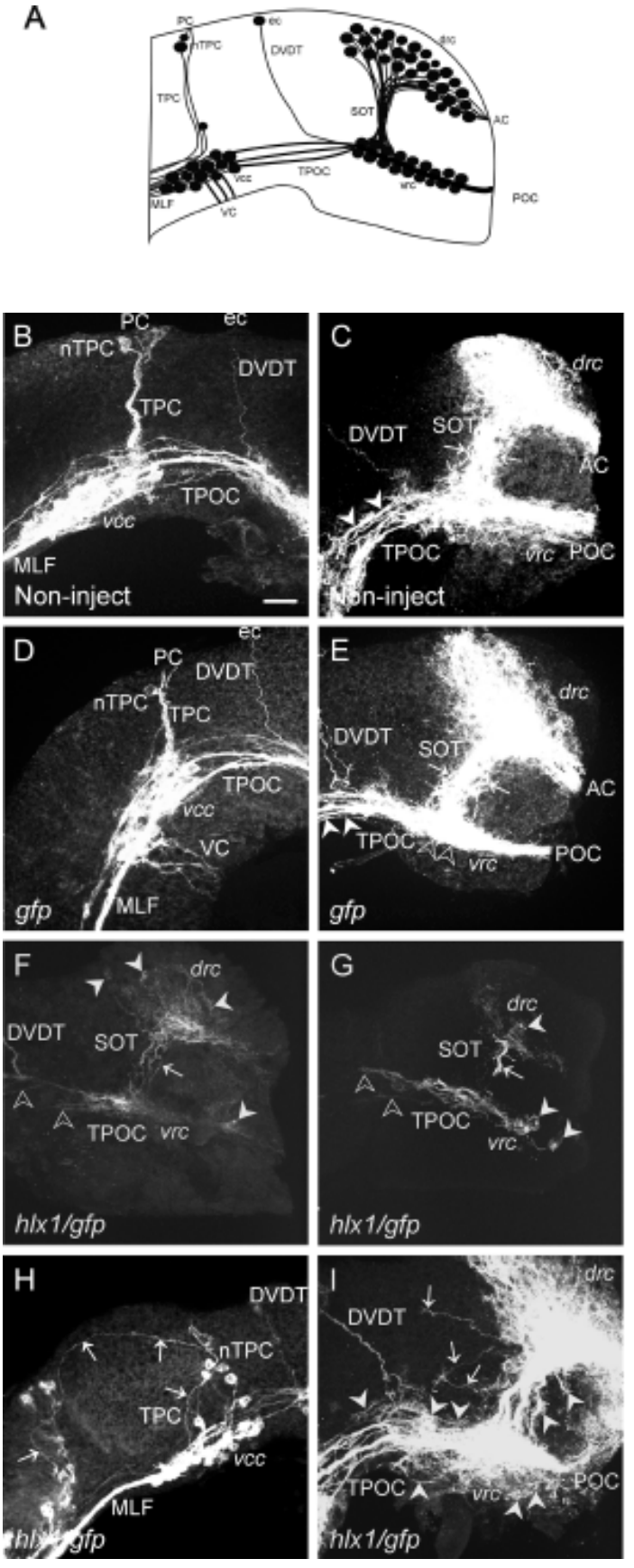

TABLE 2

MIS-EXPRESSION OF *HLX1* LEADS TO BOTH AN ABNORMAL AXON SCAFFOLD AND AN ABNORMAL NEURAL PATTERNING PHENOTYPE

RNA injected	Total number examined[a]	Normal neural phenotype	Abnormal axon scaffold[b]	Few neurons in drc and vrc	Total abnormal
Non-injected	22	22 (100%)	0 (0%)	0 (0%)	0 (0%)
gfp	20	20 (100%)	0 (0%)	0 (0%)	0 (0%)
hlx1	42	13 (31%)	15 (36%)	14 (33%)	29 (69%)

[a] Animals were injected with up to 230pg RNA as reported in Table 1.
[b] Animals were not sorted for fused brain phenotypes prior to scoring for axon tract defects.

In all *hlx1* injected animals examined, the three genetic markers were expressed appropriately, despite the morphological defects in the brain. Dorsal views of labeled embryos revealed that *krox20* was clearly expressed in rhombomeres three and five in both GFP and *hlx1* injected animals (Fig. 2 A,B; Oxtoby and Jowett, 1993). This indicates that not only are rhombomeres three and five correctly patterned, but the boundaries between rhombomeres 2/3, 3/4, 4/5 and 5/6 are maintained in *hlx1* injected embryos.

dlx2 expression was similar in both *gfp* and *hlx1* injected embryos. When viewed dorsally in *gfp* control embryos, *dlx2* expressing cells in the forebrain appear as a single patch in both the telencephalon (arrows, Fig. 2C) and the diencephalon (arrowheads, Fig. 2C) on either side of the brain. This expression was identical to the expression of *dlx2* in *hlx1* injected animals (arrows and arrowheads, Fig. 2D), although the two patches of cells expressing the gene on either side of the brain are more closely apposed.

pax6 expression marks both the midbrain and the forebrain in 24 hpf zebrafish embryos (Krauss *et al.*, 1991). In control *gfp* injected embryos *pax6* was expressed throughout the neuroepithelium in the midbrain (arrowheads, Fig. 2E), while in the forebrain it was

Fig. 3. Mis-expression of *hlx1* results in abnormalities in the axon scaffold of the zebrafish brain. *Rostral is to the right and dorsal is to the top in all panels.* **(A)** *Schematic representation of the first neuronal clusters and the early axon scaffold in the zebrafish brain at 24 hpf. At this age four neuronal clusters are present in the brain: the ventro-caudal cluster (vcc); the ventro-rostral cluster (vrc); the dorso-rostral cluster (drc); the ec, epiphyseal cluster and the nucleus of the tract of the posterior commissure (nTPC). These neuronal clusters are interconnected by a simple scaffold of axon tracts and commissures: the tract of the post-optic commissure (TPOC); the supra-optic tract (SOT); the dorso-ventral diencephalic tract (DVDT); the tract of the posterior commissure (TPC); the medial longitudinal fasciculus (MLF); the ventral commissure (VC); the post-optic commissure (POC); the posterior commissure (PC) and the anterior commissure (AC). (B-I) Projected confocal z-series images of lateral views of 24 hpf dissected zebrafish brains immunolabeled for expression of HNK-1 (white).* **(B,C)** *Non-injected embryos;* **(D,E)** *gfp injected and* **(F-I)** *hlx1/gfp coinjected embryos. (B-E) In control animals HNK-1 staining reveals a simple set of axon tracts and commissures. Axons of the SOT are tightly fasciculated (arrows), while axons of the TPOC defasciculate (arrowheads) caudal to their intersection with axons of the DVDT.* **(F,G)** *33% of animals coinjected with* hlx1 *and* gfp *RNA exhibit fewer neurons in forebrain neuronal clusters (filled arrowheads), despite axons of the SOT (arrows) and TPOC (unfilled arrowheads) projecting appropriately.* **(H,I)** *36% of animals coinjected with* hlx1 *and* gfp *RNA exhibited abnormal axon scaffolds. In some cases a subpopulation of axons in the TPC and SOT projected into inappropriate regions of the brain (arrows). In other cases axons of the TPOC and the SOT appeared to be disorganised (arrowheads). Bar, 20 μm.*

bilaterally expressed in a patch of cells (arrows, Fig. 2F). In *hlx1* injected embryos the pattern of *pax6* expression was unchanged (arrows and arrowheads, Fig. 2F).

In summary, these data suggest that even though embryos injected with *hlx1* mRNA exhibit a *fused-brain* phenotype, the correct anterio-posterior patterning of the developing brain is preserved.

Mis-Expression of hlx1 *leads to Abnormal Development of Neuronal Clusters and their Axons*

Next we examined the effect of *hlx1* mis-expression on development of specific clusters of neurons and their associated axon tracts in the embryonic brain (Fig. 3A). Immunohistochemical staining of wholemounts of zebrafish brains using antibodies against the HNK-1 carbohydrate epitope enabled the visualization of all neurons and their axons (Metcalfe *et al.*, 1990; Hjorth and Key, 2001). In 24 hpf non-injected and *gfp* injected control embryos the first neuronal clusters and associated axon tracts developed normally (Table 2; Fig. 3 B-E). At this age five neuronal clusters are present: the dorso-rostral cluster (dr*c*); the ventro-rostral cluster (vrc); the ventro-caudal cluster (vcc); the epiphyseal cluster (ec) and the nucleus of the tract of the posterior commissure (nTPC; Fig. 3 B-E; Ross *et al.*, 1992). By 24 hpf neurons in these clusters have extended axons which form a highly stereotypical scaffold of axon tracts consisting of five distinct tracts and four commissures: the supra-optic tract (SOT); the tract of the post-optic commissure (TPOC); the medial longitudinal fasciculus (MLF); the tract of the posterior commissure (TPC); dorso-ventral diencephalic tract (DVDT); the anterior commissure (AC); the post-optic commissure (POC); the posterior commissure (PC) and the ventral commissure (VC; Fig. 3 B-E).

In 69% of embryos misexpressing *hlx1* we observed abnormalities in either development of the drc and vrc in the forebrain or formation of the axon scaffolding (Table 2; Fig. 3). In 33% of animals examined, the size of the drc and vrc was consider-

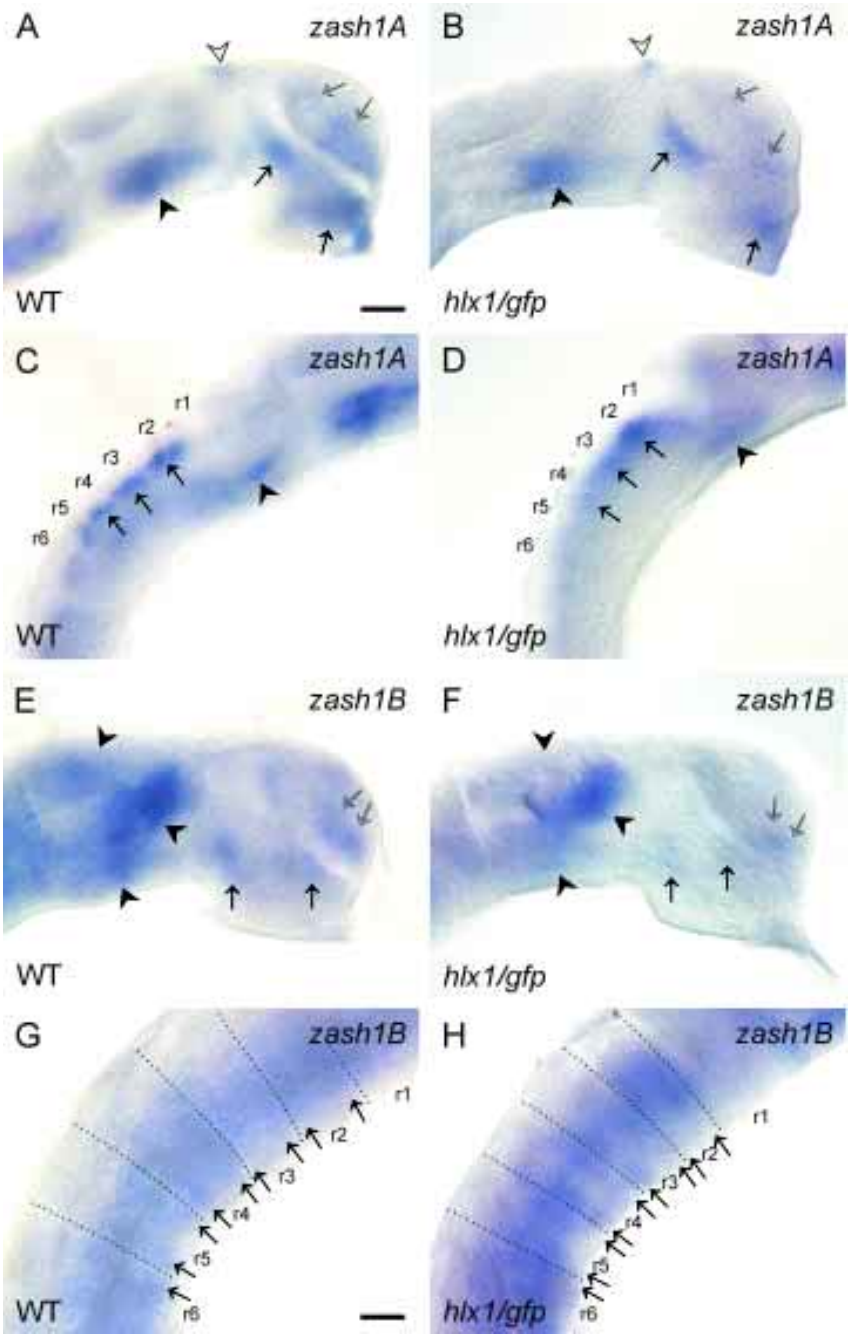

Fig. 4. Mis-expression of *hlx1* results in altered expression patterns of *zash1A* and *zash1B* in the forebrain. *Lateral views of rostral (A,B,E,F) and caudal (C,D,G,H) dissected zebrafish brains at 24 hpf labelled for* zash1A *(A-D) or* zash1B *(E-H) expression by* in situ *hybridisation. Rostral is to the right and dorsal is to the top in all panels. Stippled lines in (G,H) mark the rhombomere boundaries.* **(A)** *Rostral brain of wild-type zebrafish showing expression of* zash1A *in the ventral midbrain (filled arrowhead), the epiphysis (unfilled arrowhead), the diencephalon (filled arrows) and the telencephalon (unfilled arrows).* **(B)** *Rostral brain of* gfp/hlx1 *coinjected zebrafish showing that expression of* zash1A *is unchanged in the ventral midbrain (filled arrowhead) and the epiphysis (unfilled arrowhead).* zash1A *expression in the diencephalon (filled arrows) and telencephalon (unfilled arrows) appears to be reduced.* **(C)** *Hindbrain of wild-type animal showing expression of* zash1A *in the ventral region of rhombomere 1 (r1, arrowhead) and the dorsal regions of rhombomeres 2-6 (r2-6). This gene is more strongly expressed in more anterior rhombomeres (arrows).* **(D)** *Hindbrain of* hlx1/gfp *coinjected animal showing that expression of* zash1A *is unchanged. This gene is still expressed in the ventral region of rhombomere 1 (arrowhead) and the dorsal regions of rhombomeres 2-6, with its strongest expression occurring in more anterior rhombomeres (arrows).* **(E)** *Rostral brain of wild-type animal showing expression of* zash1B. zash1B *is expressed throughout the midbrain (arrowheads), in the diencephalon (filled arrows) and in the telencephalon (unfilled arrows).* **(F)** *Rostral brain of* hlx1/gfp *coinjected animal labelled for* zash1B *expression.* zash1B *is still expressed throughout the midbrain (arrowheads) but its expression is reduced in the diencephalon (filled arrows) and the telencephalon (unfilled arrows).* **(G)** *Hindbrain of wild-type animal labelled for* zash1B *expression. This gene is expressed at the borders of each rhombomere (arrows).* **(H)** *Hindbrain of* hlx1/gfp *coinjected embryo showing expression of* zash1B. *This gene is still expressed at the borders of each rhombomere (arrows). Bars, 70 μm (A-F); 20 μm (G,H).*

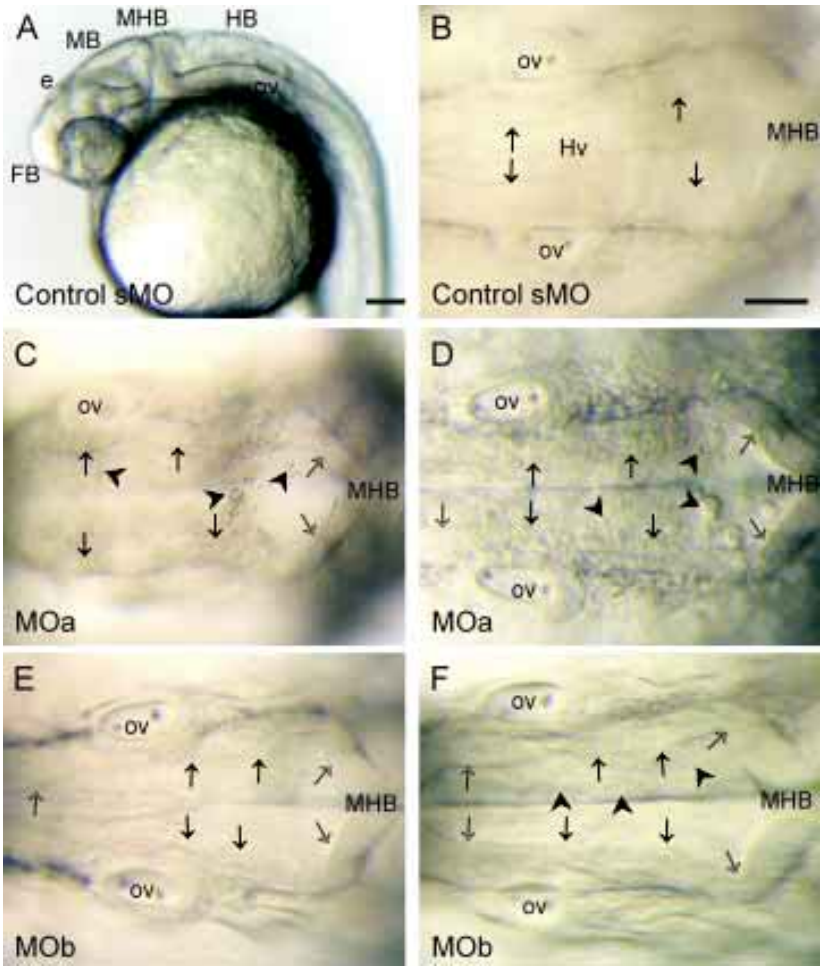

Fig. 5. Specific antisense *hlx1* morpholinos alter hindbrain morphology. (A) *Lateral view of a live 24 hpf zebrafish embryo microinjected with the control sMO. Rostral is to the left and dorsal is to the top. (B-F) Dorsal views of the hindbrain in live control sMO injected (B); hlx1 MOa injected (C,D) and hlx1 MOb injected (E,F) zebrafish embryos at 24 hpf. Rostral is to the left.* **(A)** *Embryos injected with the control sMO exhibit a normal morphology. The forebrain (FB), epiphysis (e), midbrain (MB), midbrain/hindbrain boundary (MHB), hindbrain (HB) and otic vesicle (ov) are all clearly evident.* **(B)** *Hindbrain of live control sMO injected animal. The MHB and ov are most obvious, the hindbrain ventricle (Hv) is large, while the walls of the neural tube are well defined (arrows).* **(C,D)** *Two examples of the hindbrain in MOa injected animals. The MHB and the ov are still evident, but in some cases (A) the neuroepithelium undulated along the anterio-posterior axis. The walls of the neural tube are poorly formed between rhombomeres 2-5 (filled arrows), but are normal in other regions of the hindbrain (unfilled arrows). This phenotype is also characterised by the presence of aberrant cells or vesicles in the hindbrain ventricle (arrowheads).* **(E,F)** *Two examples of the hindbrain of MOb injected embryos. The hindbrain phenotype was less severe than in embryos injected with MOa. The walls of the neural tube were poorly formed between rhombomeres 2-5 (filled arrows), but were unaffected in other regions (unfilled arrows). Cells or vesicles were still evident in the hindbrain ventricle (arrowheads in F). Bars, 400 μm (A); 200 μm (B-F).*

ably reduced (compare filled arrowheads, Fig. 3 F,G with Fig. 3 C,E). Despite this rather severe drop in size of these nuclei, the remaining neurons managed to form a rudimentary template of axon tracts. Axons of the drc grew ventrally to pioneer the SOT (arrows, Fig. 3 F,G), while axons of the vrc pioneered the TPOC (unfilled arrowheads, Fig. 3 F,G). The reduction in the number of neurons in both the vrc and the drc raises the possibility that *hlx1* is involved in a neurogenic pathway in the developing forebrain.

We also observed a less severe phenotype involving 36% of embryos. While the vrc and drc appeared to develop normally in these animals, there were noticeable defects in the trajectory of some axons (Fig. 3 H,I; Table 2). In control embryos the SOT is tightly fasciculated and all axons within this tract are compacted (arrows, Fig. 3 C,E). The TPOC is usually tightly fasciculated in its most rostral portions (unfilled arrowheads, Fig. 3 C,E), but as axons grow caudally they defasciculate into several distinct bundles immediately rostral to its intersection with the DVDT (filled arrowheads, Fig. 3 C,E). In embryos displaying the milder phenotype axons were disorganized as well as being more defasciculated in the TPOC and the SOT (arrows, Fig. 3I). However, these two tracts were still well developed, with many axons correctly coursing ventrally in the SOT and caudally in the TPOC (Fig. 3I). In these animals some axons extended out of the scaffold and into inappropriate surrounding regions. In control animals the TPC is formed by axons growing ventrally from neurons located in the dorsal midbrain (Fig. 3A; arrows, Fig. 3 B,D). In *hlx1* injected animals we observed some TPC axons coursing caudally rather than ventrally (arrows, Fig. 3H). We also observed axons of the SOT coursing caudally away from other axons of the SOT (arrowheads, Fig. 3I).

Mis-Expression of hlx1 *alters the Expression Patterns of* zash1A *and zash1B*

Next we investigated the possibility that the reduction in neuron number in the *vrc* and *drc* following *hlx1* mis-expression may be accompanied by changes in expression of zebrafish neurogenic genes. The *Drosophila melanogaster achaete-scute* complex genes are important for the correct spatial patterning of neural cells in both the peripheral and central nervous systems of the fruit fly (Skeath and Carroll, 1992). These genes, and their homologues in other species, are transcription factors which contain a basic helix-loop-helix motif and induce the expression of specific neurogenic genes within a population of proneural cells (Chitnis and Kinter, 1996; Ferreiro *et al.*, 1994; Zimmerman *et al.*, 1993). The expression pattern of two homologues of the *achaete scute* complex in zebrafish, *zash1A* and *zash1B*, within early neuronal clusters suggests that these molecules may play a role in zebrafish brain development (Allende and Weinberg, 1994). We therefore examined the expression of *zash1A* and *zash1B* in *hlx1* injected embryos (Fig. 4). In control non-injected 24 hpf zebrafish embryos *zash1A* is expressed in all major subdivisions of the brain (Fig. 4 A,C). In the forebrain it is expressed in the telencephalon (unfilled arrows, Fig. 4A), ventral diencepahlon (filled arrows, Fig. 4A) as well as in the epiphysis (unfilled arrowhead, Fig. 4A), while in the midbrain it is strongly expressed in the tegmentum (filled arrowhead, Fig. 4A). In the hindbrain *zash1A* is expressed in the dorsal regions of each rhombomere, but is more strongly expressed in rhombomeres 2-4 (arrows, Fig. 4C). Also at this age *zash1A* is strongly expressed in the ventral region of rhombomeres 1 and 2 (arrowhead, Fig. 4C). In embryos in which *hlx1* had been mis-expressed, the spatial

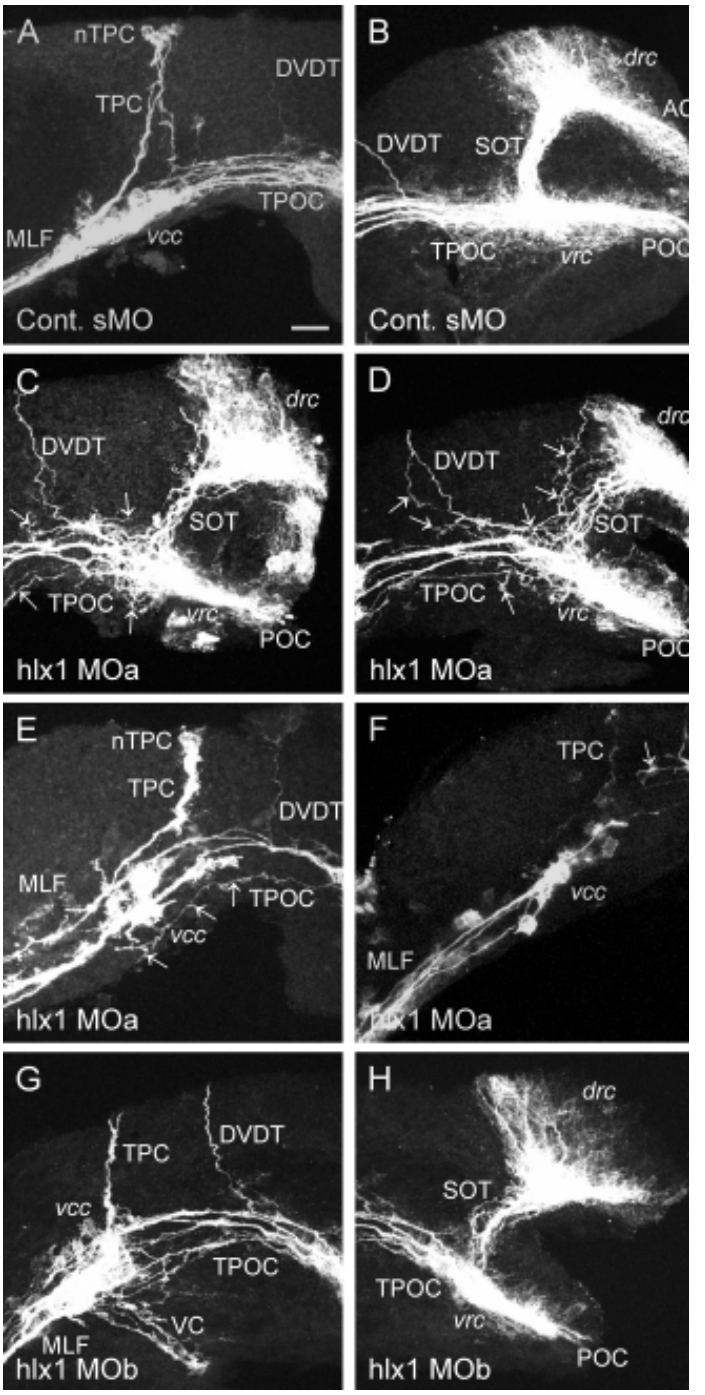

Fig. 6. Microinjection of *hlx1* specific antisense morpholinos results in some axon scaffold defects. *Projected confocal z-series images of lateral views of 24 hpf dissected zebrafish brains immunolabeled for expression of HNK-1 (white). Rostral is to the right and dorsal is to the top in all panels. Panels (A,E,F,G) are images of the midbrain, while (B,C,D,H) are images of the rostral brain.* **(A,B)** *Control sMO injected embryos exhibit normal neuronal clusters, axon tracts and commissures: the ventro-caudal cluster (vcc); the ventro-rostral cluster (vrc); the dorso-rostral cluster (drc); the ec, epiphyseal cluster and the nucleus of the tract of the posterior commissure (nTPC); the tract of the post-optic commissure (TPOC); the supra-optic tract (SOT); the dorso-ventral diencephalic tract (DVDT); the tract of the posterior commissure (TPC); the medial longitudinal fasciculus (MLF); the ventral commissure (VC); the post-optic commissure (POC); the posterior commissure (PC) and the anterior commissure (AC).* **(C-F)** *59% of* hlx1 *MOa injected animals exhibit disorganised axon tracts (arrows).* **(G,H)** *85% of animals injected with MOb exhibited no axonal scaffold defects. Bar, 20 μm.*

expression pattern of *zash1A* did not dramatically change. *zash1A* continued to be expressed in the telencephalon, ventral diencephalon, epiphysis and ventral tegmentum (Fig. 4B). However, there was clearly a reduction in the level of expression in the rostral brain, particularly in the telencephalon (unfilled arrows, Fig. 4 A,B) and ventral diencephalon (small filled arrows, Fig. 4 A,B). In the hindbrain *zash1A* continued to be expressed in the dorsal regions of rhombomeres 2-4 and the ventral regions of rhombomere 1, but its pattern was not as clearly defined as in control animals (compare arrows and arrowheads, respectively, Fig. 4 C,D).

In non-injected control animals *zash1B* is expressed by cells in all major subdivisions of the zebrafish brain (Fig. 4 E,G). It is expressed by small patches of cells in the telencephalon (unfilled arrows, Fig. 4E) and the diencepahlon (filled arrows, Fig. 4F) while it is also expressed throughout the midbrain at this age (arrowheads, Fig. 4E). In the hindbrain *zash1B* is expressed at the boundaries of rhombomeres 2-6, so that each rhombomere exhibits two stripes of expression at their anterior and posterior margin (arrows, Fig. 4G). In embryos in which *hlx1* had been mis-expressed we found that the overall expression pattern of *zash1B* did not alter (Fig. 4 F,H). This gene was still expressed in the telencephalon, diencephalon, throughout the midbrain and toward the boundaries of rhombomeres 2-6 (Fig. 4 F,H). Most notable, however, was the reduction in the level of staining in the rostral brain (compare arrows in Fig. 4 E,F).

hlx1 Antisense Morpholinos perturb Hindbrain Morphology

Two antisense morpholino oligonucleotides (MOa and MOb) designed against the 5' untranslated region and the translation start site of the *hlx1* cDNA were individually injected into zebrafish embryos at the 1-2 cell stage. A morpholino (sMO) consisting of scrambled sequence was used as a negative control. There was no difference between the survival rates of embryos injected with any of the morpholinos. In animals injected with control sMO 8% showed non-specific defects similar to those seen in animals injected with either *gfp* or *hlx1* mRNA (Table 3). The morphology of the remaining 92% of animals injected with the control sMO was indistinguishable from wild-type animals (Table 3; Fig. 5 A,B). In these animals the two sides of the hindbrain are separated by a large ventricle (Hv, Fig. 5B) and the otic vesicle (ov, Fig. 5B) is correctly positioned at the level of rhombomeres 4 and 5 (Fig. 5B). The rhombic lips are clearly visible in live wholemount embryos when viewed with oblique trans-illumination (arrows, Fig. 5B).

TABLE 3

ANTISENSE MORPHOLINOS DESIGNED AGAINST *HLX1* MRNA PRODUCE AN ABNORMAL HINDBRAIN

Injected MO	Amount injected (ng)	Total injected	Normal morphology	Non-specific defects	Hindbrain defects
control sMO	8-22[a]	203	186 (92%)	10 (8%)	0 (0%)
α*hlx1* MOa	8	139	21 (15%)	3 (2%)	115 (83%)
α*hlx1* MOb	8-22[a]	190	92 (48%)	15 (8%)	83 (44%)

[a] Data were pooled over this range since phenotypes were identical.

Injection of MOa caused a highly penetrant phenotype specifically in the hindbrain. In 83% of injected embryos the hindbrain was severely malformed (Table 3). Most notable was a deformity in the walls of the neural tube such that they were either undulating or in some cases the rhombic lips were poorly defined (Fig. 5 C,D). Small cells or membrane vesicles were scattered throughout the hindbrain of animals injected with MOa (arrowheads, Fig. 5 C,D). These structures were never observed in control animals (Fig. 5B). In all embryos which had been injected with MOa we did not observe any disruptions to the morphology of other regions of the brain apart from the hindbrain. In fact these defects were restricted to rhombomeres 2-5 whereas more rostrally the rhombic lips appeared to be well formed (unfilled arrows, Fig. 5 C,D).

In order to confirm the specificity of the effect of MOa we designed a second morpholino (MOb) against the 5' untranslated region of the *hlx1* RNA which did not overlap with the target sequence of MOa. MOb was microinjected into 1-2 cells stage zebrafish embryos and animals were again allowed to survive until 24 hpf. In these embryos 8% exhibited non-specific defects similar to those observed with our other microinjection experiments (Table 3). In these experiments MOb recapitulated the hindbrain phenotype previously obtained with MOa, however the penetrance was weaker at 44% (Table 3; Fig. 5 E,F). Although the hindbrain defects produced by MOb were also less severe than those obtained with MOa, the rhombic lips were clearly deformed and membrane vesicles were present throughout the neuroepithelium (compare Fig. 5 C,D with 5 E,F). The sharp borders of the rhombic lips were clearly evident in the rostral and caudal regions of the hindbrain (unfilled arrows, Fig. 5 E,F). In the absence of antibodies against zebrafish *hlx1* we have been unable to assess the extent of reduction of endogenous *hlx1* translation by either of the two morpholinos. However, the similarity in phenotypes and their restriction to sites of *hlx1* expression in the hindbrain indicates the potency of this approach. Moreover, the use of two distinct morpholinos directed against separate 5' regions of the *hlx1* RNA strongly supports the specificity of the phenotypic effects. In fact, the use of two morpholinos generated against separate 5' untranslated regions has emerged as the most important control for specificity (Ekker and Larson, 2001; Sumanas *et al.*, 2001).

Axon Guidance Defects following Injection of hlx1 Antisense Morpholinos

Although the morphological defects observed following injection of *hlx1* morpholinos were restricted to the hindbrain, we were interested in determining whether the scaffold of axon tracts was normal in these animals. In all control sMO injected animals examined the neuronal clusters and the axon scaffold developed normally as previously described

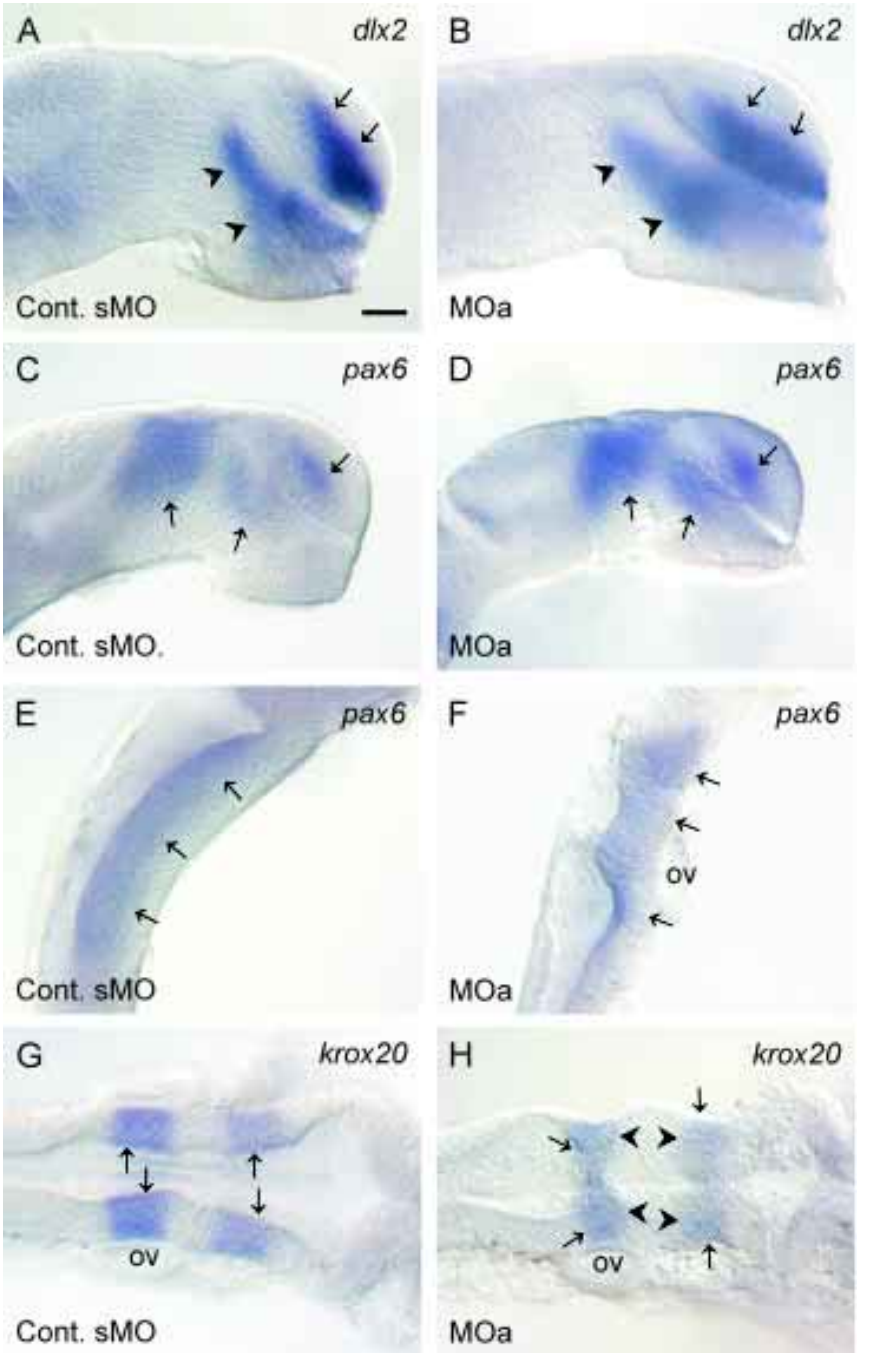

Fig. 7. Anterio-posterior marker expression is unaffected by microinjection of specific antisense *hlx1* morpholinos. *Lateral (A-D), dorso-lateral (E, F) and dorsal (G, H) views of dissected zebrafish brains at 24 hpf labelled for the expression of common marker genes by* in situ *hybridisation. Rostral in all panels, dorsal is to the top in panels (A-D).* **(A)** *Control sMO injected animal labeled for expression of* dlx2 *in the telencephalon (arrows) and diencephalon (arrowheads).* **(B)** *In MOa injected animals* dlx2 *expression is still present in the telencephalon (arrows) and diencephalon (arrowheads).* **(C)** *Expression of* pax6 *in the rostral brain of control sMO injected animals (arrows).* **(D)** *Expression of* pax6 *in the rostral brain of MOa injected animals is unchanged (arrows).* **(E)** pax6 *is expressed diffusely throughout the neuroepithelium of the hindbrain (arrows) in control sMO embryos.* **(F)** *In MOa injected animals* pax6 *is still expressed diffusely throughout the neuroepithelium (arrows).* **(G)** *In control sMO injected animals* krox20 *is expressed in rhombomeres 3 and 5 (arrows).* **(H)** *Similar expression of* krox20 *(arrows) is observed in MOa injected animals. The borders of* krox20 *expression (arrowheads) are also maintained. ov, otic vesicle. Bar, 70 μm.*

TABLE 4

***HLX1* ANTISENSE MORPHOLINO OLIGONUCLEOTIDES INDUCE AXON SCAFFOLD DEFECTS IN THE ZEBRAFISH BRAIN**

MO injected	Total number examined[a]	Normal neural phenotype[b]	Abnormal neural phenotype
control sMO	17	17 (100%)	0 (0%)
α*hlx1* MOa	22	9 (41%)	13 (59%)
α*hlx1* MOb	13	11 (85%)	2 (15%)

[a] Animals were injected with the same amounts as reported in Table 3.
[b] Animals were not sorted for hindbrain defects prior to scoring for axon tract defects

(Fig. 6 A,B). In marked contrast, 59% of animals injected with *hlx1* MOa exhibited abnormalities in the development of their axon scaffold (Table 4; Fig. 6 C-F). While all of the major axon tracts were present in these animals the normal stereotypical pattern of growth was disrupted. Axons tracts appeared disorganised as axons grew along aberrant pathways and entered and exited tracts at inappropriate positions (arrows, Fig. 6 C-F). Despite these abnormalities we did not observe any defects in either the patterning or the size of the neuronal clusters. Next we examined the effect of MOb on the development of the axon tracts in the embryonic brain. As with MOa we did not observe any effect of MOb on the formation of the neuronal clusters. In MOb injected animals only 15% exhibited an axon growth defects as was described for MOa (Table 4). Most animals (85%) injected with MOb had a normal stereotypical axonal scaffold (Fig. 6 G-H). These data are consistent with the weaker penetrance of MOb that we observed in the hindbrain.

Hindbrain Patterning is Normal following Injection of hlx1 *Antisense Morpholinos*

We next examined whether the patterning of the brain was affected by the loss of *hlx1*. Expression patterns of both *dlx2* and *pax6* in the rostral brain following injection of either MOa or MOb were identical to those observed in sMO injected controls (Fig. 7 A-D). In the hindbrain, *pax6* is expressed diffusely throughout the neuroepithelium of sMO injected embryos (arrows, Fig. 7E). In MOa injected animals *pax6* continues to be expressed throughout the neuroepithelium (arrows, Fig. 7F), even though the hindbrain is poorly developed. *krox20* is expressed in rhombomeres 3 and 5 in wild-type and control sMO injected animals (Fig. 7G). Its expression is clearly evident in both rhombomeres on either side of the neural tube (arrows, Fig. 7E). In control animals rhombomere 5 develops at the level of the middle of the otic vesicle as shown by *krox20* expression (Fig. 7G). In MOa injected embryos *krox20* continues to be expressed in two distinct bands as in controls (arrows, Fig. 7F). The posterior expression domain occurs at the level of the otic vesicle which is consistent with the position of rhombomere 5. There is also a clear boundary established between *krox20* expressing cells and *krox20* non-expressing cells in this region of the hindbrain, despite its aberrant morphology (arrowheads, Fig. 7F). These data indicate that that formation of rhombomeres or rhombomere-like compartments is independent of *hlx1* expression.

hlx1 *Antisense Morpholinos specifically affect* zash1B *Expression*

Although our earlier experiments showed that mis-expression of *hlx1* did not alter the expression pattern of either *zash1A* or *zash1B*, we were now interested in the effect of the *hlx1* antisense morpholinos on expression of these neurogenic genes. The expression pattern of *zash1A* and *zash1B* in control sMO injected animals was identical to that in control uninjected animals throughout the brain (Fig. 4). Likewise, the expression patterns of these molecules in the forebrain and midbrain were unaffected by injection of either MOa or MOb (compare arrowheads, Fig. 8 A-C and G-I). In the hindbrain of control sMO injected animals *zash1A* is diffusely expressed across all rhombomeres, although somewhat more strongly in the rostral rhombomeres (arrows, Fig. 8 A,D). In MOa and MOb injected animals we did not detect any alteration in the overall pattern of *zash1A* expression in the hindbrain, apart from a slightly more clearer definition of anterior staining in MOb injected animals (Fig. 8 D-F). In contrast, there were marked changes in the expression pattern of *zash1B* following injection of either MOa or MOb. In the hindbrain of control sMO injected animals *zash1B* is expressed in a series of bands which represent the rostral and caudal borders of rhombomeres 2-6 (arrows, Fig. 8 G,J). These stripes were absent in embryos which had been injected with MOa (Fig. 8 H,K). z*ash1B* was absent from rhombomere 5 (filled arrow, Fig. 8 H,K) and it was only expressed in the ventral region of rhombomere 3 (unfilled arrow, Fig. 8 H,K). Furthermore, in rhombomeres 2, 4, 6 and 7 cells expressing *zash1B* were no longer restricted to the border regions. Instead, *zash1B* was strongly expressed by cells throughout the entire dorso-ventral axis (unfilled arrowheads, Fig. 8 H,K). Similarly in MOb injected animals *zash1B* expression was absent from rhombomere 5 (arrow, Fig. 8 I,L) and restricted to only the ventral region of rhombomere 3 (unfilled arrow, Fig. 8 I,L). Cells expressing *zash1B* in rhombomeres 2, 4, 6 and 7 were also present throughout the dorso-ventral axis of the neural tube (unfilled arrowheads, Fig. 8 I,L).

Discussion

Two complementary approaches were used in the present study in order to assess the role of *hlx1* in zebrafish brain development. First, the spatiotemporal patterning of *hlx1* expression was disrupted by injecting mRNA for *hlx1* into the early embryo. Despite its widespread mis-expression a highly specific *fused-brain* phenotype was produced. This gross morphological abnormality was accompanied by a reduction in the size of the early neuronal cell clusters in the forebrain. Disruptions were also noted in the stereotypical arrangements of axon tracts. Despite these defects, mis-expression of *hlx1* did not disrupt the normal rostrocaudal patterning of the longitudinal neural axis. The role of *hlx1* was subsequently examined by injection of antisense morpholino oligonucleotides into the early embryo. This approach produced a highly specific hindbrain defect. Notably there were deformities in the wall of the hindbrain at the level of rhombomeres 2-5 which were complemented by disruptions to the pattern of expression of the neurogenic gene *zash1B*. While *hlx1* morpholinos did not affect the size of forebrain neuronal clusters there were abnormalities in the axon scaffold.

hlx1 *and Brain Morphogenesis*

The *fused-brain* phenotype obtained after mis-expression of *hlx1* suggests that an early developmental role for this homeodomain protein may be to regulate downstream genes controlling brain morphogenesis. The *fused-brain* is principally characterized by an absence of ventricles and a loss of neurons.

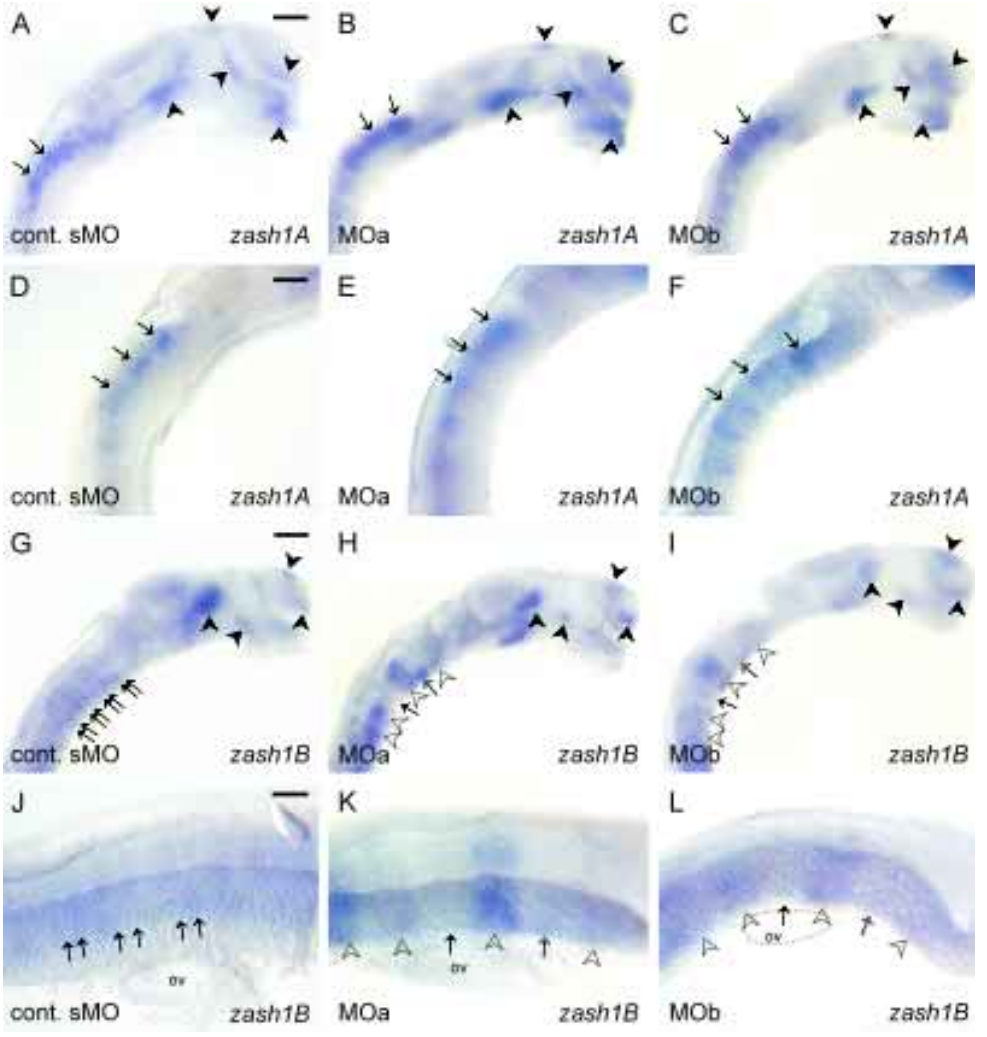

Fig. 8. Microinjection of specific *hlx1* antisense morpholinos alter the expression pattern of *zash1B* but not *zash1A* in the hindbrain. *Lateral views of dissected zebrafish brains labeled for expression of either zash1A or zas1B. Rostral is to the right and dorsal is to the top.* **(A-C)** *Low power images of* **(A)** *control sMO,* **(B)** *MOa and* **(C)** *MOb injected animals labeled for expression of zash1A. Expression of this gene in the hindbrain (arrows) and rostral brain (arrowheads) is unaffected by hlx1 morpholinos.* **(D-F)** *High power images of the hindbrain of* **(D)** *control sMO,* **(E)** *MOa and* **(F)** *MOb injected animals labeled for expression of zash1A. High expression of this gene in the dorsal regions of anterior rhombomeres (arrows) is still present in hlx1 morpholino injected animals.* **(G-I)** *Low power images of* **(G)** *control sMO,* **(H)** *MOa and (I) MOb injected animals labeled for expression of zash1B. Expression of zash1B in the rostral brain (arrowheads) is unaffected by hlx1 morpholinos.* **(J-L)** *High power images of the hindbrain of* **(J)** *control sMO,* **(K)** *MOa and* **(L)** *MOb injected animals labeled for expression of zash1B. (G, J) In the hindbrain of control sMO injected animals zash1B is expressed at the border of rhombomeres 2-6 (arrows).* **(H, I, K, L)** *In the hindbrain of MOa and MOb injected animals zash1B expression is extinguished in rhombomere 5 (filled arrows), restricted to ventral regions of rhombomeres 3 (unfilled arrows) and present throughout the neuroepithelium of other rhombomeres (unfilled arrowheads). Stippled line in panel* **(L)** *indicates the approximate location of the otic vesicle. ov, otic vesicle. Bars: 150 μm (A, B, C, G, H, I); 70 μm (D, E, F, J, K, L).*

In amphibians, chicks and mouse the rostral neural tube develops by the folding of the neural plate into a hollow tube in a process known as primary neurulation. In contrast the development of the caudal spinal cord occurs during secondary neurulation by a process of cavitation of a solid core of cells generated by the tailbud (Griffith *et al.*, 1992). In zebrafish, the rostral neural tube forms by a process similar to secondary neurulation where the neural plate folds to form a lumen-less neural keel before compacting into an axial cord of cells referred to as a neural rod (Papan and Campos-Ortega, 1994). This solid rod of cells undergoes cavitation, initially in the ventral region at 17-18 hpf to form a neurocoel. By 24 hpf the ventricles are clearly formed and their subsequent enlargement appears to depend on production of cerebrospinal fluid as the circulation becomes established (Schier *et al.*, 1996). While a number of different zebrafish mutants with circulatory defects have reduced brain ventricles at 30 hpf (Schier *et al.*, 1996), the *fused-brain* phenotype we observed occurs prior to the development of the circulation. Thus, *hlx1* may have a direct role in brain morphogenesis rather than be secondary to defects in cerebrospinal fluid production. The *fused-brain* phenotype is similar to, although far more severe, than the collapsed ventricles in the *snakehead* mutant (Jiang *et al.*, 1996; Schier *et al.*, 1996).

The morphogenetic mechanisms controlling cavitation of the neural rod are unknown but they are believed to involve complex cell movements including medial migration, radial interdigitation and intercalation (Davidson and Keller, 1999). As *hlx1* is expressed as a ventral midline of the neural rod where cavitation is initiated it is in a position to play a major role in the development of the hollow neural tube. Cavitation of the compacted mammalian embryo to produce a blastocoel depends on cell adhesion and assembly of tight junctions mediated by E-cadherin (Fleming *et al.*, 2000; Fleming *et al.*, 2001). It is tempting to speculate that *hlx1* is regulating the expression of cadherin-like molecules in the ventral neural rod which control cell movements leading to cavitation. In fact several lines of evidence indicate that cadherins are downstream targets of Hox genes (Inoue *et al.*, 1997; Packer *et al.*, 1997; Lincecum *et al.*, 1998). The expression of VN cadherin (the zebrafish homolog of cadherin-11) in the ventral neural rod during cavitation is consistent with this idea. Interestingly, overexpression of either N- or E-cadherin leads to a decrease in cell mixing and the presence of lesions in the neural tube (Detrick *et al.*, 1990; Fujimori *et al.*, 1990; Bitzur *et al.*, 1994). Mice carrying a homozygous mutation in the N-cadherin gene also exhibit severe neural tube defects (Radice *et al.*, 1997). In *Xenopus* F-cadherin is expressed

in domains located at the borders of the major subdivisions of the brain (Espeseth *et al*., 1995). One of these border regions is the sulcus limitans, which separates the alar and basal plates of the caudal neural tube. Functional analyses have revealed that F-cadherin restricts passive cell mixing and may position cells at this boundary (Espeseth *et al*., 1998). The homeobox gene *six3* is expressed in the early gastrulating zebrafish embryo as well as in the anterior neural plate (Kobayashi *et al*., 1998). Overexpression of *six3* mRNA leads to an enlarged forebrain, suggesting that this gene is a regulator of downstream targets involved in anterior brain development. (Kobayashi *et al*., 1998). Thus, both *hlx1* and *six3* may be affecting development of the brain by regulating factors controlling cavitation and ventricle enlargement. While *hlx1* gain-of-function had obvious effects on brain morphology, the morpholino oligonucleotides did not disrupt the formation of the rostral brain. The absence of a phenotype in these loss-of-function experiments suggests some form of redundancy in *hlx1* function. Obvious candidates would be the recently identified *hlx2* and *hlx3* genes (Seo *et al*., 1999) that are related to *hlx1* and share overlapping expression domains throughout embryogenesis. It is also interesting that mis-expression of *hlx1* did not perturb the anterio-posterior patterning of the neural tube. Either this gene is not involved in patterning or it acts in concert with other genes which are regulated independently to specify cell type. In the latter case mis-expression of *hlx1* would not affect patterning since co-acting factors are presumably absent.

hlx1 ***and Forebrain Neurogenesis***

Neurogenesis begins in the zebrafish brain with the development of three pairs of bilaterally symmetrical nuclei; one each in the telencephalon, diencephalon and mesencephalon (Ross *et al*., 1992). Development of all three neuronal clusters begins at approximately 16 hpf and by 24 hpf they are interconnected by a scaffold of axon tracts. In animals misexpressing *hlx1* we observed a decrease in the size of both forebrain clusters which was also reflected in the reduced expression of two homologues of the *Drosophila achaete-scute* (*ash*) gene complex, *zash1A* and *zash1B*. Interestingly, it was previously demonstrated that overexpression of *Xdbx* in *Xenopus* leads to inhibition of neurogenesis in the spinal cord due to loss of *Xash3* (Gershon, *et al*., 2000). *zash1A* and *zash1B* are not homologues of X*enopus Xash3*, but they are related, with all three being members of the *ash* gene family. Although the zebrafish homologue of *Xash3* is yet to be identified, it is plausible that *hlx1* and members of the ash related genes are in the same regulatory pathway. If this was the case, interactions between *hlx1* and *ash* family members would need to involve other factors since expression of *zash1A* and *zash1B* was unaffected in the posterior brain and spinal cord of *hlx1* injected animals. Although *hlx1* expression in the ventral region of the diencephalon overlaps with the expression of both *zash1A* and *zash1B*, it does not coincide with the location of the neuronal clusters in this region (Allende and Weinberg, 1994; Hjorth and Key, 2001). This leads to the intriguing possibility that *hlx1* is normally inhibiting neurogenesis in the ventral diencephalon. Consequently, when *hlx1* is misexpressed by precursors of the neuronal clusters neurogenesis would be inhibited. Based on these observations, we had anticipated that *hlx1* knockdown using specific antisense morpholinos would have increased neurogenesis. In contrast, however, loss of *hlx1* had no effect on either neurogenesis or the expression patterns of *zash1A* or *zash1B* in the forebrain. This absence of a phenotype suggests that redundant molecules such as *hlx2* and *hlx3* may again be acting to rescue the loss of *hlx1* expression.

hlx1 ***and the Developing Hindbrain***

hlx1 loss-of-function produced by two different morpholinos resulted in specific defects in the hindbrain. At the gross morphological level there was a loss to the integrity of the neuroepithelial wall of the hindbrain. Despite this overt phenotype there was no change in the patterning of rhombomeres as defined by expression of *krox20* and *pax6*. For instance, *krox20* continued to be expressed in rhombomeres 3 and 5. The clearly defined borders between cells expressing *krox20* and those not expressing *krox20* were still maintained, indicating that *hlx1* was probably not involved in the formation of rhombomere borders. In contrast the expression pattern of *zash1B* was clearly disrupted by loss of *hlx1*. *zash1B* expression was absent from rhombomere 5 and was only present in the ventral region of rhombomere 3. Furthermore, its expression was no longer limited to the border regions of other rhombomeres, rather it was expressed throughout each of the other hindbrain compartments. This altered expression pattern of *zash1B* was obtained with two different morpholinos. The specificity of this effect was further confirmed by the fact that there was no difference in the expression pattern of *zash1B* in any other region of the brain. Moreover, *zash1A* expression was unaffected anywhere in the brain. The observation that *hlx1* knock down did not extinguish the expression of z*ash1B* expression in all rhombomeres highlights the complex interactions occurring during patterning of the hindbrain (Krumlauf, 1994).

There is strong evidence indicating that rhombomeres are patterned by differential expression of *hox* genes (McGinnis and Krumlauf, 1992; Krumlauf, 1994). In zebrafish, co-injection of *meis3*, *pbx4* and *hoxb1b* has shown that these factors act in concert to induce the expression of anterior hindbrain markers in the rostral brain and the formation of Mauthner neurons in ectopic rostral positions (Vlachakis *et al*., 2001). The vertebrate gene *krox20* is expressed only in rhombomeres 3 and 5 and is essential for the maintenance of these two compartments (Schneider-Maunoury *et al*., 1993). Furthermore, it has been shown to regulate the expression of expression of *hoxa2* and *hoxb2* in these rhombomeres (Sham *et al*., 1993; Nonchev *et al*., 1996; Maconochie *et al*., 2001; Mark *et al*., 2001). Collectively these data indicate that patterning of the hindbrain is complex in nature and involves multiple transcription factors and regulatory elements. We have shown that inhibition of *hlx1* expression results in downstream inhibition of *zash1B* in only rhombomere 5 and the dorsal portion of rhombomere 3. The loss of expression in these rhombomeres is however independent of *krox20*.

hlx1 ***and Axon Tract Formation***

36% of embryos misexpressing *hlx1* exhibited axonal scaffold defects, while 59% of animals injected with MOa and 15% of animals injected with MOb also exhibited axon scaffold abnormalities. Although a large number of axons contribute to these tracts, only a small subpopulation of axons were affected by either gain-of-function or loss-of-function of *hlx1*. We have previously shown that within the first neuronal clusters of the zebrafish brain, neurons are mosaic for the expression of different transcription factors and regulatory proteins (Hjorth and Key, 2001). Thus, these clusters contain distinct subpopulations of neurons very early in their development. It would appear from our present results that some

of these neurons are then sensitive to *hlx1* levels which ultimately leads to axon growth defects. What is not clear and remains to be determined is whether *hlx1* is directly involved in axon guidance or whether these effects are secondary to problems with differentiation or other early events such as specification of axon polarity.

In conclusion, the present study has utilised two different approaches to understand the role of *hlx1* in the developing zebrafish brain. It is apparent that early in development *hlx1* is involved in morphogenic movements in the neuroectoderm which lead to the formation of the neural tube. Later in development it appears to be involved in neurogenesis in the forebrain potentially through a mechanism which acts to modulate the expression of two known neurogenic genes, *zash1A* and *zash1B*. *hlx1* loss-of-function leads to a morphological phenotype in the hindbrain. In particular, the expression of only *zash1B* is ablated in rhombomere 5 and reduced to only the ventral region of rhombomere 3. Taken together, these data support the hypothesis that *hlx1* is involved in multiple dynamic roles during formation of the zebrafish brain, indicative of the multifaceted roles homeobox genes play during development.

Materials and Methods

Maintenance of Adult Fish and Collection of Embryos

Zebrafish embryos were obtained from natural spawning of adult zebrafish kept in our laboratory breeding colony. Embryos were raised at 28.5ºC and staged according to Kimmel *et al.* (1995) as hours post fertilisation (hpf).

Cloning of the Full Length Coding Region of hlx1

A zebrafish cDNA library was screened using a partial clone of the coding region of *hlx1* (Fjose *et al.,* 1994). Approximately 10^6 clones were screened at high stringency. Three positive clones between 1300 and 1500 base pairs long were isolated and DNA sequence analysis confirmed that they were identical and only varied in the length of 5' untranslated region present. During these experiments Seo *et al.* (1999) published a full length coding sequence for the *hlx1* gene. Comparison of the clones we obtained and the published sequence confirmed that they were identical genes.

Microinjection of hlx1 *Capped RNA*

hlx1 or *green fluorescent protein* (*gfp*) capped RNA was synthesised using the Ambion mMessage mMachine RNA transcription kit (Ambion Inc., TX). RNA concentration was measured by spectrophotometry and the RNA stored in aliquots at –70ºC. For microinjection RNA was diluted in sterile water to the desired concentration. Control experiments were performed by microinjecting between 50-1000 pg of *gfp* RNA into different batches of zebrafish embryos. These experiments did not yield a specific phenotype, which is consistent with *gfp* being non-toxic to zebrafish embryos. *hlx1* was over expressed by microinjection into the yolk of 1-2 cell stage zebrafish embryos approximately 1 nL of injection solution using an air driven pico spritzer (World Precision Instruments Inc., FL). The volume of each injection was monitored by visualising the size of the injected bolus in the yolk. For individual injection experiments we varied the amount of *hlx1* RNA between 50-1000 pg. However, for most mis-expression experiments we microinjected between 100-300 pg of *hlx1* RNA. In all *hlx1* mis-expression experiments we coinjected approximately 100 pg of *gfp* RNA as a marker of successful injections. After microinjection embryos were incubated at 28.5ºC in egg water (60 µg/mL Aquasonic Sea Salts in sterile water) (Aquasonic, NSW, Australia). Between 6-9 hpf each batch of embryos was screened for *gfp* expression using a fluorescent stereo microscope fitted with a *gfp* filter set (Olympus Pty Ltd., Melbourne, Australia). Embryos not expressing *gfp*, or exhibiting non-specific defects similar to those observed in non-injected control animals, were removed from the batch. This screening typically removes between 5-10% of animals and we find that 98% of the remaining animals survive to 24 hpf.

Design of Specific Antisense Morpholinos against Endogenous hlx1 *mRNA*

To inhibit endogenous *hlx1* protein synthesis we utilised specific antisense morpholino oligonucleotides. Based on the cDNA sequence of *hlx1*, two separate, non-overlapping, antisense morpholinos were designed (Gene Tools LLC, OR). The first morpholino (MOa: 5' GCA ATA ACA CTA GGG ATC ATC ATG G 3') spanned the final two bases of the 5'UTR, the protein synthesis start site and 20 bases into the coding region of the *hlx1* mRNA. The second morpholino (MOb: 5' CTC CTG TTA GTA TTA CAC AAG TCT C 3') begins 56 bases upstream of the ATG start site. For control experiments the standard control morpholino available from Gene Tools LLC was obtained (sMO: 5' CCT CTT ACC TCA GTT ACA ATT TAT A 3'). To confirm specificity of the *hlx1* morpholinos and to show that the standard control morpholino did not produce any non-specific effects, we screened these sequences against the GenBank nucleotide sequence databank. The specific *hlx1* morpholinos only recognised the *hlx1* gene, while the standard control morpholino sequence did not recognise any genes in GenBank. For microinjection experiments morpholinos were diluted with 1x Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6). Approximately 1 nL was microinjected into the yolk of 1-2 cell stage zebrafish embryos. Based on previous reports which successfully used morpholinos in zebrafish to inhibit protein synthesis, we injected 8 ng of MOa (Nasevicius and Ekker, 2000). This yielded embryos exhibiting a specific hindbrain phenotype. However, at least 8 ng of MOb was required to yield a similar specific phenotype. This specific phenotype was observed when up to 22 ng MOb was injected. To control for possible morpholino toxicity, we also injected up to 22 ng of the standard control morpholino.

In Situ *Hybridisation*

Standard techniques were used to detect gene expression in wholemount zebrafish embryos (Hjorth and Key, 2001). Digoxygenin labeled riboprobes for *pax6*, *dlx2*, *krox20*, *zash1A* and *zash1B* (kindly donated by Dr. S. Wilson) were synthesised using an in vitro transcription kit (Promega Corp., Madison, WI). Probes were hybridised to zebrafish embryos at 68ºC in hybridisation buffer (1 mg/mL torula RNA, 50 ug/mL heparin, 50% formamide, 2xSSC, 0.1% Tween-20).

Immunohistochemistry

Immunohistochemistry was performed on wholemounts of zebrafish embryonic brains as previously described (Hjorth and Key, 2001). Briefly, the brains of 24 hpf zebrafish embryos were dissected and labeled with primary antibodies against the HNK-1 epitope, a marker of all neurons and axons (Metcalfe *et al.*, 1990). Secondary antibodies conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, PA) were used for visualisation of the HNK-1 epitope with the confocal microscope.

Confocal Microscopy

Following immunohistochemistry wholemount zebrafish brains were mounted between two coverslips in fluorescent mounting media (200 mg/mL propyl gallate, 5% ethanol, 95% glycerol, 25 mM sodium bicarbonate, pH 9.6). Parasagittal optical sections of zebrafish brains were collected every 0.5-1 µm to include the axon tracts on one side of the embryo using an MRC-1024 Bio-Rad confocal laser scanning microscope coupled to a Zeiss Axioplan microscope. Serial optical sections were compiled as z-series using the Bio-Rad Confocal Assistant computer program.

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