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Celsr1–3 Cadherins in PCP and Brain Development

Camille Boutin, André M. Goffinet¹, Fadel Tissir¹

Institute of Neuroscience, Developmental Neurobiology, Université Catholique de Louvain, Brussels, Belgium

¹Corresponding authors: Equal contribution. e-mail address: fadel.tissir@uclouvain.be; andre.goffinet@uclouvain.be

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Abstract

Cadherin EGF LAG seven-pass G-type receptors 1, 2, and 3 (Celsr1–3) form a family of three atypical cadherins with multiple functions in epithelia and in the nervous system. During the past decade, evidence has accumulated for important and distinct roles of Celsr1–3 in planar cell polarity (PCP) and brain development and maintenance. Although the role of Celsr in PCP is conserved from flies to mammals, other functions may be more distantly related, with Celsr working only with one or a subset of the classical PCP partners. Here, we review the literature on Celsr in PCP and neural development, point to several remaining questions, and consider future challenges and possible research trends.

Celsr1–3 genes encode atypical cadherins of more than 3000 amino acids (Fig. 7.1). Their large ectodomain is composed of nine N-terminal cadherin repeats (typical cadherins have five repeats), six epidermal growth factor (EGF)-like domains, two laminin G repeats, one hormone receptor motif (HRM), and a G-protein-coupled receptor proteolytic site (GPS). This is followed by seven transmembrane domains (classic cadherins are single-pass type I proteins) and a cytoplasmic tail (CT) that varies in size and, in contrast to the ectodomain, is poorly conserved among the three members. Celsr1–3 belong to the “adhesion receptor” family of G-protein-coupled receptors (GPCRs), also referred to as long N-terminal group B (LNB) GPCRs.
These receptors are thought to be natural chimeras of cell adhesion proteins and signaling receptors, able to convert cell–cell communication cues into intracellular signals. Studies of the latrotoxin receptor latrophilin, a member of LNB–GPCR, showed that cleavage at the GPS occurs intracellularly. However, the N-terminal (cell adhesion domain) and C-terminal (GPCR) fragments remain associated by noncovalent bonds at the plasma membrane (Krasnoperov et al., 2002; Volynski et al., 2004). It is not clear yet whether cleavage at the GPS occurs in Celsr1–3 proteins (see below). In addition to cadherin and EGF-like repeats which confer adhesive properties and facilitate cell–cell communication, the extracellular domain of Celsr1–3
contains a HRM which may bind putative ligands, although none has been identified thus far.

Celsr genes were first identified in mice (Hadjantonakis et al., 1997). They form a highly conserved family with homologs in ascidians, worms, flies, and vertebrates. The fruit fly Celsr ortholog was cloned independently by two groups and is known as Flamingo (Fmi) or Starry night (Stan) (Chae et al., 1999; Usui et al., 1999). Genetic studies have established a critical role for Fmi/Stan in planar cell polarity (PCP) events, particularly in the stereotypic organization of wing hairs (trichomes) and sensory bristles (Chae et al., 1999; Usui et al., 1999), the alignment of ommatidia in the eye (Mlodzik, 1999), and the asymmetric division of sensory organ precursors (Bellaiche, Gho, Kaltschmidt, Brand, & Schweisguth, 2001; Segalen & Bellaiche, 2009). In these processes, Fmi/Stan interacts genetically and functionally with so-called core PCP proteins. Drosophila core PCP proteins include serpentine receptors Frizzled (fz and fz2 are partially redundant in PCP); the tetraspannin Van Gogh (vang, also named strabismus); and the three cytoplasmic proteins Disheveled (dsh), Prickle (pk), and Diego (dgo) (Adler, 2002; Feiguin, Hannus, Mlodzik, & Eaton, 2001; Gubb et al., 1999; Strutt, Johnson, Cooper, & Bray, 2002; Taylor, Abramova, Charlton, & Adler, 1998; Wolff & Rubin, 1998). In the insect wing, the distribution of core PCP proteins is tightly and dynamically regulated. Initially distributed in all apical junctions, they adopt a transient polarized partition along the proximal–distal axis shortly before the growth of wing hairs, and this partition is essential to the establishment of polarity and the proper orientation of hairs. fmi/stan, fz, dsh, and dgo are enriched at the distal border of wing cells, whereas fmi/stan, vang, and pk are enriched at the proximal border (Bastock, Strutt, & Strutt, 2003; Jenny, Darken, Wilson, & Mlodzik, 2003; Usui et al., 1999). Given that fmi/stan localizes to both proximal and distal junctions, it was considered a permissive molecule with no active effect on polarity: fmi/stan homodimers would act as a scaffold, promoting cell adhesion and bridging the distal (fz-expressing) side of a cell and the proximal (vang-expressing) side of the adjacent cell. However, more recent data provide evidence that fmi/stan plays an instructive role: its central portion containing the HRM and TM domains interacts physically with fz. Furthermore, fmi/stan selectively recruits fz and vang to opposite cell boundaries, thereby initiating bidirectional polarity signals (Chen et al., 2008). In line with this, genetic analyses in flies demonstrated a mutual requirement for fmi/stan, fz, and dsh to achieve a
correct partition of polarity complexes (Das, Reynolds-Kenneally, & Mlodzik, 2002). In zebrafish, a serine acidic amino acid-rich domain (SE/D) in the CT of Celsr is required for membrane localization of the Frizzled–Disheveled complex, PCP signaling, and convergent extension (Carreira-Barbosa et al., 2009).

1. CELSR1–3 EXPRESSION PATTERNS

In mammals, the Celsr family is composed of three members, whereas birds apparently lack Celsr2 (Formstone, 2010) and fish have two, Celsr1a and 1b (Wada, Tanaka, Nakayama, Iwasaki, & Okamoto, 2006). In the mouse, Celsr1–3 genes have similar genomic organizations, with 35 (Celsr1 & Celsr3) or 34 exons (Celsr2). Apart from 3′ alternative exons in Celsr2, alternative splicing has not been described. Celsr1–3 expression is regulated spatially and temporally, indicating that they are important for development. A striking feature is the complementary pattern of Celsr1 and Celsr3 expressions in different developing systems (Formstone & Little, 2001; Shima et al., 2002; Tissir, De Backer, Goffinet, & Lambert de Rouvroit, 2002). In the nervous system, Celsr1 mRNA is heavily expressed in zones of neural stem cell (NSC) proliferation, namely, all ventricular zones during embryonic and early postnatal development, and telencephalic ependymal zones as well as subgranular layer of the dentate gyrus in the mature brain. In contrast, Celsr3 mRNA is absent from NSC and associated with most postmitotic neural cells, whereas Celsr2 mRNA is found in both NSC and postmitotic cells. Expression of Celsr1 abates during early postnatal development, in parallel to decreasing numbers of NSC. That of Celsr3 is downregulated postnatally but persists in the cerebellar granular layer, the hilus of the dentate gyrus, the rostral migratory stream, and the central region of the olfactory bulb. By contrast, Celsr2 expression remains stable throughout life. These expression patterns hint to functions of Celsr1 in NSC, Celsr3 in neural cell maturation, and Celsr2 in development and maintenance of the nervous system. Celsr1–3 mRNAs are also variably expressed in nonneural tissue, such as the skin, lungs, kidney, and digestive and reproductive systems. In the rodent testis, the spatiotemporal pattern of Celsr1–3 expression is somewhat reminiscent of that in the brain. Celsr3 is expressed exclusively in postmeiotic germ cells, and Celsr1 and Celsr2 in Sertoli cells, with a postnatal downregulation of Celsr1 and persistence of
Celsr2 in the adult (Beall, Boekelheide, & Johnson, 2005; Hadjantonakis, Formstone, & Little, 1998; Johnson, Patel, & Boekelheide, 2000).

Polyclonal antibodies against the extra- and intracellular segments of Celsr1 allowed detection of two forms, full length (p400) and cleaved (p85 kD), generated by as yet unidentified proteolysis events not involving the GPS (Formstone, Moxon, Murdoch, Little, & Mason, 2010). In the hindbrain and spinal cord, Celsr1 protein immunoreactivity was detected in the floor and roof plates, as well as in radial neuroepithelial progenitors, not only in the apical but also in their basolateral domain, particularly in end-feet abutting the pial surface (Formstone et al., 2010). In the embryonic skin, Celsr1 is asymmetrically expressed in hair germ cells and in basal layer epidermal cells, a pattern evocative of that of fmi/stan in the Drosophila wing (Devenport & Fuchs, 2008). The cellular localization of Celsr2 and Celsr3 could not be investigated thus far due to lack of antibodies suitable in immunohistochemistry.

2. CELSR1: A MAJOR PLAYER IN VERTEBRATE PCP

Two Celsr1 mutant alleles, Crash (Celsr1\textsuperscript{Crsh}) and Spin Cycle (Celsr1\textsuperscript{Scy}), were identified in an ENU screen (Curtin et al., 2003). In Celsr1\textsuperscript{Crsh}, a G-to-A mutation at nucleotide 3126 results in an aspartate-to-glycine substitution in codon 1040, within the eighth cadherin repeat. In Celsr1\textsuperscript{Scy}, a T-to-A point mutation at nucleotide 3337 results in an asparagine-to-lysine substitution in codon 1110, in a region connecting cadherin repeats. Heterozygous animals show abnormal head-shaking behavior. Both heterozygous and homozygous mice have defective organization of stereocilia bundles in inner ear hair cells. Normally, the apical surface of each cochlear hair cell is decorated with actin-filled stereocilia forming a “V” centered on a microtubular kinocilium, with all “Vs” pointing to the external aspect of the cochlear canal. In Celsr\textsuperscript{Crsh} and Celsr1\textsuperscript{Scy} mutant mice, this typical organization is altered. Stereociliary bundles are randomly oriented, some displaying up to 180\(^\circ\) rotation relative to the normal orientation (Curtin et al., 2003). The precise orientation of hair bundles in the cochlea is a classical hallmark of PCP and parallels the polarized distribution of core PCP proteins such as Fzd3, Fzd6, Vangl2, and Prickle2 (Deans et al., 2007; Montcouquiol et al., 2006; Wang, Guo, & Nathans, 2006). PCP proteins localize asymmetrically to one edge of the apical cortex of the cells by mechanisms involving selective targeting and protein stabilization and degradation. Intriguingly, studies of the distribution of PCP proteins
were carried out in late embryogenesis, starting from embryonic day 18 when the kinocilium has already migrated from the center to the lateral edge of the cell, raising the question whether the asymmetric partition of PCP proteins is the initial event that sets up the polarity and organizes the epithelium, or simply a molecular readout of polarity signals induced by as yet unidentified cues.

*Celsr1*<sup>Crsh</sup> and *Celsr1*<sup>Scy</sup> homozygotes as well as compound heterozygotes *Celsr1*<sup>Crsh/Scy</sup> exhibit craniorachischisis, a severe neural tube defect due to a failure to initiate neural tube closure in the cervical region (Curtin et al., 2003). A role for *Celsr1* in neural tube closure was recently confirmed by identification of six *Celsr1* mutations in human fetuses with craniorachischisis. In *in vitro* assays, those mutations all impair trafficking of Celsr1 protein, reducing its membrane localization (Robinson et al., 2011).

As *Celsr1*<sup>Crsh</sup> and *Celsr1*<sup>Scy</sup> homozygous mutants are embryonic lethal, a conditional allele (*Celsr1*<sup>f</sup>) was generated to allow tissue-specific inactivation upon expression of Cre recombinase (Ravni, Qu, Goffinet, & Tissir, 2009), a null allele *Celsr1*<sup>ko</sup> was obtained by crosses with germline-expressing Cre mice, and Western blot with an antibody against the C-terminal region of Celsr1 confirmed absence of Celsr1 protein in embryonic mouse brain extracts. Unlike heterozygous *Celsr1*<sup>Crsh</sup> and *Celsr1*<sup>Scy</sup>, heterozygous *Celsr1*<sup>ko/+</sup> mice have no perceptible phenotype. In contrast, homozygous *Celsr1*<sup>ko/ko</sup> mutants display abnormal behavior such as circling and hyperactivity. About 20% of them die at embryonic stages with various degrees of neural tube closure defects, and many have a looping tail. Some have striking skin hair patterning defects with whorls and crests instead of regular caudally and distally pointing hairs on the body and limbs (Fig. 7.2) (Ravni et al., 2009).

When *Celsr1* is inactivated in crosses with *Emx1-Cre* mice, inactivation of the gene in the apical ectodermal ridge induces a whorl in distal hindlimb, showing that the action of Celsr1 is ectodermal cell autonomous. The mechanism of Celsr1 action in the hair bulb has been scrutinized by the Fuchs lab (Devenport & Fuchs, 2008). They found that, prior to hair growth, Celsr1 becomes asymmetrically localized along the anterior/posterior (A/P) axis in basal epidermal cells in hair follicles and in interfollicular epithelium. This asymmetric localization is essential for A/P orientation of skin hair. Hair follicles fail to adopt the A/P orientation in skin explants isolated from E13.5 embryos, before the polarization of Celsr1. In contrast, when explants are isolated from E14.5 embryos, when Celsr1 was fully polarized, epidermis keeps the Celsr1 polarization established *in vivo*, and the A/P polarity. Consistent with this, hair follicles are misaligned in E18.5 embryos with
homozygous Crsh mutation. In mutant embryos, Celsr1 protein is produced as in the wild type, but no longer asymmetrically distributed. Moreover, the membrane recruitment of Fzd6 and the asymmetric localization of Vangl2 along the A/P axis are compromised, with some Vangl2 immunoreactivity forming intracellular puncta. Taken together, these results demonstrate that Celsr1 plays a critical role for PCP establishment in the developing skin and hair follicles (Devenport & Fuchs, 2008).

Importantly, PCP phenotypes in Celsr1 mutants were also seen in mice with mutations in other core PCP genes (Table 7.1). The inner ear phenotype was reported in mice with mutated Vangl2 (Montcouquiol et al., 2003, 2006), and Fzd3 and Fzd6 (Wang et al., 2006). Neural tube closure defects were observed in Vangl2, double Dvl1 and 2, and Fzd3 and 6 (Kibar et al., 2001; Wang et al., 2006; Ybot-Gonzalez et al., 2007). Skin hair patterning defects were described in Fzd6 and Vangl2 (Devenport & Fuchs, 2008; Devenport,
<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele</th>
<th>Phenotype</th>
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<tbody>
<tr>
<td>Celsr1</td>
<td>Celsr1 Crsh and Scy</td>
<td>Heterozygotes have inner ear PCP abnormalities, head shaking, looptail, and abnormal FBM neuron migration. Homozygotes are embryonic lethal with open neural tube.</td>
</tr>
<tr>
<td></td>
<td>Celsr1 KO</td>
<td>Heterozygotes are normal and fertile.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Homozygotes are viable but mostly sterile. About 20% die at embryonic stages with neural tube closure defects. Adults have abnormal behavioral traits, a looping tail, and abnormal skin hair patterning. The direction of FBM neuron migration is affected.</td>
</tr>
<tr>
<td>Celsr2</td>
<td>Celsr2 Dgen</td>
<td>Heterozygotes are normal and fertile.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Homozygotes are fertile, except for some females with vaginal atresia. There is abnormal trajectory of FBM neuron migration and hydrocephalus due to defective ependymal ciliogenesis.</td>
</tr>
<tr>
<td></td>
<td>Celsr2 Dgen</td>
<td>Heterozygotes are normal and fertile.</td>
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<tr>
<td></td>
<td></td>
<td>Homozygotes are fertile and have abnormal FBM neuron migration.</td>
</tr>
<tr>
<td>Celsr3</td>
<td>Celsr3 KO and Celsr3 F</td>
<td>Heterozygotes are normal and fertile.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Homozygotes die in few hours after birth and have severe axonal defects.</td>
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<tr>
<td>Fzd3</td>
<td>Fzd3 KO</td>
<td>Heterozygotes are normal and fertile.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Homozygotes die in few hours after birth and have axonal defects similar to those in Celsr3 mutants. Some have loop tail or open neural tube and PCP phenotype in the inner ear. The trajectory of FBM neuron migration is affected and mimics Celsr2 and 3 double KO.</td>
</tr>
<tr>
<td>Fzd6</td>
<td>Fzd6 KO</td>
<td>Homozygous mutants survive and breed, and have abnormal skin hair patterning.</td>
</tr>
<tr>
<td>Vangl2</td>
<td>Vangl2 Lp (Looptail)</td>
<td>Some heterozygotes have kinked or looped tails. Homozygotes do not survive due to open neural tube, loop tail, inner ear PCP defects, FBM neuron migration defects, and other defects in cardiovascular, skeletal, and respiratory systems.</td>
</tr>
<tr>
<td></td>
<td>Vangl2 KO and Vangl2 F</td>
<td>Heterozygotes are normal and fertile.</td>
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<tr>
<td></td>
<td></td>
<td>Homozygote nulls have open neural tube and other PCP defects in the inner ear.</td>
</tr>
<tr>
<td>Vangl1</td>
<td>Vangl1 gt</td>
<td>Heterozygotes are normal and fertile.</td>
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</tbody>
</table>
Oristian, Heller, & Fuchs, 2011; Guo, Hawkins, & Nathans, 2004). These data provide strong evidence that Celsr1 is involved in classical PCP together with Fzd3 and 6, Dvl1 and 2, and Vangl2.

3. CELSR2 AND 3 IN CILIOGENESIS

Cilia are appendages that extend from the cell surface to the extracellular environment. Based on their structure and motility, cilia are classified into three types: motile, primary, and nodal cilia (Davenport & Yoder, 2005). Motile cilia garnish the apical surface of epithelial cell lining airways, reproductive tracts, and cerebral ventricles. They have a central pair of microtubules surrounded by nine doublet of microtubules, an organization known as 9 + 2, and are anchored to a basal body in the apical cortex. Motile cilia form tufts and their concerted beats generate directional flow at the surface of epithelial sheets. In the airways, they are crucial for clearing mucus and debris, and in genital tracts, they assist in the transit of sperm and eggs (Salathe, 2007; Voronina et al., 2009). In the mouse brain at late embryonic stage and during the first postnatal week, neuroepithelial cells that line the cerebral ventricles differentiate into a monolayer of ependymal cells. At birth, ependymal cells are not multiciliated yet (Fig. 7.3). Motile cilia develop progressively and reach their mature shape by the end of brain maturation (Fig. 7.3B). The basal body of each cilium has a lateral extension called “basal foot” that can be detected by transmission electron microscopy. Basal feet point in the effective beat direction and are used as a hallmark of cilia polarity (Gibbons, 1961; Hagiwara, Ohwada, Aoki, Suzuki, & Takata, 2008; Wallingford, 2010). To generate an efficient directional flow, cilia coordinate their beats within the same cell (each cell has dozens of motile cilia) and in all cells in the epithelial sheet. Thus, basal feet are aligned in the same orientation with respect to the tissue polarity axis, a process referred to as “rotational polarity” (Mirzadeh, Han, Soriano-Navarro, Garcia-Verdugo, & Alvarez-Buylla, 2010). Elegant studies in Xenopus ciliated epidermal explants isolated at different developmental stages showed that motile cilia acquire their rotational polarity in two steps. Early in development, genetic cues specify a rough planar axis that allows cells to produce a directional flow, thereby inducing a positive feedback loop that tunes basal feet polarity (Mitchell, Jacobs, Li, Chien, & Kintner, 2007). In mice, Celsr1–3 are expressed in the developing ependymal layer. Mutation of Celsr2 and Celsr3 impairs ependymal ciliogenesis (Tissir et al., 2010). Although differentiation of ependymal cells occurs normally, their motile cilia never
develop in normal numbers and those that develop display abnormalities in position and planar organization. At the cellular level, basal feet are misoriented, a rotational polarity defect, and some basal bodies assemble deep in the cytoplasm. At the tissue level, ciliary tufts from neighboring cells point aberrantly in divergent directions, generating a translational polarity defect (Fig. 7.3C). Mutant cilia display the typical “9 + 2” structure. They are still able to beat with the same frequency as the controls. Yet, because of their abnormal orientation, they fail to generate a robust and directed flow (Fig. 7.3C). The membrane localization of Vangl2 and Fzd3 is disrupted in mutant ependymal cells, providing strong evidence that Celsr2 and Celsr3 regulate ciliogenesis via PCP signaling. In accord with this, downregulation of core PCP genes Disheveled1–3, and PCP effectors Inturned and Fuzzy affects the orientation of multicilia in Xenopus (Park, Haigo, & Wallingford, 2006; Park, Mitchell, Abitua, Kintner, & Wallingford, 2008). Furthermore, ependymal cells with the “looptail” mutation in Vangl2 fail to align their motile cilia in response to hydrodynamic forces in in vitro assays (Guirao et al., 2010).

Figure 7.3 Celsr2 and Celsr3 regulate rotational polarity of ependymal cilia. Representation of motile cilia anchored to basal bodies (red dots) on the apical surface of ependymal cells. Cilia develop progressively according to ventral to dorsal, medial to lateral, and caudal to rostral gradients. During the first postnatal week (P0–P8), basal feet (green arrowheads) point in different directions (A). In normal animals, they rotate and adopt a more uniform orientation between P8 and P21, in response to planar cell polarity signals and hydrodynamic forces. This uniform orientation—also referred to as rotational polarity—enables cilia to beat in a coordinated manner and to generate a directed fluid flow (B). In Celsr2 and Celsr3 mutant ependyma (C), basal feet exhibit divergent orientation and fail to generate a directed fluid flow.
4. CELSR1–3 IN NEURONAL MIGRATION

The migration of facial branchiomotor (FBM) neurons in the developing rhombencephalon is an intriguing case that combines tangential and radial migration modes. FBM neurons, which innervate muscles responsible for facial expression (Chandrasekhar, 2004; Garel, Garcia-Dominguez, & Charnay, 2000; Guthrie, 2007), are generated in medial rhombomere4 (r4) at E10.5 and immediately extend their axons laterally toward muscle targets. At E11.5, their cell bodies initiate a tangential caudal migration from r4 to r6. They migrate in the subventricular region, pass medial to the nucleus abducens (nVI) in r5 (Song et al., 2006), and then move laterally and dorsally in r6. Finally, they undergo a radial migration in r6 to reach their subpial location where they form the motor nucleus of the facial (nVII) nerve (Fig. 7.4A) (Chandrasekhar, 2004). The caudal soma translocation of FBM neurons, with looping of their axons (so-called genu of facial nerve) is conserved from fish to mammals, with important species differences. For example, it is blunted in chick (Gilland & Baker, 2005). The first indication that Celsr genes are involved in FBM neurons migration came from an ENU mutagenesis screen in zebrafish that identified four point mutations in the Celsr2/off road locus (Wada et al., 2006). In these mutants, FBM neurons fail to migrate caudally to r6, moving instead prematurely into lateral r4–r5. Morpholino knockdown experiments showed that Celsr1a and 1b have adjunct functions in FBM neuron migration. Whereas combined downregulation of Celsr1a and Celsr1b has little effect on its own, it worsens the off road mutant phenotype, with more cells stacked in r4 in Celsr1a; Celsr1b; Celsr2 triple mutants than in Celsr2 mutants. In mice, FBM neuron migration was studied using in situ hybridization for Islet1 or Tbx20, two established markers. In wild-type embryos, FBM neurons form streams from medial r4 to lateral r6, with a sharp rostral edge. In Celsr1ko/ko mice, FBM neurons are still able to move out of r4, but a subset migrate rostrally into r3 and r2 rather than caudally, a phenotype never seen in any other vertebrate. This rostral migration phenotype is fully penetrant, although with variable expressivity. Caudally directed Celsr1ko/ko neurons move through r5, medially to the abducens nucleus (nVI), before moving laterally in r6 like their wild-type counterparts. Hence, a facial (nVII) nucleus forms in its normal location in lateral r6 by E13.5. In addition, rostrally migrating FBM neurons form an ectopic nucleus adjacent or
even rostral to the trigeminal (nV) nucleus but send their axons normally in the facial nerve (Fig. 7.4B). In the hindbrain, Celsr1 is expressed in progenitors and in the floor plate, but not in postmitotic FBM neurons. Consistent with this, conditional inactivation of Celsr1 in progenitors under Nk6.2-Cre recombination induces abnormal rostral migration, whereas its deletion in FBM neurons using Isl1-Cre does not.

Figure 7.4 Migration of facial branchiomotor (FBM) neurons in normal and Celsr1–3 knockout mice. Drawings summarize the phenotype at P0. In wild-type animals (A), FBM neurons form a single nucleus (nVII) in a region that derives from r6. Their axons loop around the abducens nucleus (nVI) before exiting the rhombencephalon dorsally, caudal to the trigeminal nucleus (nV). In Celsr1ko/ko mutants (B), in addition to the normal nVII, FBM neurons form another ectopic nucleus at the level of nV, but their axons leave the rhombencephalon at the right position. This is due to aberrant migration of FBM neurons in lateral r2 during embryogenesis. In Celsr2Dgen/Dgen and Celsr2Dgen/Dgen; Celsr1ko/ko (C), the facial nerve genu is completely abnormal and axons do not loop around nVI because FBM neurons migrate prematurely in lateral r4–r5, forming lateral heterotopias. In Celsr2Dgen/Dgen; Celsr3ko/ko and in Fzd3ko/ko, in addition to the absence of the genu of the facial nerve, the size of nVII is reduced because of embryonic cell death (D). "r," rhombomere; nV, motor trigeminal nucleus; nVI, abducens nucleus; nVII, facial nerve nucleus.
In Celsr2 mutant mice (Celsr2^{Dgen/Dgen}), like in off road mutant fishes, the caudal FBM neuron migration stream is severely truncated. Cells turn laterally, rostral to the abducens nucleus, so that axons do not loop around nVI and an elongated facial nucleus forms in r4 and r5 instead of r6 (Fig. 7.4C). When Celsr2 is specifically inactivated in FBM neurons by crossing with Isl1-Cre mice, a similar phenotype is generated, suggesting that Celsr2 is required cell autonomously in FBM neurons for their caudal migration (Qu et al., unpublished). This does not rule out the possibility that Celsr2 may also be necessary in neuroepithelial cells along the migration path, as suggested in zebrafish (Wada et al., 2006). The aberrant migration of FBM neurons in Celsr2 mutant mice is reminiscent of the situation in birds, which have no facial nerve genu and, intriguingly, lack a Celsr2 gene (Formstone, 2010). Whether there is a causal link between the absence of Celsr2 and blunted FBM neuron migration remains an open question. In zebrafish, Celsr2, Celsr1a, and Celsr1b redundantly regulate FBMN caudal migration. In mouse, double inactivation of Celsr1 and Celsr2 phenocopies single Celsr2 mutation, with no obvious rostral migration of FBM neurons, suggesting that Celsr2 is epistatic to Celsr1 (Fig. 7.4C). It looks as if Celsr2 deficiency hampers or slows down neuronal migration, thus masking the Celsr1 mutant phenotype. Although Celsr3 is required for tangential migration of calretinin-positive interneurons and radial migration of calbindin-positive interneurons in the forebrain (Ying et al., 2009), its deficiency does not affect the migration of FBM neurons. It does, however, exacerbate the Celsr2 phenotype: the facial nucleus is greatly reduced in size in Celsr2^{Dgen/Dgen}, Celsr3^{ko/ko} and Celsr2^{Dgen/Dgen}; Celsr3^{Isl1cko} as compared to Celsr2^{Dgen/Dgen} mutant (Fig. 7.4C and D). Celsr3 deficiency increases apoptosis in a cell-autonomous manner. The phenotype of Celsr2^{Dgen/Dgen}; Celsr3^{ko/ko} double mutants is similar to that of Fzd3 mutants, and other PCP-related genes such as Vangl2 and Wnt5a have been implicated in FBM neuron migration, suggesting that Celsr1–3 regulate neuronal migration along the rostrocaudal axis by PCP-dependent mechanisms (Qu et al., 2010; Vivancos et al., 2009) (Table 7.1). In line with this, normal function of PCP genes such as van gogh-like 2 (vangl2), fzd3a, celsr2, prickle1a, and prickle1b is required for caudal migration of FBM neurons in fish (Bingham, Higashijima, Okamoto, & Chandrasekhar, 2002; Carreira-Barbosa et al., 2003; Jessen et al., 2002; Mapp, Wanner, Rohrschneider, & Prince, 2010; Rohrschneider, Elsen, & Prince, 2007; Wada et al., 2006).
5. CELSR2 AND CELSR3 IN BRAIN WIRING

Functional neuronal networks are crucial for brain function. Network formation is finely orchestrated at the cellular and molecular levels by genetic programs and interactions with the environment. After reaching their location, postmitotic neurons extend axons that are guided to their targets by intrinsic programs, guidepost cells, and attractive and repulsive molecular cues. They ramify receptive dendritic fields according to tiling rules. In addition to their role in neuronal migration, Celsrs are implicated in dendrite development and axon guidance, from Caenorhabditis elegans and Drosophila to mammals (Berger-Muller & Suzuki, 2011; Gao, Kohwi, Brenman, Jan, & Jan, 2000; Matsubara, Horiuchi, Shimono, Usui, & Uemura, 2011).

In flies, sensory neurons extend dendrites dorsally toward the midline. Dendrites from homologous neurons in the two opposite hemisegments avoid each other, leading to a dendrite-free zone near the dorsal midline. This reciprocal inhibition of dendrite growth, akin to tiling, is defective in fmi/stan mutants, where dendrites do grow across the midline to occupy fields overlapping those of homologous neurons (Gao et al., 2000). A role for fmi/stan in the tiling of dendrites is further supported by the identification of fmi/stan mutants in genetic screens for aberrant dendritic extension of mushroom body neurons (Reuter et al., 2003). The function of fmi/stan in dendrite growth does not rely solely on adhesion mediated by the extracellular domain because overexpression of an N-terminally truncated fini that lacks cadherin, EGF-like, and laminin G motifs rescues the dendritic phenotype partially (Kimura, Usui, Tsubouchi, & Uemura, 2006). Intriguingly, neither loss or gain of function of frizzled nor overexpression of dsh phenocopy the fmi/stan dendritic phenotype. Moreover, to our knowledge, no such phenotype has been described in other PCP mutants. In the mammalian nervous system, Celsr2 is required for the maintenance of dendritic arbors: RNAi-induced downregulation of Celsr2 in brain slices reduces the length of dendrites in cortical pyramidal neurons, and the complexity of dendritic trees of Purkinje cells (Shima, Kengaku, Hirano, Takeichi, & Uemura, 2004). By contrast, the silencing of Celsr3 results in dendrite overextension. The opposite effects of Celsr2 and Celsr3 are related to a single amino acid change in the first intracellular loop: like fmi/stan, mammalian Celsr3 has a histidine at position 2413, and both repress growth and/or induce retraction of dendrites. That histidine residue is replaced with an arginine in Celsr2, which promotes dendrite growth and maintenance (Shima et al., 2007).
Early observations showed that *fmi/stan* is essential for the development of axonal tracts. Null mutations *fmi*^{E45} and *fmi*^{E59} are embryonic lethal, and in addition to PCP defects, mutant embryos display abnormalities in longitudinal axonal tracts. Rescue of axonal, but not PCP defects, by brain specific expression of the *fmi* cDNA restores viability (Usui et al., 1999). Subsequent elegant studies in the visual system showed that *fmi/stan* mediates axon–axon and axon–target interactions required for guidance of photoreceptor axons (Lee et al., 2003; Senti et al., 2003). The *fmi*-mediated choice of postsynaptic target is dose dependent and non-cell autonomous. It looks as if growth cones “compare” their *fmi/stan* levels with that of their neighbors to “decide” whether to establish contact or not (Chen & Clandinin, 2008). In mice, *Cel3r* is crucial for axon guidance. *Cel3r* mutant mice display marked defects in major tracts such as the anterior commissure, internal capsule, medial lemniscus, and corticospinal tract (Table 7.1). *Cel3r* is also essential for the anterior–posterior organization of monoaminergic axon in the brainstem (Fenstermaker et al., 2010) and for the rostral turning of commissural axons after midline crossing in the spinal cord (Price et al., 2006; Wang & Nathans, 2007). Cel3r deficiency does not affect axonal growth *per se*, but rather guidance, resulting in stalling at intermediate targets or misrouting of axons (Tissir, Bar, Jossin, De Backer, & Goffinet, 2005). For instance, during development of corticothalamic reciprocal projections, mutant corticofugal axons travel normally in the subcortical layer but stall underneath the pallial–subpallial boundary. Reciprocally, thalamic axons never reach their cortical targets in layer 4; instead, they course ventrally along the hypothalamus and then turn externally toward the cortical marginal zone. Conditional removal of *Cel3r* in a stream that extends in the basal forebrain and ventral diencephalon precludes the entry of corticosubcortical and thalamocortical fibers in the “corridor” and results in defective internal capsule. This result demonstrates that *Cel3r* is required in intermediate targets to connect the cortex with subcortical structures. Target cells are molecularly defined by expression of Dlx5/6 and qualify as “guidepost cells” (Zhou et al., 2008). To test whether guidance of cortifugal axon is mediated by homophilic interactions of *Cel3r* in navigating growth cones and guidepost cells, *Cel3r* was specifically deleted in corticofugal axons by crossing the *Cel3r*^{f} allele with *Emx1-Cre*. Cel3r-defective corticothalamic axons develop normally, strongly suggesting that those fibers use guidance mechanisms independent of Cel3r–Cel3r homophilic interactions. Consistent with this finding, a stall phenotype of
axons at intermediate target cells was reported for fmi-defective sensory neurons in flies (Steinel & Whitington, 2009). These phenotypes can be rescued by fmi constructs that lack most of the extracellular domains, indicating that the advance of sensory axons in flies does not depend on fmi–fmi homophilic interactions (Steinel & Whitington, 2009). Contrary to corticothalamic axons, corticospinal axons do not develop in Celsr3^{Emx1cKO}, suggesting that Celsr3 mediates homophilic interactions between corticospinal growth cones and guidepost cells. In support of this, in the fly visual system, fmi mediates interactions between the growth cones of photoreceptor axons and their targets in the medulla (Hakeda-Suzuki et al., 2011). Like in flies and mammals, FMI-1, the sole C. elegans fmi/stan ortholog, enables navigation of both pioneer and follower axons in the worm’s ventral nerve cord. Rescue of FMI-1 mutant phenotype with different portions of FMI-1 revealed that the C- and N-termini are required for guidance of pioneer and follower axons, respectively (Steimel et al., 2010). Taken together, these data show that flamingo and its mammalian ortholog Celsr3 are major players in axon guidance and that their mechanisms of action are context dependent and involve both homophilic and heterophilic interactions.

5.1. Motifs of Celsr important for their functions

As mentioned in the introduction, Celsr proteins contain a large extracellular N-terminus with nine cadherin repeats, EGF-like and laminin G-like motifs, a HRM, a G-proteolysis site, followed with seven transmembrane domains and a variable intracellular C-terminal tail. Although the functional relevance of these motifs remains poorly understood, some studies have revealed a few hints. In Drosophila S2 cells—which have no self aggregation properties—expression of full-length fmi induces cell aggregation, whereas expression of a form lacking most of the ectodomain does not, showing that the extracellular domain indeed promotes homophilic cell adhesion (Usui et al., 1999). Expression of mutant forms of mammalian Celsr2 in S2 cells assigned adhesive properties to the cadherin repeats (Shima et al., 2004).

Dendritic overgrowth and tiling defects of fmi mutants as well as fly viability can be rescued by expression of full-length fmi, although the rescue can be ascribed to two different functions of the protein. In transgenic rescue experiments, ΔNfmi—lacking all extracellular domains except the HRM—can rescue the dendrite overgrowth but not the tiling phenotype. Unlike full-length fmi, ΔNfmi does not promote S2 cell aggregation in vitro.
By contrast, C-terminally deleted fmi (ΔCFmi) retains the ability to mediate homophilic binding in vitro and rescues the tiling phenotype, but not the dendritic overgrowth or fly viability, pointing to a key role of the C-terminus. Thus, a dual molecular function of Fmi plays pivotal roles in dendrite morphogenesis. In the initial growing phase, Fmi might function as a receptor for an unidentified ligand, and this hypothetical heterophilic interaction would be responsible for limiting branch elongation. At a later stage, homophilic Fmi-binding at dendro-dendritic interfaces would elicit avoidance between dendritic terminals from opposing neurons (Kimura et al., 2006). Likewise, studies in worms, flies, and mice show that the function of fmi/Celsr in axon guidance does not rely only on its ability to mediate homophilic binding of cadherin repeats but also requires interactions of the C-terminus with unknown molecules (Steimel et al., 2010; Steinel & Whittington, 2009; Zhou et al., 2008).

The concept of distinct roles of the extra- and intracellular domains of fmi/Celsr is supported by mutation analysis in vertebrates. In rodents, Celsr2 and Celsr3 have opposing roles on dendrites in brain slice in culture, with Celsr2 promoting and Celsr3 restricting dendrite growth (Shima et al., 2004, 2007). Studies of chimeric constructs in which the ectodomains of Celsr2 and Celsr3 are swapped show that, whereas homophilic interactions (Celsr2–Celsr2 and Celsr3–Celsr3) are important, the transmembrane domains and C-terminus determine the dendrite enhancing or suppressing action. Celsr2 and Celsr3 have different effects on calcium release and activate, respectively, CamKII and Calcineurin signaling, and an amino acid change (R2413H) in the first intracellular loop is crucial to these distinct functions.

Another evidence for the importance of the C-terminus comes from a study in zebrafish (Carreira-Barbosa et al., 2009). Injection of a C-terminally truncated form of Celsr (ΔC-celsr; lacking 6 TM and tail) in wild-type embryos generates epiboly defects. This is accompanied by sequestration of ΔC-Celsr in the Golgi, where the protein behaves as a dominant negative, dimerizing with and precluding trafficking of the wild-type protein. Dimerization is thought to involve a conserved arginine-rich sequence N-terminal to the first cadherin repeat. Injection of the CT of Celsr2 fused to the membrane localization signal from the Lyn tyrosine kinase (Lyn-celsr) generates convergent extension defects without affecting epiboly. Lyn-celsr perturbs the Frizzled-induced membrane localization of Disheveled and thus PCP signaling. In mammals, knock-in of the mouse Celsr1 C-tail fused to membrane localization signal Lyn in the ubiquitous Rosa26 locus also perturbs
the trafficking of Dvl2, and Vangl2 as well as the subcellular of F-actin (Trichas et al., 2011). Interestingly, when a conserved serine acidic amino acid-rich domain (SE/D) is deleted from the fish Lyn-Celsr, the membrane localization of Disheveled is restored (Carreira-Barbosa et al., 2009). Whereas the extracellular and TM domains are important for the distribution of Frizzled–Disheveled complexes at the membrane, this process depends critically on the Cter SE/D domain. The ability of Celsr to regulate epiboly is closely associated with its ability to modulate cell cohesive property, whereas its ability to interact with the PCP pathway to regulate convergent extension may not require cell adhesion mediated by the cadherin repeats.

In the mouse skin, Celsr1 is internalized during epidermal basal progenitor division, a process which is crucial for balanced distribution of PCP proteins Fzd6 and Vangl2 in daughter cells and requires a cytoplasmic dileucine motif. Whereas E-Cadherin is normally not internalized, its fusion with the Celsr1 cytoplasmic domain induces internalization during mitosis and confers to the chimeric protein the ability to recruit Fzd6 and Vangl2. When the two leucines (2748–2749) are mutated to alanines, chimeric E-Cad-Celsr1, like mutated Celsr1, no longer translocates to endosomes (Devenport et al., 2011).

The Celsr1–3 CTs display much less similarity than their extracellular and TM regions. As Celsr1 is directly implicated in PCP, and Celsr2–3 in more distantly related processes, could differences in the C-terminus account for functional differences? In Drosophila, fmi, fz, and dsh depend on each other for their membrane localization (Das et al., 2002). Fmi interacts physically with fz via a region encompassing its HRM and TM domains, and selectively recruits fz and vang (strabismus) to opposing cell boundaries (Chen et al., 2008). In mice, the membrane localization of Fzd3 and Vangl2 in ependymal cells, Fzd6 and Vangl2 in skin epithelial cells, and Dvl2 and Vangl2 in the visceral endoderm cells depends on Celsr cadherins (Devenport & Fuchs, 2008; Tissir et al., 2010; Trichas et al., 2011). In Celsr1Cre/Cre mutant mice, a single amino acid substitution results in failure of the Celsr1 protein to reach the apical membrane (Devenport & Fuchs, 2008; Formstone et al., 2010; Ravni et al., 2009). Furthermore, all six Celsr1 mutations recently identified in human fetuses with craniorachischisis impair membrane trafficking of Celsr1 in in vitro assays (Robinson et al., 2011). Although not directly useful for definition of structure–function relationships, this exquisite sensitivity to minor sequence changes underscores the importance of fmi/Celsr conformation
for their membrane insertion as well as for proper targeting of partner proteins. This raises the question of the role of putative proteins assisting in folding, like receptor-activating protein RAP does for the lipoprotein receptor family (Herz, 2006), or the beta-2 microglobulin for major histocompatibility complexes (Hansen, Connolly, Gould, & Fremont, 2010). Would Celsr, Fzd, and Vangl help each other’s folding and complex formation in the endoplasmic reticulum and traffic as a complex to the Golgi and to the membrane, or are they assisted by other unidentified partners, and what would be the role of chaperonins in the process?

REFERENCES


Celsr1–3 Cadherins in PCP and Brain Development


