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# Developmental origin of shark electrosensory organs

**Renata Freitas,[a] GuangJun Zhang,[a] James S. Albert,[b] David H. Evans,[a] and Martin J. Cohn[a,c,*]**

[a]Department of Zoology, University of Florida, PO Box 118525, Gainesville, FL 32611-8525, USA
[b]Department of Biology, University of Louisiana, PO Box 42451, Lafayette, LA 70504, USA
[c]Department of Anatomy and Cell Biology, University of Florida, PO Box 118525, Gainesville, FL 32611-8525, USA
*Author for correspondence (email: cohn@zoo.ufl.edu)

**SUMMARY** Vertebrates have evolved electrosensory receptors that detect electrical stimuli on the surface of the skin and transmit them somatotopically to the brain. In chondrichthyans, the electrosensory system is composed of a cephalic network of ampullary organs, known as the ampullae of Lorenzini, that can detect extremely weak electric fields during hunting and navigation. Each ampullary organ consists of a gel-filled epidermal pit containing sensory hair cells, and synaptic connections with primary afferent neurons at the base of the pit that facilitate detection of voltage gradients over large regions of the body. The developmental origin of electroreceptors and the mechanisms that determine their spatial arrangement in the vertebrate head are not well understood. We have analyzed electroreceptor development in the lesser spotted catshark (*Scyliorhinus canicula*) and show that *Sox8* and HNK1, two markers of the neural crest lineage, selectively mark sensory cells in ampullary organs. This represents the first evidence that the neural crest gives rise to electrosensory cells. We also show that pathfinding by cephalic mechanosensory and electrosensory axons follows the expression pattern of *EphA4*, a well-known guidance cue for axons and neural crest cells in osteichthyans. Expression of *EphrinB2*, which encodes a ligand for EphA4, marks the positions at which ampullary placodes are initiated in the epidermis, and *EphA4* is expressed in surrounding mesenchyme. These results suggest that *Eph–Ephrin* signaling may establish an early molecular map for neural crest migration, axon guidance and placodal morphogenesis during development of the shark electrosensory system.

## INTRODUCTION

Vertebrates have evolved a complex network of sensory organs to detect mechanical and electrical stimuli in their environment. The mechanosensory octavolateral system is comprised of auditory, equilibrium, and lateral line components that detect mechanical vibrations through mechanoreceptive neuromasts. The electrosensory system is used to detect weak electric signals in the aquatic environment (von der Emde 1998). Ampulla-shaped electroreceptor organs containing hair cells with cilia that can be excited by cathodal stimulation is a characteristic of crown gnathostomes (Fig. 1; Andres and von During 1988; Koyama et al. 1993; Northcutt and Bleckmann 1993; Gibbs 2004). Electroreception is absent in living myxinoids (hagfishes), however lampreys have non-ampullary electrosensory organs (Ronan and Bodznick 1986). This sense has been lost in most amniotes and neopterygians, and may have re-evolved twice in teleosts (Fig. 1; Bullock 1982; Bullock et al. 1983; Koyama et al. 1993; Gibbs 2004). Chondrichthyans are the most basal lineage of crown gnathostomes to develop a highly specialized network of ampullary organs, known as the ampullae of Lorenzini. These electrosensory organs allow chondrichthyans to detect voltage potential generated by buried prey and to navigate relative to the earth's geomagnetic field (Fig. 1; Kalmijn 1966; Heiligenberg 1993; Klimley 1993; von der Emde 1998). The ampullae of Lorenzini were first described in 1678, however both the phylogenetic and developmental origins of these organs have remained unclear (Heiligenberg 1993; Fishelson and Baranes 1998; von der Emde 1998).

Electroreceptive ampullary organs share functional and structural properties with mechanosensory organs of the lateral line; both contain hair cell integumental receptors that extend into a fluid-filled lumen, have nerve projections to brain stem medullary nuclei, and process inputs through the nuclei of the lateral lemniscus (Northcutt 1992; Conley and Bodznick 1994; Northcutt et al. 1995; Fritzsch et al. 1998). Moreover, mechanosensory and electrosensory organs play similar roles in many behavioral tasks such as prey capture, navigation and communication (Hodos and Butler 1997; Coombs et al. 2002). Differences exist, however, in the spatial distribution of mechanoreceptors and electroreceptors; mechanosensory organs are organized in tracts on the head and trunk, whereas electrosensory organs are organized in clusters and are generally restricted to the head (Schellart and Wubbels 1998; von der Emde 1998).

Developmentally, mechanoreceptive neuromasts and electroreceptive ampullary organs have been suggested to arise strictly from epidermal placodes in amphibians (Northcutt et al. 1995). However, cell lineage studies have shown that mechanoreceptor development in amphibians and teleosts also involves neural crest cells, which have been fate-mapped to lateral line neuromasts (Collazo et al. 1994). These cell lineage relationships have been conserved during mechanoreceptor evolution, as neural crest cells also give rise to melanocytes that contribute to the fish macula and to the lining of mammalian cochleae (*stria vascularis*), within which sensory hair cells detect mechanical stimuli in the ear (Torres and Giraldez 1998; Whitfield 2002). Morphogenesis of electroreceptive organs is somewhat less well understood (Gibbs 2004), and this represents a important gap in our understanding of the evolution of sensory system development.

We have investigated cephalic laterosensory system development using a variety of molecular markers in the catshark (*Scyliorhinus canicula*), the ampullary organs of which retain many plesiomorphic features (Fig. 1). Here we report that neural crest cells contribute to formation of electroreceptors, highlighting a new developmental fate of the neural crest. Given that neural crest cells participate in the formation of electroreceptors, normal development of the ampullary network must depend upon coordinated pathfinding by neural crest cells and the sensory axons that innervate ampullary organs. Members of the Eph class of receptor tyrosine kinases and their ligands, Ephrins, have been implicated in the regulation of both neural crest cell migration and axon guidance in a number of developmental contexts (Gale et al. 1996; Kullander and Klein 2002) and are therefore good candidates for coordinating morphogenesis of the composite electrosensory system. Consistent with this hypothesis, we find that axons of the mechanosensory and electrosensory system navigate along pre-established tracts of *EphA4* expression. In addition, we report that ampullary placodes express *EphrinB2*, and the periodicity of these organs corresponds to focal gaps in the *EphA4*-expressing domain. Taken together, these findings suggest that *Eph–Ephrin* signaling may establish an early molecular map of the cephalic laterosensory system during the development of the shark head.

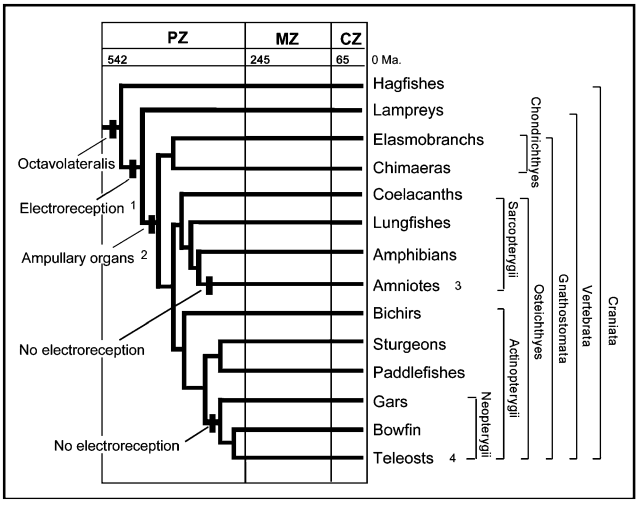


**Fig. 1.** Phylogenetic distribution of electroreception in craniates. Numbers on tree refer to the following: (1) electroreception as characterized by hair cell receptors with cathodal stimulation, lateral line afferents, and central processing via the lateral lemniscus; (2) ampullary organs with hair cells bearing an apical kinocilium; (3) absence of electroreception in amniotes with the exception of monotremes with trigeminal nerve electrosensory system; (4) absence of electroreception in teleosts with the exception Mormyriformes, Siluriformes, and Gymnotiformes with lateral line nerve electrosensory systems, hair cells with apical microvilli, and anodal stimulation. CZ, Cenozoic; MZ, Mesozoic; PZ, Palaeozoic; Ma, millions of years ago. Phylogeny and dates after Bullock (1982), Donoghue and Smith (2003) and Janvier (1996).

## MATERIALS AND METHODS

### Collection and staging of embryos

*S. canicula* eggs were collected from the Menai Strait (North Wales). Embryos were isolated from egg cases and dissected from the yolk sac in ice-cold phosphate-buffered saline (PBS). The specimens were staged according to Ballard et al. (1993), before being frozen in liquid nitrogen for RNA extraction with Tri-Reagent (Sigma, St. Louis, MO, USA) or fixed and processed as described below.

### Isolation of catshark genes

Degenerate RT-PCR reactions were performed to amplify fragments of *Sox8* (673 bp) and *EphrinB2* (595 bp) from a *S. canicula* cDNA library. PCR products were cloned into pDrive vector (Qiagen, Valencia, CA, USA) and sequenced in both directions. Sequence identity was determined by Blast (NCBI) searches, protein alignments (ClustalX), (Thompson et al. 1997) and molecular phylogenetics using both neighbor joining (MEGA3; Kumar et al. 2004) and maximum likelihood (TreePuzzle; Schmidt et al. 2002). The *EphA4* clone was described previously (Freitas and Cohn 2004). The new sequence data have been submitted to GenBank (Accession numbers: DQ190443-DQ190442).

### Whole-mount in situ hybridization and immunochemistry

Whole-mount in situ hybridization was performed in catshark embryos during early development of the cephalic laterosensory system (stages 30–32) using digoxigenin-labeled riboprobes for *EphA4, EphrinB2* and *Sox8*. Embryos were processed for in situ hybridization as described in Freitas and Cohn (2004); however, for stage 32 embryos, the hydrogen peroxide treatment was increased to 2 h and the proteinase K to 40 min. Immunochemistry also was performed as described in Freitas and Cohn (2004). Primary antibodies 3A10 and HNK1 were used at concentrations of 1:500 and 1:70, respectively, and peroxidase-conjugated secondary antibodies were used at concentration of 1:500.

### Frozen sections of whole-mount embryos

After in situ hybridization or immunochemistry, embryos were equilibrated in graded sucrose (15%, 30%) at 4°C and graded

gelatine (20% gelatine in 30% sucrose, followed by 20% gelatine) at 50°C. The specimens were then frozen on dry ice and mounted in TissueTek OCT (Torrence, CA, USA) for cryosectioning (15–20 μm).

## RESULTS

### Neural crest cells contribute to shark electroreceptors and mechanoreceptors

In the catshark *S. canicula,* the ampullae of Lorenzini surround three mechanosensory tracts, the supraorbital, infraorbital, and preopercular–mandibular canals (Fig. 2A and Al-Zahaby et al. 1996). The primordia of the ampullary organs develop as epidermal placodes between stages 31 and 32 (Fig. 2B(1)). These cells form rosette-shaped structures (Fig. 2B(2)) that then invaginate to give rise to the ampullary vesicles (Fig. 2B(3)). By stage 34, each ampulla forms an elongated tube that connects the surface of the skin to a basal cluster of electrosensory hair cells (Fig. 2B(4)). Morphogenesis of catshark electroreceptors bears a striking resemblance to mechanoreceptor development (Gibbs and Northcutt 2004). Based on these similarities, and evidence that neural crest cells

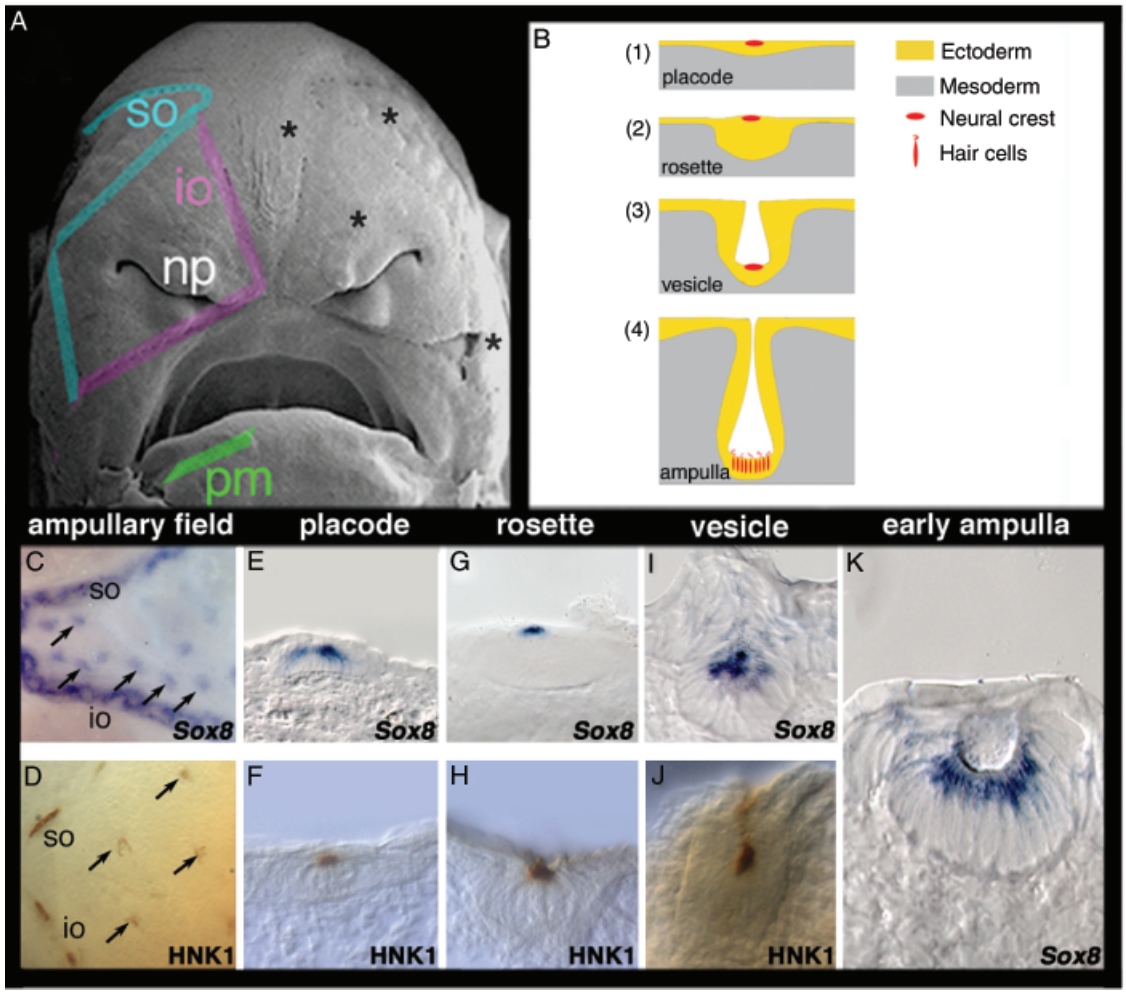


**Fig. 2.** Neural crest cells contribute to electrosensory and mechanosensory organs in the catshark head. Mechanosensory tracts are supraorbital canal (so), infraorbital canal (io), preopercular–mandibular canal (pm). (A) Scanning electron micrograph of a catshark face, ventral view, at stage 32. Mechanosensory tracts are pseudocolored on the left side of the rostrum in blue (so), pink (io), and green (pm). Nasal pit is indicated by np. Asterisks indicate four clusters of ampullary organs on ventral right side of the rostrum. (B) Schematic representation of four stages of ampullary organ development. (C) *Sox8* expression in mechanosensory tracts (so, io) and electrosensory ampullary organs (arrows). (D) Immunolocalization of neural crest cell marker HNK1 in mechanosensory neuromasts (so, io) and electrosensory ampullary organs (arrows). (E–K) Histological sections showing distribution of *Sox8* (E, G, I, K) and HNK1 (F, H, J) during development of ampullary organs. (E, F) Cells positive for HNK1 and *Sox8* were detected in the apical layer of the ampullary placode. (G, H) HNK1 and *Sox8* persisted in the apical cells of the placodal rosettes. (I, J). During formation of ampullary vesicles, HNK1 and *Sox8* were detected in cells lining the ampullary lumen. (K) *Sox8* expression in the sensory layer of the early ampulla.

participate in mechanoreceptor development in at least two osteichthyans (Collazo et al. 1994), we hypothesized that electroreceptors also may have a dual embryonic origin in the neural crest and epidermal placodes. We tested this hypothesis by examining the neural crest contribution during development of catshark electrosensory system using two molecular markers. The SRY-related gene *Sox8* marks neural crest cell derivatives (McKeown et al. 2005) and the monoclonal antibody HNK1 is a well-characterized marker of neural crest cells in a broad range of species, including sharks (Tucker et al. 1988; Epperlein et al. 1990; Sadaghiani and Vielkind 1990; Kuratani and Horigome 2000). We detected *Sox8* and HNK1-positive cells in both mechanosensory and electrosensory placodes at stage 32 (Fig. 2, C and D). Histological analysis over the course of ampullary organ development revealed that *Sox8* and HNK1 positive cells were positioned initially at the apical region of the early placode (Fig. 2, E and F) and expression of both markers persisted apically as the placode thickened to form a rosette (Fig. 2, G and H). During invagination of the ampullary vesicle, *Sox8* and HNK1 were detected along the lining of the lumen (Fig. 2, I and J), and *Sox8* expression was localized to sensory cells in the early ampulla (Fig. 2K). The dynamics of *Sox8* and HNK1 expression during ampullary placode morphogenesis suggest that neural crest-derived hair cell progenitors are transported from the epidermal surface to the base of the ampullary lumen during placodal invagination (Fig. 2B). These data provide the first evidence for neural crest involvement in electrosensory organogenesis.

## Mechanosensory tract development follows the spatiotemporal pattern of *EphA4* expression

Although formation of mechanosensory epidermal placodes has been described in chondrichthyans and osteichthyans (Johnson 1917; Northcutt et al. 1995; Gibbs and Northcutt 2004), the question of how neural crest cells and axons are guided to specific topographic positions within the laterosensory fields remains unknown. Our finding that neural crest cells participate in shark laterosensory organogenesis suggested that development of this organ system may be coordinated by a signal capable of guiding both neural crest cells and sensory axons, which led us to examine expression of *EphA4*. As the cephalic laterosensory tracts were being laid down at stage 30, we observed mechanosensory axons growing along narrow, intense bands of *EphA4* expression (Fig. 3, A and B). Both infraorbital and supraorbital sensory axons were observed extending towards the rostral limits of *EphA4* expression domains around the nasal capsule (Fig. 3, A and B). Similarly, in the preopercular-mandibular tract, mechanosensory axons migrated towards the *EphA4* domains at the tip of each pharyngeal arch (Fig. 3, A and B). Thus, the laterosensory nerve tracts

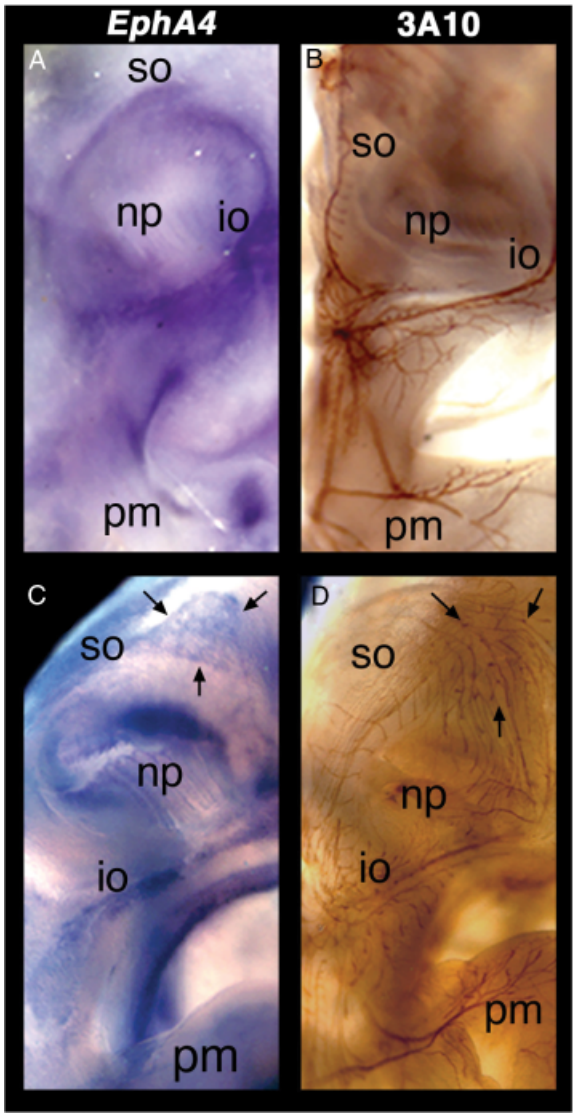


**Fig. 3.** Electrosensory and mechanosensory development follows the patterns of *EphA4* expression in the catshark head. Ventral views of the face, anterior is to top. Mechanosensory tracts are labelled (so, io, pm). (A) *EphA4* expression in mechanosensory tracts at stage 30. (B) Immunolocalization of 3A10 showing the distribution of mechanosensory nerves at stage 30. (C) *EphA4* expression has expanded into the prospective electrosensory network by stage 32 (arrows). (D) Immunolocalization of 3A10 shows innervation of the ampullary fields at stage 32 (arrows).

expand through the head following pre-established domains of *EphA4* expression.

## *EphA4* and *EphrinB2* expression in the ampullary fields mark the sites of electroreceptor development

Mechanosensory tracts branched into electrosensory ampullary fields at stage 32, following the expansion of *EphA4*

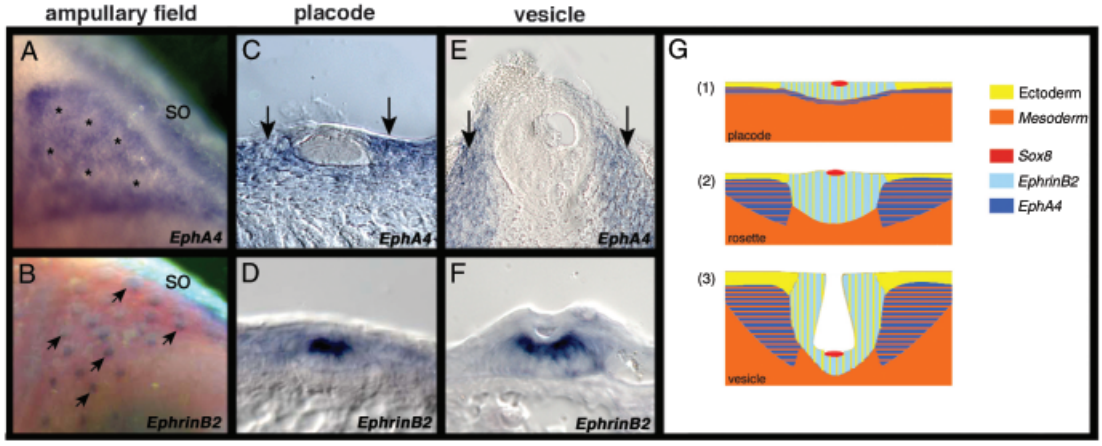


**Fig. 4.** *EphA4* and *EphrinB2* are expressed in complementary patterns during ampullary organ development. The rostral ampullary field (A, B) and individual ampullary placodes (C–F) shown at different developmental phases in a stage 32 embryo. (A) *EphA4* expression in the ampullary field medial to the supraorbital canal (so). Asterisks indicate absence of *EphA4* expression at sites of ampulla formation. (B) *EphrinB2* expression in the ampullary placodes (arrows) medial to the supraorbital canal. Note complementary expression of *EphA4* and *EphrinB2* in (A) and (B). (C–F) Histological sections showing *EphA4* expression in the mesenchyme around the ampullary placode (C, E), and *EphrinB2* expression within the epidermal cells (D, F) at the placode and vesicle stages of ampulla development. (G) Schematic representation summarizing gene expression at three stages of ampullary organ development.

expression domains (Fig. 3, C and D). These new sites of *EphA4* expression marked each group of ampullary organs (Fig. 3, C and D). Interestingly, within each group, we observed regularly spaced gaps in the *EphA4* domain (Figs 3C and 4A). A comparison with *EphrinB2* expression at this stage indicated that *EphrinB2* was restricted to epidermal cells within these gaps (Fig. 4, A and B). Histological analysis confirmed that each localized spot of *EphrinB2* expression marked an ampullary placode (Fig. 4D), which was surrounded by mesenchyme that expressed *EphA4* (Fig. 4C). These mutually exclusive domains of expression were maintained as the ampullary placode invaginated into the underlying mesenchyme to form a vesicle (Fig. 4, E and F). Thus, *EphrinB2* and *EphA4* are expressed in a complementary pattern in the ampullary placode and adjacent mesenchyme, respectively, during ampullary organ development (Fig. 4G).

## DISCUSSION

The origin of vertebrates was marked by the emergence of the neural crest. The multipotency of neural crest cells facilitated the evolution of numerous craniofacial innovations (Shimeld and Holland 2000). In this study, we have presented the first direct evidence that neural crest cells contribute to electrosensory organogenesis, highlighting a novel developmental potential for these cells. We also found that mechanosensory neuromasts of sharks express neural crest markers, consistent with the cell lineage studies of Collazo et al. (1994), who provided the first demonstration that the neural crest gives rise to hair cells of the lateral line neuromasts in teleosts and amphibians. Thus, neural crest cell involvement in laterosensory organ development may be a plesiomorphic condition for jawed vertebrates. Given that lampreys also have mechanoreceptors and electroreceptors, it is possible that the neural crest involvement in development of these organs maps to a node deeper than Gnathostomata (Fig. 1). However, significant structural differences exist between lamprey and gnathostome electroreceptors; the peripheral electrosensory system of adult lampreys consists of small swellings called "end buds" rather than ampullary organs, which are innervated by axons of the laterosensory system (Ronan and Bodznick 1986). Moreover, unlike the electrosensory and mechanosensory organs of gnathostomes, lamprey hair cells possess apical microvilli, instead of cilia. Whether these structural differences reflect differences in cell lineage will require further studies of laterosensory organogenesis in lampreys.

During shark laterosensory development, *EphA4* expression marks the ampullary fields, and placode development within these fields is restricted to localized domains in which *EphA4* is absent. Given that *EphA4* directs neural crest migration by repulsion from *EphA4*-positive domains (Kullander and Klein 2002), this may result in the guidance of neural crest cells to *EphA4*-negative sites for ampullary organogenesis. We also find that *EphrinB2* is expressed in ampullary placodes. This was somewhat unexpected, as *EphrinB2* has been shown to repel neural crest in other contexts (Krull et al. 1997). Nonetheless, the complementary expression patterns of *EphrinB2* and *EphA4* were strikingly similar to that found in the mammalian cochlea, where they

have been suggested to mediate bidirectional signaling between different cell layers and to maintain cell segregation (Pickles et al. 2002; Pickles 2003).

Our finding that the rostral spread of the *EphA4* expression domain prefigures the routes taken by mechanosensory and electrosensory axons is reminiscent of mouse ear innervation, in which *EphA4* is expressed in the cells lining the auditory nerve pathway, where it directs axons to the cochlea (Pickles 2003). If this function is conserved in the shark laterosensory system, then *EphA4* may be involved in guidance of sensory axons to electroreceptors and mechanoreceptors. Indeed, absence of *EphA4* expression from ampullary placodes may also relate to the termination of growth cones at these positions. This may be important for both function and development of electroreceptors, as it has been suggested that the arrival of nerve fibers may induce formation of electroreceptive organs (although it is also possible that the placodes attract axons; Fritzsch et al. 1998).

Expression of *EphA4* in the shark laterosensory system may represent a deeply conserved mechanism for establishing topographic maps of peripheral sensory inputs in vertebrates. In the mouse, *EphA4* and *EphrinA5* regulate development of the somatotopic map of projections from sensory whiskers to the barrel fields on the cortex (Vanderhaeghen et al. 2000). *EphA4* has been shown to regulate thalamocortical projections, as well as the topographic projections of motor neurons from the spinal cord to the limb (Eberhart et al. 2000, 2002). Similar spatial patterning occurs in the auditory system, where topographic projections originating from the cochlea project to the *nucleus magnocellularis*, which in turn, innervates the *nucleus laminaris* in the brain to form a tonotopic map of high- to low-frequency sounds. Interestingly, *EphA4* is expressed in a tonotopic gradient at the time when *nucleus magnocellularis* axons are forming synapses on the *nucleus laminaris* (Person et al. 2004). The association between expression of *EphA4* and development of the shark electrosensory system suggests that *EphA4* could play a role in establishing the topographic relationships between peripheral electroreceptors and their primary central targets. Regulation of *EphA4* expression during development of the cephalic electrosensory system would therefore underlie how sharks localize the position of electrical stimuli relative to their spatial map of the body. This hypothesis is consistent with *EphA4* playing a general role in the establishment of topographic maps during vertebrate embryogenesis (Vanderhaeghen et al. 2000; Yue et al. 2002; Dufour et al. 2003; Person et al. 2004).

Taken together, the above results reveal a novel role for neural crest cells in the development of electroreception, and suggest that *EphA4–EphrinB2* signaling may coordinate both electroreceptor and mechanoreceptor development in sharks by establishing an early molecular map for neural crest cells, sensory axons, and placodal morphogenesis. These findings highlight an underlying conservation of *Eph–Ephrin* expression during the evolutionary diversification of vertebrate sensory systems.

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