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The Atypical Cadherin *Celsr1* Functions Non-Cell Autonomously to Block Rostral Migration of Facial Branchiomotor Neurons in Mice

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Abstract

The caudal migration of facial branchiomotor (FBM) neurons from rhombomere (r) 4 to r6 in the hindbrain is an excellent model to study neuronal migration mechanisms. Although several Wnt/Planar Cell Polarity (PCP) components are required for FBM neuron migration, only *Celsr1*, an atypical cadherin, regulates the direction of migration in mice. In *Celsr1* mutants, a subset of FBM neurons migrates rostrally instead of caudally. Interestingly, *Celsr1* is not expressed in the migrating FBM neurons, but rather in the adjacent floor plate and adjoining ventricular zone. To evaluate the contribution of different expression domains to neuronal migration, we conditionally inactivated *Celsr1* in specific cell types. Intriguingly, inactivation of *Celsr1* in the ventricular zone

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Author Contributions

D.M.G. designed and performed all experiments except those indicated, analyzed the data, and co-wrote the manuscript. W.P. performed the sFRP experiments, and the Wnt bead experiments, which L.R. initiated and standardized. Y.Q., A.M.G. and F.T. performed the Nkx6.2 cKO experiments. K.M. provided some sFRP mutant embryos, and both sFRP knockout lines. M.D.B. and M.S. generated and provided the r4-Cre line before publication. B.F. performed the NeuroVue labeling experiments with assistance from D.M.G. All authors commented on and contributed to manuscript revisions. A.C. conceived the project, supervised the experiments and co-wrote the manuscript.

of r3-r5, but not in the floor plate, leads to rostral migration of FBM neurons, greatly resembling the migration defect of *Celsr1* mutants. Dye fill experiments indicate that the rostrally-migrated FBM neurons in *Celsr1* mutants originate from the anterior margin of r4. These data suggest strongly that *Celsr1* ensures that FBM neurons migrate caudally by suppressing molecular cues in the rostral hindbrain that can attract FBM neurons.

Keywords

Hindbrain; Facial Branchiomotor Neuron; Neuronal migration; *Celsr1*

Introduction

Neuronal migration is a fundamental process in nervous system development, aiding in the establishment of complex neuronal architecture and underlying neural circuitry. All central nervous system neuron cell bodies undergo radial migration, usually along radial glial fibers, as they move from their birthplaces in the ventricular zone to their final locations near the pial surface. In addition, many types of neurons undergo tangential migration, which involves movement orthogonal to the radial direction within specific brain regions. In contrast to radial migration, which establishes distinct neuronal layers and is part of a neuron's maturation process, tangential migration helps generate neuronal diversity within a given brain region (Marín and Rubenstein, 2003; Valiente and Marín, 2010). The facial branchiomotor (FBM) neurons are a particularly intriguing neuron type that undergoes both radial and tangential migration. These neurons, which control facial and jaw movements, are born in rhombomere (r) 4 of the developing hindbrain. In most species studied, they migrate tangentially (caudally) into r6, followed by radial migration to form the facial motor nucleus near the pial surface of r6 (Chandrasekhar, 2004; Guthrie, 2007; Wanner et al., 2013).

Some, but not all, components of the non-canonical Wnt/planar cell polarity (PCP) pathway regulate tangential migration of FBM neurons in zebrafish and mice. While many PCP genes such as *Celsr1-3*, *fzd3a*, *Pk1*, *Ptk7*, *Scrb*, *Vangl2*, and *Wnt5a* are necessary for proper migration of FBM neurons into r6 (Carreira-Barbosa et al., 2003; Glasco et al., 2012; Mapp et al., 2010; Qu et al., 2010; Vivancos et al., 2009; Wada et al., 2006, 2005; Yang et al., 2014), other genes such as *Dvl1-2* and *glypican4/6* are not (Bingham et al., 2002; Glasco et al., 2012; Jessen et al., 2002; however, see also Davey et al., 2016), suggesting that caudal migration of FBM neurons is a PCP-independent process. Interestingly, one PCP gene, the atypical cadherin *Celsr1*, has been shown to regulate the directionality of FBM migration in mice (Glasco et al., 2012; Qu et al., 2010). In *Celsr1*-deficient mice, many FBM neurons migrate normally, yet a subset migrates rostrally into r2 instead of caudally into r6, completing their radial migration in r2. This reverse migration is not due to defects in neuronal specification or hindbrain patterning, suggesting that *Celsr1* acts specifically to dictate the direction of migration, and unlike other Wnt/PCP genes, does not affect their ability to migrate (Qu et al., 2010). The *Celsr1* mutant is the first mutant in zebrafish or mouse to exhibit a rostral migration phenotype, and provides a handle to investigate the mechanisms regulating directionality of neuronal migration.

Celsr1 is expressed in a dynamic fashion in the developing hindbrain, but is excluded from migrating FBM neurons (Formstone and Little, 2001; Qu et al., 2010; Shima et al., 2002; Tissir and Goffinet, 2006; Tissir et al., 2002). To understand how Celsr1 regulates the directionality of FBM neuron migration, we investigated the spatial requirement for Celsr1 function through tissue-specific knockouts by utilizing several tissue-specific Cre lines whose activities overlap with different aspects of the Celsr1 expression domain. Our data suggest strongly that Celsr1 functions in the ventricular zone of multiple rhombomeres to suppress the rostral migration of FBM neurons.

MATERIALS AND METHODS

Animals

Mouse colony maintenance and embryo collection were carried out using protocols approved by the Animal Care and Use Committee at the University of Missouri and the Animal Ethics Committee at the University of Louvain.

Mouse lines and genotyping

The Celsr1^{Crsh}, Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)Luo (ROSA26^{mT/mG}; gift from D. Cornelison, University of Missouri), Gt(ROSA)26Sor^{tm1}(EYFP)^{Cos} (ROSA26-EYFP), Tg(Nkx6-2-icre)1Kess/SshiJ (Nkx6.2-Cre), and Tg(Isl1-EGFP)2Slp (SE1::GFP) mouse lines and genotyping protocols have been described previously (Curtin et al., 2003; Fogarty et al., 2007; Glasco et al., 2012; Muzumdar et al., 2007; Shirasaki et al., 2006; Soriano, 1999; Srinivas et al., 2001). Shh^{tm1}(EGFP/cre)^{Cjt} (Shh^{Cre}) mice (Harfe et al., 2004) were obtained from Jackson Laboratories (stock #005622) and genotyped via their recommended protocols. Tg(Hoxb1-cre)r4Mist (r4-Cre) mice were genotyped as previously described (Di Bonito et al., 2013). Egr2^{tm2}(cre)^{Pch} (Krox20^{Cre}) mice (Voiculescu et al., 2000) were a gift from Susan Dymecki (Harvard University) and were genotyped with primers 5'-CTTTACACAGCATCGCCAAG-3', 5'-TTGACCAGATGAACGGAGTG-3', and 5'-ATCAGGACATAGCGTTGGCT-3', the Krox20^{Cre} and wild type alleles producing 500- and 659-bp bands, respectively. Sfrp1^{tm1}Bsk (Bodine et al., 2004) and Sfrp2^{tm1}Sato (Kobayashi et al., 2009) mutant mice were described previously. To obtain litters containing double mutant embryos, sFRP1^{+/-}; sFRP2^{+/-} adults were intercrossed, and embryos were genotyped as described (Misra and Matisse, 2010).

The Celsr1 conditional mutant mouse line was described previously (Ravni et al., 2009). Genotyping assays to distinguish between Celsr1^{WT}, Celsr1^{tm1}Fati (Celsr1^{floxed}, fl), and Celsr1^{tm1.1}Fati (Celsr1^{KO}, KO) alleles were designed by the University of Missouri-Columbia Research Animal Diagnostic and Investigative Laboratory (RADIL, now a part of IDEXX Laboratories). DNA was extracted from forelimb buds, which do not express Celsr1. The primer set 5'-CCACTCTGCTAACGGTAGG-3' and 5'-GAAAGAGACTGTTGGTGAAGC-3' produces a 596-bp band for the Celsr1^{fl} allele and a 389-bp band for the Celsr1^{WT} allele. The primer set 5'-CTCTGTTGACTTCTGACTGG-3' and 5'-GAAAGAGACTGTTGGTGAAGC-3' produces a 3504-bp band for the Celsr1^{KO} allele, and a 389-bp band for the Celsr1^{WT} allele. Since the assay to detect the Celsr1^{fl} allele

does not amplify the *Celsr1*^{KO} allele and vice versa, both assays were performed concurrently in order to identify mice with a *Celsr1*^{KO/fl} genotype.

In situ hybridization and immunohistochemistry

Synthesis of digoxigenin-labeled probes (*Celsr1*, *Islet1*, *Tbx20*, *Wnt5a*) and whole mount in situ hybridization were carried out as described previously (Glasco et al., 2012; Qu et al., 2010; Song et al., 2006). Methods for demarcation of rhombomere boundaries were described previously (Glasco et al., 2012).

For immunohistochemistry, the following antibodies were used: mouse anti-Is11 (1:500, DSHB 39.4d5), rabbit anti-GFP (1:500, Molecular Probes A11122), Alexa Fluor® 568 goat anti-mouse (1:250, Invitrogen A11004), and Alexa Fluor® 488 chicken anti-rabbit (1:250, Invitrogen A21441). 30-µm frozen tissue sections were prepared from OCT-embedded embryos on a Leica CM1900 cryostat, and slides were dried for 30 minutes at room temperature. Subsequent steps were carried out in a humidified chamber adapted from guidelines described elsewhere (Harlow and Lane, 2006a, 2006b, 2006c). The sections were permeabilized with PBT (1% Triton X-100 in PBS) three times for 10 minutes each. Non-specific binding was blocked with 10% filtered HIGS in PBT for one hour at room temperature. The diluted primary antibody was applied overnight at 4 °C. Following three ten-minute washes in PBT at room temperature, the sections were incubated in the diluted secondary antibody overnight at 4 °C. After three ten-minute PBT washes at room temperature, each slide was mounted in 175 µL of Mowiol mounting medium and imaged on a Zeiss LSM 510 confocal microscope. Images were processed using Zeiss software.

Neuronal tracing with lipophilic dyes

Protocols previously described (Fritsch et al., 2005; Qu et al., 2010) were adapted for anterograde labeling of hindbrain neurons. Small pieces of eyebrow hairs were soaked in liquid NeuroVue dyes (Molecular Targeting Technologies) and placed into the appropriate positions of intact embryo hindbrains. NeuroVue Jade (508 nm), Maroon (667 nm), and Red (588 nm) were used in this study. After dye diffusion was visually confirmed, hindbrains were dissected out, flat-mounted in 100% glycerol, and stored at 4 °C until imaging. Images were captured on a Leica SP5 confocal microscope and processed using Leica software.

Results

***Celsr1* functions non-cell autonomously, and independently of the floor plate, to regulate FBM neuron migration**

We showed previously that a subset of FBM neurons migrates rostrally into r3 and r2 in *Celsr1*^{Crsh/+} and *Celsr1*^{KO/KO} embryos (Qu et al., 2010). Interestingly, *Celsr1* is primarily expressed in midline tissues, and does not appear to be expressed in FBM neurons (Qu et al., 2010). Consistent with this, conditional inactivation of *Celsr1* in FBM neurons did not result in rostral migration, as seen in whole animal knockouts, suggesting strongly that *Celsr1* functions non-cell autonomously to suppress the rostral migration of FBM neurons.

Celsr1 is expressed in midline tissues adjacent to the migrating FBM neurons (Fig. 1A), including the ventral aspects of the ventricular zone at all axial levels in the hindbrain (Qu et al., 2010). It is robustly expressed in floor plate cells throughout the period of FBM neuron migration (Formstone et al., 2010; Qu et al., 2010). Since floor plate cells play a critical role in FBM neuron migration in mice and zebrafish (Sittaramane et al., 2013), we wondered whether Celsr1 may act in the floor plate to prevent the rostral migration of FBM neurons. We tested this hypothesis by specifically inactivating Celsr1 in floor plate cells using the Shh^{Cre} (Fig. 1B) and $Celsr1^{floxed}$ mouse lines.

To confirm that Cre activity was restricted to floor plate cells, we crossed Shh^{Cre} mice (Harfe et al., 2004) with $ROSA26^{mT/mG}$ reporter mice (Muzumdar et al., 2007) to generate $Shh^{Cre}; ROSA26^{mT/mG}$ embryos expressing membrane-targeted EGFP in tissues with active Cre recombinase. In addition to GFP immunostaining on sections (to label Cre-expressing cells), we examined the expression of the motor neuron marker *Islet1* (*Isl1*) to provide landmarks along the dorsal-ventral and rostral-caudal axes. At the onset of FBM neuron migration (E10.5) and later (E12.5), Cre-mediated GFP expression was robust in floor plate cells at all axial levels of the hindbrain (Fig. 2A-D) (n=6; 3 whole mount, 3 sectioned). Next, we generated *Celsr1* floor plate conditional mutants (FP-cKO) using a breeding strategy designed to produce two FP-cKO genotypes, $Shh^{Cre}; Celsr1^{KO/fl}$ and $Shh^{Cre}; Celsr1^{fl/fl}$ (Fig. S1). All embryos were assayed for FBM neuron migration defects by *Tbx20* in situ hybridization (ISH). In FP-cKO embryos, FBM neurons migrated only in the caudal direction in all embryos tested (n=14) (Figs. 2E, F and 5D), demonstrating that *Celsr1* does not function in the floor plate to suppress rostral migration of FBM neurons.

Celsr1 partly functions in rhombomere 4 to suppress rostral neuronal migration

Since *Celsr1* function in the floor plate is dispensable, we reasoned that its expression in the ventricular zone in r4, where the FBM neurons are born, could play a role. Therefore, we conditionally inactivated *Celsr1* in r4 and its derivatives using the r4-Cre mouse line (Di Bonito et al., 2013). To confirm Cre expression within r4, we crossed r4-Cre mice (Fig. 1B) with $ROSA26^{mT/mG}$ reporter mice and immunostained for *Isl1* and GFP at E10.5. Cre-mediated GFP was localized exclusively to r4-derived cells, including FBM neurons along their migration route in r4-r6 (Fig. 3A-D) (n=9; 2 whole mount, 7 sectioned). Notably, Cre-mediated GFP expression was robust throughout the neuroepithelium of r4, and was tightly restricted to this rhombomere. The only cells expressing GFP outside r4 in E10.5-12.5 wild type embryos were located in r5, likely representing the caudally migrating stream of FBM neurons (Fig. 3A, D).

In *Celsr1* r4-cKO embryos, a subset of *Tbx20*+ve FBM neurons migrated rostrally instead of caudally (Figs. 3E-F and 5D) (9/12 embryos), as seen in $Celsr1^{Crsh/+}$ (also referred to as $Celsr1^{Crsh}$) and $Celsr1^{KO/KO}$ embryos (also referred to as $Celsr1^{KO}$). Interestingly, these aberrantly migrating FBM neurons were found exclusively in r3, typically in clusters near the r3/r4 boundary. Therefore, although FBM neurons migrated rostrally in r4-cKO embryos, it was less severe than the $Celsr1^{Crsh}$ and $Celsr1^{KO}$ mutant phenotypes, where FBM neurons migrate two rhombomeres in the rostral direction and settle in r2 adjacent to

the trigeminal motor nuclei (Qu et al., 2010). These data suggest that *Celsr1* function in r4 contributes to the ability of *Celsr1* to suppress rostral migration of FBM neurons.

Celsr1 functions in rhombomeres 3 and 5 to regulate FBM neuron migration

Since r4-specific inactivation generated only a partial rostral migration phenotype, we tested whether *Celsr1* inactivation in more anterior rhombomeres like r3 could generate a similar phenotype. Since an r3-specific Cre driver is not available, we employed the *Krox20^{Cre}* knock-in mouse line, where Cre recombinase driven by the endogenous *Krox20* promoter is expressed in rhombomeres 3 and 5 (Voiculescu et al., 2000). To confirm Cre expression in r3 and r5, we crossed *Krox20^{Cre}* mice (Fig. 1B) with *ROSA26^{mT/mG}* reporter mice and immunostained for *Isl1* and GFP at E10.5. Cre-mediated GFP expression was seen at all dorsal-ventral levels of the neuroepithelium in r3 and r5 (Fig. 4A-D) (5 whole mount, 5 sectioned). Notably, Cre-mediated GFP was excluded from r4-derived FBM neurons (*Isl1*+ve) migrating caudally within r5 (Fig. 4D).

Next, we generated *Celsr1* r3/r5 conditional mutant embryos (r3/r5-cKO) with the *Krox20^{Cre}*; *Celsr1^{KO/fl}* or *Krox20^{Cre}*; *Celsr1^{fl/fl}* genotypes, and assayed for FBM neuron migration defects by *Tbx20* ISH. In some *Celsr1* r3/5-cKO embryos (3/12), a subset of *Tbx20*+ve FBM neurons migrated rostrally into r2 instead of caudally into r6 (Fig. 4F), similar to *Celsr1^{Crsh}* and *Celsr1^{KO}* mutants (Qu et al., 2010). In several r3/r5-cKO embryos (9/12), some caudal migration defects were seen, where FBM neurons exhibited dorsolateral migration in r5 instead of r6 (Fig. 4F). To rule out the possibility that the FBM neuron migration defects in r3/r5-cKO embryos might be due to *Krox20^{Cre}* activity in the male germ line (Fig. S1), we implemented an alternate breeding strategy using *Krox20^{Cre}*; *Celsr1^{KO/+}* males and *Celsr1^{fl/fl}* females to generate *Krox20^{Cre}*; *Celsr1^{KO/fl}* (r3/r5-cKO) embryos. In most r3/r5-cKO embryos (8/9; Fig. 5D), FBM neurons migrated rostrally (data not shown), confirming that the phenotype resulted from the conditional deletion of *Celsr1* in r3/r5.

Together, these results indicate that loss of *Celsr1* function in r3/r5 can generate all of the migration defects seen in *Celsr1^{Crsh}* and *Celsr1^{KO}* embryos, albeit at a lower frequency. However, rostral migration defects are seen in >90% of *Celsr1^{Crsh}* and *Celsr1^{KO}* mutants (Qu et al., 2010; unpublished data), indicating that *Celsr1* function in tissues other than r3 (and/or r5) normally contributes to the suppression of rostral migration. Given our data for a contribution of r4 in this process (Fig. 3), an attractive possibility is that loss of *Celsr1* function in the ventricular zone at all axial levels from r2 to r5, but excluding the floor plate, is sufficient to generate the migration defects seen in *Celsr1^{KO/KO}* mutants. We tested this hypothesis by conditional inactivation of *Celsr1* using an *Nkx6.2-Cre* driver, which is expressed in the appropriate tissues.

Celsr1 functions in the ventricular zone along the ventral midline of the hindbrain to regulate FBM neuron migration

We deleted *Celsr1* in *Nkx6.2*-expressing cells using *Nkx6.2-Cre* mice (Fogarty et al., 2007). Throughout FBM neuron specification (E9.5) and at the onset of their migration (E10.5), *Nkx6.2* is expressed broadly in the neural tube at all axial levels of the hindbrain (Moreno-

Bravo et al., 2010; Qiu et al., 1998) but is mostly absent from the floor plate (Fig. 5A). Thereafter (E11.5), Nkx6.2 expression is largely restricted to pre-migratory FBM neurons in r4 (Pattyn et al., 2003).

As predicted, deletion of *Celsr1* in Nkx6.2-expressing cells generated the rostral migration phenotype seen in *Celsr1*^{KO/KO} mutants and *Celsr1*^{Crsh/+} embryos. In many Nkx6.2-Cre; *Celsr1*^{KO/fl} embryos (6/15; 40%), a subset of Isl1+ve FBM neurons migrated rostrally into r2 instead of caudally into r6 (Fig. 5B, C). In other embryos (7/15; 47%), a subset of FBM neurons also migrated rostrally, albeit to a lesser extent, into r3. Overall, 87% of Nkx6.2-Cre embryos contained FBM neurons migrating rostrally into r2 and/or r3, which is similar to the fraction (~93%) of *Celsr1*^{Crsh} and *Celsr1*^{KO} embryos exhibiting rostral migration defects (Qu et al., 2010; unpublished data). The summarized data from the various conditional knockouts (Fig. 5D) clearly demonstrate the importance of *Celsr1* function in the ventricular zone in the anterior hindbrain up to rhombomere 5 for suppressing rostral migration of FBM neurons.

Rostral migration of FBM neurons in *Celsr1*^{Crsh} embryos may result from the loss of local guidance cues

We wondered why, in *Celsr1* mutants, only a subset of FBM neurons migrates rostrally, while the majority undergoes normal caudal migration. One possibility is that loss of *Celsr1* function leads to failure of PCP and loss of cell polarity in pre-migratory FBM neurons in r4 (even though they do not express *Celsr1*) such that they migrate either rostrally or caudally in a random fashion. In this case, rostrally migrating FBM neurons would be expected to originate at various positions, spanning the rostral-caudal extent of r4. Alternatively, since disruption of *Celsr1* function in r2 to r5 facilitates rostral migration of FBM neurons, this phenotype may result from the loss of local cues that normally act at the r3/r4 boundary to suppress rostral migration of the FBM neurons. In this case, rostrally migrating FBM neurons would be predicted to originate preferentially from the anterior aspects of r4 adjacent to the r3/r4 boundary.

To distinguish between these putative mechanisms, we placed NeuroVue lipophilic dyes into the regions of the hindbrain neuroepithelium corresponding to both the normally-migrating (caudal) and aberrantly-migrating (rostral) populations of FBM neurons in *Celsr1*^{Crsh} mutants. By E9.5, FBM axons extend to the r4 exit point at various rostralcaudal levels corresponding to their soma positions within r4, generating a fan shape (Fritzscht et al., 1993; Rossel and Capecchi, 1999; Schneider-Maunoury et al., 1997). Therefore, through anterograde labeling of migrating FBM neurons from outside of r4, we could infer their starting positions within r4 prior to migration (Fig. 6A).

In E12.5 WT embryos, dye application in the caudally migrating stream in r5/r6 anterogradely labeled FBM axons projecting to the r4 exit point from various points spanning the rostral-caudal extent of r4 (5 injections; 3 embryos) (Fig. 6B). Dye application into r2/r3 did not label any axons extending to the r4 exit point. The medial longitudinal fascicle and reticular formation were consistently labeled in all embryos, since they run parallel to the floor plate at all axial levels. Dye application into the caudally migrating stream of *Celsr1*^{Crsh/+} embryos consistently labeled a smaller number of FBM axons

projecting to the r4 exit point (Fig. 6C). Importantly, dye application into the rostrally migrating stream in r2/r3 labeled FBM axons extending to the r4 exit point (4 injections; 3 embryos). Furthermore, the two axon populations were completely segregated (no mixing), with the rostrally labeled axons originating entirely near the r3/r4 boundary, and the caudally labeled axons originating from the central and caudal aspects of r4 (Fig. 6C, D). These results are consistent with our predictions based on putative underlying mechanisms (Fig. 6A), and suggest that rostral migration of FBM neurons in *Celsr1* mutants is a response of mutant neurons at the r3/r4 boundary to local (short range) attractive cues that are normally suppressed in wild type embryos (Fig. 7).

We also noted differences between embryos in the number and location of crossing axons (reticulo- and vestibulo-bulbar projections in rhombomeres 2-6; Straka et al., 2014) (Fig. 5B, C). However, there was no reproducible difference between WT and *Celsr1*^{Crsh/+} embryos, suggesting that the observed differences are likely due to variability in NeuroVue dye application and extent of anterograde labeling. However, we cannot rule out that some crossing axons may be differentially affected in *Celsr1* mutants.

Potential roles for Wnt signaling in generating the rostral migration phenotype of *Celsr1* mutants

Since the rostrally migrating subset of FBM neurons originates from the anterior part of r4, adjacent to the r3/r4 boundary, we wondered whether these FBM neurons may be responding to a chemoattractant in r3 whose expression/function is unmasked by *Celsr1* inactivation (Fig. 7). *Wnt5a*, a Wnt/PCP ligand, is expressed in a rhombomere-specific fashion, and can serve as a chemoattractant for caudally migrating FBM neurons (Vivancos et al., 2009). In E11.5 control embryos, *Wnt5a* was expressed strongly in the hindbrain neuroepithelium posterior to r5, virtually absent from r4, and strongly expressed in the floor plate anterior to the r3/r4 boundary (Fig. S2A, C) (Vivancos et al., 2009). *Wnt5a* expression was unaffected in r3/r5-cKO or r4-cKO hindbrains (Fig. S2B, D), indicating that the rostral migration phenotype in *Celsr1* mutants is not caused by ectopic or increased expression of *Wnt5a*.

While *Wnt5a* expression is not affected, its activity in the anterior hindbrain may be unmasked in *Celsr1* mutants, and induce chemotactic migration of FBM neurons into r2 and r3 (Fig. 7). Vivancos et al. (2009) showed that FBM neurons can respond to and migrate toward *Wnt5a*-soaked beads placed laterally in r4, consistent with a role for *Wnt5a* in directing caudal migration of FBM neurons. Interestingly, we found that *Wnt5a*-soaked beads placed in r3 in wild type hindbrain explants could induce the rostral migration of FBM neurons from r4 (Fig. S3), suggesting that excess *Wnt5a* can overcome putative inhibitory factors that normally suppress rostral migration. We hypothesized that two Wnt-binding proteins sFRP1 and sFRP2 (secreted frizzled-related proteins 1 and 2) act as putative inhibitory factors (Bodine et al., 2004; Bovolenta et al., 2008; Jones and Jomary, 2002; Kobayashi et al., 2009; Lei et al., 2006; Leimeister et al., 1998; Misra and Matise, 2010). The two genes are expressed in partially overlapping patterns (Fig. 8A, B and Fig. S4), with sFRP1 showing strongest expression medially and sFRP2 more laterally. sFRP1 is also expressed weakly along the midline in r3 and r2 (Fig. S4), overlapping with *Celsr1* and

Wnt5a expression domains. If sFRPs act in r3 to block Wnt5a activity (in a Celsr1-dependent fashion) and suppress rostral migration of FBM neurons, we predicted that loss of sFRP function would recapitulate the migration defect of Celsr1 mutants.

Therefore, we examined FBM neurons by Tbx20 ISH in sFRP compound mutants lacking two or more copies of these genes (Fig. 8C-H). Weak streams of Tbx20+ve putative FBM neurons were seen in r3 in over 50% of E11.5 mutants (Fig. 8C, D, G; 12/21 embryos), but the “rostral migration” phenotype was much weaker than seen in Celsr1 mutants. No Tbx20+ve cells were seen in these locations in any E12.5 mutant (Fig. 8E, F, G; 0/14 embryos), suggesting that sFRPs play a minor role, if any, in suppressing the migration of FBM neurons into r3. Interestingly, lateral streams of FBM neurons in r5, indicative of precocious dorsolateral migration, were seen in sFRP compound mutants, sometimes at E11.5 (Fig. 8H, 5/29 embryos) and frequently at E12.5 (Fig. 8E, F, H; 11/14 embryos), suggesting that sFRP1 and 2 help establish the medial pathway of the caudal migratory stream.

Discussion

The caudal migration of FBM neurons in the developing hindbrain is a tightly regulated process involving diverse signaling pathways, including non-canonical Wnt/PCP signaling. Though several genes are required for the caudal migration of FBM neurons (Chandrasekhar, 2004; Song, 2007; Wanner et al., 2013), our studies indicated for the first time that some genes like Celsr1 function to suppress rostral migration (Glasco et al., 2012; Qu et al., 2010). Our conditional knockout studies demonstrate that Celsr1 function is required in a relatively small expression domain, the Nkx6.2+ve ventricular zone spanning rhombomeres 2-5, to suppress rostral migration of FBM neurons. This analysis provides a starting point for elucidating the molecular mechanisms that control the directionality of FBM neuron migration.

No discernable function for floor plate-derived Celsr1 in FBM neuron migration

Given the strong expression of Celsr1 in floor plate cells (Qu et al., 2010), it was surprising to find that loss of Celsr1 function in the floor plate did not affect FBM neuron migration. Celsr1 is expressed in the floor plate at the onset of migration, and persists even after its downregulation at the ventricular surface. However, a minor role for Celsr1 in the floor plate cannot be ruled out for two reasons. First, in both r3/r5-cKO and r4-cKO embryos, which exhibit partially overlapping rostral migration phenotypes, Celsr1 function is inactivated in the entire neuroepithelium of specific rhombomeres including the floor plate. Second, the weaker ability of FBM neurons in Nkx6.2-Cre (VZ-cKO) embryos to migrate into r2 (Fig. 5) compared to that in Celsr1^{Crsh} and Celsr1 KO embryos (Qu et al., 2010) may reflect a functional role for Celsr1 in floor plate cells in anterior rhombomeres. Therefore, although the floor plate itself plays an important role in regulating FBM neuron migration (Sittaramane et al., 2013; however see also Davey et al., 2016), there is only a minor role, if any, for floor plate-derived Celsr1 in this process. Importantly, floor plate-derived Celsr1 may regulate other floor plate-dependent processes such as neural tube closure (Doudney and Stanier, 2005; Greene et al., 1998; Murdoch et al., 2001), orientation of floor plate

primary cilia (Borovina et al., 2010; Okada et al., 2005), guidance of commissural axons (Serafini et al., 1996), migration of inferior olivary, lateral reticular, and external cuneate nuclei (de Diego et al., 2002), or the contralateral projection of olivocochlear efferent neurons (Fritzsch et al., 1993). While there were no obvious neural tube closure defects in *Celsr1* floor plate cKO embryos, potential roles for *Celsr1* in these processes have not been directly examined.

Celsr1 suppresses rostral migration of FBM neurons

Our analysis of *Celsr1* mutants (Qu et al., 2010) suggests that FBM neurons possess the ability to migrate in either the rostral or caudal direction, but that *Celsr1* normally mediates directionality cues that steer them caudally. Rhombomere specification and patterning occur normally in *Celsr1* mutants indicating that the reversed migration is not a consequence of aberrant rhombomere boundary formation (Qu et al., 2010). One possibility is that loss of *Celsr1* function results in the random orientation of FBM neurons in rhombomere 4 due to loss of tissue polarity in the *Celsr1*-expressing cells in their environment, causing FBM neurons to migrate rostrally or caudally in random fashion. In this case, one would expect about 50% of FBM neurons to migrate rostrally out of r4; however, in all *Celsr1* mutants analyzed, the rostrally migrating stream contains substantially fewer neurons. While functional redundancy between *Celsr1* and related genes may account for this “weak” rostral migration phenotype, another plausible explanation is that it is the consequence of the inactivation of *Celsr1*-dependent local cues that normally suppress rostral migration. Given that *Wnt5a* is expressed in a gradient in rhombomeres 5-7 and plays a role in caudal migration of FBM neurons (Vivancos et al., 2009), it might play a similar role to induce rostral migration in *Celsr1* mutants. Consistent with this, *Wnt5a* is expressed in midline tissues in the rostral hindbrain up to r3, overlapping in expression with *Celsr1* (Fig. S2). Furthermore, loss of *Dvl2* function in *Celsr1*^{Crsh/+} embryos suppresses the rostral FBM neuron migration phenotype (Glasco et al., 2012), suggesting that the ability to migrate rostrally involves activation of the Wnt/Dvl signaling pathway. Based on these observations, we propose that *Wnt5a* in r3 can potentially act as a chemoattractant to induce FBM neurons to migrate rostrally from r4 (Fig. 7A). Further, we suggest that in the wild type hindbrain, *Celsr1* functions to suppress the activity of *Wnt5a* in inducing neuronal migration. In *Celsr1* mutants, this suppression is relieved, resulting in rostral migration (Fig. 7B). Since the Wnt-mediated chemoattraction would decrease with increasing distance from the source in r3, this model also explains why the rostrally migrating FBM neurons in *Celsr1* mutants originate exclusively from near the r3/r4 boundary (Fig. 6), where chemoattraction would be the strongest. Consistent with this model, placement of numerous *Wnt5a*-soaked beads in r3 in wild type hindbrain explants results in rostral migration of FBM neurons (Fig. S3), suggesting that excess *Wnt5a* can overcome *Celsr1*-mediated suppression.

Although a model invoking the loss of local cues at the r3-r4 boundary adequately explains the rostral migration phenotype of *Celsr1* mutants, and is consistent with several independent observations discussed above, the differing phenotypes between r3/r5-cKO and r4-cKO embryos suggest that the polarity of *Celsr1*-expressing cells in r3 may also be important. When *Celsr1* is inactivated solely in r4 (r4-cKO embryos), a subset of FBM neurons migrates rostrally into r3, but not further into r2 (Fig. 3). In this case, *Celsr1* is not

inactivated in r3, which may represent a non-permissive (polarized?) environment that prevents FBM neurons from migrating further rostrally into r2. In r3/r5-cKO embryos, however, FBM neurons may be able to migrate rostrally into r2, since inactivation of *Celsr1* in the cells adjacent to the FBM neurons in r3 may represent a permissive (non-polarized) environment.

Mechanism of *Celsr1*-mediated suppression of rostral migration of FBM neurons

While our genetic and bead-explant data suggest strongly that *Celsr1* normally suppresses rostral migration, likely by antagonizing Wnt function (Fig. 7), potential mechanisms remain obscure. In *Celsr1* mutants, even a small change in the Wnt concentration gradient could in principle affect only the rostral-most FBM neurons in r4, and induce them to migrate rostrally. Alternatively, *Celsr1*-mediated changes in the adhesion properties of the ventricular zone in r3 (adjacent to the rostral-most FBM neurons) may modify the local “non-migratory” environment, causing the rostral-most FBM neurons to migrate into r3. Expression profiling using RNA-seq of hindbrain fragments from wild type and *Celsr1*^{Crsh} embryos have identified candidate secreted molecules and receptors that are being validated.

Independently of this analysis, we investigated potential roles for secreted Frizzled related proteins (sFRPs) due to their well-documented roles in antagonizing Wnt function, and their expression patterns in the developing hindbrain (Bodine et al., 2004; Bovolenta et al., 2008; Jones and Jomary, 2002; Kobayashi et al., 2009; Lei et al., 2006; Leimeister et al., 1998; Misra and Matise, 2010). If sFRPs were mediating the suppressive effect of *Celsr1* on Wnt5a, we would predict that FBM neurons would migrate rostrally in sFRP mutants. While there was a weak transient effect reminiscent of rostral migration in sFRP1/2 compound mutants (Fig. 8), it is unlikely that the putative inhibition of Wnt activity by *Celsr1* is mediated through sFRPs. However, we did observe a strong effect on caudal migration in sFRP mutants, consistent with a role in modulating Wnt5a activity. In *Wnt5a*^{-/-} hindbrains, caudally migrating FBM neurons frequently undergo precocious dorsolateral migration, resulting in the formation of ectopic streams in r5 (Vivancos et al., 2009), and suggesting that when Wnt signaling is reduced, FBM neurons may prematurely respond other cues mediating dorsolateral migration. Interestingly, precocious dorsolateral streams are also seen in sFRP compound mutants (Fig. 8E, F), in which Wnt signaling is likely to be enhanced. These results suggest that Wnt signaling must be maintained in an optimal range to prevent precocious dorsolateral migration. In addition, the exclusion of sFRP gene expression from the FBM migratory pathway (Fig. 8A, B) suggests that sFRPs may help establish a medial Wnt5a-enriched pathway that is permissive for FBM neuron migration.

We provide here novel insight into the cellular mechanism by which the atypical cadherin *Celsr1* regulates the direction of migration of FBM neurons. Our data suggest strongly that *Celsr1* functions in a relatively small expression domain in the ventricular zone, rather than in a more prominent floor plate domain, to suppress rostral migration. We propose that *Celsr1* suppresses rostral migration by antagonizing a Wnt5a chemoattractive source in the anterior hindbrain. How *Celsr1* mediates its putative effects on Wnt activity, and whether this mechanism is conserved between mice and zebrafish are important issues for further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Celsr1 regulates the directionality of mouse facial branchiomotor neuron migration.
- Celsr1 functions specifically in the ventricular zone of rhombomeres 3-5.
- In Celsr1 mutants, rostrally-migrated neurons originate from anterior rhombomere 4.
- Celsr1 may suppress attractive cues to prevent inappropriate rostral migration.

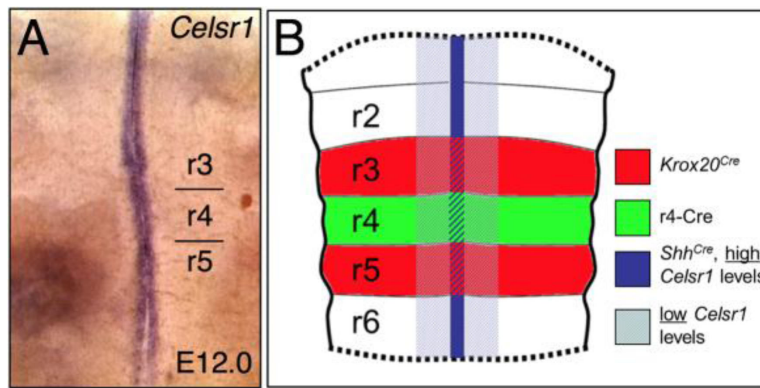


Figure 1. Overlap of the Celsr1 expression pattern with tissue-specific Cre driver domains
 (A), Flat-mounted E12 hindbrain processed for Celsr1 in situ hybridization (ISH). Celsr1 is expressed in the floor plate at all axial levels. B, Overview of the tissue-specific Cre lines used in this study showing overlap of the Cre domains with Celsr1 expression. “High” and “low” refer to the relative levels of Celsr1 expression in the floor plate and ventricular zone, respectively.

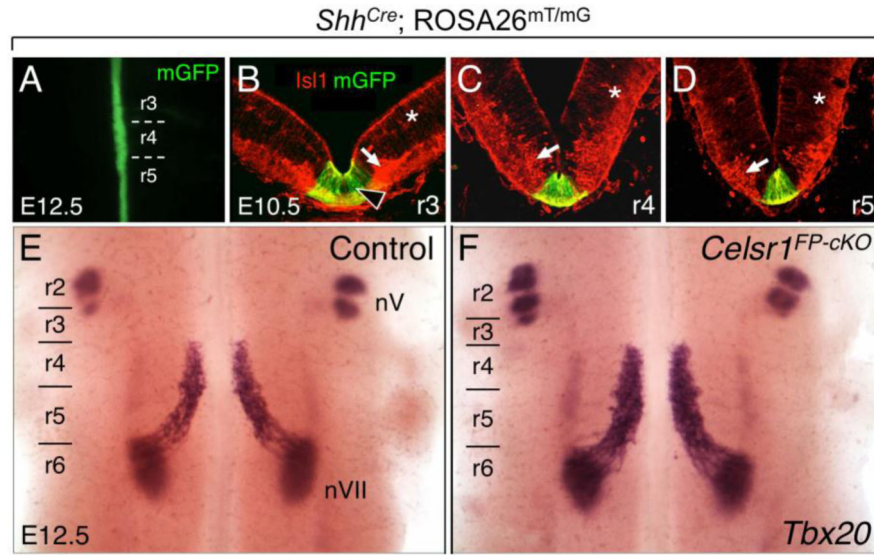


Figure 2. Disruption of Celsr1 function in the floor plate does not affect FBM neuron migration
 A, E, F, Dorsal views of E12.5 embryos visualized live for GFP (A) or processed for Tbx20 ISH (E, F). GFP expression along the midline in floor plate cells in a *Shh^{Cre}; ROSA26^{mT/mG}* embryo (A) labels the expression domain of Cre used for the floor plate-specific knockout of Celsr1 function (FP-cKO). B-D, 30-μm coronal sections of an E10.5 wild type hindbrain processed for anti-GFP (green) and anti-Isl1 (red) immunostaining to mark Cre expression (GFP), and FBM neurons (arrow), respectively. The red background signal (asterisks) is due to mTomato expression in all tissues of the *ROSA26^{mT/mG}* mouse where Cre is not active. At all axial levels, Cre activity is restricted to floor plate cells (arrowhead). E, F, In control embryos (*Shh^{Cre}; Celsr1^{fl/+}* or *Shh^{Cre}; Celsr1^{KO/+}*, 8/8 embryos), Tbx20 labels trigeminal BM neurons (nV) in r2 and r3, and FBM neurons (nVII) migrating caudally from r4 to r6 (E). FBM neurons also migrate normally in FP-cKO embryos (F) (*Shh^{Cre}; Celsr1^{KO/fl}* or *Shh^{Cre}; Celsr1^{fl/fl}*, 14/14 embryos).

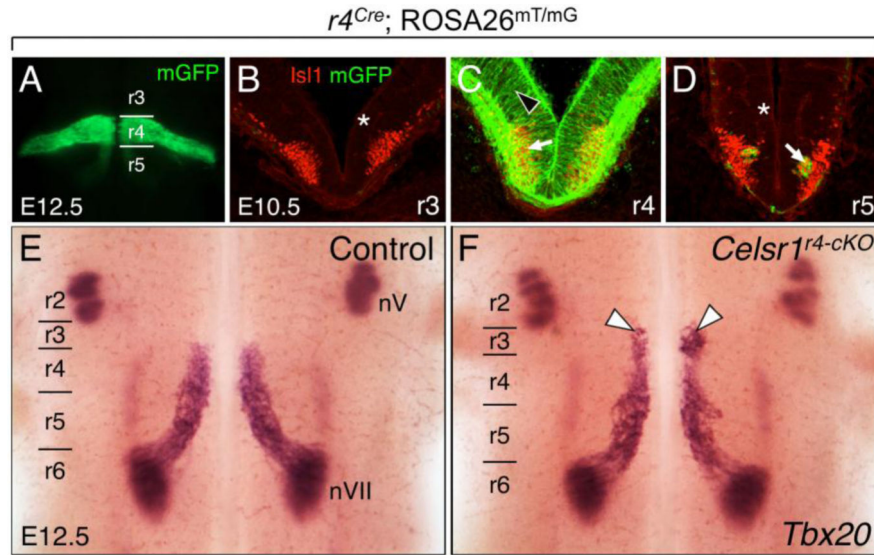


Figure 3. Some FBM neurons migrate rostrally into rhombomere 3 (r3) following disruption of *Celsr1* function in r4

A, E, F, Dorsal views of E12.5 embryos visualized live for GFP (A) or processed for *Tbx20* ISH (E, F). GFP expression in r4 in a r4-Cre; ROSA26^{mT/mG} embryo (A) labels the expression domain of Cre used for the r4-specific knockout of *Celsr1* function (r4-cKO). BD, 30-µm coronal sections of an E10.5 wild type hindbrain processed for anti-GFP (green) and anti-*Isl1* (red) immunostaining to mark r4-Cre expression (GFP), and FBM neurons (arrow), respectively. The light red background signal (asterisks) is due to mTomato expression in all tissues of the ROSA26^{mT/mG} mouse where Cre is not active. Cre activity is restricted to tissues within r4 (black arrowhead). The GFP-expressing cells in D (arrow) are the earliest caudally-migrating FBM neurons originating in r4. E, F, In control embryos (r4-Cre; *Celsr1*^{fl/+} or r4-Cre; *Celsr1*^{KO/+}, 20/21 embryos), *Tbx20* labels trigeminal BM neurons (nV) in r2 and r3, and FBM neurons (nVII) migrating caudally from r4 to r6 (E). A small but significant number of FBM neurons (arrowheads) migrates rostrally into r3 in a majority of r4-cKO embryos (F) (r4-Cre; *Celsr1*^{KO/fl} or r4-Cre; *Celsr1*^{fl/fl}, 9/12 embryos).

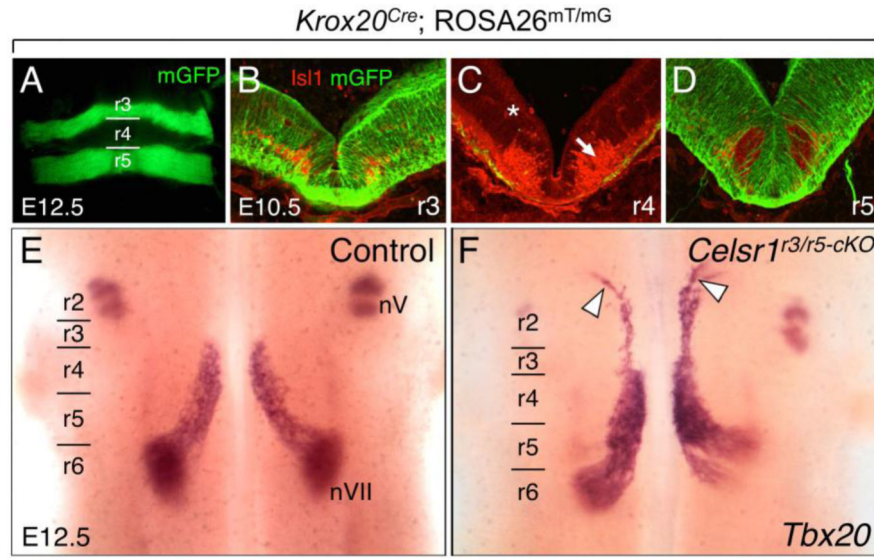


Figure 4. Some FBM neurons migrate rostrally following inactivation of Celsr1 in r3 and r5
 A, E, F, Dorsal views of E12.5 embryos visualized live for GFP (A) or processed for Tbx20 ISH (E, F). GFP expression in r3 and r5 in a *Krox20^{Cre}; ROSA26^{mT/mG}* embryo (A) labels the expression domain of Cre used for the r3/r5-specific knockout of Celsr1 function (r3/r5-cKO). B-D, 30- μ m coronal sections of an E10.5 wild type hindbrain processed for anti-GFP (green) and anti-Isl1 (red) immunostaining to mark Cre expression (GFP), and FBM neurons (arrow), respectively. The light red background signal (asterisk) is due to mTomato expression in all tissues of the ROSA26^{mT/mG} mouse where Cre is not active. In r3 (B) and r5 (D), Cre activity is ubiquitous, including in floor plate cells. In r4 (C), Cre is inactive except for a few cells along the pial surface, which was seen in all sections (5/5 embryos). E, F, In control embryos (*Krox20^{Cre}; Celsr1^{fl/+}* or *Krox20^{Cre}; Celsr1^{KO/+}*, 18/18 embryos), Tbx20 labels trigeminal BM neurons (nV) in r2 and r3, and FBM neurons (nVII) migrating caudally from r4 to r6 (E). A small but significant number of FBM neurons migrate rostrally into r2 in several r3/r5-cKO embryos (F, white arrowheads) (*Krox20^{Cre}; Celsr1^{KO/fl}* or *Krox20^{Cre}; Celsr1^{fl/fl}*, 3/12 embryos).

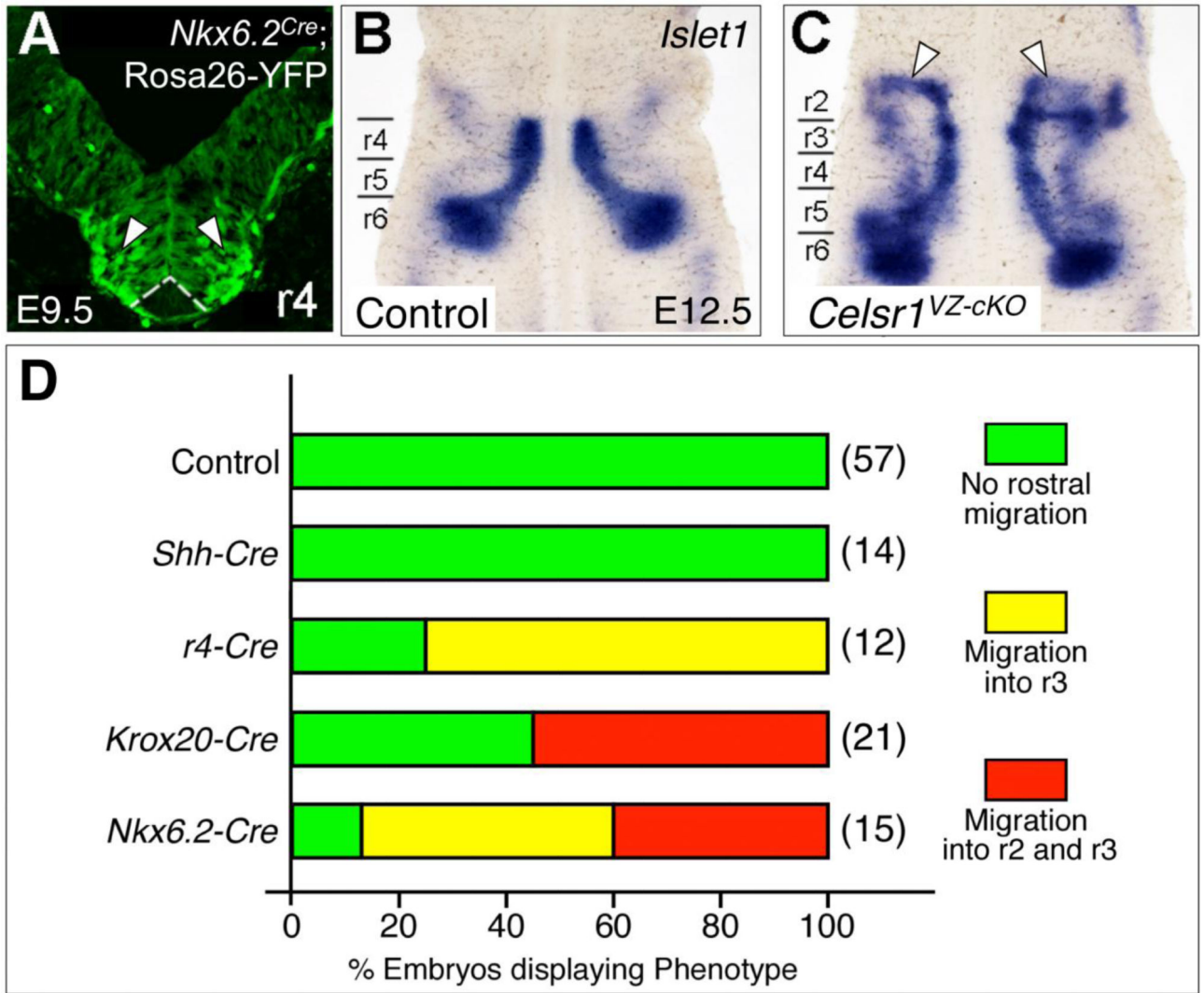


Figure 5. Conditional *Celsr1* inactivation in the *Nkx6.2* expression domain generates a similar rostral migration phenotype to *Celsr1* knockout

A, 30-µm coronal section of an E9.5 ROSA26-EYFP; *Nkx6.2-Cre* hindbrain in r4, imaged to directly visualize YFP fluorescence, which indicates the expression domain of Cre used for the ventricular zone-specific knockout of *Celsr1* function (VZ-cKO). *Nkx6.2-Cre* is expressed in the motor neuron progenitor domain at all axial levels (arrowheads), and is excluded from the floor plate (dotted lines). B-C, Dorsal views of E12.5 hindbrains processed for *Isl1* ISH. In a control embryo (*Nkx6.2-Cre; Celsr1^{fl/+}*, 11/11 embryos), *Isl1*-expressing FBM neurons migrate caudally from r4 to r6 (B). In VZ-cKO embryos (C), (*Nkx6.2-Cre; Celsr1^{fl/fl}*), significant numbers of FBM neurons (arrowheads) migrate rostrally into r3 and r2 in a majority of embryos (13/15 embryos). D, Quantitation of rostral migration phenotypes in various tissue-specific *Celsr1* knockouts. In *Krox20-Cre* and *Nkx6.2-Cre* embryos, embryos displaying the r2 migration phenotype also have some FBM neurons in r3.

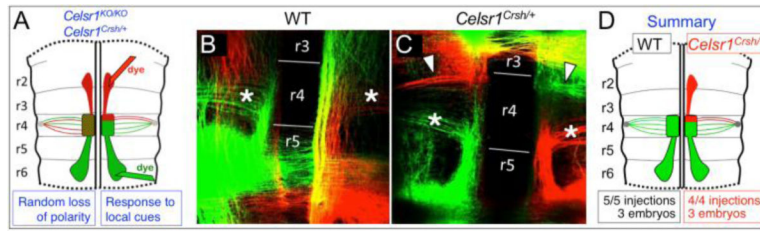


Figure 6. The rostrally migrating FBM neurons in *Celsr1^{Crsh/+}* embryos originate in the rostral portion of r4, adjacent to the r3/r4 boundary

A, Schematic of a flat-mounted E12.5 hindbrain indicating the dye application sites within the rostral (NeuroVue Maroon, red) and caudal (NeuroVue Jade, green) migrating streams of FBM neurons to anterogradely label the FBM axons exiting in r4. Two hypotheses (blue boxes) and predicted phenotypes are shown. If the rostral migration phenotype of *Celsr1* mutants results from a loss of polarity in FBM neurons in r4 (left side), a newborn FBM neuron in any location within r4 (mixed red/green stipples) could potentially migrate rostrally, resulting in a mixed collection of FBM axons exiting from r4. However if rostrally migrating neurons arise exclusively from the anterior margin of r4 in response to guidance cues unmasked in the mutants (right side), the FBM axons extended by the rostrally and caudally migrating neurons would be clearly segregated in r4. B-C, Flat-mounted views of dye-labeled WT and *Celsr1^{Crsh/+}* hindbrains. To maximize data collection from each embryo, the migratory streams were labeled on both sides (caudal left-green; rostral left-red; caudal right-red; rostral right-green). In a WT embryo (B), dye application in r5/r6 anterogradely labels FBM axons (asterisks) spanning the anterior-posterior extent of r4. Dye application in r2/r3 does not label any axon projections into the r4 exit points. In a *Celsr1^{Crsh/+}* embryo (C), dye application in r5/r6 anterogradely labels FBM axons in r4 (asterisks). Importantly, dye application in r2/r3 anterogradely labels FBM axons projecting toward the r4 exit points, extending along the anterior margin of r4 (arrowheads). Axons crossing the midline in r5 (B) and r3 (C) are commissural axons that were inadvertently labeled in these particular embryos, and are not relevant to the rostral migration defect. D, Schematic summarizing the results shown in B and C.

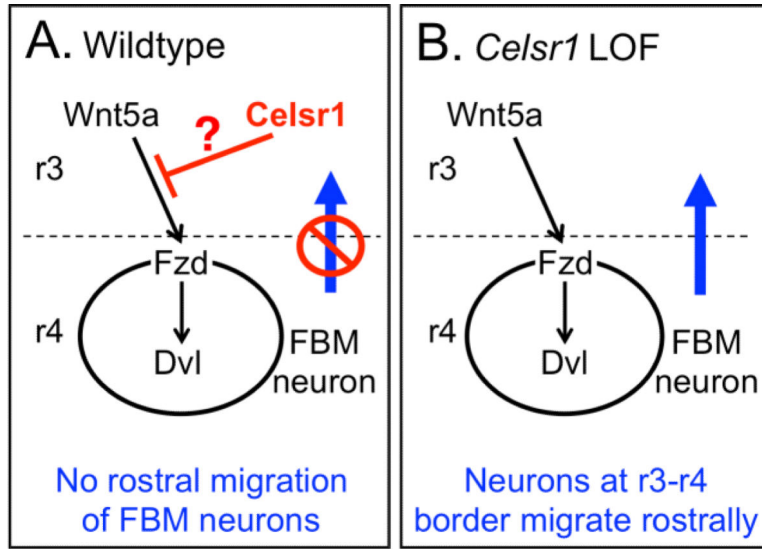


Figure 7. Model of Celsr1 function in repressing rostral migration of FBM neurons
 We propose that Wnt5a, which is expressed along the midline in r3 and more anterior regions of the hindbrain, can act as a chemoattractive cue to facilitate the migration of FBM neurons located in anterior r4, adjacent to the r3/r4 boundary, rostrally into r3 and r2. A, In a wild type hindbrain, Celsr1 expression in the ventricular zone inhibits Wnt5a-dependent activation through an unknown mechanism (?), blocking the rostral migration of FBM neurons into r3. B, In a Celsr1-deficient hindbrain, the inhibition is relieved and FBM neurons migrate rostrally into r3 toward the Wnt5a source.

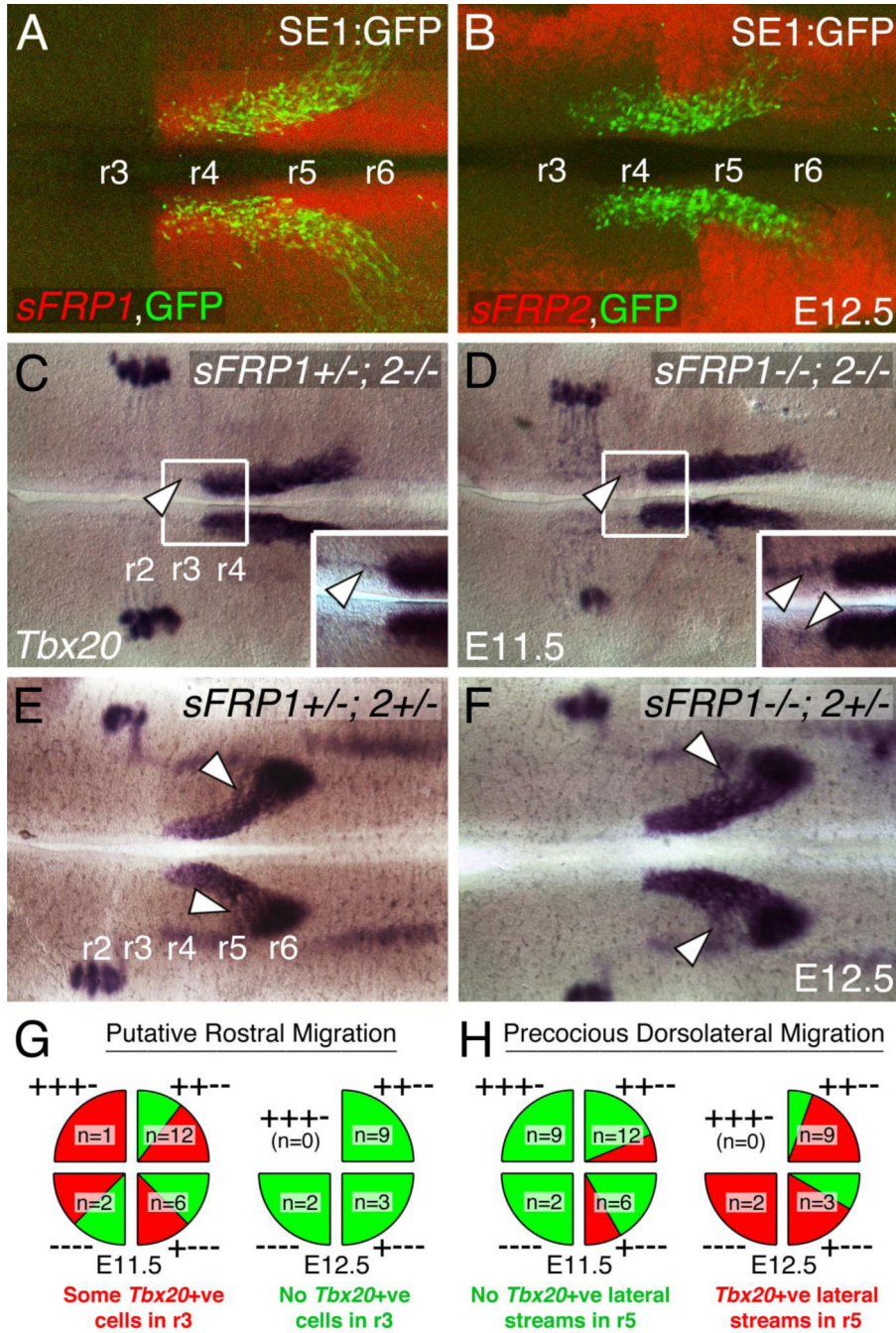


Figure 8. Wnt antagonist genes sFRP1/2 modulate caudal migration of FBM neurons
 A-F, Flat-mounted hindbrains (left is rostral) processed for ISH with sFRP1 (A), sFRP2 (B) and Tbx20 (C-F). The distribution of GFP-expressing FBM neurons in A, B (from SE1::GFP embryos) helps to delineate rhombomere boundaries. A, At E12.5, sFRP1 is strongly expressed caudal to the r3/r4 boundary in the entire neuroepithelium. Although not apparent in fluorescent ISH, sFRP1 is also expressed weakly in r3 and r2. B, sFRP2 is weakly expressed in medial regions, and strongly expressed along the lateral margins of the hindbrain at all axial levels (r3-r6). C-D, In E11.5 hindbrains, when 3 or more copies of

sFRP1/2 are removed, a small number of Tbx20-expressing cells (arrowheads) appear to migrate rostrally into r3 (see insets). There are no defects in the caudally migrating streams of FBM neurons. E-F, In E12.5 hindbrains, when 2 or more copies of sFRP1/2 are removed, the caudal migration streams frequently contain ectopic streams indicating precocious dorsolateral migration (arrowheads). Interestingly, there are no rostral migration defects. G-H, Summary data showing distribution of migration defects among various sFRP1/2 compound mutants at E11.5 and E12.5. The four quarters in each “pie” show the breakdown of two phenotypes (red and green sectors) in the indicated sFRP1; sFRP2 genotypes. For example, ++— corresponds to sFRP1+/+; sFRP2-/- . G, While over 50% of E11.5 mutants (12/21 embryos) contain some rostrally migrating Tbx20+ve cells, these cells are no longer evident in E12.5 mutants (14/14 embryos). H, While less than 20% of E11.5 mutants (5/29 embryos) contain precocious lateral streams of Tbx20+ve cells, most E12.5 mutants (11/14 embryos) contain such streams.