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Review article

Eph/ephrin signalling in the development and function of the mammalian cochlea

Jean Defourny

GIGA-Neurosciences, Unit of Cell and Tissue Biology, University of Liège, C.H.U. B36, B-4000, Liège, Belgium

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ABSTRACT

In mammals, the functional development of the cochlea requires the tight regulation of multiple molecules and signalling pathways including fibroblast growth factors, bone morphogenetic proteins, Wnt and Notch signalling pathways. Over the last decade, the Eph/ephrin system also emerged as a key player of the development and function of the mammalian cochlea. In this review, we discuss the recent advances on the role of Eph/ephrin signalling in patterning the cochlear sensory epithelium and the complex innervation of mechanosensory hair cells by spiral ganglion neurons. Finally, we address the issue of a syndromic form of hearing loss caused by a deficient member of the Eph/ephrin family.

1. Introduction

Hearing requires an optimal innervation of mechanosensory hair cells (HCs), which transduce the acoustic signal. In mammals, HCs and several types of non-sensory supporting cells are organized in a regular mosaic pattern to form the sensory epithelium of the cochlea (the organ of Corti). The primary afferent innervation of sensory HCs includes two functionally distinct neuronal populations conveying sound information from the cochlea to the central nervous system. In the organ of Corti, each inner HC (IHC) is connected by multiple type I spiral ganglion neurons (SGNs) (about 90–95% of the neuronal population) and represent the principal encoder of the auditory signal (Rubel and Fritzsch, 2002) (Fig. 2A). However, recent studies revealed a greater heterogeneity among type I SGNs than previously reported. Based on their expression of $Ca2^+$ binding proteins, ion channel regulators, guidance molecules and transcription factors, type I SGNs can be divided in three subgroups (type Ia, b, c) (Petitpré et al., 2018; Shrestha et al., 2018; Sun et al., 2018). This sensory neuron diversity is shaped by HC mechanotransduction and spontaneous SGN activity during the pre-hearing period (Shrestha et al., 2018; Sun et al., 2018).

While the outer hair cells (OHCs) considerably outnumber the IHCs, their afferent innervation is much more limited. Type II SGNs form "en passant" contacts with OHCs and represent 5–10% of the total neuronal population (Rubel and Fritzsch, 2002) (Fig. 2A). Rather than transmitting information, type II afferent SGNs could drive medial olivocochlear efferent reflex suppression of the gain of the "cochlear amplifier". Such efferent feedback is important for promoting discrimination of sounds in background noise, sound localization and protecting the cochlea from acoustic overstimulation (Froud et al., 2015). However, the role of type II afferents still remains controversial, since another group concluded that type II SGNs do not drive the olivocochlear efferent reflex (Maison et al., 2016).

The development of the cochlea requires the tight regulation of multiple molecules and signalling cascades including fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), Wnt and Notch signalling pathways (Groves and Fekete, 2012; Wu and Kelley, 2012). Over the last decade, the Eph/ephrin system also emerged as a key player of the development and function of the mammalian cochlea. In the late 1990s/early 2000s, studies on Eph/ephrin signalling in cochlear development were roughly limited to immunohistochemical analysis, semi-quantitative RT-PCR and in vitro experiments (Bianchi and Gale, 1998; Bianchi and Gray, 2002; Bianchi and Liu, 1999; Bianchi et al., 2002; Brors et al., 2003; Lee et al., 1996; Pickles, 2003; Pickles et al., 2002; Van Heumen et al., 2000). However, hearing assessment in Eph or ephrin knockout mouse models progressively revealed altered auditory brainstem response (ABR), ABR peak I amplitude (reflecting the summed activity of cochlear afferents) or decreased levels of distortion product otoacoustic emissions (DPOAEs, used to assess OHC integrity), highlighting the functional relevance of Eph/ephrin signalling in the cochlea (Defourny et al., 2013; Howard et al., 2003; Ingham et al., 2017; Kim et al., 2016; Lee et al., 2015; Miko et al., 2008; Yates et al., 2014) (Table 1).

E-mail address: jean.defourny@uliege.be.

Table 1

Hearing phenotype of Eph/ephrin deficient mice. The equals sign (=) indicates no change relative to wild-type mice. ABR = auditory brainstem response; DPOAE = distortion product otoacoustic emission; n.d. = not determined; n.m. = not mentioned.

	ABR threshold	Peak 1 amplitude	DPOAE levels	Genetic background	Reference
$Ephrin\text{-}A2^{-/-}$	=	=	n.d.	C57BL/6	Ingham et al. (2017)
	↓ (at high frequency stimuli)	↑	n.d.	C57BL/6J	Yates et al. (2014)
$Ephrin\text{-}A5^{-/-}$	=	↓	n.d.	C57BL/6	Defourny et al. (2013)
	=	↓	n.d.	C57BL/6J	Yates et al. (2014)
$Ephrin\text{-}A2^{-/-}/Ephrin\text{-}A5^{-/-}$	↓ (at high frequency stimuli)	=	n.d.	C57BL/6J	Yates et al. (2014)
$EphA4^{-/-}$	↑	↓	n.d.	C57BL/6J	Miko et al. (2008)
$EphA7^{-/-}$	=	↓	n.d.	n.m.	Kim et al. (2016)
$Ephrin\text{-}B2^{+/lacZ}$	↑	↓	n.d.	CD-1/129	Miko et al. (2008)
$Ephrin\text{-}B3^{-/-}$	n.d.	n.d.	=	CD-1	Howard et al. (2003)
$EphB1^{-/-}$	n.d.	n.d.	↓	CD-1	Howard et al. (2003)
$EphB2^{-/-}$	n.d.	n.d.	=	CD-1	Howard et al. (2003)
$EphB3^{-/-}$	n.d.	n.d.	↓	CD-1	Howard et al. (2003)
$EphB1^{-/-}/EphB2^{-/-}/EphB3^{-/-}$	↑	n.d.	n.d.	n.m.	Lee et al. (2015)

2. Eph/ephrin signalling

Eph receptors represent the largest family of receptor tyrosine kinases identified to date. Eph receptors and their membrane-bound ephrin ligands are both divided into two classes on the basis of sequence homology and binding affinity (Gale et al., 1996). Glycosylphosphatidylinositol-anchored ephrin-As bind to and activate EphA receptors, whereas transmembrane ephrin-Bs preferentially interact with EphB receptors. However, some cross-class interactions are possible, as EphA4 can also bind to ephrin-B ligands (Bowden et al., 2009; Qin et al., 2010). One of the unique features of the Eph/ephrin system is the fact that both receptors and ligands are competent to transduce a signalling pathway. Upon binding, receptor clustering initiates a "forward signalling", but receptor-ligand interaction can also stimulate a "reverse signalling" downstream of the ephrin ligand (Kania and Klein, 2016; Kullander and Klein, 2002; Lisabeth et al., 2013).

At first, the main function attributed to Eph receptors was to guide growing neuronal processes during development towards their targets through repulsive effects (Tessier-Lavigne, 1995). However, since their discovery three decades ago (Hirai et al., 1987), Eph and ephrin genes have been implicated in an increasing number of physiological and pathological processes in many cell types and different organs. Eph/ephrin interactions may have diverse consequences, including widespread effects on the actin cytoskeleton, cell-substrate adhesion, intercellular junctions, cell shape and movement (Egea and Klein, 2007; Pasquale, 2005). Eph/ephrin signalling pathways are key determinants not only of neural development, but also of cell migration and proliferation, cell sorting and positioning, cell fate specification and differentiation, tissue patterning and morphogenesis, angiogenesis and neural plasticity (Kania and Klein, 2016; Klein, 2004; Pasquale, 2008; Poliakov et al., 2004; Wilkinson, 2014). In addition, effects on cell survival, immune function, secretion and repair after injury have also been described (Kania and Klein, 2016; Pasquale, 2008).

3. Differentiation and patterning of the cochlear sensory epithelium

3.1. Sensory/non-sensory cell fate specification

Within the organ of Corti, HCs and several types of specialized non-sensory supporting cells are organized in a regular mosaic pattern that extends along the basal-to-apical axis of the cochlear duct. HCs and adjacent supporting cells arise from a common progenitor that acquires a HC or supporting cell phenotype via lateral inhibition mediated by the Notch signalling pathway (Haddon et al., 1998; Lanford et al., 1999; Kelley, 2006). One of the most striking aspects of this mosaic is that specific cell types are arranged in discrete rows. This organization,

coupling cell positioning and fate specification in the organ of Corti, is at least partly driven by the EphA4/ephrin-B2 signalling pathway. In late embryonic stages, ephrin-B2 is specifically expressed in supporting cells, whereas EphA4 is expressed in adjacent HCs. At the interface between HCs and supporting cells, the disruption of ephrin-B2 signalling results in supporting cell translocation into HC layers and switch in cell identity (Defourny et al., 2015). These data fit with the idea that cell contact-dependent Eph/ephrin signalling induces a distinct cell fate at the interface of their expression domains (Wilkinson, 2014). How such cell fate change precisely occurs in the cochlea still remains unclear. However, a hypothesis could be that, as shown during vasculogenesis (Adams and Alitalo, 2007), ephrin-B2 is upregulated by Notch signalling in supporting cells, and required to segregate the latter cells from adjacent HCs. As a consequence, the loss of a Notch pathway component could weaken the supporting cell fate and make easier the switch towards the HC phenotype. This hypothesis supposes that Notch signalling pathway couples cell segregation and differentiation in the organ of Corti, as it has been shown to occur at the hindbrain boundaries (Cheng et al., 2004).

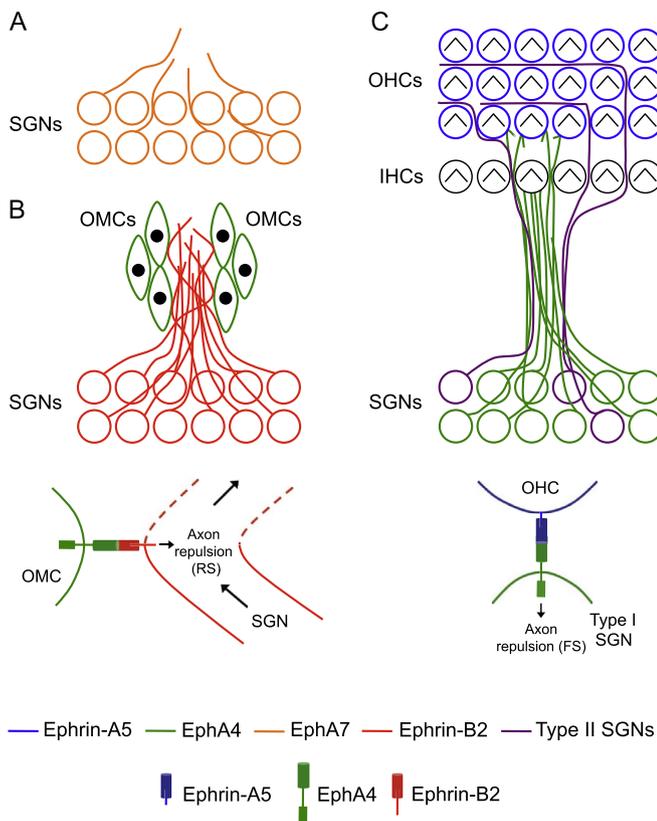### 3.2. Postnatal patterning of the sensory epithelium

Besides sensory HCs, the organ of Corti contains a functionally important triangular fluid-filled space between adjacent pillar cells referred to as tunnel of Corti. The somatic motility of OHCs produces oscillatory fluid flow in the tunnel of Corti, which is critical for cochlear amplification (Karavitaki and Mountain, 2007). At birth, all cells of the organ of Corti are closely connected and inner pillar cells abut on adjacent outer pillar cells through E-cadherin-based homophilic adhesion interactions (Johnen et al., 2012; Whitlon, 1993). As development progresses, the apical ends of the pillar cells remain connected, forming the reticular lamina, while the lateral membranes become no longer apposed, being separated with fluid spaces (Ito et al., 1995). This local inner/outer pillar cell detachment is likely controlled by Eph/ephrin signalling, as recently proposed. Except at the apical extremity of the cells, EphA4 and its ligand ephrin-B2 are co-expressed on both sides of the inner/outer pillar cell junction from early postnatal stages. These cells fail to separate from each other, and E-cadherin abnormally persists at the pillar cell junction in EphA4 forward-signalling-deficient mice (Defourny et al., 2019a). Using immunolabelling and an *in situ* proximity ligation assay, it was shown that EphA4 forms a complex with E-cadherin and its sheddase ADAM10, which could be activated by ephrin-B2 across the pillar cell junction to promote the local disruption of adherens junctions (Defourny et al., 2019a). Together, these data suggest a key role for Eph/ephrin signalling in the postnatal patterning of the cochlear sensory epithelium.

component of auditory dysfunction.

Beside the afferent system, the Eph/ephrin system also controls the outgrowth of efferent fibers. A DiI neuronal tracing revealed that inner ear efferents fail to extend into the sensory structures of the cochlea of *EphB2*[lacZ/lacZ] mice (Cowan et al., 2000).

4.2. Neurite refinement and innervation of sensory hair cells

After reaching the organ of Corti, SGNs arborize and likely project simultaneously to both IHCs and OHCs at each radial position. Each fiber emits temporary additional collateral branches to HCs, which are pruned at later stages (Druckenbrod and Goodrich, 2015; Huang et al., 2007). In the meantime, type I fiber tracts are segregated and connected to IHCs (Huang et al., 2007). The patterning of radial SGN projections occurring at early perinatal stages is controlled by the Eph/ephrin system. Ephrin-As and EphA receptors are often expressed in complementary gradients in target cells and projecting neurons, respectively (Flanagan, 2006). In the cochlea, the process of axon refinement and HC innervation requires the complementary expressions of ephrin-A5 in OHCs and EphA4 in type I SGNs throughout the perinatal period (Defourny et al., 2013) (Fig. 1C). In the absence of *ephrin-A5* or EphA4 forward signalling, a subset of type I projections aberrantly overshoot the IHC layer and invade the OHC area, thereby suggesting an Eph/ephrin-mediated mutual repulsion mechanism responsible for specific afferent targeting to cochlear HCs (Defourny et al., 2013) (Fig. 2D).

Beside the EphA/ephrin-A system, class B ephrins and EphB receptors also control the innervation pattern of sensory HCs. The latter cells express EphB1 and EphB2, whereas SGNs express ephrin-B1, ephrin-B2, EphB1 and EphB2 (Bianchi and Gale, 1998; Pickles et al., 2002; Zhou et al., 2011). In mice lacking *ephrin-B1* or *EphB1/EphB2/EphB3*, SGN axons aberrantly grow beyond the third row of OHCs (Zhou et al., 2011). This suggests that EphB/Ephrin-B signalling delimits the epithelial region through which SGN axons should grow.

Little is known about the molecular mechanisms that guide type II projections towards the OHC area. However, several genes of the Eph/ephrin families are particularly enriched in type II SGNs, likely participating in their axon growth and guidance within the OHC area (Petitpré et al., 2018).

5. Hearing loss caused by a deficient ephrin ligand in humans

Beside the potential involvement of EphA4, as a product of Pou3f4 transcriptional activity, in X-linked deafness DFNX2, a recent study has shown that *EFNB2* haploinsufficiency likely causes a sensorineural hearing loss in humans (Lévy et al., 2018). A familial deletion at chromosome 13q33 encompassing only *ARGLU1* and *EFNB2*, which encodes ephrin-B2, has been shown to provoke a syndromic neurodevelopmental disorder including developmental delays, intellectual disability, seizures, congenital heart defects, and sensorineural hearing loss (Lévy et al., 2018). To date, the mechanisms causing deafness in this case still remain unclear. However, some hypotheses can be suggested. On the one hand, ARGLU1 is a transcriptional coactivator and splicing regulator important for stress hormone signalling and development (Magomedova et al., 2019). Although hearing function was not assessed in *Arglu1*^{+/−} heterozygous mice, these animals appear phenotypically normal (Magomedova et al., 2019). On the other hand, an ABR audiometry test revealed that not only the hearing threshold is elevated in *ephrin-B2*[+/lacZ] heterozygous mice lacking one ephrin-B2 cytoplasmic domain, but also the peak I amplitude reflecting the function of the cochlea is significantly reduced (Miko et al., 2008) (Table 1). This means that a defective activity of the cochlea in auditory processing is at least partly responsible for the hearing deficit. In addition to the cochlea, the mammalian inner ear contains another sensory system, the vestibule (the organ of balance), which shares a similar embryological origin, the otic vesicle. Both organs also share morphological and physiological properties, such as HCs and the mechanical transduction mechanism, which requires the tight

regulation of extracellular K$^+$ concentration and endolymphatic potential. Interestingly, it has been shown that ephrin-B2 regulates the ionic homeostasis of the vestibular endolymph. In the vestibular sensory epithelium, ephrin-B2 is expressed in transitional and supporting cells, which are involved in endolymph homeostasis, likely by controlling K$^+$ recycling or resorption (Cowan et al., 2000; Dravis et al., 2007; Wangemann, 2002a,b). The K$^+$ concentration and the endolymphatic potential are significantly reduced in *ephrin-B2*[+/lacZ] heterozygous mice lacking one ephrin-B2 cytoplasmic domain, leading to vestibular dysfunction and hyperactive circling locomotion in a subset of *ephrin-B2*[+/lacZ] mice (Dravis et al., 2007). These findings could explain to some extent why psychomotor development is delayed in patients suffering from *EFNB2* haploinsufficiency (Clark et al., 1977; Lévy et al., 2018). Ephrin-B2 contains a cytoplasmic C-terminal PDZ-binding motif through which it can interact with several PDZ domain proteins (Mäkinen et al., 2005). Thus, it is possible that ephrin-B2, through its C-terminal PDZ-binding motif, forms multi-protein complexes with other molecules involved in endolymph K$^+$ cycling and fluid regulation. This would be consistent with the idea that ephrin-B2 can exert biological functions independently of Eph-receptor binding (Bochenek et al., 2010).

In the cochlea, connexin 26 (Cx26) and connexin 30 (Cx30) gap junction proteins allow the rapid removal of K$^+$ away from the base of sensory HCs, resulting in the recycling of this ion back to the endolymph to maintain cochlear homeostasis (Ahmad et al., 2003; Kikuchi et al., 2000; Sun et al., 2005). In humans, mutations in *GJB2* or *GJB6* which encode CX26 and CX30 respectively, disrupt cochlear homeostasis and induce hearing loss (Grifa et al., 1999; Kelsell et al., 1997). Cx26 and Cx30 assemble in two types of homomeric and heteromeric channels, which form a syncytium extending from the spiral limbus to the cochlear spiral ligament, via the supporting cells. Although these channels were lately shown to exhibit distinct assembly mechanisms (Defourny et al., 2019b), little is known about their molecular regulation. In this context, it is useful to remind that, in a mouse embryo fibroblast cell line, the distribution of connexin 43 (Cx43) at cell-cell interface is controlled by the transmembrane protein ephrin-B1 (Davy et al., 2006). Interestingly, ephrin-B2, which belongs to the same family, is specifically expressed in cochlear supporting cells (Defourny et al., 2015). Since gap junction communication is promoted at ephrin/ephrin interfaces within a cell compartment (Davy et al., 2006), it is tempting to speculate that ephrin-B2 might regulate Cx26 and/or Cx30 distribution and gap junction communication between adjacent supporting cells.

6. Conclusion

The data we have presented here provide evidence that Eph/ephrin signalling plays diverse roles during cochlear development in a context-dependent manner. Different or redundant combinations of ephrin ligands and Eph receptors pattern the sensory epithelium and the subsequent innervation of mechanosensory HCs. Functional impairments resulting from defective Eph/ephrin family members confirm the relevance of such proteins during different stages of cochlear development. Further exploration of the Eph/ephrin signalling will be especially needed to decipher the molecular mechanisms causing hearing loss in humans.

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