belladonna (lhx2) is required for neural patterning and midline axon guidance in the zebrafish forebrain


An error in this article was not corrected before publication.

The GenBank Accession Number quoted on p. 726 for the lhx2-coding region should read DQ146407 (not 725255).

The authors apologise to readers for this mistake.
belladonna/(lhx2) is required for neural patterning and midline axon guidance in the zebrafish forebrain

Anandita Seth¹, James Culverwell¹, Mitchell Walkowicz¹, Sabrina Toro³, Jens M. Rick², Stephan C. F. Neuhauss², Zoltan M. Varga³ and Rolf O. Karlstrom¹.*

Some of the earliest axon pathways to form in the vertebrate forebrain are established as commissural and retinal axons cross the midline of the diencephalon and telencephalon. To better understand axon guidance in the forebrain, we characterized the zebrafish belladonna (bel) mutation, which disrupts commissural and retinal axon guidance in the forebrain. Using a positional cloning strategy, we determined that the bel locus encodes zebrafish Lhx2, a limb-homeodomain transcription factor expressed in the brain, eye and fin buds. We show that bel(lhx2) function is required for patterning in the ventral forebrain and eye, and that loss of bel(lhx2) function leads to alterations in regulatory gene expression, perturbations in axon guidance factors, and the absence of an optic chiasm and forebrain commissures. Our analysis reveals new roles for lhx2 in midline axon guidance, forebrain patterning and eye morphogenesis.

KEY WORDS: AC, Chiasm, Commissure, Ephrin, Gfap, Glial bridge, Morpholino, Netrin, POC, Semaphorin, Slit

INTRODUCTION
The anterior commissure (AC), post-optic commissure (POC) and the optic chiasm are the first major axon pathways to cross the midline of the vertebrate forebrain during embryonic development. The POC is formed as neurons in the lateral diencephalon extend axons anteriorly and across the midline (reviewed by Bak and Fraser, 2003), whereas the AC forms as lateral telencephalic neurons extend axons anteriorly across the midline of the ventral/anterior telencephalon (reviewed by Chitnis and Kuwada, 1990; Wilson et al., 1990). Soon after the formation of these forebrain commissures, retinal ganglion cell (RGC) axons from the eye cross the midline of the diencephalon near the POC to form the optic nerve, optic chiasm and optic tract (Burrill and Easter, 1995). In monocular organisms such as zebrafish, all retinal axons cross the ventral midline, whereas in binocular organisms some retinal axons turn ipsilaterally at the midline and innervate ipsilateral targets in the brain (reviewed by Williams et al., 2004). In humans, congenital defects in chiasm formation lead to visual defects, including nystagmus and loss of depth perception (Apkarian and Bour, 2001).

The cellular and molecular cues that guide midline-crossing axons in the forebrain are poorly understood. Glial cells may provide the cellular substrate for midline crossing axons and help establish the position of commissures, as glial structures have been found associated with many brain commissures, including the corpus callosum (Shu et al., 2003), chiasm (Marcus et al., 1995), AC and POC (Barresi et al., 2005). This role in guiding commissural axons may be conserved through evolution, as midline glial cells are known to help guide axons toward the midline in the Drosophila CNS (Chotard and Salecker, 2004; Hidalgo and Booth, 2000).

Compared with these cellular cues, more is known about the molecular guidance cues that influence commissure formation. In the vertebrate spinal cord, a combination of attractive and repulsive cues regulates commissural axon crossing (Dickson, 2002). Netrin and Sonic hedgehog (Shh) act as attractants for spinal commissural axons (Salinas, 2003), whereas Slit molecules prevent non-commissural axons from crossing the midline (Brose et al., 1999). In the vertebrate forebrain, netrin expression in the telencephalon is consistent with a possible role in AC formation; however, the lack of netrin expression in the diencephalon suggests it does not play a role in POC or chiasm formation (Lauderdale et al., 1997). Slit genes are expressed in bands across the midline of both the telencephalon and diencephalon, and these proteins act to channel Robo-expressing retinal axons during chiasm formation (reviewed by Rasband et al., 2003; Richards, 2002). This repellent function also helps to position glial cells and axons in the POC region (Barresi et al., 2005). The transcription factor Zic2 is expressed in a subset of retinal axons that grow ipsilaterally in binocular organisms, and Zic2 may regulate EphB1 receptors that receive the repulsive EphrinB2 cues from the midline, thus preventing these axons from crossing the midline (Herrera et al., 2003).

The precise expression of axon guidance cues in the eye and forebrain is dependent upon complex cellular differentiation events that lead to an exquisitely patterned neural tube. A large number of transcription factors interact to help pattern the forebrain (Dodd et al., 1988; Herrera et al., 2004; Shimamura et al., 1997). These include several members of the Lim-Homeodomain (LHD) transcription factor family that are involved in the related processes of neural patterning, cell fate determination and axon pathfinding (reviewed by Sockanathan, 2003). Among these, Lhx2 is required for mouse forebrain patterning and eye formation, with Lhx2 knock-out (KO) mice having a highly reduced telencephalon and no eyes (Porter et al., 1997). Although the telencephalic and eye phenotypes in Lhx2 KO are well documented, little information is available about the role of Lhx2 in forebrain axon guidance or in the formation of the diencephalon, a region where it is also strongly expressed.

Here, we show that the zebrafish axon guidance mutant belladonna (bel) encodes Lhx2. In bel mutants, both POC and RGC axons fail to cross the midline of the forebrain and no optic chiasm forms. bel(lhx2) mutant embryos have subtle eye defects, but have no other morphological defects and can grow to adulthood (Karlstrom et al., 1996). bel(lhx2) mutants also have a reversed...
optokinetic response, similar to defects in human achiasmats (Rick et al., 2000). We show that bel(lhx2) mutants have subtle forebrain patterning defects that are restricted to regions of the forebrain where the AC, POC and optic chiasm form. Our detailed analysis of forebrain defects in bel(lhx2) mutants indicates that disorganization of midline glia and the misexpression of a subset of known axon guidance molecules accompany retinal and commissural axon guidance defects. These results demonstrate a role for bel(lhx2) in forebrain axon guidance and in the patterning of the diencephalon and eye, and help to characterize the guidance substrate for commissural and retinal axons in the forebrain.

MATERIALS AND METHODS

Fish lines and genetic mapping

bel<sup>ph0</sup> was isolated in a screen for retinotectal axon guidance mutants (Karlstrom et al., 1996), whereas bel<sup>ph0</sup> was isolated in a genetic screen for forebrain patterning mutations (Z.M.V., unpublished). For genetic mapping, bel<sup>ph0</sup>-<sup>42</sup> heterozygotes in the Tü background were crossed into polytomorphic TL and WIK strains (Rauch et al., 1997). Mutant embryos were identified by axon guidance defects or visible eye defects at 5 dpf. PCR was performed on embryonic DNA using simple sequence repeat (SSR) markers (Knapik et al., 1998). Polymorphisms were visualized on 2% agarse gels or by single-strand conformation polymorphism (SSCP) analysis (Karlstrom et al., 1999).

In situ hybridization and immunohistochemistry

Embryos were maintained at 28.5°C and 0.003% 1-phenyl-2-thiourea (PTU) to label glial cells (Nona et al., 1989), and anti-phosphohistone H3 tubulin (1:1000, Sigma) to label axons (Wilson et al., 1990), anti-GFAP monoclonal antibody (1:400) to label glial cells (Laessing et al., 1994), anti-acetylated tubulin (1:1000, Sigma) (Barth and Wilson, 1995), pa2.1 (Krauss et al., 1991), sema3d (Halloran et al., 1998), slit2 (Ye et al., 2001), vulx (Takeuchi et al., 2003) and zic2.1 (Grimblat and Sive, 2001). Antisense lnx2 probes were generated against the full-length cDNA cloned into the pCR4TOPO vector (see below). Immunohistochemistry was performed as described for whole-mount embryos (Karlstrom et al., 1999) and frozen sections (Devoto et al., 1996). Antibodies used were: ZN-5 (1:25) to label RGCs (University of Oregon Monoclonal Antibody Facility) (Laessing et al., 1994), anti-acetylated tubulin (1:1000, Sigma) to label axons (Wilson et al., 1990), anti-GFAP (1:400) to label glial cells (Nona et al., 1989), and anti-phosphohistone H3 (pH3) (1:100, Sigma) to label mitotic cells (Nechiporuk and Keating, 2002). Individual pH3-labeled cells were counted in the telencephalon (dorsal to the optic recess and first ventricle) and preoptic area of the diencephalon. Cell numbers were compared between wild type and bel mutants using Student’s paired t-test. Sections (7 μm) of 5 dpf larvae and adult eyes were embedded in pion/araldite (Mollenhauer, 1964) and counterstained with Toluidine Blue.

Positional cloning of the bel locus

The zebrafish CHORI-211 BAC library (RZPD, Germany) was screened by PCR, using closely linked zMarkers, according to manufacturer’s instructions. BAC ends were sequenced directly or obtained from the Sanger zebrafish genome database (http://www.sanger.ac.uk/Projects/D_rerio/). PCR primers were designed using these sequenced ends and the library was re-screened until a BAC was identified that spanned the bel genetic interval (zC14218). Genescan analysis (http://genes.mit.edu/GENSCAN.html) of the zC14218 sequence revealed a single coding sequence encoding Lhx2.

The Lhx2 coding region was amplified from first-strand cDNA (Clonetech RT-PCR kit) using 5′UTR and 3′UTR PCR primers (Lhx2.5Fw, 5′-GG-GTTGCGAATCTGACGCG-3′; Lhx2.21Rv, 5′-GCATTGCTGAAATAATCGTG-3′). The gel-purified 1191 bp PCR product was cloned into the PCR4TOPO cloning vector (Invitrogen) and sequenced (GenBank Accession number 725255). The predicted Lhx2 protein sequence was compared with Lhx2 sequences from other species using ClustalW analysis (Biology Workbench, http://workbench.sdsc.edu). To sequence the lhx2 gene in the two bel alleles, primers flanking each of the five lhx2 exons were used to amplify genomic DNA from bel<sup>ph0</sup> siblings, bel<sup>ph42</sup> mutants, bel<sup>ph0</sup> siblings and bel<sup>ph42</sup> mutants.

For genotyping bel carriers, fin clip or embryo DNA was amplified using allele specific primers. bel<sup>ph42</sup> genotyping primers (tv42.GT.Fw, 5′-GC-TGCAATAGAAGAG-3′; and tv42.GT.BsmAL.Rv, 5′-CTCAAGACTCTCAGTGTTACAGTC-3′) amplify a 248 bp fragment with the mutant sequence containing a restriction site for BsmAL bel<sup>ph0</sup> genotyping primers (lhx2.37Fw, 5′-CAATCCACCCGATGTTAGC-3′; and lhx2.18Rv, 5′-CAGTTAACCCGACCAAC-3′) flank the 22 bp deletion. DNA fragments were resolved on 3.5% Metaphor (Cambrex) or 4% agarose (Gel) gels.

Antisense oligonucleotide injections and cell transplantation

Phosphorothioated antisense oligonucleotides (S-oligos) (Stenkamp and Frey, 2003) were generated against three different regions in zebrafish lhx2 coding sequence. S-oligo sequences were: CCTTgacagctgttg, GCAgtgctctgtgtcctg, and GCctacagccagctgttg (Sigma Genosys). Capital letters signify bases joined by thioester bonds. An lhx2 splice blocking morpholino (MO) (GeneTools) (5′-CTTTTCTCCTCTGCG-3′-CTGGTTC3′; 8-15 ng), an unrelated mismatch control MO (10 ng), or a cocktail of the three S-oligos (1-1.5 pg each) was injected into one- to two-cell embryos. Embryos were incubated at 28.5°C until the desired time points, fixed in 4% paraformaldehyde, and processed for in situ labeling.

For cell transplantation, wild-type or bel embryos were injected with rhodamine-dextran (2.5%) at the one- to two-cell stage and used as donors. Ten to 20 cells were transplanted from the donor animal pole at the dome stage into the same region of unlabeled wild-type or mutant hosts. Donor and host embryos were maintained as pairs in 24-well dishes in Danieau solution [1 × Danieau: 58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO<sub>4</sub>, 0.6 mM Ca(NO<sub>3</sub>)<sub>2</sub>, containing penicillin and streptomycin (1% each)]. At the Prim-5 stage (24 hpf), donors and hosts were analyzed for dtx2 expression by in situ hybridization. Genotypes were determined by dtx2 expression or by PCR-based genotyping of tail tissue, as described above.

FGF signaling inhibition

To block Fgf signaling, embryos were treated with 20 μM SU5402 (Calbiochem) diluted in embryos raising medium for described time intervals at 28.5°C. Control embryos were treated with DMSO (SU5402 carrier). Embryos were placed in 12-well plates (30 embryos per well) with 0.5 ml of medium. After treatments, embryos were fixed and processed for in situ hybridization. As a control for SU5402 efficacy, we examined expression of the Fgf gene ermA (Raible and Brand, 2001) in similarly treated embryos.

RESULTS

Belladonna mutations affect midline axon guidance in the forebrain

We initially identified belladonna in a large-scale screen for mutations that affect axon guidance in the zebrafish retino-tectal system. In 5-day-old bel mutants, retinal axons fail to cross the midline and instead project ipsilaterally. We initially identified belladonna in a large-scale screen for mutations that affect axon guidance in the zebrafish retino-tectal system. In 5-day-old bel mutants, retinal axons fail to cross the midline and instead project to the ipsilateral tectal lobe where they find their correct topographical target (Karlstrom et al., 1996) (Fig. 1A,B). To understand the nature and onset of bel axon guidance defects, we examined retinal axon growth at earlier time-points. As retinal axons grew toward the midline (32-36 hpf) two distinct phenotypes were seen in bel mutants; retinal axons either projected ipsilaterally immediately after leaving the eye [16/39 optic nerves (ONs) examined], or they projected toward the midline (23/39 ONs; Fig. 1D, inset). When examined at 38-48 hpf of development, RGC axons failed to cross the midline in 88% of bel mutants (n=276 embryos; Fig. 1F) and almost no midline growth was seen. Thus, in bel mutants RGC axons often approached the midline at early stages of development, but subsequently axons failed to cross the midline and instead projected ipsilaterally.
We next examined the formation of the forebrain commissures in bel mutants (Chitnis and Kuwada, 1990; Wilson et al., 1990). In 28 hpf wild-type embryos, axons from the nucleus of the tract of the post-optic commissure (ntPOC) and axons of the nucleus of the tract of the AC (ntAC) have crossed the midline to form the POC and the AC, respectively (Fig. 1G) (Bak and Fraser, 2003). In bel mutants, both ntPOC and ntAC axons were present but failed to extend axons across the midline and no forebrain commissures were formed (Fig. 1H). All other major axon pathways appeared to be normal in bel mutants. These include the posterior commissure, hindbrain commissures, Mauthner cells, spinal cord commissures and peripheral axons (data not shown). Thus mutation of bel affects midline axon crossing only in the forebrain.

**bel mutations disrupt zebrafish lhx2**

To understand the molecular mechanisms underlying the bel phenotype we next identified the gene encoded by the bel locus. Using simple sequence repeat (SSR or Z) markers we linked bel to chromosome 8. Fine mapping using a mapping panel that represented more than 2500 meioses identified two Z markers closely linked to bel on either side of the bel locus, z44909 (0.6 cM) and z24272 (0.3cM; Fig. 2A). A chromosomal walk identified two overlapping clones (zC142I8 and zC95F8) that spanned the bel locus (Fig. 2A). Genescan analysis of zC142I8 sequence identified only one potential gene with high sequence homology to chick Lh2A and mouse and human Lhx2 (Fig. 2B,C). Similar to other LHD family members, the predicted Lhx2 sequence contains two LIM domains followed by a highly conserved homeobox domain (Fig. 2E). Based on the sequence homology and expression analysis (see below), we concluded that bel is more closely related to Lhx2 than the closely related family member Lhx9.

A single nucleotide polymorphism (SNP) in the first intron of the predicted lhx2 sequence co-segregated with the bel locus (0 recombination events in 2500 meioses). We therefore sequenced the lhx2 gene in two bel alleles and found genetic lesions leading to severe protein truncations in both cases. In bel<sup>b700</sup>, a point mutation (C to A) in the second exon introduces a premature stop codon that would truncate the Lhx2 protein in the first LIM domain (Fig. 2E). In bel<sup>b542</sup>, a 22 bp deletion in the third exon results in a frame-shift that leads to a stop codon after the second LIM domain (Fig. 2E).

To verify that lhx2 is the gene mutated in bel embryos, we used antisense thioester oligos (S-oligos) and a splice blocking morpholino (MOs) to reduce Lhx2 function in wild-type embryos. Injection of the MO or of a cocktail of three S-oligos (SO) led to reduced dlx2 (see Fig. 5A inset, Table 1) and sema3d (see Fig. 6D inset) expression in the ventral forebrain that was very similar to that seen in homozygous bel mutants. These results further support the idea that bel disrupts lhx2 and suggest that these two bel alleles result in a loss of Lhx2 function. These antisense injections also led to subtle and variable axon defects, including POC defasciculation and RGC guidance errors at the midline (data not shown). Ipsilateral projections were not seen, suggesting antisense injections may not reduce Lhx2 function as completely as do the bel mutations.

**Expression of lhx2 and regulation by Fgf signaling**

To determine whether lhx2 is expressed in regions disrupted by bel(lhx2) mutations, we analyzed the expression pattern of lhx2 during zebrafish embryonic development. lhx2 mRNA was first detectable at shield stage, with no maternal message detectable by RT-PCR at the four-cell stage (data not shown). At tailbud stage (10 hpf), lhx2 expression was seen in the anterior CNS corresponding to the forebrain, including the eye fields (Fig. 3A, inset). By 23 hpf, lhx2 was strongly expressed in the forebrain, including in the epiphysis (Fig. 3A). At 26 hpf, lhx2 expression was seen in two bands in telencephalon, in the preoptic area and in the hypothalamus (Fig. 3B). In bel(lhx2) mutants, lhx2 expression was severely reduced in the preoptic area but was unaffected elsewhere in the embryo (Fig. 3B, inset). At 32 and 48 hpf, lhx2 was also expressed in the tectum and the midbrain-hindbrain boundary; expression was more discrete in the hindbrain (Fig. 3C-E). In the hindbrain, lhx2 expression was seen in distinct cells at the rhombomere boundaries at 48 hpf (Fig. 3F). In the eyes, lhx2 was initially expressed globally in the eye fields from 15 hpf (Fig. 3G) to 21 hpf (Fig. 3H), then became restricted to the amacrine cells in the inner nuclear layer by 28 hpf.
32 hpf (Fig. 3I), and became further restricted to ventral amacrines cells at 3 dpf (Fig. 3J). lhx2 expression was also seen in entire fin buds at 26 hpf, and this expression became restricted to the posterior half of the fin bud by 48 hpf (Fig. 3K,L).

Because of the timing of lhx2 expression and the known role of Hedgehog (Hh) and Fibroblast growth factor (Fgf) signaling in regulating forebrain patterning, we next examined whether Fgf and/or Hh signaling regulate lhx2 expression. We first blocked all Fgf signaling using the small molecule SU5402 (Mohammadi et al., 1997), starting prior to the onset of lhx2 expression (6 hpf). This SU5402 treatment nearly eliminated lhx2 expression in the forebrain at 10 hpf, with expression remaining in only a few ventral cells (Fig. 4A,B). Treating embryos with SU5402 starting at 10 hpf led to a major reduction of lhx2 expression at 24 hpf, showing a continued role for Fgf in the expression of lhx2 (Fig. 4C,D). In contrast to these results, elimination of Hh signaling, either in Shh pathway mutants or using the alkaloid Hh signal blocker CyA, had little affect on lhx2 expression (data not shown). These results show that Fgf signaling is required for both the onset and maintenance of lhx2 gene expression.

zebrafish lhx2 function is required cell autonomously for forebrain patterning

The identification of bel as a LHD forebrain transcription factor led us to look for forebrain patterning defects that might underlie the observed axon guidance defects, focusing on the regions where commissural and retinal axons cross the midline. The distal-less related transcription factor dlx2 is expressed in the ventral telencephalon adjacent to the AC, and in the preoptic area of the diencephalon adjacent to the POC and chiasm prior to commissure formation (Fig. 5A) (Akimenko et al., 1994; Ellies et al., 1997). In bel mutants, dlx2 expression is absent from the diencephalon in the preoptic area (Fig. 5B). The homeodomain transcription factor nk2.1b is also expressed in the anterior telencephalon and diencephalon (Fig. 5C) (Rohr et al., 2001). In bel mutants, nk2.1b expression is subtly disrupted in the preoptic area, with a small region adjacent to the optic recess ectopically expressing nk2.1b and a small region in lateral diencephalon adjacent to the tract of the POC lacking nk2.1b expression (Fig. 5D). In the telencephalon, expression of dlx2 is slightly reduced in the AC region (Fig. 5B), whereas expression of nk2.1b appears...
unaffected (Fig. 5D), indicating that lhx2 is also required for ventral telencephalon formation. These forebrain patterning defects are apparent starting at 24 hpf, as POC axons are crossing the midline, suggesting that bel(lhx2) is required for patterning the neural growth substrate that provides guidance cues for retinal and commissural axons.

Mutations affecting the Hh and Fgf signaling pathways lead to forebrain patterning defects and axon guidance defects similar to those seen in bel mutants (Barresi et al., 2005; Culverwell and Karlstrom, 2002; Karlstrom et al., 1999; Karlstrom et al., 2003; Shammugalingam et al., 2000; Tuurina et al., 2005; Walsh and Mason, 2003). Furthermore, it was previously suggested that bel might mediate Fgf and Hh signaling in the regulation of vax expression in the preoptic area (Take-uchi et al., 2003). To determine whether the forebrain defects seen in bel may be due to defects in Hh or Fgf signaling, we examined the expression of Hh and Fgf signaling molecules, or their downstream targets, in bel mutants. fgf8 expression is lost in the preoptic area of bel mutants (Fig. 5). By contrast, preoptic expression of the Hh target genes ptc1 and nk2.2 was unaffected in bel mutants (data not shown), which suggests that loss of Lhx2 function does not affect Hh signaling in the POA. Thus bel(lhx2) might affect forebrain patterning and axon guidance, at least partially, via Fgf8 signaling.

We next examined optic stalk expression of vax2, zic2.1 and pax2.1. As previously documented, we showed that bel mutants lack vax2 expression only in preoptic area and medial optic stalk (data not shown) (Take-uchi et al., 2003). pax2.1 is expressed prior to vax2 expression in the optic stalk and eye (Krauss et al., 1991). Similar to vax2, pax2.1 was missing in bel mutants in the medial regions, but was unaffected in more lateral regions and the ventral retina (Fig. 5G,H). Another transcription factor involved in establishing retinal projections in mice is Zic2, acting both in the retina (Herrera et al., 2003) and ventral diencephalon (Williams et al., 2004). In zebrafish, Zic2.1 is expressed in the optic stalk but not in RGCs. Similar to pax2.1, bel mutants lack expression of zic2.1 in the optic stalk (Fig. 5L,J).

Given the multiple roles for Lhx genes in cell differentiation and axon guidance, we next wondered whether lhx2 affected cell differentiation cell autonomously in the brain. When transplanted into a wild-type forebrain, bel mutant cells were unable to correctly express dlx2 (Fig. 5S,U). Conversely, wild-type cells were able to express dlx2 appropriately when transplanted into a bel mutant forebrain (Fig. 5R,T). These results indicate that lhx2 function is cell-autonomously required for cell differentiation in the forebrain.

Reduced cell proliferation in bel(lhx2) mutants

Loss of lhx2 function in mouse leads to a highly reduced cortex and reduced cell proliferation in the telencephalon (Porter et al., 1997). We therefore examined bel mutants to determine whether zebrafish lhx2 similarly affects cell proliferation in the forebrain. Labeling mitotic cells with the anti-phosphohistone3 antibody revealed that bel mutants have regionally reduced cell proliferation in the ventral forebrain (Fig. 5K,L). In the diencephalon, bel mutants had less than half the number of proliferating cells when compared with the wild-type siblings. However, in the telencephalon, there was no statistical

<table>
<thead>
<tr>
<th>Antisense oligo injection</th>
<th>dlx2 reduced* in POA (%)</th>
<th>Wild-type dlx2</th>
<th>Number of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>lhx2 S-oligo cocktail</td>
<td>100 (91)</td>
<td>10</td>
<td>110</td>
</tr>
<tr>
<td>(4.5 pg total)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lhx2 S-oligo cocktail</td>
<td>12 (44)</td>
<td>15</td>
<td>27</td>
</tr>
<tr>
<td>(3 pg total)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lhx2 MO (15 ng)</td>
<td>81 (86)</td>
<td>5</td>
<td>86</td>
</tr>
<tr>
<td>lhx2 MO (8 ng)</td>
<td>13 (61)</td>
<td>16</td>
<td>29</td>
</tr>
<tr>
<td>Control MO</td>
<td>0 (0)</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Uninjected</td>
<td>0 (0)</td>
<td>70</td>
<td>70</td>
</tr>
</tbody>
</table>

*Similar to bel mutants.

POA, preoptic area of the diencephalon.
difference in the number of proliferating cells (Fig. 5M). No differences were seen in cell death in the forebrain (data not shown). Thus defects in ventral forebrain patterning, including loss of dlx2, vax2 and pax2.1, may, at least partially result, from the failure of precursor cells to proliferate in the preoptic area of the forebrain.

bel(lhx2) is required for the formation of forebrain glial bridges and for proper expression of axon guidance molecules

Given the restricted forebrain patterning defects documented above and the lack of midline axon crossing in bel(lhx2) mutants, we wondered how bel(lhx2) affects the cellular substrate for commissural and retinal axon growth. GFAP expressing glial cells span the midline forming a glial bridge prior to commissure formation and may thus be the cellular substrate for midline growth. GFAP expressing glial cells was also observed at later ages, and AC and POC axons appear to grow in association with the mis-placed glial cells (Fig. 6A,B inset).

Fig. 4. Regulation of lhx2 expression. (A,B) Wild-type embryos were treated with SU5402 (B) or DMSO carrier (A) from 6 hpf to 10 hpf and labeled to show lhx2 expression. Blocking Fgf signaling during the onset of lhx2 expression nearly eliminated lhx2 expression in the embryo (B, arrowhead). Insets show complete loss of the Fgf-regulated gene emr in the forebrain and hindbrain of identically treated embryos (arrows). (C,D) Treating embryos with SU5402 from 10 hpf to 24 hpf reduced lhx2 expression in the telencephalon (arrowheads) and diencephalon (arrow). All panels show lateral views of the head, anterior to the left. Eyes were removed in C and D.

differences were seen in cell death in the forebrain (data not shown). Thus defects in ventral forebrain patterning, including loss of dlx2, vax2 and pax2.1, may, at least partially result, from the failure of precursor cells to proliferate in the preoptic area of the forebrain.

bel(lhx2) is required for eye development

belladonna mutants have a ‘dilated pupil’ phenotype at 5 dpf (Karlstrom et al., 1996) and thus they were named after the plant whose extract causes pupil dilation in humans (Duncan and Collison, 2003; Feinsod, 2000). To understand the eye defects in bel mutants, we sectioned 5 dpf wild-type and mutant eyes. This analysis showed that the pigmented epithelium (PE) fails to contact the lens in mutants, leaving a gap between PE and the lens (Fig. 7A,B). bel mutant eyes are also shorter in the dorsoventral axis and wider in the mediolateral axis than are wild-type eyes; the ratio of eye width (mediolateral axis) to eye height (dorsoventral axis) is 15% greater in bel mutants than in wild types. Finally, in nearly all bel eyes we observed an acellular aggregate near a normal looking lens that was labeled with the lens protein-specific Zl-1 antibody (Fig. 7A,B insets).

Although all cell layers appear to be present in bel mutant eyes, labeling with in situ probe to apoE (Babin et al., 1997) revealed that amacrine cells are mostly absent, with only a few cells remaining in the ventral retina (Fig. 7C,D). Labeling of Müller glial cells showed that these cells are disorganized (data not shown). These subtle eye morphogenesis defects become more severe in those bel mutants that survived into adulthood. bel mutant eyes are much smaller than in wild type, with highly disorganized retinal layers that appear to fold over on themselves, most likely due to the failure of the fluid filled posterior compartment to form (Fig. 7E,F). In some adults, vascularized retinal tissue protrudes from the eye adjacent to the lens (Fig. 7F, left inset).

DISCUSSION

We have identified a zebrafish Lmx-homeodomain protein that appears to be orthologous to Lhx2 based both on sequence similarity and embryonic expression pattern (Bachy et al., 2001; Nohno et al., 1997). Our analyses show that lhx2 is disrupted in bel mutants and that two bel alleles encode severely truncated Lhx2 proteins that lack the highly conserved homeodomain. Both alleles appear to be loss-of-function mutants based on two criteria: first, neither allele appears to have any dominant activity; and second, bel forebrain patterning defects are similar to those seen following MO- or S-oligo-induced loss of Lhx2 function. Analysis of the bel phenotype reveals that zebrafish Lhx2 function is required for proper patterning of the anterior forebrain and eye, and for the proper expression of several axon guidance molecules in the preoptic area of the diencephalon. Coincident with these localized gene expression defects, retinal axons fail to cross the midline and instead project ipsilaterally, and forebrain commissural neurons fail to cross the midline.
Lhx2 and neural patterning

Our analysis shows that, similar to in mouse, Lhx2 is required for both proper gene expression and for cell proliferation in the forebrain in zebrafish (Fig. 5). fgf8, one of the genes downregulated in the forebrain of bel mutants, is known to regulate cell proliferation (Xu et al., 1993). Thus, reduced fgf8 signaling may account for the reduced proliferation observed in bel mutants (Fig. 5K,L). However, because the loss of the optic stalk marker pax2.1 occurs prior to the loss of fgf8 expression in this region (data not shown), it appears that cell differentiation defects may precede these cell proliferation defects. Furthermore, although it is possible that regional reductions in cell proliferation could account for the observed losses in forebrain gene expression (Fig. 5), we show that the ventral telencephalic markers nk2.1b and netrin1 are expanded into the preoptic area of the diencephalon. Thus, lhx2 may directly regulate cell specification and regional identity. This is consistent with the model for Lhx2 function in the mouse cortex, in which Lhx2 is initially thought to function in the specification of cortical cells, as distinct from the cortical hem, and later is required for the proliferation of cortical precursors (Bulchand et al., 2001). Whether lhx2 and other forebrain patterning genes regulate neural patterning primarily by affecting precursor cell differentiation or by selectively regulating precursor proliferation, or both, is an important issue that remains to be determined.

---

**Fig. 5. Forebrain patterning defects in bel mutants.** (A,B) In bel mutants (B), dlx2 expression is extremely reduced in anterior/dorsal diencephalon (arrows) and is slightly reduced in the telencephalon (brackets), compared with in wild type (A). Inset in A shows a similar loss of dlx2 expression in the anterior diencephalon after injection of lhx2 antisense MO into a wild-type embryo. (C,D) In bel mutants (D), nk2.1b expression is regionally disrupted in the diencephalon (arrows) and expanded at a small region at the optic recess (arrowhead), compared with in wild type (C). (E,F) fgf8 expression is extremely reduced in the optic stalk region (arrowheads) in bel mutants (F), compared with wild type (E). Insets show anterior views of the head, with fgf8 expression absent at the midline (arrowheads) in bel mutants. (G,H) In bel mutants (H), pax2.1 expression is absent in the medial optic stalk (arrowheads) but remains laterally. (I,J) zic2.1 expression is similarly absent across the midline (arrowheads) in bel mutants (J). (K,L) The number of phospho-histone-labeled mitotic cells is reduced in the dorsal/anterior diencephalon of bel mutants (brackets). (M) Graph showing a significant reduction in the number of proliferating cells in the mutant diencephalon (asterisk, P<0.01). (N,O) Anterior views showing the loss of dlx2 expression in the anterior diencephalon of bel mutants (arrows, compare with lateral views in A,B). (P-U) Fluorescein dextran-labeled cells (red/brown) were transplanted between wild-type and bel mutant embryos. (P,R,T) Transplanted wild-type cells express dlx2 (blue) in a wild-type (P) and bel mutant (R,T) background (seven embryos). (Q) Transplanted bel mutant cells do not express dlx2 in the ventral midline in bel mutants. (S,U) In a wild-type background (19 embryos), bel mutant cell clones do not express dlx2, but populate the midline and are intermingled with wild-type cell clones. (T,U) Higher magnification view of transplanted cells in the diencephalon. (T) dlx2 transcripts (blue) and lineage tracer (red) are present in wild-type cells (purple arrowheads) in a bel mutant forebrain. (U) By contrast, alternating blue (dlx2-positive wild type) and red (lineage tracer containing dlx2-negative bel mutant) cells occupy the ventral midline in a wild-type background (red and blue arrowheads). (A-F,K,L) Lateral views of the forebrain, anterior left, eyes removed; (G-J) ventral views of the forebrain, anterior up; (N-U) anterior views of the forebrain, dorsal up; (T,U) Black dots mark the position of the optic recess. Scale bars: 15 μm.
In the spinal cord, combinatorial expression of LHD and Lim domain binding (LDB) proteins generates a ‘LIM code’ that determines motoneuron identity (Allan and Thor, 2003; Bach, 2000; Gill, 2003; Lumsden, 1995; Sockanathan, 2003). Similarly, overlapping expression of a large number of LHD and Lim domain only (LMO) genes in the forebrain helps to define prosomeric boundaries (Rubenstein and Beachy, 1998), and combinatorial Lhx function may control the specification of postmitotic thalamic neurons, as well as thalamocortical axon projections (Nakagawa and O’Leary, 2001). Overlapping expression may also result in overlapping functionality. In fact, although zebrafish lhx2 is expressed in the midbrain, hindbrain, epiphysis and fin buds, we did not observe any early developmental defects in these structures. This is similar to the situation in mice, where it has been speculated that the highly related LHD gene lhx9 may compensate for the loss of lhx2 function in tissues that express both genes (Bachy et al., 2001; Retaux et al., 1999). Consistent with this idea, our search of the zebrafish genome sequence database uncovered a zebrafish expressed sequence tag (EST) on chromosome 22 that is highly similar to lhx9 (Fig. 2) and that may compensate for the loss of lhx2 function in regions of overlapping expression.

**Lhx2 and diencephalon development**

We show that the optic stalk and preoptic area of the diencephalon are major targets of Lhx2 function in zebrafish. The expression of several patterning and axon guidance molecules is disrupted in this region (Figs 5, 6), glial cells are disorganized (Fig. 6), and commissural and retinal axons are unable to cross the midline (Fig. 1). lhx2 is expressed throughout this region in all vertebrates examined, including mice (Bachy et al., 2001; Nohno et al., 1997), but no gene expression or axon guidance defects have yet been reported in lhx2 mutant mice (Bulchand et al., 2003; Vyas et al., 2003). Although development of the anterior diencephalon and optic stalk have not yet been explicitly described in mouse lhx2 mutants, published reports show disrupted dlx2 expression in more posterior regions of the diencephalon (Vyas et al., 2003), suggesting lhx2 function is likely to be conserved in this region.

Some aspects of zebrafish Hh (Culverwell and Karlstrom, 2002) and Fgf (Shamugalingam et al., 2000) pathway mutant phenotypes resemble those seen in bel mutants. Furthermore, previous studies suggested that bel might act downstream of Hh and in parallel to Fgf signaling (Take-uchi et al., 2003). We thus examined whether lhx2 is regulated by, or regulates, Fgf and Hh signaling in the diencephalon. lhx2 expression is largely unaffected in yot and dtr mutations that block Hh signaling (Karlstrom et al., 2003), and bel mutants have normal expression of the Hh regulated genes nk2.2 and ptc1 (data not shown). This indicates that lhx2 is not regulated by Hh and strongly suggests that defects seen in bel mutants are not due to defects in Hh signaling. By contrast, we show that Fgf signaling is required for both the induction and maintenance of lhx2 expression.
Lhx2 and telencephalon development

Lhx2 function is best studied in the mammalian cortex, where loss of Lhx2 function leads to a general reduction in the dorsal telencephalon (neocortex or pallium), but not more medial and ventral telencephalic structures (paleocortex or subpallium) (Vyas et al., 2003). Loss of cortical tissue in mouse lhx2 mutants was originally attributed to a general reduction in telencephalic cell proliferation (Porter et al., 1997). More recent work showed that the cortical hem is expanded at the expense of cortex in lhx2 mutant mice, suggesting that Lhx2 may also pattern the cortex through selectively influencing precursor cell proliferation (Bulchand et al., 2003). It was also suggested that Lhx2 influences cell fate decisions at later times (Porter et al., 1997). In zebrafish, loss of Lhx2 function subtly disrupts ventral telencephalon patterning but does not grossly affect the telencephalon. However, because teleosts lack a cortex, this difference is likely to reflect the structural differences between species.

More ventrally, our analysis of bel mutants revealed a slight reduction in dlx2 expression in the subpallium (Fig. 5) that precedes the failure of AC axons to cross this region of the forebrain (Fig. 1). This suggests that patterning defects may underlie axon guidance defects in the telencephalon, and shows that Lhx2 is required for normal development of this tissue. In mouse, this region includes the medial and lateral ganglionic eminences (MGE and LGE) that are just ventral to the pallium/subpallium border (PSB). Similar to bel(lhx2) mutants, dlx2 expression appears subtly disrupted in the MGE and LGE of mouse lhx2 mutants (Vyas et al., 2003), although this phenotype has not been described in detail. A more detailed analysis of ventral forebrain development in lhx2 mutant mice may thus reveal a conserved role for Lhx2 in neural patterning in this region.

Lhx2 and eye development

Lhx2 is one of the six known eye field transcription factors (EFTFs) that establish the presumptive eye field and can induce ectopic eyes in Xenopus (Zuber et al., 2003). Mice lacking lhx2 completely lack eyes at birth (Porter et al., 1997). However, this phenotype does not represent a complete loss of eye development, as pax6 expressing optic vesicles are formed in these embryos. Eye development arrests before the optic cup forms, at which time the defective eye tissue is reabsorbed (Porter et al., 1997). By contrast, bel mutants have relatively well-developed, functional eyes (Karlstrom et al., 1996; Rick et al., 2000) (Fig. 7). Although this seemingly large phenotypic difference may represent a divergence in Lhx2 function between species, it is more likely explained by the fact that zebrafish embryos do not reabsorb defective tissue, as is the case for mouse embryos.

Our analysis of bel mutants has thus allowed us to identify new roles for lhx2 in vertebrate eye development. Although most cell layers form normally in bel(lhx2) mutants, amacrine cell numbers are extremely reduced (Fig. 7D). The most obvious defect in bel mutant adults is the small size of the eye and the disorganization of the retinal layers (Fig. 7). In teleosts, the eye grows throughout life, with new cells being added at the ciliary marginal zone (CMZ). We did not observe major cell proliferation defects in the eye using the phosho-histone antibody (data not shown), and at 5 dpf bel mutant eyes are normal in size (Fig. 7), suggesting that the small size of bel eyes at later ages is not due to proliferation defects but is due to the failure of the posterior compartment to form. The posterior compartment is filled with vitreous humor, which is produced by cells of the CMZ (reviewed by Bishop et al., 2002). CMZ cells appear to differentiate appropriately based on a-collagen expression in bel mutants (data not shown); however, this region of the eye is clearly disrupted, as evidenced by the gap between the PE and the lens, and by the presence of ectopic lens proteins (Fig. 7B). Thus, failure of the posterior chamber to form in bel mutants may result from defects in CMZ cell function, and/or from defects in the formation of the barrier to contain the vitreous humor in the eye.

Lhx2 and forebrain axon guidance

Despite the fact that numerous guidance systems are affected by mutations in bel, the observed axon guidance defects are remarkably specific. Axon defects are limited to the midline, with retinal axon...
pathways forming normally on the ipsilateral side of the brain. This indicates that dorsal guidance systems are unaffected by defects in midline axon crossing and is in contrast to other mutations affecting single axon guidance systems in the forebrain. In mutants affecting only Robo/Slit-mediated repulsion, defects in axon crossing are accompanied by axon wandering in the rostrocaudal axis and the formation of ectopic chiasm (Plump et al., 2002; Richards, 2002). Loss of Netrin results in lack of the hippocampal commissure, the corpus callosum and the anterior commissure in the forebrain (Mitchell et al., 1996). Similarly, mice with disrupted ephrin function show a wide range of defects, including guidance errors, incorrect mapping in the tectum or lateral geniculate nucleus, and defasciculation (O’Leary and McLaughlin, 2005).

Despite extensive work on LHD proteins and forebrain patterning, little is known about how LHD-mediated forebrain specification affects neural connectivity. LHD transcription factors have been shown to regulate the expression of the axon guidance molecule receptor Epha in motoneurons, thus directly affecting the response to the guidance molecule EphrinA (Kania and Jessell, 2003). Although it is possible that cell fate changes or changes in guidance receptors in commissural and retinal neurons could account for the lack of midline crossing in bel/llx2 mutants, RGCs appear to be specified correctly in bel mutants and can form functional projections on the incorrect tectal lobe (Rick et al., 2000) (Fig. 1). Combined with the observed gene expression defects in the preoptic area, this strongly suggests that forebrain patterning defects underlie the observed axon defects in bel/llx2 mutants.

The midline defects seen in bel mutants are in fact surprisingly similar to those seen in the hedgehog pathway mutant yot-foo (yot) (Barresi et al., 2005). Both mutants have similar defects in midline-spanning glial bridges that provide the cellular substrate for commissural and retinal axons (Barresi et al., 2005). In addition, slit guidance molecule expression is expanded across the commissure regions in both yot and bel mutants. slit2 and slit3 have been shown to directly influence the position of the forebrain commissures (Barresi et al., 2005) and the optic chiasm (Rasband et al., 2003) by a surround/repulsion mechanism. Expanded slit expression appears to be the major cause of axon defects in yot, as reducing Slit function in yot mutants largely rescues commissure formation (Barresi et al., 2005). Because slit repulsion also helps to position the glial bridges (Barresi et al., 2005), the expansion of slit genes across the midline in bel mutants could affect midline crossing by disrupting the cellular substrate for axon growth and/or by directly repelling midline crossing axons. Finally, misexpression of the axon guidance genes sema3d and netrin1 in bel mutants may also contribute to the observed axon guidance defects. Further analysis of the causes of axon guidance defects in bel thus promises to shed light on the relative importance of multiple guidance systems in the vertebrate forebrain.

We wish to thank the zebrafish community for its patience. We thank Jeanne Thomas for expert technical assistance and Judy Bennett for fish care. Many thanks to Carol Mason, Abbie Jensen, and the members of the Karlstrom Laboratory for thoughtful critique. This work was supported in part by F.R.I.A. Belgium (S.T.), SFB 592 A5 (Z.M.V.) and NIH NS39994 (R.O.K.).

References


Karlstrom, R. O., Trowe, T., Klostermann, S., Baiher, H., Brand, M., Crawford,


