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Cochleovestibular nerve development is integrated with migratory neural crest cells

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ABSTRACT

The cochleovestibular (CV) nerve, which connects the inner ear to the brain, is the nerve that enables the senses of hearing and balance. The aim of this study was to document the morphological development of the mouse CV nerve with respect to the two embryonic cells types that produce it, specifically, the otic vesicle-derived progenitors that give rise to neurons, and the neural crest cell (NCC) progenitors that give rise to glia. Otic tissues of mouse embryos carrying NCC lineage reporter transgenes were whole mount immunostained to identify neurons and NCC. Serial optical sections were collected by confocal microscopy and were compiled to render the three dimensional (3D) structure of the developing CV nerve. Spatial organization of the NCC and developing neurons suggest that neuronal and glial populations of the CV nerve develop in tandem from early stages of nerve formation. NCC form a sheath surrounding the CV ganglia and central axons. NCC are also closely associated with neurites projecting peripherally during formation of the vestibular and cochlear nerves. Physical ablation of NCC in chick embryos demonstrates that survival or regeneration of even a few individual NCC from ectopic positions in the hindbrain results in central projection of axons precisely following ectopic pathways made by regenerating NCC.

Introduction

During embryogenesis the vertebrate inner ear begins as a simple flat otic placode that invaginates to become a spherical epithelial sac known as the otic vesicle (reviewed in (Bok et al., 2007; Chen and Streit, 2013; Groves and Fekete, 2012; Ladher et al., 2010; Schlosser, 2010)). This vesicle grows and remodels to form a complex structure of interlinked compartments known as the membranous labyrinth (Kopecky et al., 2012; Streeter, 1906). In mammals these compartments consist of a spiraling cochlea, which is the auditory organ, a saccule, utricle and three semicircular canals, which together form the vestibular system. These structures contains patches of mechano-sensory receptors that detect sound or gravity and motion, each of which is connected to the brain via the cochleovestibular (CV) nerve (reviewed in (Appler and Goodrich, 2011; Fekete and Campero, 2007; Yang et al., 2011)). The CV nerve originates initially during embryogenesis as a simple unified entity that progressively develops into an elaborate branching structure (Kopecky et al., 2012; Streeter, 1906). In mammals, the mature CV nerve is subdivided into distinct nerve trunks; a superior and inferior vestibular nerve, which together innervate the vestibular system, and a cochlear nerve, which innervates the auditory organ.

All peripheral nerves are composed of neurons supported by glial cells, and both cell types are important for nerve function (reviewed in (Hanani, 2005)). Peripheral nerve glia include Schwann cells, which support neuronal axons and neurites, and satellite cells, which surround and myelinate ganglionic neuronal cell bodies. As one of the cranial peripheral nerves, the CV nerve is likewise composed of both neurons and glia (Rosenbluth, 1962). These two cell types arise developmentally from distinct sources, the glial cells being derived from neural crest cell (NCC) progenitors (D'Amico-Martel and Noden, 1983; Harrison, 1924; Yntema, 1943b), while the neurons originate almost exclusively from the otic placode (Breuskin et al., 2010; D'Amico-Martel and Noden, 1983; van Campenhout, 1935), with the exception of rare neurons of the vestibular ganglia. The exclusive placodal derivation of CV neurons makes the nerve somewhat unique among cranial sensory nerves, most of which contain neurons derived from two

Abbreviations: 3D, 3-dimensional; CV, cochleovestibular; E, embryonic day; GFP, Green Fluorescent Protein; NCC, neural crest cells; P, postnatal day; R, rhombomere

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sources, the sensory placodes and NCC (D'Amico-Martel and Noden, 1983). While experiments in amphibian, avian, and mammalian systems have each indicated CV neurons derive almost exclusively from placode progenitors, a recent study of transgenic lineage reporter mice has posed a contradictory scenario wherein NCC or a related population of migratory neuroepithelial cells give rise to a significant fraction of CV neurons and also to cells of the otic epithelium (Freyer et al., 2011).

The neuronal cells of the CV nerve develop by delaminating from the otic vesicle, proliferating and aggregating to form the CV ganglion (Altman and Bayer, 1982; Carney and Silver, 1983). As development proceeds CV neurons project central axons to the hindbrain and project peripheral neurites to sensory targets developing within the otic epithelium (reviewed in (Fritzsch, 2003)). The glial cells of the CV nerve derive from NCC that emigrate from the hindbrain at the level of rhombomere 4 (R4) (D'Amico-Martel and Noden, 1983). Based on dye labeling analysis, CV neurons extend peripheral neurites to the developing vestibular sensory epithelium as early as embryonic day 11.5 (E11.5) and central axons to the hindbrain as early as E12.5 (Fritzsch, 2003; Matei et al., 2005). Maturation of CV neurons, as measured by final cell division, indicates vestibular and cochlear neurons mature at embryonic day E11.5 and E13.5, respectively (Matei et al., 2005; Ruben, 1967), earlier than the associated Schwann cell progenitors, which continue dividing up to the time of birth (Ruben, 1967).

A growing body of evidence suggests that interactions between glial and neuronal progenitors may be important for development of the CV nerve. In mouse, disturbance of ERBB2, a receptor that mediates neuron–glia interactions via the ligand Neuregulin 1 (reviewed in (Corfas et al., 2004)) compromises development of the CV nerve. Complete loss of *ErbB2* severely disrupts formation of cranial nerves (Lee et al., 1995) but is lethal at E10.5 owing to cardiac defect. *ErbB2* mutants rescued to later stages exhibit abnormalities in development of the CV nerve, including altered migration of neuronal cell bodies, abnormal targeting of peripheral neurites and reduced neuron number (Morris et al., 2006). Whereas the phenotype of *ErbB2* mutants indicates that migration and targeting of CV neurons depends upon signaling interactions with NCC glial progenitors, the relatively normal development of CV nerves in embryos lacking *Sox10,* a transcription factor important for development of peripheral glial NCC, suggests otherwise (Breuskin et al., 2010). The reason for the differing effect of *ErbB2* mutation versus *Sox10* mutation is not yet clear, but, because *Sox10* mutation results in loss of NCC glial progenitors after E10.5, the normal growth and guidance of CV neurons in those mutants may indicate that critical interactions occur earlier.

That interactions between NCC glial progenitors and neurons are important for development of cranial nerves is supported also by chick and zebrafish studies in which NCC are eliminated or signaling interactions between NCC and neurons is blocked. Molecularly blocking Semaphorin/Neuropilin signaling in chick disrupts NCC migratory pathways and impairs the inward movement of epibranchial placodal neurons (Osborne et al., 2005). In some studies ablation of NCC migration by physical or molecular methods in chick results in reduced numbers of neuroblasts migrating from epibranchial ganglia and abnormal projection of central axons (Begbie and Graham, 2001; Freter et al., 2013; Yntema, 1944), although other studies reported NCC removal did not disrupt formation of ganglia (Begbie et al., 1999). In zebrafish also elimination of specific sub-populations of cranial NCC disrupts formation of the epibranchial nerves (Culbertson et al., 2011).

Visualization of early developmental association between neuronal and glial progenitors of the facial ganglion in mouse and chick indicates that NCC form a corridor surrounding placodal neuroblasts (Freter et al., 2013). In mouse, genetic ablation of NCC causes some abnormalities in growth of peripheral projections of the facial nerve, but delamination of neuroblasts and formation of the ganglion is not impaired (Coppola et al., 2010).

Despite the many important insights regarding cranial sensory nerve development that have been made, owing to the dynamic remodeling and morphological complexity of the embryonic inner ear, many details of CV nerve formation remain obscure. To understand development of neuronal and glial progenitors in the embryonic CV nerve, we examined immunostained inner ears of mouse transgenic NCC lineage reporter embryos and chick embryos by confocal microscopy, compiling multiple optical sections into virtual 3D renderings of developing CV nerves. By this method we gain insight into the coordinated development of otic vesicle-derived neurons and NCC-derived glial cells of the CV nerve.

Materials and methods

Mice

Mouse strains utilized in this study included the following:

"*Z/EG*", official name, Tg(CAG-Bgeo/GFP)21Lbe/J, Jax stock # 003920;

"*R26R*", official name, FVB.129S4(B6)-Gt(ROSA)26Sortm1Sor/J, Jax stock # 009427,

"*Wnt1Cre*", official name, Tg(Wnt1-cre)11Rth Tg(Wnt1-GAL4) 11Rth/J, Jax stock #003829,

"*6.5Pax3Cre*", novel stable transgenic Cre driver line in FVB/NJ background. Transgene contains 6.5 kb NotI-HindIII fragment of genomic regulatory DNA 5' of mouse *Pax3* start site.

Images to emphasize embryo morphology

In order to enhance visualization of morphology of embryos stained for β-galactosidase activity, color images of β-galactosidase stained embryos were overlain onto greyscale images of same embryos stained for DAPI to label all cell nuclei. Imaging of whole embryos stained with DAPI or other nuclear fluorescent dye reveals details of embryo morphology not visible with white light, as previously described (Sandell et al., 2012). Overlay of color images of β-galactosidase stain embryos with greyscale image of embryo morphology yields improved visualization of β-galactosidase signal relative to embryonic structures.

Chick NCC ablation

Fertile chicken eggs (*Gallus gallus domesticus*) were preliminarily incubated at 37 °C to allow embryos to develop to HH stage 8 to 9+ (6–9 somites). Eggs were then windowed and R4 NCC were ablated by microsurgical removal of the neural tube from R3 through R5. Operated embryos were re-sealed and allowed to continue development for an additional 24 or 48 h.

Whole mount immunostain

For E10.5 mouse and chick, specimens were immunostained as whole embryo. For E11.5 mouse inner ear, embryo head specimens were bisected sagittally prior to immunostain to permit penetration of antibody and wash solutions. For E12.5 specimens, inner ears were isolated by dissection. Embryo specimens were fixed in 4% paraformaldehyde (formaldehyde) phosphate buffered saline (PBS), overnight at 4 °C, then washed in PBS and transferred (through a graded series) into 100% methanol. Tissues and embryos to be immunostained were permeabilized in Dent's bleach (MeOH:DMSO:30%H_2O_2, 4:1:1) for 2 h at room temperature, then washed with 100% methanol and transferred (through a