INTRODUCTION

Some current cochlear implant arrays incorporate strategies to bring the stimulating electrodes toward the modiolus and thus closer to the spiral ganglion cells. Studies with perimodiolar electrodes in both animals and humans have demonstrated lower evoked auditory brainstem response thresholds.1–5 In addition, this strategy has lead to reductions in threshold and comfort level stimuli intensity.6–8 as well as stapedial reflex thresholds.9 However, the apparent physiologic benefits of perimodiolar electrodes unfortunately have not translated to improved speech perception performance.10–13 Therefore, simply decreasing electrode to neuron distance for more focal stimulation may not improve speech performance. However, creating a contact-mediated stimulation paradigm may better approximate the normal physiology and fine modulation of the innate ribbon synapse connecting hair cells to spiral ganglion neurons and enable improved speech recognition and sound quality for cochlear implant users. Achieving controlled neurite outgrowth for contact of electrodes and ganglion cells that maintains the precise tonotopic organization requires an understanding of the molecular cues spiral ganglion cells use to find appropriate hair cell synaptic targets.

Ephrins are a family of membrane bound axon guidance molecules that bind to Eph receptors. This interaction leads to signaling that guides growing axons to appropriate synaptic targets, in most cases, by an inhibitory or repelling mechanism.14 Ephrins are separated in two classes based on their attachment to cell membranes via a glycosal-phosphatidylinositol (GPI) linkage (ephrin-A) or a transmembrane domain (ephrin-B). These proteins are of particular interest as they are expressed in gradients in the retina and tectum and have a well-demonstrated role in guiding retinal ganglion projections to establish the topographic organization of the visual system.15–17

Previous studies have demonstrated that these proteins are expressed in spiral ganglia and cells in and near the organ of Corti of chicks and rodents.18–23 In addition, ephrin-A2, normally expressed in acoustic ganglion cells, is suppressed during reinnervation of hair cells during regeneration in the chick,22 suggesting a role for these proteins in axon guidance in the auditory system. Furthermore, studies in knockout mice with...
function null ephrin and Eph proteins have demonstrated abnormal phenotypes, indicating roles for these molecules in the auditory and vestibular systems. Specifically, evaluation of distortion product otoacoustic emissions (DPOAE) revealed diminished levels in Eph B1 and Eph B3 receptor knockout mice relative to wild-type littermates, suggesting the potential of aberrant inner ear efferent innervation patterns.

We are interested in further investigating if the auditory deficits in the Eph B receptor mutated mice are attributable to axon path finding errors in the organ of Corti. We have used mutant mice with the B-galactosidase gene inserted in to various ephrin-B and Eph B coding regions to determined expression of these proteins in the cochlea. We have also developed a functional coculture assay to investigate the effect of Eph B2 on spiral ganglion neurite outgrowth in vitro. Last, we used a technique to label nerve fibers innervating the cochlear hair cells with lipophilic dyes to determine in vivo cochlear innervation patterns in normal as well as knockout mice with disrupted ephrin/Eph signaling. The results may have important implications for future design of the next generation of cochlear implants.

**METHODS**

**Lac Z Staining**

Postnatal days 6 to 14 (P6–P14) CD1 mice were sacrificed with CO2 asphyxiation and decapitated. Their temporal bones were isolated and rinsed in 0.1 M phosphate buffer and then fixed for 30 minutes with 25% gluteraldehyde with 100 mM EGTA, 1 M magnesium chloride, and 0.1 M sodium phosphate. Samples were washed three times with 1 M magnesium chloride, 2% Nonidet-P40, and 0.1 M sodium phosphate (wash buffer). The temporal bones were stained overnight at 37°C in 25 mg/mL X-gal, then rinsed in wash buffer and postfixed with 4% paraformaldehyde, decalcified with 10% EDTA for 5 days, placed in 15% sucrose for 2 hours, then 30% sucrose overnight at 4°C, then mounted in O.C.T. compound. Subsequently, 14-μm cryosections were cut and mounted on glass slides and coverslipped. The slides were viewed on a light microscope and images captured with a Nikon digital camera.

**Spiral Ganglion Cell and COS-1 Cell Cocultures**

Postnatal day 7 (P7) wild-type CD-1 or ephrin B1 null mice were sacrificed with CO2 asphyxiation and decapitated. Their spiral ganglia were microdissected and isolated from the temporal bone and cut in to several small fragments. The neuronal tissue was digested in Hibernate A medium with L Glutamine (Invitrogen, Carlsbad, CA) with 5% papain, 5% cysteine, and 10% EDTA for 30 minutes at room temperature then 37°C for 30 minutes. The supernatant was removed and tissue pellet resuspended in 1 mL of Neurobasal A growth medium with B27 supplement, L Glutamine, Penicillin, and Streptomycin (Invitrogen), fortified with Brain Derived Neurotrophic Factor (BDNF), Neurotrophin 3 (NT-3), Leukemia Inhibitory Factor (LIF) (all from Sigma, St. Louis, MO), and triturated 30 times. The cell suspension was then plated on sterile glass coverslips precoated with poly-DL-ornithine and laminin (Sigma). Neurons were cultured for 48 hours at 37°C then underwent a 50% medium change with fresh growth medium described above and cultured for an additional 24 hours. For neurons cocultured with COS-1 cells, which endogenously express Eph B2, various concentrations ranging from 5 to 80 × 10⁻⁴ mL/mL of COS-1 cells determined by measurement with a Hemacytometer cell density counter (Fisher Scientific, Pittsburgh, PA) were added to the cultures after 48 hours at the time of the 50% medium change. After a total of 72 hours in culture, the neurons were fixed and immunolabeled with neuron specific marker Tuj-1 (2 mg/mL diluted 1:500, mouse monoclonal anti-βIII tubulin antibody, Covance, Berkeley, CA), followed by biotinylated secondary antibody and the ABC Elite Kit with diaminobenzidine detection (Vector Laboratories, Burlingame, CA). These specimens were viewed with a light microscope, images digitized with a Nikon camera and neurite lengths were quantified using NIH Image J software. Statistical analysis using non-parametric Student’s t test was performed using Prism 5 (Graph Pad Software, San Diego, CA).

**RESULTS**

**Eph and Ephrin Expression in the Cochlea**

Expression patterns of Eph B1, B2, and ephrin B2 in the cochlea were identified using Lac Z mutant mice and β-gal staining. All results of expression are shown in Figure 1. Eph B1 expression was identified in the border cells, inner and outer hair cells, and spiral ligament, and in a subset of spiral ganglion cells. Eph B2 expression was found in the border cells, inner hair cells, Hensen’s cells, stria vascularis, and in the spiral ganglion cells, but not in the outer hair cells. Ephrin B2 was expressed throughout the cochlea and was found in spiral ganglion cells, spiral limbus, border cells, inner and outer hair cells, Hensen’s cells, Claudius’s cells, spiral ligament, and Reissner’s membrane. Ephrin B3 expression, however, was not identified in the cochlea.

**Spiral Ganglion Cell Response to Eph B2 In Vitro**

COS-1 cells that endogenously expressed Eph B2 and were cocultured with dissociated mouse spiral ganglion cells to determine the effect of Eph B2 on neurite extension in culture. After 72 hours in culture, the dissociated neurons from wild-type mice were immunolabeled and the length of spiral ganglion cell neurites when cocultured with Eph B2 expressing COS-1 cells were
notably shorter compared to similar neurons cultured alone (Fig. 2A and B). When COS-1 cells were added to the cultured dissociated spiral ganglion neurons at varying concentrations, there was a dose-dependent decrease in neurite length with increasing concentration of COS-1 cells in culture. Compared to a mean neurite length of 162.5 μm (±14.4 SEM) when cultured alone, spiral ganglion cells cocultured with approximately 5, 25, and 75 /L of COS-1 cells per mL of culture medium, demonstrated mean neurite lengths of 84.7 μm (±8.6 SEM), 61.9 μm (±8.7 SEM), and 46.9 μm (±7.1 SEM), respectively. The differences of all of these means compared to that of wild-type neurons cultured without COS-1 cells were all statistically significant, with P-values all being <.005 (Fig. 2C). This experiment was repeated using dissociated spiral ganglion neurons from mice with null function of ephrin B1, a ligand of the Eph B2 receptor known for mediating the repelling signal used for axon guidance. With functional disruption of the ephrin B1/Eph B2 interaction, the growth inhibitory effect was not seen (Fig. 2D and E). At varying concentrations of COS-1 cells, there were no significant differences in neurite lengths after 72 hours in coculture with approximately 8, 30, and 80 × 10^-4 μL of COS-1 cells/mL of culture medium (36.9 μm (±4.0 SEM), 36.5 μm (±4.0 SEM), and 37.3 μm (±4.1 SEM), respectively) compared to the 38.0 μm (±4.1 SEM) seen in neurons from ephrin B1 knockout mice cultured without COS-1 cells (Fig. 2F).

**Analysis of Cochlear Innervation Patterns In Vivo**

Neurovue dye was placed in the internal auditory canals of wild-type mice, triple knockout mice with null function of Eph B1, Eph B2, Eph B3 in combination, as well as ephrin B1 null function single mutants. The temporal bones were incubated to allow the lipophilic fluorescent dye to migrate along the cochlear nerve fibers (Fig. 3). Subsequent analysis of the tissue with confocal microscopy allowed visualization of the nerve projections in the cochlea and determination of innervation patterns in the organ of Corti (Fig. 4). To determine the relative position of the nerve fiber terminations the hair cells in the organ of Corti were labeled with FITC conjugated phalloidin. Cochlear innervation patterns in wild-type mice show that the nerve fibers terminate on the basal surfaces of inner hair cells and outer hair cells (Fig. 5). However, in knockout mice with null function of 3 Eph receptors (Eph B1, B2, and B3), some nerve fibers extend beyond the third row of outer hair cells in to the area of Hensen’s cells (Fig. 6). This over projection of the nerve fibers to terminate beyond the level of the last row of outer hair cells was also seen in the cochlea of ephrin B1 single mutants (Fig. 7).
DISCUSSION

We were able to successfully identify the expression patterns of Eph B1, B2, and ephrin B2 in the cochlea of Lac Z mutant mice using β-gal staining. Studies that previously demonstrated the expression of these proteins in the auditory system were performed using antibodies.18–21,23,25 Although immunostaining with antibodies can provide some indicated of expression patterns, it is limited by the lack of specificity. In our studies, we used X-gal staining with Lac Z mutant mice, which is the gold standard for localizing gene expression as it is a much more reliable technique.

In our COS-1 coculture experiments we found that the endogenously expressed Eph B2 in the COS-1 cells inhibited the outgrowth of wild-type spiral ganglion neurites in culture. Furthermore, they did so in a concentration dependent manner (Fig. 2C). When the same experiment was repeated using spiral ganglion cells from knock out mice with null function of ephrin B1, the growth inhibitory effect observed in the wild-type cultures was not present. In fact, there were no significant differences in neurite lengths after 72 hours even with the highest concentration of COS-1 cells (Fig. 2F). In addition, spiral ganglion cells from ephrin B2

Fig. 2. Eph B2 inhibits spiral ganglion cell neurite extension in culture. Spiral ganglion cells from wild-type mice were cultured alone (A) and in coculture with Eph B2 expressing COS-1 cells (B). The lengths of the neurites were measured by tracing from digitized images of immunohistochemically labeled neurons, and the results are shown in a chart that demonstrates a dose-dependent inhibition of neurite outgrowth in culture (C, error bars represent standard error of the means). Disruption of ephrin B1/Eph B2 interaction results in loss of inhibition of neurite lengthening when spiral ganglion neurons are cocultured with COS-1 cells. Spiral ganglion neurons from knock out mice with null function of ephrin B1 were cultured alone (D) and in the presence of COS-1 cells (E). Measurement of neurite lengths from these spiral ganglion cells did not demonstrate shorted lengths even in the presence of the highest concentration of COS-1 cells (F).

Fig. 3. Neurovue dye was place in the internal auditory canal (IAC) of mouse temporal bones and allowed to migrate along the auditory nerve into the cochlea to trace the spiral ganglion terminal fibers in the organ of Corti. Gross views of temporal bone specimens are shown with the IAC, facial nerve (FN), and otic capsule (OC) labeled in an intact cochlea (A) and with the OC opened, which shows the dye migrating along the nerve fibers into the modiolus of the cochlea (B).
mutated mice were also cocultured with the highest concentration of Eph B2 expressing COS-1 cells used in similar experiments with ephrin B1 mutated and wild-type mice ($80.4 	imes 10^{-4}$), and the lengths of the neurites from these cultures were not significantly different from those of cultured ephrin B2 mice cultured alone (data not shown). These results further support the hypothesis that the inhibition of neurite growth seen in the wild-type experiment was mediated by ephrin/Eph interactions.

It was observed that the mean neurite length of the ephrin B1 knock out control group (no COS-1 cells added) were substantially shorter than those from the wild-type control group 162.5 µm versus 38.0 µm. We hypothesize this may indicate that the neurons from ephrin B1 knock out mice may be less robust in neurite extension secondary to lack of ephrin B1 signaling. However, we often see substantial variability in mean neurite lengths from neuronal preparation to neuronal preparation and thus cannot rule out that these mean differences simply reflect the variability from experiment to experiment. However, all comparisons of the quantitative data were done using neurons from the same preparation to control for this variability and ensure that differences seen reflect the effect of coculturing with the COS-1 cells.

The most interesting finding in this study is the in vivo tracing of the nerve fibers in Eph B1, B2, and B3 triple receptor knock out and ephrin B1 single ligand knockout mice showing overshooting of the fibers beyond the third row of outer hair cells. This finding is consistent with known effect of ephrin/Eph interaction resulting in a repelling signal that prevents axon extension to inappropriate synaptic targets. When this signal is disrupted or lost, neurons extend their neurite to cells that are not their intended targets as seen in our Fig. 4. Confocal photomicrograph demonstrating dye-labeled spiral ganglion cell bodies (SGC), nerve fibers (NF), the innervation of the inner hair cells (IHC), and fibers crossing the tunnel of Corti (TOC) to innervate the three rows of outer hair cells (OHC).

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Fig. 6. Cochleae of Eph B1, B2, and B3 triple receptor knock out mice double labeled with Neurovue and Phalloidin shows overshooting of the nerve fibers beyond the last row of outer hair cells. Nerve fibers (NF) shown in low and high magnification (A and D, respectively). Position of outer hair cells (OHC) demonstrated with Phalloidin in low and high magnification (B and E, respectively). Images merged to confirm position of NF relative to OHC (C and F).

Fig. 7. Cochleae of ephrin B1 single ligand knock-out mice double labeled with Neurovue and Phalloidin shows overshooting of the nerve fibers beyond the last row of outer hair cells. Nerve fibers (NF) shown in low and high magnification (A and D, respectively). Position of outer hair cells (OHC) demonstrated with Phalloidin in low and high magnification (B and E, respectively). Images merged to confirm position of NF relative to OHC (C and F).
experiments with the over projection of spiral ganglion neurites.

Interestingly, the presence of over projection of nerve fibers beyond the third row of outer hair cells into the supporting Hensen cells has been described previously in the cochlea of guinea pigs as well as isolated finding in the human cochlea. The role of such fibers and synapses in guinea pigs still remains unclear but may simply be due to species variability. Nevertheless, we have only observed similar over projections in our triple knock out (Eph B1, B2, and B3 null) and ephrin B1-deficient mice. This overprojection was never seen in any cochlea of our wild-type mice.

CONCLUSION

In our experiments, we have demonstrated the presence of Eph receptors and ephrin ligands in the cochlea of wild-type mice. We have also shown that the disruption of this signaling pathway known to provide a mechanism for axon guidance results in functional consequences in spiral ganglion cells. These results lead us to believe that these proteins play an important role in establishing normal innervation patterns in the cochlea. Further investigation and understanding of Eph/ephrin activity in the cochlea could provide the basis for developing innate axon guidance mechanisms to facilitate a contact mediated stimulation paradigm that may improve cochlear implant function in the future.

BIBLIOGRAPHY