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Eph proteins and the assembly of auditory circuits

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Abstract

Many kinds of information are carried in the acoustic signal that reaches auditory receptor cells in the cochlea. The analysis of this information is possible in large part because of the neuronal architecture of the auditory system. The mechanisms that establish the precise circuitry that underlies auditory processing have not yet been identified. The Eph receptor tyrosine kinases and their ligands are proteins that regulate axon guidance and have been shown to contribute to the establishment of topographic projections in several areas of the nervous system. Several studies have begun to investigate whether these proteins are involved in the formation of auditory system connections. Studies of gene expression show that Eph proteins are extensively expressed in structures of the inner ear as well as in neurons in the peripheral and central components of the auditory system. Functional studies have demonstrated that Eph signaling influences the assembly of auditory pathways. These studies suggest that Eph protein signaling has a significant role in the formation of auditory circuitry.

Keywords: Auditory pathways; Auditory nerve; Axon guidance; Eph receptor; Ephrin

1. Introduction

Neuronal connections in the auditory system convey detailed information about the timing, intensity, and frequency of sounds. These features are used to compute more complex aspects of acoustic input, such as interaural phase and intensity differences, which are used to determine the location of sound sources. The ability of the auditory nervous system to make these computations depends on precision in the arrangement of auditory circuitry.

One of the principal features of this circuitry is tonotopy. The orderly arrangement of best frequency in the cochlea is preserved at the level of the cochlear ganglion, which sends tonotopic projections peripherally to hair cells, as well as centrally, to the cochlear nucleus of the brainstem (Rubel and Fritzsch, 2002). Within the brainstem, the cochlear nucleus in turn makes tonotopic connections with its targets. A second feature is that contralateral targets differ from ipsilateral targets (Cant and Benson, 2003), a distinction important in sound localization. For example, the chick nucleus magnocellularis (NM) projects to its target, nucleus laminaris (NL), bilaterally. The auditory brainstem circuitry of chicks is shown schematically in Fig. 1A. The ipsilateral branch of NM axons contacts the dorsal dendrites and cell bodies of NL, while the contralateral branch contacts ventral dendrites of NL and cell bodies. This arrangement aids in the computation of interaural phase differences (Young and Rubel, 1983; Carr and Konishi, 1990; Overholt et al., 1992). In an analogous pathway in the mammalian brainstem (Fig. 1B), neurons in the anteroventral cochlear nucleus (AVCN) project to the medial superior olive (MSO) on both sides of the brain, with ipsilateral

Abbreviations: AVCN, anteroventral cochlear nucleus; GPI, glycosyl-phosphatidylinositol; LSO, lateral superior olive; MGB, medial geniculate body; MNTB, medial nucleus of the trapezoid body; MSO, medial superior olive; NL, nucleus laminaris; NM, nucleus magnocellularis

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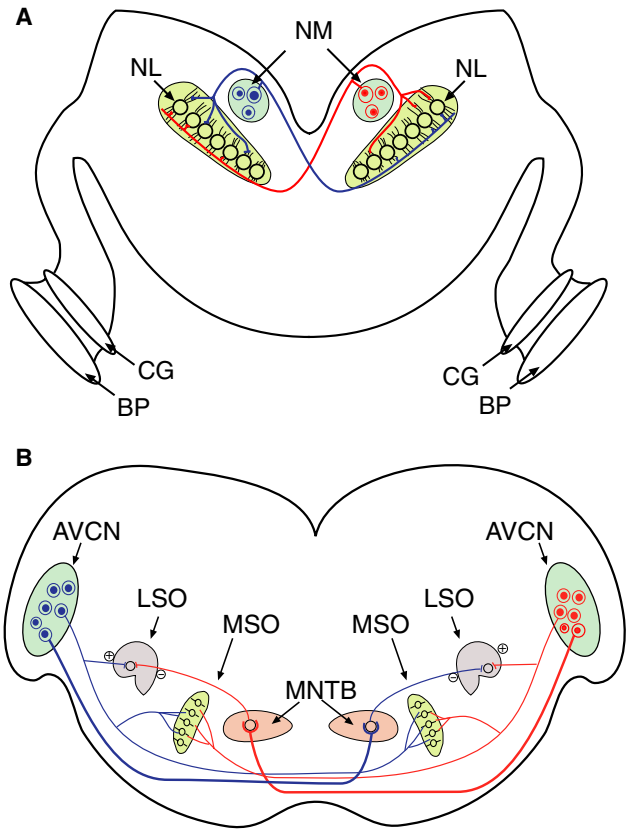

Fig. 1. Schematic illustrations of some auditory pathways in avian and mammalian brainstems. (A) Chick auditory brainstem connections. The basilar papilla (BP) and NM both receive tonotopic input from cochlear ganglion (CG) neurons. NM in turn projects tonotopically to NL. Each NL neuron receives segregated inputs from the ipsilateral and contralateral NM. This circuit computes interaural phase differences, used to localize sound sources in the low frequency ranges. (B) Mammalian auditory brainstem connections. The anteroventral cochlear nucleus (AVCN) is homologous to NM. Spherical bushy cells in AVCN project to ipsilateral and contralateral MSO, which is analogous to NL. This pathway computes interaural phase differences. This computation in MSO also relies on inhibitory inputs (not shown), which arise from MNTB and LNTB. In addition, globular bushy cells in AVCN make strictly contralateral projections to MNTB, which sends inhibitory ipsilateral connections to LSO. LSO cells receive a tonotopically matched input from spherical bushy cells in ipsilateral AVCN. These inhibitory and excitatory projections to LSO neurons aid in the computation of interaural intensity differences, which are used to localize high frequency sounds.

axons contacting the lateral dendrites of MSO and contralateral axons contacting the medial dendrites (Cant, 1992; Cant and Benson, 2003). The mammalian MSO differs from NL in that inhibitory projections contribute significantly to the computation of interaural intensity differences (Brand et al., 2002; Grothe, 2003). These inhibitory projections arise from the lateral and medial nucleus of the trapezoid body (Cant and Hyson, 1992; Kuwabara and Zook, 1992; Grothe and Sanes, 1993; Smith et al., 2000). In another mammalian brainstem pathway, AVCN neurons project to MNTB on the contralateral side, but not on the ipsilateral side (Fig. 1B). MNTB neurons in turn make inhibitory projections to the lateral superior olive (LSO). LSO receives these tonotopic projections in register with excitatory ipsilateral inputs from AVCN spherical bushy cells (Glendenning et al., 1985). The balance between inhibitory and excitatory projections to LSO neurons contributes to the computation of interaural intensity differences, which are used to localize high frequency sounds.

An important challenge for auditory neuroscience is to understand how these circuits are assembled during embryonic and postnatal development. Patterns of connectivity are in essentially correct locations from their initial arrival at target regions, with some projections and synaptic weights refined by activity-dependent processes (Sanes and Rubel, 1988; Friauf and Lohmann, 1999; Leake et al., 2002; Rubel and Cramer, 2002). Moreover, tonotopic connections between the cochlea and spiral ganglion form even in the absence of differentiated hair cells (Xiang et al., 2003). It is thus likely that auditory circuits form largely through activity-independent processes. Several axon guidance molecules have recently been identified that have roles in many regions of the nervous system and are thus good candidates within the auditory region. How does the auditory system make use of developmental molecules during the formation of its specialized structures and connectivity? The roles of one class of molecules, the Eph family proteins, are discussed here, with special emphasis on the formation of the auditory regions of the nervous system and their connectivity in the periphery and brainstem.

2. Eph proteins

The Eph proteins consist of Eph receptor tyrosine kinases and their ligands, called ephrins. Eph receptors are the largest known family of receptor tyrosine kinases (for review, see Flanagan and Vanderhaeghen, 1998). Eph-ephrin binding mediates cell–cell interactions because ephrin ligands are membrane-associated. The ephrin-A ligands are associated with the membrane through a glycosyl-phosphatidylinositol (GPI) linkage, while the ephrin-B ligands have a transmembrane domain. Eph receptors are also classified into A and B classes. In general, ephrin-A ligands bind EphA receptors, while ephrin-B ligands bind EphB receptors. Two exceptions to this rule have been identified. EphA4 binds ephrin-B ligands (Gale et al., 1996), and EphB2 binds ephrin-A5 (Himanen et al., 2004). While interactions are often promiscuous within a class, there are differences in the affinity of a ligand for the different receptors.

The Eph family proteins are especially promising in the study of auditory circuitry because they have a well-established role in the formation of topographic maps

elsewhere in the nervous system. The most abundant evidence comes from studies in the visual system (for review, see O'Leary et al., 1999). In the retinotectal map, nasal retinal ganglion cell axons project to posterior tectum, while temporal axons project to anterior tectum. During the time that retinotopic maps form in the chick, EphA3 receptors are expressed in temporal-nasal gradients in retinal ganglion cell axons, and ephrin-A2 and ephrin-A5 are expressed in opposing posterior–anterior gradients in the tectum (Cheng et al., 1995). Thus, axons with high levels of EphA3 project to areas with low levels of ephrin-A, while areas with low levels of EphA3 can innervate regions with high levels of ephrin-A. In vitro assays of retinal cells on cultured tectal membranes have demonstrated that interactions between these proteins are repulsive at higher ephrin concentrations, but at low ephrin concentrations retinal growth is promoted (Hansen et al., 2004). The retinotectal map forms as a consequence of these opposing gradients of proteins. This mechanism has been demonstrated in the mammalian retinogeniculate pathway as well (Feldheim et al., 1998, 2000). In addition to these studies in the visual pathway, Eph/ephrin signaling has been shown to be necessary for development of topographic maps in the thalamocortical projections of the somatosensory system (Prakash et al., 2000; Vanderhaeghen et al., 2000; Dufour et al., 2003), the hippocamposeptal pathway (Gao et al., 1996, 1999; Yue et al., 2002), in motor axon projections (Helmbacher et al., 2000; Eberhart et al., 2004), and, in conjunction with odorant receptors, in the glomerular map in the olfactory bulb (Cutforth et al., 2003). In these pathways, while several other signaling molecules may also be involved, Eph proteins have a significant role in establishing topographic connections.

An important feature of Eph-ephrin signaling is that it can be bidirectional. That is, in addition to forward signaling in which ephrins signal through Eph receptors, reverse signaling mechanisms have also been identified. Eph receptors can act as ligands that signal through ephrins and result in tyrosine phosyphorylation of ephrins (Holland et al., 1996; Bruckner et al., 1997; Knoll and Drescher, 2002; Kullander and Klein, 2002) and subsequent downstream signaling. In the case of the transmembrane ephrin-B ligands, phosphorylation sites at tyrosine residues have been identified (Kalo et al., 2001) but it is not clear which tyrosine kinases phosphorylate these residues. This phosphorylation results in recruitment of the Src-homology-2 adaptor protein Grb4, which then allows signaling that alters cytoskeletal elements and adhesion (Cowan and Henkemeyer, 2001). Reverse signaling can also occur through ephrin-A ligands, where GPI linkages associate these proteins with lipid rafts. EphA signaling through these ligands facilitates integrin-mediated cell adhesion; this response requires recruitment of Fyn tyrosine kinase (Davy et al., 1999; Huai and Drescher, 2001; Kullander and Klein, 2002). Bidirectional signaling between Eph receptors and ephrins thus allows cell–cell interactions to independently influence growth and adhesion in both cells. Moreover, it allows both Eph receptors and ephrins to act cell autonomously and non-cell autonomously. These factors add to the diversity of Eph protein functions in development.

The cell–cell interactions of Eph proteins have an important role in both cell migration and axon guidance. Both forward and reverse signaling have a demonstrated role, and these interactions may be attractive or repulsive. In the visual system both mechanisms have a role in establishing topography. While the temporonasal-anteroposterior axis is formed with repulsive actions mediated by ephrin-A2 and ephrin-A5, the dorsoventral projections make use of attractive interactions, in this case between ephrin-B ligands and EphB receptors (Braisted et al., 1997; Holash et al., 1997; Hindges et al., 2002; Mann et al., 2002). Often several Eph proteins are expressed within a single structure, and Eph receptors and ephrins can both be expressed on the same cells (Holash et al., 1997; Hornberger et al., 1999; Menzel et al., 2001). To some extent, these expression patterns provide redundancy in which one family member can assume roles of another. Moreover, ephrins can signal through receptors in *cis*, that is, within the same cell, through extracellular binding domains (Yin et al., 2004). Together, these studies show that Eph proteins are important in developmental mapping of projections, but also that the interactions and molecular mechanisms may be very complicated and difficult to understand completely, even for a single neuroanatomical pathway.

Several studies have begun to explore the role of Eph proteins in the formation of auditory structures and synaptic pathways. These proteins appear to be an important component of molecular mechanisms of auditory development.

3. Early development

During early development, Eph receptors and ephrins have distinct patterns of expression in the rhombomeres, which are hindbrain segments found in all vertebrate embryos. Progenitor cells within rhombomeres give rise to structures in the brainstem and cerebellum. In some cases, Eph proteins are the first axon guidance molecules downstream of the homeobox transcription factors that largely define segment identity. Migration of cells across rhombomere boundaries is limited. This restriction is due, at least in part, to inhibitory interactions between EphA4 in rhombomere 3 (r3) and r5, and ephrin-B2 in r2, r4, and r6. These interactions are mediated by bidirectional signaling (Mellitzer et al., 1999).

The progenitor cells that give rise to the auditory brainstem nuclei of the chick are found in r4 through r7, and all of the auditory brainstem nuclei have

progenitors in r5 (Cramer et al., 2000a). EphA4 is expressed uniformly through r5 at embryonic day 2 (E2), then becomes divided into longitudinal bands by E5 (Cramer et al., 2000b; Kury et al., 2000). It has been proposed that these bands of expression are related to distinct migration pathways for NM versus NL progenitors (Cramer et al., 2000a). Some support for this view comes from the fact that misexpression of EphA4 at E2 results in disrupted NL morphology at E10 (Cramer et al., 2004); however, studies that examine cells at intermediate time points will be necessary to evaluate this hypothesis more rigorously.

4. Expression studies

An important component of our understanding of the function of Eph proteins in the development of the auditory system is the identification of protein expression patterns in the different compartments of the developing system. While specific antibodies are not available for all of the family members, additional information on protein expression can be obtained with the use of truncated ligand or receptor fusion proteins. Mutant mice with reporter proteins such as β-galactosidase have provided an additional method for describing protein expression patterns. These mice have been helpful in confirming the specificity of antibodies (Cowan et al., 2000; Bianchi et al., 2002). Data on the distribution of messenger RNA have been obtained using Northern blot analysis (Bianchi and Gale, 1998), in situ hybridization and reverse-transcription polymerase chain reaction (Pickles, 2003). Eph proteins are expressed in subsets of auditory structures. The expression patterns can overlap or complement those of other family members, and in some cases expression patterns are found in gradients along topographic axes.

4.1. Mammalian auditory system

A number of studies have examined the expression of Eph proteins in the developing and mature inner ear and central projections of mammals. In mice EphB2 is expressed in developing statoacoustic nerve fibers (Henkemeyer et al., 1994) and EphA6 is expressed in the developing and mature spiral ganglion (Lee et al., 1996). EphA7 is expressed in the early mouse cochlea and in the ventral cochlear nucleus in the hindbrain (Ellis et al., 1995; Rogers et al., 1999). A detailed study in rats and gerbils using immunohistochemistry and Northern blot analysis (Bianchi and Gale, 1998) showed the expression of several family members in the cells of the developing cochlea, with complementary expression patterns of ephrin-A2 and one of its receptors, EphA4, in the developing spiral limbus. EphA4 is expressed in interdental cells, and ephrin-A2 is expressed in regions adjacent to these cells. EphA4 is expressed in the cochlear nucleus of the gerbil but not the rat. In addition, the ligand ephrin-B1 and the receptor EphB1 are expressed in the statoacoustic ganglion. Eph protein expression remains throughout adulthood in the inner ear (Bianchi and Gale, 1998). Ephrin-B1 and ephrin-B2 proteins are expressed in the peripherally directed processes of statoacoustic ganglion cells (Bianchi and Gray, 2002). EphA4 protein is expressed in the early guinea pig statoacoustic ganglion and in the spiral ligament in mice and guinea pigs (van Heumen et al., 2000). In the mouse inner ear, EphA4 has complementary expression with one of its ligands, ephrin-A2 (Pickles et al., 2002). Moreover, EphB1, ephrin-B1, and ephrin-B2 are expressed in spiral ganglion neurons, and in the periphery these ligands have layered expression patterns, with adjacent regions expressing distinct Eph proteins. While there are similarities across species for some of the cell types in the developing cochlea, numerous differences were observed, and in some cases the methods used to evaluate expression yielded conflicting results within a species (van Heumen et al., 2000). Differences in the reported expression patterns may arise from differences in the sources of antibodies, the tissue fixation, or the methods used to label tissue.

The expression of EphB1 is greater in vestibular neurons than in auditory neurons, suggesting a potential role in the specification of these neuronal cell types or their projections (Bianchi and Gale, 1998). While Eph proteins are axon guidance molecules that may influence the choice of auditory or vestibular pathways, other molecules that may have a strong role in this distinction include transcription factors. In the developing mouse ear GATA3 is expressed in auditory components and NeuroD is expressed in vestibular components (Karis et al., 2001; Lawoko-Kerali et al., 2004). Mice lacking these transcription factors have deficits in ear morphogenesis and axon pathfinding (Karis et al., 2001; Kim et al., 2001), and NeuroD appears to influence the expression of neurotrophin receptors, which have a substantial role in ear maturation (Kim et al., 2001). An interesting possibility is that these transcription factors influence Eph expression as well. The interplay between these proteins represents an important area of future studies.

Overall, these studies show that there are similiarities in the expression patterns of Eph proteins in rodents, but that species differences may occur. These differences could signify that individual Eph proteins perform distinct roles in the development of different species. These studies show that several family members are expressed in distinct regions in the developing mammalian ear. Often cells expressing a ligand are adjacent to cells expressing a receptor. This alternating pattern is reminiscent of that seen in the hindbrain, and also resembles patterns of Eph protein expression that define compartments within the striatum (Janis et al., 1999). Alternation

of Eph receptors and ephrins may thus represent a general mechanism by which Eph proteins regulate cell migration and neural target selection.

While the expression of Eph proteins has been determined in some detail in the mammalian peripheral auditory structures, less is known about the expression patterns in auditory regions of the central nervous system. A panel of antibodies has been used to identify expression patterns in the E12.5 hindbrain of the mouse (Cowan et al., 2000) in order to examine interactions at the midline. EphB1, EphA4, ephrin-B1, and ephrin-B2 are all expressed in the floor plate region of the midline. In addition, EphB1 and EphB6 are expressed in hindbrain regions containing neurons that project to the inner ear. In preliminary studies, early postnatal EphA4^{lacZ} mice showed β-galactosidase reporting of EphA4 expression in the auditory brainstem nuclei, including AVCN and MNTB (Cramer et al., 2002a).

At higher levels in the auditory system, ephrin-A5 messenger RNA has been demonstrated in the E16 mouse inferior colliculus (Zhang et al., 1996), and Eph protein expression has been demonstrated in the mouse medial geniculate body (MGB) of the thalamus and in auditory cortex. In the MGB, fusion protein analysis and in situ hybridization were used to demonstrate a gradient of expression of ephrin-A5, and to a lesser extent, ephrin-A2 (Lyckman et al., 2001). These proteins have a similar graded expression pattern in the visual thalamus, where Eph/ephrin signaling is necessary for the formation of appropriate retinotopic maps (Feldheim et al., 1998). It is not known whether these Eph protein cues are used in a similar manner to form tonotopic maps in the projections from the inferior colliculus to MGB, but the gradients of expression suggest that a similar mechanism may regulate these projections.

4.2. Avian auditory system

Several studies have addressed the expression of Eph proteins and their potential role in the formation of pathways in the avian ear and brainstem. In the chick auditory system, several Eph proteins are expressed during development. As in rodents, axons from the statoacoustic ganglion express ephrin-B1 (Bianchi and Gray, 2002; Siddiqui and Cramer, 2005). Auditory axons from the VIIIth nerve invade the brainstem at about embryonic day 4.5 (E4.5), slightly later than vestibular axons, and reach their target areas in the brainstem beginning at about E6 (Kubke and Carr, 2000; Molea and Rubel, 2003). During development of connections several other Eph proteins are also expressed in the statoacoustic ganglion and in centrally projecting axons to both vestibular and auditory targets (Siddiqui and Cramer, 2005). Ephrin-A2, ephrin-B1, and EphB1 are expressed throughout the VIIIth nerve. In contrast, EphA4 is expressed more heavily in auditory regions of the nerve, with higher expression in the areas that will later contain low frequency-selective fibers. A similar expression pattern was seen in the ganglion and in peripherally directed fibers. The expression of EphB2 is complementary to that of EphA4. Protein levels are higher in vestibular axons and in high frequency regions of the auditory nerve. These expression patterns suggest that Eph proteins are involved in auditory versus vestibular targeting as well as in tonotopic mapping in the brainstem. While GATA3 and NeuroD transcription factors have distinct expression patterns in mouse auditory and vestibular components of the ear, it is not known whether these patterns are also seen in chicks. It is interesting to note that while EphB1 has higher levels of expression in vestibular neurons than in cochlear neurons in gerbils (Bianchi and Gale, 1998), EphB1 has a more uniform distribution in the chick statoacoustic ganglion and VIIIth nerve. Thus, while both of these classes of animals may use Eph signaling to specify auditory versus vestibular cells, different species may use different Eph proteins to accomplish this specification.

The auditory nuclei of the chick brainstem also express several Eph proteins at the ages coinciding with synapse formation in this circuitry. EphA4 expression from E9 to E11 is heavy in the dorsal, but not ventral NL dendrites (Cramer et al., 2000b). The immunolabeled region is the area that receives ipsilateral, but not contralateral, input. During this time, the expression in the dorsal NL dendrites is higher in the rostromedial part of the nucleus and declines toward the caudolateral region (Person et al., 2004). This expression gradient thus varies along the tonotopic axis, with high frequency areas showing greater levels of expression than low frequency areas. From E12 to later development, EphA4 expression is high in both dorsal and ventral NL dendrites. Because of the asymmetric distribution of EphA4 in NL, one possible role might be to facilitate binaural segregation in the NM–NL projection. Moreover, the tonotopic expression patterns suggest a role in forming a tonotopic arrangement of connections.

The pattern of Eph protein expression in the chick auditory brainstem are summarized in Fig. 2. EphB2 and ephrin-B1 are both expressed in dorsal and ventral NL neuropil, in the midline of the brainstem, and in NM neuropil during embryonic development (Cramer et al., 2002b). EphB5 has a similar pattern, with much less pronounced staining in NM and NL, but significant expression in the midline. EphA4 and ephrin-B2 are not expressed in the midline. Midline expression thus appears to vary from that seen in the mouse brainstem, in which both EphA4 and ephrin-B2 have high levels of expression in the floor plate region of the midline (Cowan et al., 2000). Ephrin-B2 is expressed in chick in axons that project from NM to NL. It is also expressed in NL cell bodies and in the glial margin surrounding NL. In both of these regions ephrin-B2 expression varies along the tonotopic axis, with high frequency regions of NL showing greater expression than low frequency

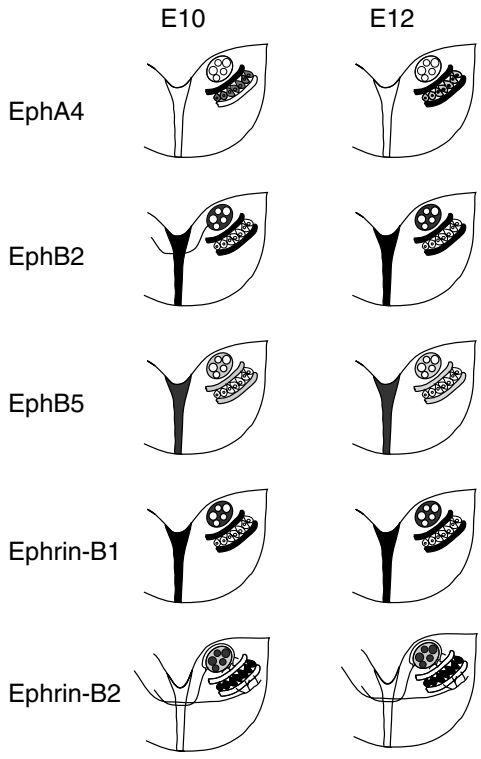

Fig. 2. Summary of Eph protein expression in the chick auditory brainstem. During the ages that NM–NL projections are forming, several family members are expressed in various compartments of the auditory circuit. EphA4 is expressed in dorsal but not ventral NL neuropil, while EphB2, EphB5, and ephrin-B1 have a symmetric expression pattern including both dorsal and ventral regions. Ephrin-B2 and to a limited extent, EphB2, are expressed in NM axons that project to NL. EphB2, EphB5, and ephrin-B1 are expressed in the midline region. Adapted with permission from Cramer et al. (2002b).

regions (Person et al., 2004). This expression pattern suggests a role in the formation of projections from NM to NL. For example, gradients of ephrin-B2 in the ventral glial margin may form permissive or inhibitory pathways for axon growth; the strength and direction of the signal might vary with tonotopic position.

5. Functional studies

In order to dissect the mechanisms that establish auditory circuits and to evaluate the extent to which Eph proteins participate in this process, it is necessary to perturb expression levels and evaluate the effects on auditory pathways. The behavior of auditory neurons in response to Eph proteins has been examined in vitro. In addition, in vivo functional studies of mammalian auditory development have been carried out primarily in mice, where the availability of mutant "knockout" lines provides a loss-of-function paradigm. Functional studies in the chick are aided by the accessibility of the embryo for perturbations and the use of in ovo electroporation to introduce DNA into developing embryos.

5.1. Mammalian auditory development

Functional studies using cultured spiral ganglion neurons have revealed important insights into how Eph protein expression patterns lead to directed growth of neuronal processes toward their target. In the rat, outgrowth of processes from cultured spiral ganglion cells is repelled on stripes of EphA4 (Brors et al., 2003). Processes grow toward the edge then turn away from the border with EphA4. In this study, EphA4 was shown to interact with ephrin-B2 and ephrin-B3 mediate the repulsion in neurites emanating from spiral ganglion explants. When blocking antibodies were used against either ligand, the effect was reduced. When the blocking antibodies were used together, the effect was abolished. This result demonstrates that ephrin-B2 and ephrin-B3 mediate repulsion of spiral ganglion processes by EphA4, which is expressed in the surrounding osseous spiral lamina. It is not known whether EphB receptors also have a role in the outgrowth of these neurons, or whether EphA4 is sufficient for the targeting of this pathway. Nonetheless, the knowledge of protein expression patterns in individual components of the developing ear (Bianchi and Gale, 1998; van Heumen et al., 2000; Bianchi et al., 2002; Pickles et al., 2002; Pickles, 2003) together with these functional perturbations has resulted in a feasible model of how spiral ganglion neurons use Eph-ephrin signaling to find their appropriate peripheral targets (Brors et al., 2003). In this model, EphA4 limits growth of spiral ganglion dendrites by signaling in the reverse direction through ephrin-B2 and ephrin-B3 on spiral ganglion neurons, and a region free of EphA4 provides a channel through which peripheral processes can grow toward their targets. The guidance of these neurites thus depends critically on the spatial arrangement of Eph proteins in the developing inner ear.

Studies in mice lacking one or more Eph receptors have provided further data on how Eph signaling may be important in establishing mature connectivity. EphB2 mutant mice have vestibular dysfunction and exhibit circling behavior (Cowan et al., 2000). These mice have an embryonic defect in inner ear efferent projections from the hindbrain. Moreover, EphB2/EphB3 double mutants have abnormally small semicircular canals. EphB2 can interact with aquaporin1, which may have important consequences for the regulation of endolymph in the inner ear (Cowan et al., 2000). This study demonstrates an important role for EphB2 in axon pathfinding in the vestibular system, and highlights the fact that Eph proteins may have roles in vestibular function that extend beyond axon guidance.

Similarly, Eph proteins may have roles in the maintenance of auditory function. Tests of distortion product otoacoustic emissions were carried out in mature mice with mutations in EphB1, EphB2, EphB3, or ephrin-B3 (Howard et al., 2003). Deficits were found in mice lacking EphB1 or EphB3, but not mice lacking EphB2 or

ephrin-B3. These results suggest that EphB1 and EphB3 are necessary for normal cochlear function. The effects in EphB3 mutants are unlikely to be due to axon pathfinding errors, because deletion of EphB3 does not cause pathfinding errors in the VIIIth nerve or inner ear efferents (Cowan et al., 2000).

In the central nervous system, very little is known about the role of Eph proteins in the formation of mammalian auditory circuits. In the visual system, EphA signaling is necessary for retinotopic projections to the tectum and visual thalamus (Feldheim et al., 1998, 2004). While the molecular regulation of normal inputs to MGB is not understood, Lyckman et al. (2001) examined retinal inputs that were surgically redirected to innervate MGB. In ephrin-A2/ephrin-A5 double knockout mice, the extent of the novel retino-MGB projection is expanded, suggesting that these proteins normally limit the growth of retinal axons. It is not known whether the topographic arrangement of the novel projection is influenced by topographic gradients of ephrin-A proteins. However, the discovery that Eph proteins influence cross-modal projections supports the idea that these proteins have a function that may be generally operative across sensory modalities.

Further support for a role of Eph proteins in sensory system development comes from studies of thalamic projections to sensory cortical areas (Dufour et al., 2003). Mice lacking EphA4 and ephrin-A5 have abnormal organization of thalamocortical somatosensory projections, and in addition, have disordered projections to appropriate cortical areas as a result of misrouting of thalamocortical axons. While these studies do not specifically address ordering of auditory thalamocortical projections, they provide evidence that Eph proteins act on early thalamocortical axons and guide them to their cortical targets.

5.2. Chick auditory development

Recent studies in vitro and in vivo have provided evidence that Eph proteins regulate growth of axons in the developing chick auditory system. In a study of cultured statoacoustic ganglion cells, Bianchi and colleagues (2002) showed that soluble EphB fusion proteins inhibit neurite outgrowth. These neurites express ephrin-B1. The results suggest that EphB receptors signal through ephrin-B1 to inhibit growth. This finding is similar to that seen in rat spiral ganglion explants (Brors et al., 2003) in that ephrin-B ligands receive reverse, inhibitory signals. However, in rats, neurites continue to grow, but turn away from the region containing the receptors. The actions of Eph signaling and the individual family members involved may thus vary with species.

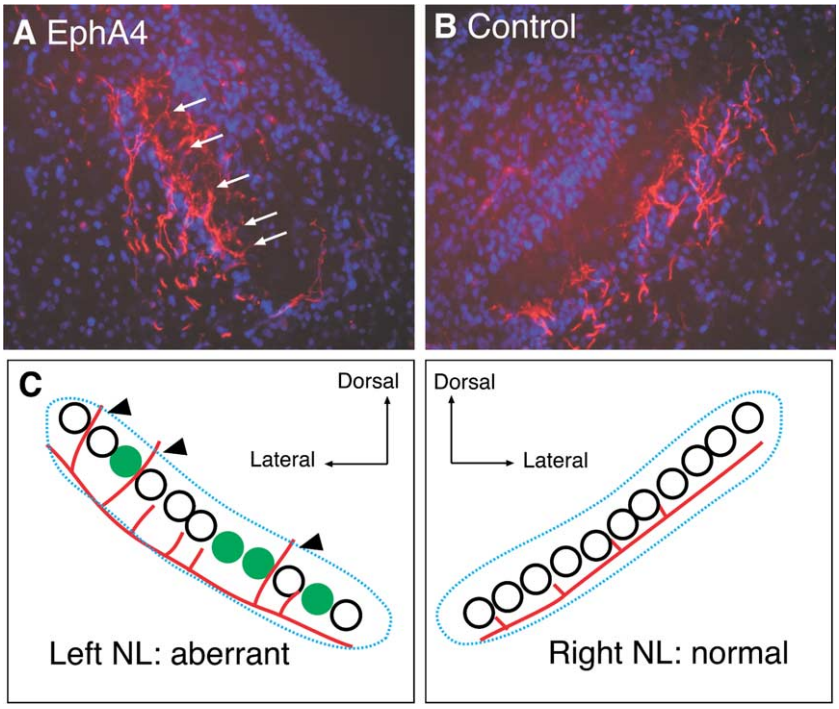

Fig. 3. Functional role for EphA4 in establishing correct projections from NM to NL. (A) In a section through NL misexpressing EphA4, projections arising from contralateral NM (labeled with rhodamine dextran amine) cross the cell body line and grow in the inappropriate dorsal NL neuropil. Targeting errors are indicated by arrows. (B) The contralateral side in not transfected, and contralateral axons are restricted to the ventral portion of NL. (C) Schematic illustration of the effects of misexpression on NM axon targeting. Segregation is disrupted in the NM–NL projection when EphA4 is misexpressed. Adapted with permission from Cramer et al. (2004).

The role of Eph proteins in central connections of the avian auditory system has also begun to be addressed (Cramer et al., 2004). The chick embryo can be subjected to gene misexpression for extended periods of development using in ovo electroporation of plasmid DNA (for review, see Krull, 2004). This technique allows chick cells to express exogenous genes, and because of the accessibility of the embyro, distinct regions can be targeted. Because EphA4 is preferentially expressed in the dorsal NL neuropil (Cramer et al., 2000b), this protein was a candidate gene for functional studies. When EphA4 was misexpressed in NM and NL, there was a significant increase in the number of targeting errors in NM axons (Cramer et al., 2004). These studies are illustrated in Fig. 3. This effect was also observed when a kinase inactive form of EphA4 was used. These errors allowed axons to grow past the line of cell bodies in NL into the inappropriate region of the neuropil. Errors occurred preferentially near transfected NL neurons, and the number of errors was not increased when NM alone was transfected. These studies suggest that the EphA4 signals to growing NM axons, which express ephrin-B2. This reverse signaling may be attractive, because an increase in the extracellular domain of EphA4 is associated with increased axon growth. This study shows that an understanding of expression patterns coupled with targeted electroporation can reveal details of how proteins may interact to assemble precise circuits in the brain. This approach can be used to evaluate whether other Eph proteins are also involved, and whether Eph proteins act cooperatively to shape the auditory pathways.

6. Conclusions

The studies reviewed here provide evidence that Eph proteins are an important component of the developmental mechanisms responsible for maturation of the auditory system. These proteins may function at many levels. They are expressed early in development and may regulate cell fate specification and migration in the hindbrain and in the otic epithelium. The differential expression of individual Eph proteins in the statoacoustic ganglion and VIIIth nerve suggest a role in establishing auditory versus vestibular phenotypes. Moreover, several studies in both mammals and birds suggest that Eph proteins regulate axon outgrowth and target choice in the auditory pathways. Together, these results show that Eph proteins shape auditory structures and connectivity during development.

Important questions remain about the function of these molecules in the auditory system. These include the identification of mechanisms of action, and an investigation into whether the proteins are necessary for the formation of tonotopic maps. While tonotopic gradients of expression have been identified, functional studies are required to evaluate whether these gradients are necessary for the establishment of the tonotopic projections. Topography is a general organizing principal in the brain, and is seen in the auditory system as well as in other sensory systems. Thus an investigation into the role of Eph receptors and other molecules that establish circuitry elsewhere in the brain will provide an important starting point for understanding the formation of the auditory system as well. In addition, several other axon guidance molecules may significantly influence auditory circuitry (Rubel and Fritzsch, 2002; Gu et al., 2003). Investigation of the coordinated activities of these different factors will be essential to completing our understanding of auditory circuit assembly.

Some considerations should be noted in the study of how Eph proteins contribute to the assembly of auditory circuitry. First, the expression of the proteins is overlapping and may include redundancy of function, so that single gene knockouts may fail to reveal important functions for individual family members. Second, because these proteins have several roles at different times during development, phenotypes expressed early in development could mask roles in the formation of auditory pathways. Third, the complexity of interactions among members of the family presents a challenge in understanding the types of signaling required. These proteins can signal in the forward and reverse direction, and can promote or inhibit axon outgrowth.

Several strategies can be used to overcome these challenges. The use of double knockouts can reveal phenotypes not evident in single knockouts; this approach has already yielded important results on pairs of Eph family proteins in the visual system and vestibular system. Conditional mutations that can be induced at later time points can be useful when early phenotypes are manifested. Focal electroporation in chick embryos represents an important advance in our ability to dissect the molecular interactions between Eph proteins during development. Carefully designed experiments to misexpress Eph proteins in individual compartments of the auditory circuit, combined with axon tracing studies, provide an important tool for understanding how this family of proteins can collectively shape the circuitry essential for auditory function.

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