Frizzled3a and Celsr2 function in the neuroepithelium to regulate migration of facial motor neurons in the developing zebrafish hindbrain

Hironori Wada¹, Hideomi Tanaka¹-², Satomi Nakayama¹, Miki Iwasaki¹-² and Hitoshi Okamoto¹-²,*

Migration of neurons from their birthplace to their final target area is a crucial step in brain development. Here, we show that expression of the off-limits/frizzled3a (olt/fz3a) and off-road/celsr2 (ord/celsr2) genes in neuroepithelial cells maintains the facial (nVII) motor neurons near the pial surface during their caudal migration in the zebrafish hindbrain. In the absence of olt/fz3a expression in the neuroepithelium, nVII motor neurons extended aberrant radial processes towards the ventricular surface and mismigrated radially to the dorsomedial part of the hindbrain. Our findings reveal a novel role for these genes, distinctive from their already known functions, in the regulation of the planar cell polarity (i.e. preventing integration of differentiated neurons into the neuroepithelial layer). This contrasts markedly with their reported role in reintegration of neuroepithelial daughter cells into the neuroepithelial layer after cell division.

KEY WORDS: Zebrafish, frizzled, celsr, Facial motor neuron, Neuroepithelium

INTRODUCTION
Migration of immature neurons from their site of origin to their final destination is a crucial step in the development of the vertebrate nervous system. Many neurons migrate tangentially through one cell layer at a specific depth within the brain. Some neurons migrate through the subventricular region (e.g. the GABAergic neurons into the olfactory bulb); others migrate near the pial surface (e.g. the granule cells into the cerebellar cortex), or through the intermediate zone (e.g. the GABAergic neurons into the cerebral cortex) (reviewed by Kriegstein and Noctor, 2004; Hatten, 2002). The mechanisms by which neurons select specific layers as their migrating pathway remain largely unknown.

The zebrafish is a good model to address this issue. In the developing zebrafish hindbrain, the facial (nVII) motor neurons originate in rhombomere (r)4 and migrate caudally to r6, where they form the facial motor nucleus (Chandrasekhar et al., 1997; Higashijima et al., 2000). These neurons migrate near the pial surface of the hindbrain (Wada et al., 2005). Several molecules that regulate the migration of nVII motor neurons have been identified in our, and other, laboratories. The triolobite/strabismus (tri/stbm; stbm is also known as vangl2 – Zebrafish Information Network) and prickle1 (pk1) genes, originally identified in Drosophila as planar cell polarity (PCP) genes (reviewed by Tree et al., 2005; Klein and Mlodzik, 2005), regulate caudal migration of the nVII motor neurons (Bingham et al., 2002; Jessen et al., 2002; Carreira-Barbosa et al., 2003). We have previously shown that a cytoplasmic protein Landlocked/Scrubble1 (Llk/Scrb1) is required for migration of the nVII motor neurons and that it genetically interacts with Tri/Stbm during convergent extension (CE) movements (Wada et al., 2005). A recent study has shown that the tri/stbm gene controls the anterior-posterior polarity of the neuroepithelial cells to regulate their reintegration in the zebrafish spinal cord after cell division (Ciruna et al., 2006). Thus, the neuroepithelial cells may regulate migration of nVII motor neurons through their polarized activity mediated by these gene products. However, the mechanisms by which these molecules regulate neuronal migration have not been investigated.

Here, we demonstrate that expression of the off-limits/frizzled3a (olt/fz3a) and off-road/celsr2 (ord/celsr2) genes in neuroepithelium maintains the nVII motor neurons near the pial surface during their caudal migration in the zebrafish hindbrain. In the absence of olt/fz3a expression in the neuroepithelium, the nVII motor neurons failed to migrate caudally; instead, they migrated radially into the dorsomedial part of the hindbrain by extending aberrant radial processes. Mosaic analyses showed that expression of the olt/fz3a gene in the surrounding neuroepithelial cells prevented integration of the nVII motor neurons into the neuroepithelial layer.

MATERIALS AND METHODS

Zebrafish strains and mutagenesis
Zebrafish (Danio rerio) were maintained according to standard protocols (Westerfield, 2000). The zebrafish Isl1-GFP transgenic line (registered as Tg(CM-isl1:GFP)w34) in the Zebrafish National BioResource Project of Japan, http://www.shigen.nig.ac.jp/zebra/index_en.html) (Higashijima et al., 2000) was derived from the RIKEN-Wako wild-type strain. The WIK strain was used for genetic mapping (Shimoda et al., 1999). Mutagenesis using N-ethyl-N-nitrosourea (ENU) has been described previously (Wada et al., 2005). One allele for the olt locus (olt/606) and four alleles for the ord locus (ordw3, ordw153, ordw166 and ordw300) were identified and used in this study. All embryos used in this study carried the Isl1-GFP transgene. Images were captured using a fluorescent dissecting microscope (Leica MZFLIII) with a CCD camera (Hamamatsu C5810). All strains are available from the Zebrafish National BioResource Project of Japan.

Mapping of the mutant loci
Genetic mapping of the mutant loci was carried out as described previously (Wada et al., 2005). In total, 463 olt and 1027 ord homozygous embryos were used to assign the locus to a linkage group. Expressed sequence tags (ESTs) and genomic sequences were obtained from the T51 radiation hybrid panel (Research Genetics, http://www.resgen.com/) and the Sanger Centre genome database (http://www.ensembl.org/index.html). The SSLP markers mrck1 and wz12343 were generated based on the sequences of a putative gene annotated as myotatin-related Cdc42-binding kinase (mrck) and an EST (wz12343), respectively (Table 1).
Table 1. Nucleotide sequences of the primers and antisense morpholin oligonucleotides MOs used in this study

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<th>Primers</th>
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<tr>
<td>mrcr1</td>
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<td>5'-CACTATCGACCAAGGTGAG-3' (antisense)</td>
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Identification of the genes

To isolate the f3a, f3b and celsr2 genes, total RNA was extracted from 24-hours post-fertilization (hpf) homzygous mutant embryos using an RNA extraction kit (Nippon gene). cDNAs were amplified by PCR using a first-strand cDNA synthesis kit (Takara) and specific primers designed according to genomic sequences from the Sanger Centre genome database. The amino acid sequences were deduced from the nucleotide sequences of partial cDNAs. To confirm the nucleotide changes in the mutant alleles, genomic DNA from grandparents of the family carrying the mutations were also sequenced. The phylogenetic relationship among family genes was analyzed using the CLUSTAL W program at the DDBJ (http://www.ddbj.nig.ac.jp/search/clustalw-e.html). The following amino acid sequences were used for comparison: mouse (m) Fz3 (Accession number, Q61091); mFz3 (Q61086); human (h) Fz3 (AAAF9088); Xenopus (x) Fz3 (ZAA04977); mCelsr1 (NP_536685); mCelsr2 (NP_059088); and mCelsr3 (NP_034016). In the analyses, total lengths were used for the Frizzled family proteins and the extracellular regions were used for the Celsr family proteins. For in situ hybridization, we used partial cDNA fragments PCR-amplified from the f3a gene (517 bp corresponding to the 3'-terminal region), the celsr2 gene (1057 bp encompassing exons 8-11), the celsr1a gene (565 bp corresponding to the N-terminal region), and the celsr3 gene (674 bp encompassing exons 8-11) (see Table 1). The PCR products were cloned into the TA cloning vector, pCRII-TOPO (Invitrogen), and sequenced using a BigDye terminator cycle sequencing kit (PE Applied Biosystems) with a DNA sequencer (ABI PRISM/3100 Genetic Analyzer). The accession numbers of celsr2, celsr3, f3a and f3b are AB246774, AB246775, AB246776 and AB246777, respectively.

Immunohistochemistry, in situ hybridization and retrograde cell labeling

Immunohistochemistry was performed according to standard protocols (Westerfield, 2000), using anti-acetylated α-tubulin antibody (Sigma, diluted 1:1000), en-5-antibody (Oregon Monoclonal Bank, diluted 1:100) (Trevorrow et al., 1990), anti-β-catenin antibody (Sigma, diluted 1:500) and a secondary antibody conjugated to Alexa-533 (Santa Cruz Biotechnology, 1:500). The samples were viewed under confocal microscopy (Zeiss LSM 510). Isl1-GFP signals were captured simultaneously in the samples. In situ hybridization using RNA probes was carried out as described (Westerfield, 2000). To monitor hindbrain differentiation, we used RNA probes for tagl (also known as csn2 – Zebrafish Information Network) (Warren, Jr et al., 1999), hoxb1a (Prince et al., 1998), krox20 (Octoby and Jowett, 1993) and valm/mbq (Moens et al., 1998). Images were captured using a differential interference contrast microscope (Zeiss Axioplan2) with a CCD camera (Olympus DP50). Retrograde labeling of the reticulospinal neurons and the octavolateral (OLe) neurons was carried out as described previously (Wada et al., 2005). At least ten embryos were monitored in each experiment.

Gene knockdown

Antisense morpholin oligonucleotides (MO) to target the first exon/intron boundary of f3a, and the second exon/intron boundary of celsr2, celsr1a and celsr1b were designed by Gene Tools (see Table 1 and Fig. S2D,I in the supplementary material). Approximately 1 nl of MO (5 mg/ml) was injected into one-cell-stage embryos (5 ng per embryo), as described (Nasevicius and Ekker, 2000). RT-PCR assay was carried out to confirm the splicing defects of the MOs, as described (see Fig. S2E,J in the supplementary material) (Goutel et al., 2000).

Injection of mRNA and detection of the expressed proteins

The wild-type f3a, the mutated f3a<sup>ΔC</sup> (1515 bp, which lacks the cytoplasmic region) cDNAs were amplified by RT-PCR and subcloned into pcS2 expression vectors. Sense-capped mRNA was synthesized using a Message Machine kit (Ambion). Approximately 1 nl of mRNA (0.5 mg/ml) was injected into one-cell-stage embryos (0.5 ng per embryo). To monitor the subcellular localization of the expressed proteins, Venus-fused cDNAs (f3a-venus and f3a<sup>ΔC</sup>-venus) were generated (Nagai et al., 2002) and the corresponding mRNA was injected into one-cell-stage embryos, as described. To monitor the subcellular selection of Deshevelled (Dsh), mRNA encoding Xdsh:GFP (Rothbacher et al., 2000) was co-injected with f3a or f3a<sup>ΔC</sup> mRNAs. For each construct, five embryos were monitored under confocal microscopy at 10-12 hpf.

Cell transplantation

Cell transplantation was performed as described (Wada et al., 2005; Moens et al., 1996). MZ-ort<sup>ΔM</sup> and MZ-ort<sup>ΔO</sup> embryos were produced by homzygous crosses. Live mosaic embryos were analyzed at 36-48 hpf. To confirm that the transplanted donor cells developed into nVII motor neurons, we monitored peripheral axons from donor cells labeled with rhodamine, as described previously (Wada et al., 2005). We used IMARIS 4.2 software (Bitplane) to visualize morphism and optical sections in the neuroepithelium.

Time-lapse observations

The procedures for time-lapse observations were essentially the same as described (Langenberg et al., 2003; Bingham et al., 2005) with modifications (H.T. and H.O., unpublished). Briefly, the hindbrain regions were excised with a fine blade from embryos at 18 hpf. The resulting hindbrain explants were embedded in 1.5% agarose in a small chamber and covered with L-15 medium (Gibco BRL) supplemented with penicillin/streptomycin cocktail (Gibco BRL). The chamber was sealed with a cover slip and observed under
confocal microscopy every 10 minutes at 18-33 hpf (lateral views) or 18-22 hpf (cross-sections). Five samples were analyzed in each experiment. Aberrant radial processes of the nVII motor neurons were counted in live embryos at 20-22 hpf.

RESULTS

The genes affected in the olt and ord mutants are specifically required for caudal migration of the nVII motor neurons

We previously used the zebrafish Isl1-GFP transgenic line, which expresses green fluorescent protein (GFP) in the branchiomotor neurons of the hindbrain (Higashijima et al., 2000), to identify novel mutants with perturbed caudal migration of the nVII motor neurons (Wada et al., 2005). The olt and ord mutations were further characterized, and one allele for the olt locus (olt\(^{rw689}\)) and four alleles for the ord locus (ord\(^{w71}\), ord\(^{w135}\), ord\(^{w166}\) and ord\(^{w380}\)) were used in this study.

In wild-type embryos, the nVII motor neurons begin to express GFP in r4 at 16 hours post-fertilization (hpf), after which they start migrating caudally towards r6 (Fig. 1D,G) (Chandrasekhar et al., 1997; Higashijima et al., 2000; Wada et al., 2005). The olt and ord embryos display impairment in migration of the nVII motor neurons, albeit differently. In the olt embryos, none of the nVII motor neurons migrated caudally, but they all migrated dorsally into r4 (Fig. 1E,H). By contrast, in the ord embryos, some of the nVII motor neurons also migrated into the r5 region (Fig. 1F,I). Embryos homozygous for each of the four alleles of ord showed similar impairment in migration of the nVII motor neurons. The other morphological features of the olt and ord embryos were normal as compared to those of wild-type embryos (Fig. 1A-C), and the adult mutants were viable and fertile. We obtained maternal-and-zygotic (MZ) embryos by mating homozygous fish. Because the MZ-olt\(^{rw689}\) and MZ-ord\(^{w71}\) embryos were also viable and displayed the same defects in neuronal migration, we used them in the subsequent analyses. In the following experiments, we showed that the ord and olt genes are barely expressed maternally (see below and Fig. S2C,G in the supplementary material). These results suggest that these genes are not required in the early stages of development, supporting the viability of the MZ-olt\(^{rw689}\) and MZ-ord\(^{w71}\) embryos.

Despite their ectopic localization, nVII motor neurons in the mutant embryos expressed \(tag-1\) mRNA and extended peripheral axons normally to the correct target muscles (see Fig. S1A-F in the supplementary material). Moreover, the expression patterns of the rhombomere-specific genes and the formation of hindbrain neurons were unaffected in the mutant embryos (see Fig. S1G-U in the supplementary material). These results suggest that the overall patterning and differentiation of the hindbrain neurons were unaffected by the mutation, except for the aberrant migration of the nVII motor neurons.

olt encodes zebrafish Frizzled3a

The olt locus was genetically mapped to the linkage group 20 (Fig. 2A). This genomic region contains a gene similar to mammalian frizzled3 (Fig. 2B,C) (Wang et al., 2002). Because another frizzled3 homolog was identified (Fig. 2D), we termed the gene associated with the olt locus zebrafish frizzled3a (\(fz3a\)) and the other homolog frizzled3b (\(fz3b\)). Sequence analyses showed that the allele olt\(^{rw689}\) carries a missense mutation resulting in substitution of the second conserved cysteine of the extracellular cysteine-rich domain (CRD) of Fz3a (C38S) (Fig. 2C; see also Fig. S2A in the supplementary material). This amino acid substitution may disrupt proper folding, and thereby the function, of the Frizzled family proteins, because the second and fourth conserved cysteines of the CRD form a disulfide bond (Dann et al., 2001). Indeed, in Drosophila, the mutated \(Dfz2\) allele, which carries a substitution of the fourth conserved cysteine of the CRD to serine (C118S), shows a reduced capacity to reach the cell surface (Chen et al., 2004). Ectopically expressed Frizzled family proteins recruit the downstream effector Dsh to the plasma membrane (Axelrod et al., 1998), and recruitment of Dsh to the plasma membrane is essential for downstream signaling (Park et al., 2005). Thus, to determine whether the amino acid substitution resulting from the mutation in the ord\(^{rw689}\) allele leads to functional disruption of the Fz3a protein, we examined the subcellular localization of Fz3a and mutated Fz3arw689, and of Xenopus Dsh (Xdsh), when Fz3a or Fz3arw689 is overexpressed. We found that the mutated Fz3arw689 protein was not associated with the plasma membrane and that it lost its capacity to recruit Xdsh to the plasma membrane (see Fig. S2B in the supplementary material).

To confirm that the loss of function of the \(fz3a\) gene is responsible for the olt phenotype, we used an antisense MO (\(fz3a\)-MO) (see Fig. S2D,E in the supplementary material). The resulting morphant phenotype was identical to that of the olt mutant embryos (Fig. 2F-H), with impaired migration of the nVII motor neurons (100% of MO-injected embryos; \(n=47\)). Control MO (\(fz3a\)-MO-5mhs) did not impair migration of the nVII motor neurons in the injected embryos (\(n=30\)). There was little expression of \(fz3a\) mRNA up to the gastrula
Migration of the nVII motor neurons was specifically impaired (arrow in injected with mRNA into the wild-type or injection of embryos. However, neuronal migration defects in the nVII motor neurons are migrating (Fig. 2E,E).

Next, we analyzed whether injection of mRNA could rescue the neuronal migration defects in the olt gene. The mis-sense amino acid substitution in the Fz3arw689 gene was indicated. Genetic map of the olt locus. (A) Lateral view of a wild-type embryo reacted with the protein is indicated. (B) Cross section of E at r5. (F-H) Wild-type isl1-GFP embryo injected with fz3a-MO (H) showing the same neuronal migration defects as those observed in an olt embryo (G). The embryos are shown in dorsal view and the images are composite stacks of serial optical sections. (I-I') Wild-type isl1-GFP embryo injected with fz3a-ΔC mRNA. Migration of the nVII motor neurons was specifically impaired (arrow in I'). Lateral views. Scale bars: 50 μm.

To confirm that loss of function of the celsr2 gene is responsible for the olt phenotype, we used an antisense MO (celsr2-MO) (see Fig. S2J in the supplementary material). The resulting morphant phenotype was identical to that of the olt mutant embryos (Fig. 3H-J), with impaired migration of the nVII motor neurons (95% of MO-injected embryos; n=99), confirming that the olt gene encodes Celsr2. Migration of the nVII motor neurons was not impaired in 74 embryos injected with control MO (celsr2-MO-5mis). The celsr2 mRNA was expressed throughout the embryo at the gastrula stages, and became restricted to the CNS thereafter (see Fig. S2G in the supplementary material). The celsr2 mRNA was expressed throughout the brain at 24 hpf, when the nVII motor neurons were migrating (Fig. 3E,E').

We designed celsr1a-MO and celsr1b-MO (see Fig. S2J in the supplementary material). Injection of celsr1a-MO or celsr1b-MO alone into wild-type embryos did not cause any defect in neuronal migration (Fig. 3L). However, co-injection of celsr1a-MO and celsr1b-MO gave rise to a phenotype similar to that of the olt embryos, showing impaired migration of the nVII motor neurons (34% of injected embryos, n=82; Fig. 3L). Interestingly, injection of stages (see Fig. S2C in the supplementary material), but fz3a mRNA was expressed throughout the hindbrain at 24 hpf, at the time when the nVII motor neurons are migrating (Fig. 2E,E').

This region contains a gene coding for Celsr2 (for cadherin, EGF-like, LAG-like and seven-pass receptor), which is a vertebrate homolog of Drosophila Flamingo (Fmi, also known as Stan – Flybase) (Fig. 3B-D) (Usui et al., 1999; Formstone and Little, 2001; Shima et al., 2002). Sequence analyses showed that each of the four alleles of the olt locus carries a point mutation in the celsr2 gene. The alleles olt m, olt m-166 and olt m-380 carry a mis-sense mutation resulting in amino acid substitution (C1835S, E582K and V1168D, respectively) in the extracellular domain, and the olt allele carries a premature stop codon (L288Stop) in the extracellular domain (Fig. 3C, see also Fig. S2F in the supplementary material).

We showed that this genomic region contains a gene coding for Celsr2, an ortholog exists in vertebrates. The celsr1a orthologs and one celsr3 ortholog exists in the zebrafish genome (Fig. 3D). The celsr1 orthologs have been described and referred to previously as flamingo (fmi1a and fmi1b) (Formstone and Mason, 2005). To abide by the rule of the Zebrafish Nomenclature Committee (http://zfin.org/zf_info/nomen.html) to follow the mammalian terminology, we refer to these genes as celsr1a (equivalent to fmi1a) and celsr1b (equivalent to fmi1b) in this study. We demonstrated that celsr1a and celsr1b mRNAs, but not celsr3 mRNA, were also expressed in the developing hindbrain (Fig. 3F,F',G,G' and Fig. S2H in the supplementary material). We therefore analyzed the function of the celsr1a and celsr1b genes.

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ord encodes zebrafish Celsr2

The olt locus was genetically mapped to linkage group 22 (Fig. 3A). We showed that this genomic region contains a gene coding for Celsr2 (for cadherin, EGF-like, LAG-like and seven-pass receptor), which is a vertebrate homolog of Drosophila Flamingo (Fmi, also known as Stan – Flybase) (Fig. 3B-D) (Usui et al., 1999; Formstone and Little, 2001; Shima et al., 2002). Sequence analyses showed that each of the four alleles of the olt locus carries a point mutation in the celsr2 gene. The alleles olt m, olt m-166 and olt m-380 carry a mis-sense mutation resulting in amino acid substitution (C1835S, E582K and V1168D, respectively) in the extracellular domain, and the olt allele carries a premature stop codon (L288Stop) in the extracellular domain (Fig. 3C, see also Fig. S2F in the supplementary material).

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celsr1a and celsr1b function redundantly with ord/celsr2 in regulating migration of the nVII motor neurons

The celsr1, celsr2 and celsr3 mRNAs are differentially expressed in developing mouse CNS (Formstone and Little, 2001; Shima et al., 2002). To understand the function of zebrafish celsr homologs in migration of the nVII motor neurons, we identified celsr family genes. A BLAST homology search and phylogenetic tree analyses revealed that two celsr1 orthologs and one celsr3 ortholog exists in the zebrafish genome (Fig. 3D). The celsr1 orthologs have been described and referred to previously as flamingo (fmi1a and fmi1b) (Formstone and Mason, 2005). To abide by the rule of the Zebrafish Nomenclature Committee (http://zfin.org/zf_info/nomen.html) to follow the mammalian terminology, we refer to these genes as celsr1a (equivalent to fmi1a) and celsr1b (equivalent to fmi1b) in this study. We demonstrated that celsr1a and celsr1b mRNAs, but not celsr3 mRNA, were also expressed in the developing hindbrain (Fig. 3F,F',G,G' and Fig. S2H in the supplementary material). We therefore analyzed the function of the celsr1a and celsr1b genes.
celsr1a-MO or celsr1b-MO into the MZ-ord embryos enhanced the severity of neuronal migration defects. In most of the resulting embryos, the nVII motor neurons did not migrate at all (79% of embryos injected with celsr1a-MO, n=85; 85% of embryos injected with celsr1b-MO, n=72; Fig. 3L). Moreover, co-injection of celsr1a-MO and celsr1b-MO resulted in complete loss of neuronal migration in all the MZ-ord embryos, as observed in the olt embryos (97%, n=151; Fig. 3L). Control MOs did not enhance the severity of neuronal migration defects in the MZ-ord embryos (none of the 71 embryos injected with celsr1a-MO-5mis, or of the 60 embryos injected with celsr1b-MO-5mis). These results suggest that celsr1a and celsr1b act in concert with ord/celsr2 to regulate migration of the nVII motor neurons.

**In mutant embryos, nVII motor neurons migrate aberrantly away from the pial surface of the hindbrain**

To understand the function of the fz3a and celsr2 genes, we characterized the migration of the nVII motor neurons in further detail. Wild-type and mutant embryos were stained with anti-

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**Fig. 3. Identification of the ord gene as zebrafish celsr2 gene.** (A) Genetic map of the ord locus. (B) Genomic structure of the zebrafish celsr2 gene. The nucleotide substitution resulting from each mutation is indicated. (C) Schematic drawings of the mouse and zebrafish Celsr2 proteins. Amino acid sequence similarity (%) is shown for each domain. The amino acid substitution resulting from each mutation is indicated. (D) The phylogenetic tree for celsr-family genes. (E-G) Lateral views of wild-type embryos reacted with RNA probes for celsr2 (E), celsr1a (F) and celsr1b (G) at 24 hpf. (E’, F’, G’) Cross sections at r5 for each embryo, E-G, respectively. (H-K) A wild-type Isl1-GFP embryo injected with celsr1a/1b-MOs shows complete loss of migration of the nVII motor neurons. The embryos are shown in dorsal view and the images are composite stacks of serial optical sections. (L) The neuronal migration phenotype in each experiment was scored as follows: r4, complete loss of migration, as shown in K; r5, partial disruption of migration, as shown in I; and r6, normal migration, as shown in H. Scale bars: 50 μm.
Fig. 4. Defective migration of the nVII motor neurons in mutant embryos. (A–K) Wild-type (A,D,G,J), olt\(^{rw699}\) (B,E,H) and ord\(^{rw71}\) (C,F,I,K) Isl1-GFP embryos were stained with anti-acetylated α-tubulin antibody. (A–C) Composite stacks of serial optical sections, shown in dorsal view. (D–K) Images are single focal planes of cross sections at the rhombomeric regions indicated by broken lines in A–C. Hindbrain regions are outlined by broken lines. (H,I,K) Higher magnifications of E, G, I, respectively. In the mutant embryos, some of the neurons reach the ventricular surface (arrowheads in H and K). However, these mismigrated neurons extend axons normally (shown by arrows in H and J). GFP-expression in single axons was barely detectable (H). Therefore, the yellow signals of the axons of the nVII motor neurons in the wild-type embryos (J) are technical artifacts caused by superimposition of the red signals of the axons and the green signals of the cell bodies of the overlapping neurons. (L–P) Aberrant radial processes in the mutant embryos (arrowheads in M). Frontal views of the live wild-type (L) and olt (M) Isl1-GFP embryos at r4, and a dorsal view of the olt Isl1-GFP embryo (N) at 24 hpf. Higher magnification of the boxed region is shown in the inset. (L–N) Images are composite stacks of serial optical sections. (O) Scoring of aberrant processes in the wild-type (WT), olt and ord embryos injected with celsr1a/Hb-MOs (ord-MOs). Bars represent S.D. (P) Direction of the aberrant processes was quantified in the olt embryos. The angle of each process was measured as the deviation from the right angle to the midline. Scale bars: 50 μm.

Acetylated α-tubulin antibody (red), and the Isl1-GFP signals (green) were captured simultaneously (Fig. 4A–K). The peripheral axons of the nVII motor neurons and the medial longitudinal fascicles (MLF) were immunoreactive to anti-acetylated α-tubulin antibody (Fig. 4A–K). In the wild-type embryos, the migrating motor neurons were localized close to the pial surface of the hindbrain and were connected to the MLF (Fig. 4A,D,G). Some of the migrating nVII motor neurons appeared to be in direct contact with the MLF; others, which were located a short distance away from the MLF, had axons projecting to the MLF tracts (Fig. 4J), showing that migrating motor neurons also extend axons. A time-lapse study using hindbrain explants (Bingham et al., 2005) (see Materials and methods) confirmed that the motor neurons were always located near the pial surface of the hindbrain during migration (Fig. 5A, see also Movie S1 in the supplementary material).

By contrast, in the olt and ord embryos, the nVII motor neurons did not migrate caudally near the pial surface of the hindbrain. Instead, they mismigrated towards the ventricle of the hindbrain at r4 (Fig. 4B,C,E,F). Some of the mismigrating neurons almost reached the midline at r4 or r5 (arrowheads in Fig. 4H,K; compare with the wild-type embryo in Fig. 4J). However, the nVII motor neurons located away from the pial surface of the hindbrain still projected axons to the MLF (arrows in Fig. 4H). The peripheral axons extended dorsally and laterally from the cell bodies of the nVII motor neurons, and they exited the hindbrain at r4 in the olt embryos (see Fig. S4 in the supplementary material). These results suggest that the fz3a and celsr2 genes regulate the pathway of migrating nVII motor neurons without affecting their axogenesis.

**nVII motor neurons migrate in aberrant directions by extending radial processes in the mutant embryos**

In the wild-type embryos, the migrating nVII motor neurons showed biased caudal protrusive activity (see Movie S1 in the supplementary material) (Jessen et al., 2002). Our time-lapse observations revealed that the nVII motor neurons migrated dorsally in the hindbrain explants from the olt embryos (arrow in Fig. 5B, see Movie S2 in the supplementary material). Moreover, the motor neurons migrated aberrantly by extending long processes towards the ventricle at r4 (Fig. 5C, Movie S5 in the supplementary material). On average, 1.7 processes per embryo were observed at a single time point (Fig. 4M,O; 27 olt embryos were observed). A large proportion (47%; n=47) of these aberrant radial processes reached the ventricular surface (Fig. 4M). By contrast, radial processes were seldom observed in the wild-type embryos (Fig. 4L,O, see Movie S4 in the supplementary material). Only two aberrant processes were detected in 39 wild-type embryos and they did not reach the ventricular surface. The ord embryos injected with celsr1a-MOs and celsr1b-MOs showed an identical phenotype to that of the olt embryos (Fig. 4O; on average, 1.7 processes per embryo were observed in 24 morphants). These results suggest that the fz3a and celsr genes function in the same manner.

Next, we quantified the direction of the aberrant processes in the olt embryos. Most of the nVII motor neurons extended aberrant processes at right angles to the ventricular surface (Fig. 4N,P). Mismigrating motor neurons sometimes reached the ventricles (arrowhead in Fig. 4H), indicating that these aberrant processes could steer the migrating motor neurons in the wrong direction.
Incomplete migration of nVII motor neurons in \textit{ord} embryos

Next, we performed time-lapse observations of hindbrain explants from \textit{ord} embryos. As described above, some of the nVII motor neurons migrated into r5 in the \textit{ord} embryos because of the functional redundancy of \textit{celsr1a} and \textit{celsr1b} (Fig. 3H-L). These incompletely migrated neurons were not associated with the pial surface of the hindbrain (Fig. 4K).

The time-lapse observations revealed that some of the early-born nVII motor neurons migrated near the pial surface of the hindbrain in the \textit{ord} embryos (Fig. 5D; also see Movie S3 in the supplementary material). By contrast, most of the late-born nVII motor neurons became detached from the pial surface of the hindbrain, and these motor neurons still migrated caudally to some extent (arrows in Fig. 5D; also see Movie S3 in the supplementary material). However, the migration of these nVII motor neurons in the \textit{ord} embryos was by no means normal in nature, and most of the motor neurons stopped migrating at r5 and failed to reach r6.

We previously traced cell movements in wild-type embryos and showed that nVII motor neurons migrate independently of other neuroepithelial structures (Wada et al., 2005). To compare the behavior of nVII motor neurons with neighboring neuroepithelial cells, we observed mosaic embryos in which several neuroepithelial cells were randomly labeled with rhodamine-conjugated dextran (see Fig. S3A,B in the supplementary material). We confirmed that, in the \textit{ord} embryos, some of the nVII motor neurons became detached from the pial surface of the brain and migrated caudally relative to the neighboring cells (see Fig. S3B in the supplementary material). These results demonstrated that the nVII motor neurons can migrate caudally to some extent without keeping direct contact with the pial structure of the hindbrain in the \textit{ord} embryos. However, we cannot exclude the possibility that some component of the pial structures, such as the MLF, may have a long-distance influence.

\textit{fz3a} and \textit{celsr2} genes mainly act in a non-cell-autonomous manner during migration of nVII motor neurons

During the development of zebrafish embryos, the hindbrain everts, and its ventricle opens at 18-22 hpf and begins to expand at 24 hpf (Lowery and Sive, 2005). The \textit{olt} embryos also showed rapid expansion of the ventricle (as observed in the wild-type embryos) when they were stained with anti-β-catenin antibody at 24 hpf and 33 hpf (Fig. 6A,B) (Lowery and Sive, 2005). As a consequence, the orientation of the neuroepithelial cells became more parallel to the
midline, and the nVII motor-neuron clusters changed their positions to be relatively further away from the ventricle and the neurons were located laterally in the olt embryos (Fig. 6B).

We investigated the cell autonomy of the mutations by mosaic analysis (see Fig. S3C,C in the supplementary material for imaging of the shapes of the transplanted neuroepithelial cells). As described above, none (or almost none) of the nVII motor neurons had reached the r6 region in the olt and ord mutant embryos at 48 hpf (Fig. 1D-F). However, a significant proportion of the MZ-olt-derived neurons (45% of 29 neurons monitored in four mosaic embryos), or of the MZ-ord-derived neurons (56% of 75 neurons monitored in nine mosaic embryos), had migrated into r6 in the wild-type host embryos at 36 hpf (Fig. 6C,E, also see Fig. S5A,C in the supplementary material for observations of other mosaic embryos). These results indicate that the fz3a and celsr2 genes mainly act in a non-cell-autonomous manner during migration of nVII motor neurons.
**Fig. 7. Schematic drawing of nVII motor-neuron migration in the wild-type and olt embryos.** In the wild-type embryos, the nVII motor neurons (green) migrated caudally near the pial surface of the hindbrain. By contrast, in the olt embryos, because the neuroepithelial cells (red) had lost their ability to prevent integration of the nVII motor neurons, the motor neurons migrated towards the ventricle by extending aberrant processes radially to the ventricle. Directions of migration of the nVII motor neurons are indicated by arrows. See text for details.

**fz3a is required in the neuroepithelial cells to restrict the nVII motor neurons near the pial surface of the hindbrain.**

We then investigated the cell type responsible for regulating the migration of the nVII motor neurons in the developing hindbrain. Because fz3a and celsr2 mRNAs are expressed widely in the hindbrain (Fig. 2E,E’; Fig. 3E,E’), the neuroepithelial cells surrounding the nVII motor neurons are good candidates as regulators of nVII motor-neuron migration. In the mutant embryos, the nVII motor neurons migrated into the neuroepithelial layer at r4 (Fig. 4E,F). A function of wild-type neuroepithelium may, therefore, be to prevent integration of the nVII motor neurons into the neuroepithelium and restrict them near the pial surface of the brain.

To test this hypothesis, we observed the behavior of nVII motor neurons in wild-type embryos in which the neuroepithelium contained MZ-olt-derived cells in mosaic analyses (Fig. 6G,1). In these experiments, we analyzed MZ-olt embryos because the ord embryos showed complex migrating behavior, as shown in Fig. 5D. The MZ-olt-derived cells (red) were incorporated into the neuroepithelium of the wild-type host embryos at r4 (Fig. 6G). In the intact side (left side of the embryo shown in Fig. 6G), the wild-type nVII motor neurons (green) were located normally near the pial surface of the brain, as described (Fig. 4A,D). However, in the mosaic region (right side of the embryo shown in Fig. 6G), the wild-type nVII motor neurons migrated into the MZ-olt-derived cell cluster (Fig. 6G,1; the example shown is representative of six mosaic embryos). Because of the eversion of the hindbrain as described above (Fig. 6A,B), these migrated nVII motor neurons were located dorsally rather than medially in the hindbrain (Fig. 6G). These results showed that the mutant neuroepithelial cells allowed invasion of the wild-type nVII motor neurons into the neuroepithelial layer (Fig. 6I’).

Next, we observed the behavior of nVII motor neurons in olt embryos in which the neuroepithelium contained wild-type-derived cells (Fig. 6H-J). In this case, the wild-type-derived cells (red) were incorporated into the neuroepithelium of the MZ-olt host embryos at r4 and r5 (Fig. 6H). In the intact side (left side of the embryo shown in Fig. 6H), the nVII motor neurons (green) mismigrated dorsally in the olt mutant neuroepithelium, as described (Fig. 4B,E). However, in the mosaic region (right side of the embryo shown in Fig. 6H), the MZ-olt-derived nVII motor neurons failed to invade the wild-type cell cluster (Fig. 6H,J,J’; the example shown is representative of ten mosaic embryos; also see Fig. S6 in the supplementary material for observations of other mosaic embryos). These results showed that the wild-type neuroepithelial cells prevented the nVII motor neurons from invading the neuroepithelial layer (Fig. 6I’). In Fig. 6H, it is apparent that several nVII motor neurons in the mosaic regions migrated slightly caudally in comparison with the intact side, suggesting that the caudal migration may be rescued by the associated wild-type neuroepithelium. However, we could not introduce wild-type large-cell clusters encompassing the r4 and r5 regions in the other mosaic embryos. Therefore, we could not make sufficient observations to provide statistical support for this possibility.

Together, the above results demonstrate that the fz3a gene acts in the neuroepithelium to regulate the direction of neuronal migration by preventing integration of nVII motor neurons into the neuroepithelium and by restricting them to a region near the pial surface of the brain (Fig. 7).

**DISCUSSION**

**Neuroepithelial cells prevent integration of nVII motor neurons into the neuroepithelium.**

Previous studies from our, and other, laboratories revealed that the normal migration of the nVII motor neurons is guided by two mechanisms. In mouse hindbrain, guidance cues emanate from r5 and r6 to attract the nVII motor neurons (Studer, 2001). These may include Reelin (Ohshima et al., 2002; Rossel et al., 2005) and Sdf-1 (Sapele et al., 2005) signaling. In the present study, we have clearly shown that the neuroepithelial cells function in preventing integration of the nVII motor neurons into the neuroepithelial layer of the hindbrain, and that this radial exclusion is essential for normal migration of the nVII motor neurons. The mosaic experiments using tri/stbm (Jessen et al., 2002), llk/scrb1 (Wada et al., 2005), olt/fz3a and ord/celsr2 (present study) genes showed that the wild-type-derived nVII motor neurons completely failed to migrate caudally in the mutant host embryos. We showed that the nVII motor neurons of MZ-olt host embryos did not invade the wild-type neuroepithelial cell cluster but that, conversely, the nVII motor neurons of wild-type host embryos did invade the mutant neuroepithelial cell cluster (Fig. 6G-J). These findings indicate that normal migration requires a functional olt/fz3a gene mainly in the surrounding neuroepithelial cells. However, as a non-negligible proportion of the mutant embryo-derived nVII motor neurons also failed to migrate in the wild-type host embryos (Fig. 6C), we can not exclude the possibility that these genes may also act in the migrating nVII motor neurons themselves.

We observed that some of the early-born nVII motor neurons mismigrated radially away from the pial surface in the olt embryos (Fig. 5C). However, we can still not exclude the possibility that some other late-born neurons may have stalled during migration on their way to the pial surface, before they started expressing GFP.

**Neuroepithelial cells play a contrasting role for the re-integration of newly divided neuroepithelial cells.**

It has been shown that the tri/stbm gene in neuroepithelial cells regulates the re-integration of daughter cells into the neuroepithelial layer after cell division (Ciruna et al., 2006). By contrast,
neuroepithelial cells prevent integration of nVII motor neurons into the neuroepithelial layer, as shown in the present study. Hence, we propose opposite roles for the neuroepithelial cells depending on the cell type that they act upon—integration of neuroepithelial daughter cells into the neuroepithelial layer and exclusion of nVII motor neurons from the neuroepithelial layer. Fz3a and Celsr2 together with Stbm may control cell-adhesion or cell-repulsion molecules that are specifically required for the integration or exclusion of cells from the neuroepithelium.

Radial exclusion and caudal migration of the nVII motor neurons are interdependent mechanisms

We showed that in the *otl* embryos, the mismigrated nVII motor neurons extended aberrant processes towards the ventricle at right angles to the anterior-posterior axis (Fig. 4N,P). These observations suggest that the nVII motor neurons in the *otl* embryos lose their ability to migrate caudally. There was some, albeit incomplete, migration of the nVII motor neurons in the *ord* embryos, apparently because of functional redundancy with the *celsr1a* and *celsr1b* genes. However, the migration of the nVII motor neurons in the *ord* embryos was by no means normal in nature, and these neurons stopped migrating at r5 and failed to reach r6. In the *ord* embryos, the nVII motor neurons also invaded the neuroepithelial layer. These results suggest that radial exclusion and caudal migration are interdependent mechanisms of normal nVII motor neuron migration. A recent study has shown that the *tristbm* gene is essential for localization of Prickle to the anterior membrane of neuroepithelial cells (Ciruna et al., 2006), suggesting that these genes may control the anterior-posterior polarity of these cells (Ciruna et al., 2006). This might provide some basis for caudal migration of the nVII motor neurons.

In a previous study, we showed that the r4-derived nVII motor neurons are composed of the branchiomotor neurons and the OLe neurons (Higashijima et al., 2000; Wada et al., 2005), and that the OLe neurons are possibly among the population to first migrate out of r4 (Fig. 5D). However, by labeling the OLe neurons with Dil retrogradely, we demonstrated that the OLe neurons failed to migrate caudally and remained in r4 in the *ord* embryos (n=4, see Fig. S7 in the supplementary material).

Possible roles of the *fz3a* and *celsr* genes in the neuroepithelium

We showed that maternal and zygotic impairment of Fz3a, or overexpression of Fz3a-ΔC, specifically disrupted nVII motor-neuron migration without affecting the early pattern formations or CE movements during gastrulation (Fig. 1B; Fig. 2I). These results suggest that Fz3a interacts with only a specific ligand to regulate the neuroepithelial functions. Conversely, Silberblick (Slb)/Wnt11 and Pipetail (Ppt)/Wnt5a regulate CE movements but do not regulate nVII motor-neuron migration (Bingham et al., 2002; Jessen et al., 2002). Our data, taken together with these results, suggest that the genetic cascades regulating the neuroepithelial functions adopt a different ligand-receptor system to that used for the regulation of CE movements.

It remains unclear as to how the other downstream effectors of Fz3a, including Dsh, are involved in the regulation of neuroepithelial functions. We showed that simultaneous overexpression of Fz3a and Xenopus Dsh (Xdsh) led to the recruitment of Xdsh to the plasma membrane at the blastula stages (see Fig. S2B in the supplementary material). These results suggest that Fz3a can interact with Xdsh. By contrast, overexpression of a mutant form of Xdsh, Xdd1, which lacks a PDZ domain disrupts CE movements in a dominant-negative manner but does not impair migration of the nVII motor neurons (Jessen et al., 2002). Therefore, there may be functional specialization among different Dsh proteins in zebrafish, as described above for Wnts and Fzs, and *Xenopus*-derived Xdd1 may antagonize a special Dsh that is required for CE movements only. Alternatively, the domains of Dsh required for the neuroepithelial functions may be different from those regulating CE movements, and Xdd1 may still be able to transmit signals that normally regulate neuroepithelial functions. We also do not exclude the possibility that Fz3a may act independently of Dsh in its regulation of neuroepithelial functions.

It has been suggested that homophilic interaction of Fmi through the extracellular cadherin-repeat domain plays an important role at cell–cell boundaries in epithelial planar polarity (Usui et al., 1999). Thus, it is possible that the Celsr proteins also regulate cell adhesion between adjacent neuroepithelial cells to restrict invasion of the nVII motor neurons into the neuroepithelium.

The Eph receptors and their ligands, the ephrins, are other good candidate mediators of cell repulsion (for reviews, see Wilkinson, 2001; Kullander and Klein, 2002). In the mouse and chick hindbrain, Eph receptors and ephrin ligands are expressed in the nVII motor neurons and neuroepithelial cells, respectively (Cowan et al., 2000; Kury et al., 2000). Recent studies have shown that Dsh forms a complex with EphrinB to mediate cell repulsion in *Xenopus* embryos (Tanaka et al., 2003; Lee et al., 2006). Thus, it is possible that Fz and Celsrs may regulate the activity of cell-repulsive molecules, such as ephrins, on the surface of the neuroepithelial cells.

Novel roles of the frizzled and celsr family genes in brain development

Our present finding that neuroepithelial cells are involved in positioning specific neurons near the pial surface suggests a fundamental role for the neuroepithelium in brain development. In the mammalian cortex, neurons are generated in ventricular germinal zones and migrate radially towards the pial surface to form architectual layered structures. In mouse embryos, Reelin signaling regulates the positioning of neurons during layer formation of the cerebrum (reviewed by Tissir and Goffinet, 2003), and is essential for radial migration of the nVII motor neurons (Ohshima et al., 2002; Rossel et al., 2005). These data suggest that similar mechanisms regulate the proper positioning of both the hindbrain motor neurons and the cortical layer neurons.

In the mouse cerebral cortex, many wnt and frizzled family genes are expressed in gene-specific regional and lamina patterns (Shimogori et al., 2004). Such patterned expression suggests the possibility that these genes are involved in other aspects of brain development. Recent studies have shown that functional *fzd3* and *celsr3* genes are required for the development of the anterior commissure, and the cortico-subcortical, thalamocortical and corticospinal tracts (Wang et al., 2002; Tissir et al., 2005). It is possible that the mouse *fzd3* and *celsr3* genes regulate neuroepithelial cells to guide these axonal tracts to the proper region in a similar manner to that by which the zebrafish *fz3a* and *celsr* genes act in neuroepithelial cells to restrict the migrating nVII motor neurons near the pial surface of the hindbrain. Our demonstration of a role for neuroepithelial cells in preventing integration of differentiated neurons into the neuroepithelial layer may provide new insights into the general mechanisms underlying the formation of layered structures in the mammalian brain, such as in the cerebral cortex.

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Supplementary material
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