Expression of nerve growth factor (NGF) receptors in the developing inner ear of chick and rat

CHRISTOPHER S. VON BARTHELD1,2, SUSAN L. PATTERSON2, JOSEF G. HEUER2, ESTHER F. WHEELER2, MARK BOTHWELL2, and EDWIN W. RUBEL1,2.*

1Hearing Development Laboratories RL-30 and 2Department of Physiology and Biophysics SJ-40, University of Washington School of Medicine, Seattle, WA 98195, USA

*Author for correspondence

Summary

The expression of nerve growth factor receptors (NGFRs) was studied in the developing inner ear with in situ hybridization in chick embryos and with immunocytochemistry in rat embryos to determine sites of possible functions of NGF or NGF-like molecules in inner ear development. NGFR expression in the chick otocyst and acoustic ganglion is compared with epithelial differentiation and the onset of afferent innervation as determined with fluorescent carbocyanine tracers.

In the inner ear of the chick embryo, NGFR mRNA expression shows an alternating pattern in mesenchymal and epithelial tissues. NGFR mRNA is heavily expressed in the mesenchyme surrounding the otocyst (E2–3), ceases at E3–5, and reappears in a thin layer of mesenchymal cells surrounding the membranous epithelia (E5–13). In the otocyst epithelium, NGFR mRNA expression develops in one anterior and one posterior focus at E3–4.5. NGFR mRNA is expressed in the primordia of the ampullary cristae (E5–7) and possibly the anlage of the utricle; label transiently concentrates in the planum semilunatum of the cristae ampullares and in superior portions of the semicircular canals at E9, but is not seen in differentiating hair cells. In the acoustic ganglion, NGFR mRNA expression begins at E4; at the same time, the first peripheral acoustic nerve processes penetrate the otic epithelium (E4–4.5). The acoustic ganglia remain weakly NGFR mRNA-labeled in the posthatch animal.

In the rat embryo, NGFR immunoreactivity is present in the auditory placode at E9, in the periotic mesenchyme at E9–10, and in the medial half of the otocyst at E10–11. At E12, epithelial NGFR expression becomes restricted anteriorly and posteriorly in a pattern similar to that of the chick otocyst and ceases at E13. NGFR immunoreactivity appears transiently in pillar cells of the cochlea in the third week of gestation. NGFR and NGF mRNA is expressed after E11 in the acoustic ganglia. While NGFR transcripts are expressed in the cochlear ganglion cell bodies, NGFR protein becomes restricted to neuronal processes by the third week of gestation. The vestibular, but not the cochlear (spiral) ganglia remain NGFR-labeled in the adult rat.

Onset of NGFR mRNA expression in the acoustic ganglion during the period of afferent fiber ingrowth into the otocyst epithelium is consistent with the hypothesis that NGF-like molecules may have a neurotrophic function for acoustic ganglion cells. Transient expression of NGFRs in secretory cells of the vestibular endorgan and pillar cells in the organ of Corti implicate a role for neurotrophins in the differentiation of these epithelial cell types.

Key words: nerve growth factor (NGF) receptor, otic vesicle, otocyst, cochleovestibular ganglion, in situ hybridization, chick embryo, rat embryo, immunocytochemistry, carbocyanine tracers.

Introduction

The vertebrate inner ear is an unusually complex structure. The inner ear labyrinth is formed by a series of morphogenetic events that begin with the differentiation of a seemingly simple otic anlage (Fuchs, 1923; Romanoff, 1960; Rubel, 1978). Transplantation experiments indicated that the otic epithelium becomes polarized and determined at early stages in development (Harrison, 1936; Hall, 1939, 1941; Yntema, 1955; Rubel, 1978). The molecular mechanisms that regulate the differentiation of the inner ear anlage are poorly understood. Normal development of the otocyst appears to require extrinsic inducing factors (trophic factors, chemoattractants) (Harrison, 1936; Yntema, 1955; Ard et al. 1985; Van De Water, 1976, 1988). Interest in the factors that regulate proliferation and differentiation of acoustic sensory receptors has re-
cently gained impetus from work that demonstrated hair cell regeneration in the avian cochlea (Corwin and Cotanche, 1988; Ryals and Rubel, 1988).

The trophic molecule nerve growth factor (NGF) appears to be involved in the differentiation of several neuronal and non-neuronal cell types in development. NGF receptors (NGFRs) are expressed differentially in the avian otocyst during early development (Raivich et al. 1987; Bernd and Represa, 1989; Hallböök et al. 1990). Raivich et al. (1987) localized NGF-binding primarily to the non-sensory otic epithelium in 3- to 6-day-old chick embryos, whereas Bernd and Represa (1989) described NGF-binding in the ventromedial ('neurogenic') otic epithelium in 3-day-old quail embryos and suggested that NGF may be involved in the differentiation of acoustic ganglion cells and hair cells. Hallböök et al. (1990), on the other hand, located NGFR mRNA expression in lateral portions of the chick otocyst at 3 days of incubation. NGF may serve as a mitogen in the otic epithelium (Represa and Bernd, 1989) and, contrary to previous belief (Ard, 1982; Pearson et al. 1983; Davies and Lindsay, 1985; Lindsay et al. 1985), as a survival and neurite-promoting factor for the acoustic ganglion (Lefebvre et al. 1990; Van De Water et al. 1991).

For the assessment of possible functions of NGF (or NGF-like molecules BDNF and NT-3, Rodriguez-Tebas et al. 1990; Hohn et al. 1990; Maisonpierre et al. 1990), it is necessary to know the location and timing of NGFR expression. Are NGFRs expressed in hair cell precursors (Represa and Bernd, 1989), differentiating hair cells or in non-sensory otic epithelial cells (Raivich et al. 1987)? Are NGFRs expressed in precursors of acoustic ganglion cells (Bernd and Represa, 1989), in cells migrating towards the ganglion and is NGFR expression maintained in postmitotic, differentiated ganglion cells? Are NGFRs involved in afferent innervation of the organ of Corti, or may expression of these receptors be restricted to the efferent system (Després et al. 1991)?

In the present study, we investigate the spatial and temporal expression of NGFRs in the developing inner ear of chick and rat. Antibodies against avian NGFR are not yet available; therefore, we used in situ hybridization to determine sites of NGFR expression in the chick. Both procedures are sensitive techniques (Koh et al. 1989); in situ hybridization facilitates the identification of cell types with NGFR expression, because only the cell bodies are labeled. Immunocytochemistry, on the other hand, renders information about the spatial distribution of the protein. We compare expression of NGFR mRNA in chick with NGFR immunocytochemistry in rat embryos. Three-dimensional reconstructions from serial sections show that NGFR expression forms a heterogeneous, 'mosaic' pattern with an anterior—posterior polarization in both the chick and rat otocyst, possibly reflecting an early determination and differentiation ('chemodifferentiation', Van De Water, 1976) of the otic epithelium (Yntema, 1955; Rubel, 1978). Coincidence of NGFR expression in the acoustic ganglion and onset of afferent innervation supports the hypothesis that NGF may have a neurotrophic function for the acoustic ganglion (Lefebvre et al. 1990). Alternating patterns of mesenchymal/epithelial NGFR expression suggest that NGF or NGF-like molecules may play a role in mesenchymal—epithelial interactions. Preliminary reports of part of our work have been presented in abstract form (von Bartheld et al. 1990b; Wheeler et al. 1990).

Materials and methods

A total of 89 White Leghorn chick embryos, 3 posthatch chicks, 30 Sprague-Dawley rat embryos and one adult rat were used in the present study. Eggs were incubated at 37–38°C in 50–60% humidity and chick embryos were staged according to Hamburger and Hamilton (1951). We will refer to these stages as hours or days of incubation. For rat embryos, noon on the day of vaginal plug observation is 0.5 days post coitum (E0.5).

Chick NGFR mRNA in situ hybridization

Twenty-two chick embryos were fixed in 4% paraformaldehyde. Embryos older than E7 were anesthetized with Nembutal (approximately 20 mg kg⁻¹ body weight) and perfused intracardially with fixative. In hatchlings, pieces of the head including the inner ear were decalcified in 4N formic acid and 0.5 M sodium formate for 2 days. Whole embryos or heads of animals were dehydrated and embedded in paraffin. Serial sections of 6–10 μm were cut on a microtome in the transverse plane, including embryos staged E2 (HH13), E2.5 (HH17), E3 (HH20), E4 (HH24), E5 (HH27), E5.5 (HH28), E7 (HH31), E8.5 (HH35), E9 (HH35), E11 (HH37), E13 (HH39), and one 10-day-old chick (P10). In addition, some embryos were sectioned in the sagittal plane (E3, 4, 7), and horizontal plane (E3, 3.5, 4, 4.5, 6). Planes of sectioning are relative to the neuraxis (neural tube) at the level of the inner ear or inner ear anlage. Sections were collected on polylysine-coated slides, baked overnight at 42°C and deparaffinized in xylene.

Pretreatment for hybridization included rehydration of sections, fixation for 10 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), washes for 5 min in PBS and treatment with 20 μg ml⁻¹ Proteinase K for 7.5 min. The sections were fixed again in 4% paraformaldehyde in PBS for 5 min, treated with acetic anhydride and dehydrated according to Cox et al. (1984). 35S-UTP-labeled single-stranded sense and antisense RNA riboprobes were prepared according to Melton et al. (1984). pGEM4Z plasmid containing a 436 bp fragment of the chick NGFR cDNA was linearized with EcoRI and used as a template with T7 RNA polymerase to generate an NGFR antisense transcript (Heuer et al. 1990). The same plasmid, linearized with BamHI and used as a template with SP6 polymerase, provided a sense transcript and was applied in adjacent sections as a control for nonspecific binding (data not shown). Transcripts were degraded to an average length of 150 base pairs using alkaline hydrolysis according to Cox et al. (1984) and then were ethanol precipitated.

Hybridization was carried out in 2X sodium chloride—sodium phosphate—ethylenediaminetraacetate (SSPE: 0.3 M NaCl, 10 mM Na2HPO4 and 1 mM EDTA), 50% formamide, 20 mM Tris–HCl, 5 mM disodium ethylenediaminetetraacetate (EDTA), 10% dextran sulfate, 5X Denhardt's solution, 20 μM dithiothreitol (DTT), 0.5 μM ml⁻¹

Materials and methods

A total of 89 White Leghorn chick embryos, 3 posthatch chicks, 30 Sprague-Dawley rat embryos and one adult rat were used in the present study. Eggs were incubated at 37–38°C in 50–60% humidity and chick embryos were staged according to Hamburger and Hamilton (1951). We will refer to these stages as hours or days of incubation. For rat embryos, noon on the day of vaginal plug observation is 0.5 days post coitum (E0.5).

Chick NGFR mRNA in situ hybridization

Twenty-two chick embryos were fixed in 4% paraformaldehyde. Embryos older than E7 were anesthetized with Nembutal (approximately 20 mg kg⁻¹ body weight) and perfused intracardially with fixative. In hatchlings, pieces of the head including the inner ear were decalcified in 4N formic acid and 0.5 M sodium formate for 2 days. Whole embryos or heads of animals were dehydrated and embedded in paraffin. Serial sections of 6–10 μm were cut on a microtome in the transverse plane, including embryos staged E2 (HH13), E2.5 (HH17), E3 (HH20), E4 (HH24), E5 (HH27), E5.5 (HH28), E7 (HH31), E8.5 (HH35), E9 (HH35), E11 (HH37), E13 (HH39), and one 10-day-old chick (P10). In addition, some embryos were sectioned in the sagittal plane (E3, 4, 7), and horizontal plane (E3, 3.5, 4, 4.5, 6). Planes of sectioning are relative to the neuraxis (neural tube) at the level of the inner ear or inner ear anlage. Sections were collected on polylysine-coated slides, baked overnight at 42°C and deparaffinized in xylene.

Pretreatment for hybridization included rehydration of sections, fixation for 10 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), washes for 5 min in PBS and treatment with 20 μg ml⁻¹ Proteinase K for 7.5 min. The sections were fixed again in 4% paraformaldehyde in PBS for 5 min, treated with acetic anhydride and dehydrated according to Cox et al. (1984). 35S-UTP-labeled single-stranded sense and antisense RNA riboprobes were prepared according to Melton et al. (1984). pGEM4Z plasmid containing a 436 bp fragment of the chick NGFR cDNA was linearized with EcoRI and used as a template with T7 RNA polymerase to generate an NGFR antisense transcript (Heuer et al. 1990). The same plasmid, linearized with BamHI and used as a template with SP6 polymerase, provided a sense transcript and was applied in adjacent sections as a control for nonspecific binding (data not shown). Transcripts were degraded to an average length of 150 base pairs using alkaline hydrolysis according to Cox et al. (1984) and then were ethanol precipitated.

Hybridization was carried out in 2X sodium chloride—sodium phosphate—ethylenediaminetetraacetate (SSPE: 0.3 M NaCl, 10 mM Na2HPO4 and 1 mM EDTA), 50% formamide, 20 mM Tris–HCl, 5 mM disodium ethylenediaminetetraacetate (EDTA), 10% dextran sulfate, 5X Denhardt's solution, 20 μM dithiothreitol (DTT), 0.5 μM ml⁻¹.
yeast tRNA and 10 million cts min⁻¹ ml⁻¹ (10 ng ml⁻¹) of RNA probe in a volume of 50 μl at 50°C for 16–20 h. After hybridization, sections were washed in 4x sodium chloride–

sodium citrate (SSC: 0.15 M NaCl; 0.015 M sodium citrate) for 15 min at room temperature, in 2xSSC for an additional 15 min, after which they were immersed in 50% formamide,

2xSSPE at 65°C for 10 min. The sections were washed in

2xSSC for 30 min at room temperature, 0.1xSSC 50°C for 15 min, and 0.1xSSC for 30 min at room temperature, dehydrated and coated with nuclear track emulsion (NTB-2, Eastman Kodak Co.) for autoradiography (Angerer and Angerer, 1981). Coated sections were exposed at 4°C for 10–14 days and were developed, counterstained with cresyl violet or thionin, and coverslipped with di-n-butyl-phthalate-
xylene (DPX) or Permount. In some embryos, adjacent sections were collected and processed on different sets of slides; hybridization of adjacent sections rendered virtually identical labeling. Antisense and sense (control) sections were examined under bright-field as well as dark-field illumination.

Otocysts from ages E2.5 to E4 were reconstructed three-
dimensionally from serial hybridized sections through the otocyst to gain a synoptic view of NGFR expression (Fig. 2). Autoradiographic grains over 20 randomly chosen cells from the eighth nerve ganglion, in which vestibular and cochlear portions can be distinguished, as the vestibulocochlear ganglion (after E4 in the chick embryo; D’Amico-Martel and Angerer, 1990). Coated sections were exposed at 4°C for 2–6 months at room temperature, the embryos were cryoprotected in 30% sucrose, cryosectioned at 25–35 μm in the transverse plane and sections were collected on gelatin-coated slides. The sections were dried on slides and observed without coverslips on a fluorescence microscope using standard rhodamine filters for Dil, and fluorescein isothiocyanate (FITC) filters for DiO (von Bartheld et al., 1990a). Dil and DiO were dissolved in dimethylformamide (1%) and injected into the brainstem (E3–7), or into the otocyst (E2–7) by using a Picospritzer (General Valve Corp.). Following incubation for 2–6 months at room temperature, the embryos were cryosectioned at 30 μm, cryosectioned at 25–35 μm in the transverse plane and sections were collected on gelatin-coated slides. The sections were dried on slides and observed without coverslips on a fluorescence microscope using standard rhodamine filters for Dil, and fluorescein isothiocyanate (FITC) filters for DiO (von Bartheld et al., 1990a). Representative series of sections were charted and documented on TMAX 400 film (Kodak).

**Neuronal tracing with Dil and DiO**

Sixty-five chick embryos of 2–7 days of incubation were immersion-fixed with 2% paraformaldehyde and 0.5% glutaraldehyde. Tracers were injected and the tissue was processed as described previously (von Bartheld et al., 1990a). Dil and DiO were dissolved in dimethylformamide (1%) and injected into the brainstem (E3–7), or into the otocyst (E2–7) by using a Picospritzer (General Valve Corp.). Following incubation for 2–6 months at room temperature, the embryos were cryoprotected in 30% sucrose, cryosectioned at 25–35 μm in the transverse plane and sections were collected on gelatin-coated slides. The sections were dried on slides and observed without coverslips on a fluorescence microscope using standard rhodamine filters for Dil, and fluorescein isothiocyanate (FITC) filters for DiO (von Bartheld et al., 1990a). Representative series of sections were charted and documented on TMAX 400 film (Kodak).

**Rat NGFR immunocytochemistry**

Pregnant Sprague-Dawley rats were anesthetized with Equithesin (0.5 ml/100 g body weight) and rat embryos were obtained via Cesarian section. Thirty embryos ranging in age from E9 to E22 were immersion-fixed in methyl Carnoy’s fixative. Fixed tissues were dehydrated, embedded in paraffin, and sectioned at 8–10 μm onto gelatin-coated slides. In addition, one adult rat was anesthetized, perfusion-fixed with 4% paraformaldehyde and 0.1% glutaraldehyde, and the head was decalcified in 4M formic acid and 0.5M sodium formate for 2 months. The inner ears were dissected and one ear was cryosectioned at 30 μm; the other ear was embedded in paraffin and sectioned at 12 μm. Sections were processed with 0.3% hydrogen peroxide in methanol for 5 min to inactivate endogenous peroxidase activity. Sections were rehydrated and rinsed in PBS. Nonspecific binding was blocked by preincubation with 2.5% horse serum and 2.5% rat serum in PBS for 2 h in a humidified chamber. Primary and secondary antibodies were diluted in the same solution.

The monoclonal antibody against rat NGFR, 192-IgG (Chandler et al., 1984), was purified from ascites fluid from mice with hybridoma 192 ascites tumors. Tissue sections were incubated with the primary antibody at 6 μg ml⁻¹ for 2–3 h at room temperature, and then overnight at 5°C. Control sections were incubated in nonimmune IgG (ICN Immunobiologicals), diluted to the same concentrations as the 192-IgG. After washing in PBS, secondary antibody (biotinylated horse anti-mouse, Vector Laboratories) was applied at 1:400 dilution and incubated 2–3 h at room temperature, then overnight at 5°C. After washing, sections were incubated with streptavidin aminohexanol-biotin complex (Zymed Laboratories) at 1:100 dilution for 3–4 h at room temperature. Slides were washed in 0.1M sodium acetate (pH 6.1) and incubated with 0.003% hydrogen peroxide, 34 μg ml⁻¹ diaminobenzidine, and 0.025 g ml⁻¹ nickel sulfate in 0.1M sodium acetate (pH 6.1) for 2 min at 37°C. Sections were rinsed in sodium acetate, stained with neutral red or methyl green, or left without counterstain, dehydrated and coverslipped. Otocysts from ages E11 and E12 were reconstructed three-dimensionally from serial sections through the otocyst to gain a synoptic view of NGFR expression (data not shown) and for comparison with similar reconstructions from chick otocysts (Fig. 2).

**Rat NGFR in situ hybridization**

Sagittal sections from E17 and E18 rat embryos were subjected to NGFR in situ hybridization. A cDNA containing the first two exons of the low-affinity NGFR was provided by Moses Chao (Radeke et al., 1987). A 250 bp EcoRI–BamHI fragment encoding the 5’ end of the receptor was subcloned into pGEM3Z (f+) (Promega). 35S-UTP-labeled single-stranded antisense and sense probes were generated by using SP6 polymerases on templates linearized with SP6 polymerases on templates linearized with EcoRI (sense). In situ hybridization was carried out as described above for the chick NGFR.

**Results**

In our description of inner ear development, we will refer to the early (E2–4) eighth nerve ganglion as the *acoustic ganglion*, and to the differentiated eighth nerve ganglion, in which vestibular and cochlear portions can be distinguished, as the *vestibulocochlear ganglion* (after E4 in the chick embryo; D’Amico-Martel and Noden, 1983).

**Chick embryo**

NGFR mRNA expression in the otic mesenchyme and epithelium

The otic anlage becomes visible as a thickening of the ectoderm at E2, followed by an invagination at E2.5 (otic pit stage), closure of the otic vesicle around E3.5, and separation of the otic epithelium from the surface ectoderm after E4 (Meier, 1978; Alvarez et al. 1989). The distribution and developmental changes in expression of NGFR mRNA in the chick otocyst are illustrated in Figs 1–2. Periotic mesenchymal and otic epithelial tissues express NGFR mRNA in distinct, developmentally regulated patterns. The mesenchyme subjacent to the otocyst is heavily labeled at E2 and E2.5 (Fig. 1A, D); the otic epithelium is NGFR mRNA-negative at this age. At E3, NGFR expression
develops in two opposite areas in the otocyst epithelium, one is located anteriorly, the other posteriorly. Expression appears to be concentrated in the transition zone of the thinner lateral epithelium (dorsolaterally, where the otic vesicle has just closed) and the thickened epithelium (cf. Alvarez et al. 1989). Ventral portions of
NGF receptors in the inner ear

The otocyst is the anlage of the inner ear. As it elongates and expands, the otocyst forms the future sensory epithelium of the vestibular endorgans. The developing cristae are heavily labeled at E9. At this age, it is not clear whether label is restricted to the developing plana semilunata, presumptive secretory cells which circumscribe the sensory epithelium of the ampullary organs (Amerlinck, 1950; Dohlman, 1964; Kimura et al. 1964), or may also involve peripheral portions of the developing neuroepithelium. At E11, label is restricted to the plana semilunata of the cristae (Fig. 3C, D) and to cells of the epithelium lining the superior portions of the semicircular canals. NGF expression was not observed in the plana semilunata at E13 or at P10. Also, NGFR mRNA label was not observed in the cochlea/lagena, saccule, or in the paratympanic organ, a hair cell-organ in the avian middle ear (von Bartheld, 1990), at any age examined. Grain counts over hair cells in the cochlear duct or cochlea from ages E7, E9, E11, E13 and P10 did not exceed background levels (Fig. 5); therefore, NGF-binding in the sensory epithelium of the avian cochlea (Represa et al. 1991) may be due to NGF that binds to NGFR on nerve fibers within the sensory epithelium.

As mentioned earlier, NGFR mRNA expression ceases in the mesenchyme surrounding the inner ear epithelia from E4 to E5, but later gradually reappears from E5 to E11 in a distinct pattern. At E5–7 only the mesenchyme surrounding the primordium of the endolymphatic duct is labeled; after E7 the mesenchymal cells surrounding the maculae and cristae organs also express NGFRs, and at E11 the entire membranous labyrinth is circumscribed by a thin layer (between 40 and 60 μm in diameter) of NGFR mRNA-expressing cells (Figs 3C, D, 4). Some of the labeled regions referred to here as mesenchyme may contain nerve fiber pathways innervating the sensory endorgans, and thus may include Schwann cells. It is possible that Schwann cells contribute to the NGFR mRNA label, but the label is not restricted to innervating pathways (Fig. 3A), and is intense in mesenchymal regions that lack nerve fibers. Mesenchymal label is absent in tissue adjacent to NGFR mRNA-labeled epithelium; a reciprocal relationship exists between mesenchymal and epithelial label. Mesenchymal cells surrounding the cochlea express NGFR mRNA at E9–13, but components of the sensory epithelium of the cochlea are not labeled at any stage investigated, including hair cells (Fig. 5), support cells, hyaline cells, cuboidal cells and the tectorial membrane, which can be identified after E8 (Romanoff, 1960).

Expression of NGFR mRNA in acoustic ganglia

The ventromedial wall of the otic vesicle is the source of the acoustic ganglion cells (Alvarez et al. 1989). This part of the otic epithelium remains unlabeled between E2.5 and E4 (Fig. 2), and NGFR mRNA label was not detected in the path of migrating ganglion precursor cells in the early stages of ganglion formation, when these cells migrate from the thickened, ventromedial portion of the otocyst at E2.5–3.5 (Fig. 1A,B,D). In the acoustic ganglion, NGFR mRNA appears at E4 (Fig. 1B), which coincides with the onset of afferent innervation of the otocyst (E4–4.5, see below).
Fig. 3. (A–D) Transverse sections through the membranous labyrinth of 9- and 11-day-old chick embryos hybridized in situ for NGFR mRNA. (A) Section through saccule (s), utricle (u), and crista (c) of the superior semicircular canal at E9 shows heavy mesenchymal label subjacent to sensory epithelia (stippled) and epithelial label on the slopes of the crista organ (dorsal slope boxed). Scale bar: 0.5 mm. (B) Higher magnification of the region boxed in A. Arrows depict heavy expression of NGFR mRNA in the marginal epithelium of the crista. Note that label is sparse in the subjacent mesenchyme. Scale bar: 20 μm. (C) Section through the crista (c) of the posterior ampulla and the semicircular canals at E11. Note label in the mesenchyme subjacent to the epithelium, but lack of label in the sensory epithelium. The boxed region is shown at higher magnification in D. Scale bar: 0.5 mm. (D) Higher magnification of the crista (boxed in C). Arrows depict sensory epithelium that lacks NGFR mRNA label at this age. The planum semilunatum (PS) and the mesenchyme subjacent to the epithelium show autoradiographic label. Scale bar: 20 μm.

The vestibular and cochlear ganglia show moderate expression of NGFR mRNA between E4 and E13. At E4–7, neuronal ganglion cells could not be distinguished from stem cells or satellite (glial) cells. After E7, it appears that Schwann cells as well as neuronal ganglion cells may express NGFR mRNA. Throughout development, the cochleovestibular ganglion cells, though labeled above background, are less intensely labeled than some other cranial ganglia (e.g. the nodose and trigeminal ganglia, Fig. 5). Grain counts from cranial ganglia at E9.5 show that the number of autoradiographic grains above cochlear ganglion cells is less than 25% of that observed above trigeminal ganglion cells in tissue hybridized on the same slide (Fig. 5). Cochleovestibular ganglia remain lightly NGFR mRNA-positive in the posthatch chick. There
was no apparent difference in labeling intensity of vestibular and cochlear portions of the eighth nerve ganglion at P10. Due to low levels of NGFR mRNA expression, it was not possible to determine if Schwann cells contribute to the label in the posthatch ganglion.

Onset of afferent innervation

Earlier studies using silver impregnations rendered conflicting results about the time at which the acoustic nerve fibers enter the medulla in the chick embryo, between E2.5 and E3 (Windle and Austin, 1939) or after E3.5 (Whitehead and Morest, 1985b), and when they enter the otocyst (see Discussion). To determine how the onset of NGFR mRNA expression in the acoustic ganglion may relate to the onset of central and peripheral target innervation, we injected fluorescent carbocyanine tracers (Dil and DiO, von Bartheld et al. 1990a) into chick otocysts and medullas, respectively.

Dil-labeled central processes of the eighth nerve penetrate the ventral surface of the neural tube between 65–69 h (HH stage 18) and occupy a ventrolateral position in the medulla. At 70 h, labeled fibers extend rostrally in the brain to the level of the trigeminal ganglion. Some labeled fibers project caudally (50 μm) in the medulla. At E4.5, ascending eighth nerve fibers extend to the level of the anlage of the cerebellum, and descending fibers of the eighth nerve reach the level of the glossopharyngeal nerve in the medulla (data not shown). Labeled processes up to E4 obviously belong to vestibular but not cochlear portions of the acoustic ganglion, because cochlear ganglion cells have not yet become postmitotic at this age; the earliest final mitoses ('birthdays') of cochlear ganglion cells occur in chick embryos at E4 (D'Amico-Martel, 1982).

Because of the early ingrowth of central acoustic nerve fibers into the brain, the peripheral eighth nerve projections can be 'retrogradely' labeled by injections of Dil or DiO into the medulla after E2.5. The earliest labeled acoustic dendritic processes (peripheral to the ganglion) were observed at E3.5–4. Presumptive sensory epithelia can be recognized as an epithelial thickening and the appearance of an increased number of pyknotic cells (Knowlton, 1967). Epithelial thickenings develop anteroventrally (the anlage of the anterior and lateral cristae and macula utriculi; Knowlton, 1967) and postero-ventromedially (saccular region', Norris, 1892). The latter region divides subsequently and forms a posteroventral thickening (the anlage of the posterior crista and the macula neglecta; Knowlton, 1967), and a ventromedial thickening (the anlage of the papilla acustica basilars, the maculae sacculi and the lagenae; Knowlton, 1967). In the anteroventral and posteroventral anlagen, ingrowing eighth nerve fibers penetrate the basal lamina of the thickened epithelium between E4 and E4.5 (Fig. 6A; cf. Obata and Tanaka, 1988). The anterior ventral nerve fiber bundle divides and one portion grows medially, the other laterally (Fig. 6A). The posterior nerve fascicle passes the cochlear duct dorsally and enters the posteroventral anlage at E4.5. At this age, labeled nerve fibers extend ventrally and abut the ventromedial sensory anlage of the cochlear duct, but do not penetrate the sensory epithelium (Fig. 6B), as they do at 5.5 days of incubation (Fig. 6C). The first nerve fibers of the 'cochlear' branch begin to enter the otic epithelium of the cochlear duct between E5 and E5.5. Thus, the onset of NGFR mRNA expression in the acoustic ganglia coincides with the time at which their peripheral nerve fibers penetrate the otocyst epithelium.

Rat embryo

Expression of NGFR in the otic epithelium and mesenchyme

In rat embryos of 9 days of gestation the otic placode forms a pit. The otic epithelium invaginates at E10,
NGFR immunoreactivity in rostral portions of the otic epithelium (Fig. 7A), but absent in the ventral and dorsal pole of the otocyst including the endolymphatic projection. NGFR immunoreactivity in rostral portions of the otocyst extends laterally (Fig. 7A). At 12 days of gestation, the otocyst epithelium develops a mosaic pattern of NGFR expression. NGFR protein expression becomes restricted to a large area at the anterior pole and a smaller area at the posterior pole (Figs 7B). Maximal immunoreactivity is seen in the center of these two areas; it decreases towards the margins. Immunoreactivity appears to be 'intrinsic' to the epithelial cells, and does not resemble the immunoreactivity on nerve fibers expressing NGFRs that penetrate the epithelium as seen at later stages of development (Fig. 7C). At E13–15, neither extrinsic nor intrinsic immunoreactivity was observed in the otic epithelium.

In the third week of gestation, NGFR-labeled nerve fibers approach and contact the epithelia of the inner ear. Immunoreactive nerve fibers within the sensory epithelium are first seen in the ampullary organs (E16, Fig. 7C), but not until E18 in the otolith organs and the organ of Corti (data not shown). At E17, only pillar cells are NGFR-labeled in the developing organ of Corti (Fig. 8B, C). Pillar cells are specialized support cells in the organ of Corti that form the tunnel of Corti (Angelborg and Engström, 1972). The pillar cells show immunoreactivity throughout the cytoplasm, but reactivity is most intense in the apical portion of the cell facing the luminal surface (Fig. 8C). In sections through the cochlea processed for NGFR mRNA, we observed a similar distribution of label. NGFR mRNA is expressed in the pillar cells, with the most abundant levels of NGFR transcripts located in the luminal part of these cells (Fig. 8E). Neither NGFR protein nor NGFR mRNA was observed in the tectorial membrane (cf. Despré et al. 1991). Cochlear nerve fibers grow and pass the pillar cells on their way towards the outer hair cells at this age (E17). With Nomarski optics, presumptive nerve fibers are seen close to the base of the pillar cells at E17. Within the epithelium these nerve fibers do not appear to express NGFR, either in the cochlea (Fig. 8C) or in the epithelia of the otolith endorgans at this age (Fig. 7D). Outside the epithelium, cochlear nerve fibers are strongly NGFR-positive (Fig. 8A–C). At E18, NGFR-positive fibers approach both the inner hair cells and the outer hair cells (data not shown), as previously described for the innervation of outer hair cells in the organ of Corti of the postnatal rat (Despré et al. 1991). NGFR expression is not restricted to the efferent component of the cochlear nerve in the prenatal rat (see Discussion). NGFR immunoreactivity in developing pillar cells is transient, as evidenced by lack of label in the mature organ of Corti (Fig. 9E).

Numerous vestibular nerve fibers are strongly immunopositive for NGFR in the adult inner ear, including terminal fibers within the vestibular sensory epithelia (see below).

Expression of NGFR in the acoustic ganglia
NGFR immunoreactivity is faint in the acoustic
NGF receptors in the inner ear

Fig. 7. (A–D) Sections through the otic vesicle (OV) and through vestibular endorgans of rat embryos (11 to 17 days of gestation) immunolabeled for nerve growth factor receptor (NGFR). (A) Horizontal section. At 11 days, NGFRs are expressed in the rostromedial part of the otocyst. The acoustic ganglion (AG) shows faint label. No labeled fibers are seen that enter the otic epithelium or the neural tube (NT). (B) Horizontal section. At 12 days, NGFRs are expressed in a mosaic in the otic epithelium: the rostral pole is labeled more heavily than the caudal pole. The acoustic ganglion is labeled strongly; label is seen in fibers entering the neural tube (arrowheads), but not the otic epithelium. (C) Sagittal section. At 16 days, the first NGFR-labeled nerve fibers enter the sensory epithelium of the ampullary organs. No intrinsic label remains in epithelial cells after E13. (D) Sagittal section. Labeled nerve fibers approach the sensory epithelium of the utricle. Utricular hair cells have ciliary bundles (arrows) at this age. Note the absence of NGFR label within the sensory epithelium. Scale bars: 50 μm.

ganglion at E11 (Fig. 7A), and increases at E12 (Fig. 7B). Central processes leaving the acoustic ganglion and entering the neural tube are NGFR-positive at E12 (Fig. 7B). Nerve fibers approaching the otocyst were not seen at this age in the NGFR-labeled material. The acoustic ganglion is labeled differentially at E13. The peripheral portions of the ganglion are strongly NGFR-positive, sharply contrasting the non-reactive central portions of the ganglion. Peripheral acoustic nerve fibers are NGFR-positive at E13; labeled fibers reach the otic epithelium but do not extend significantly into the epithelium (data not shown). A similar pattern of NGFR protein expression was observed at E15. At this age, the resolution of immunoreactivity at the light microscopic level does not allow us to distinguish label on neuronal ganglion cells from label on Schwann cells, in either the ganglion or the acoustic nerve. At E17, NGFR immunoreactivity is confined to peripheral portions of the acoustic ganglia and the peripheral nerve; the cores of the vestibular and cochlear ganglia are not labeled (Fig. 8A). To determine if NGFR is expressed in cochlear ganglion cells but may be differentially distributed in cell bodies and nerve fibers, in situ hybridization was performed in adjacent sections from E17 and E18 rat embryos. NGFR mRNA is expressed throughout the vestibular and cochlear ganglia (Fig. 8D, F). Substantial levels of NGFR mRNA expression are found over ganglion cell bodies of the spiral (cochlear) ganglia, including those in the core of the ganglion (Fig. 8D, F). Whether or not Schwann cells also were labeled could not be determined with certainty.

In the adult rat, most vestibular ganglion cell bodies are NGFR-positive (Fig. 9A). In these ganglion cells, immunoreactivity is prominent in the cytoplasm surrounding the nucleus (data not shown); the large majority of Schwann cells is not labeled. Numerous NGFR-positive vestibular nerve fibers of various diameters can be traced into the sensory epithelia of both the otolith organs and the ampullary organs where they branch and terminate (Fig. 9B). Reaction product
Fig. 8. (A–F) Nerve growth factor receptor (NGFR) immunoreactivity (A–C) and NGFR mRNA expression (D–F) in the cochlea of a 17-day-old rat embryo. (A) Label is prominent in the cochlear nerve (CN), including the periphery but not the core of the cochlear (spiral) ganglia (CG). Small arrows point to the heavily labeled luminal projections of pillar cells (see B and C). Adjacent section through the area boxed on the right is shown at higher magnification in B. (B) The developing organ of Corti shows strong label in the cochlear nerve (CN) and in the pillar cells. See higher power micrograph in C. TM, tectorial membrane; V, spiral vessel. (C) Labeled pillar cell (PC) between the rows of unstained inner hair cells (IHC) and outer hair cells (OHC). Hair cells in the cochlea have not yet developed ciliary bundles at E17. Immunoreactivity is intense in the apical portion near the luminal surface of the PC. Nerve fibers approaching the base of the PC (pointer) and penetrating the sensory epithelium are not NGFR-labeled. (D) Dark-field view of NGFR mRNA in situ hybridization showing basal turn of the cochlea. Note substantial label throughout the spiral (cochlear) ganglion (CG) and in one distinct portion of the epithelium (arrow), the pillar cells (see E). (E) Bright-field image of the pillar cells (PC) showing heavy expression of NGFR mRNA. Note that highest levels of expression are in the apical portion of the PC adjacent to the outer hair cells (OHC). (F) Bright-field image of the cochlear ganglion (CG). Ganglion cell bodies throughout the ganglion show substantial numbers of autoradiographic grains (arrows). The NGFR protein appears to be distributed in the nerve fibers rather than the cell bodies (compare with A). Scale bars: 100 µm (A, D), 10 µm (B, C, E, F).

in thick fibers is often restricted to the outer portion of the nerve fiber; the core remains unlabeled. In the adult cochlear (spiral) ganglia, NGFR immunoreactivity was not detected in the cell bodies. The majority of cochlear nerve fibers also lacks NGFR expression in the mature cochlea; only a small subpopulation of cochlear nerve fibers is strongly immunopositive (Fig. 9D). We counted approximately 40 thick and 150 thinner labeled fibers in one horizontal section through the basal turn of the cochlea; a smaller number of labeled fibers was observed in the apical turns of the cochlea. Some of these labeled fibers were traced peripherally towards the habenular area (Fig. 9E); immunoreactivity did not extend into sites of termination in the organ of Corti so
NGF receptors in the inner ear

that we do not know if these fibers contact inner hair cells, outer hair cells or both. Centrally, the pathway of labeled and unlabeled cochlear nerve fibers merges with the vestibular nerve. It is not clear whether the NGF-labeled cochlear nerve fibers are efferent or afferent fibers. Lack of NGF immunoreactivity in cochlear (spiral) ganglion cell bodies and in the large majority of cochlear nerve fibers at this age may suggest that these fibers are efferent; however, regions of origin of the olivocochlear bundle (White and Warr, 1983; Warr et al. 1986) do not appear to express NGFRs in the adult rat brain (Koh et al. 1989; Pioro and Cuello, 1990) as they do in the perinatal rat (Després et al. 1991). NGFR-positive nerve fibers projecting to the adult organ of Corti may originate from ganglion cells located in the vestibular ganglion, and some of these fibers may be identical with the 'giant fiber type' described by Perkins and Morest (1975).

Discussion

Trophic and inducing factors may play an important role in the formation of the inner ear. The development of the otocyst is influenced by the neural crest, mesoderm and the neural tube (Harrison, 1936; Hall, 1939, 1941; Trampusch, 1941; Detwiler and van Dyke, 1950; Yntema, 1950, 1955; Rubel, 1978). Both the otic vesicle and medulla provide trophic support for the acoustic ganglion (Van De Water and Ruben, 1984; Ard et al. 1985; Zhou and Van De Water, 1987; Van De

Fig. 9. (A–E) Nerve growth factor receptor (NGFR) immunoreactivity in the inner ear of the adult rat. Sections were lightly counterstained with methyl green. (A) Cryosection shows strongly labeled vestibular ganglion (VG) cells. (B) Immunoreactive fiber branches (arrowhead) in the sensory epithelium of the crista (ampullary organ). (C) Paraffin section of the cochlear ganglion (CG). Note absence of label in the cell bodies. One labeled nerve fiber (arrow) heads towards the habenular region. (D) Many thick and a few thin nerve fibers (arrows) are labeled in the cochlear nerve (CN). (E) Paraffin section through the organ of Corti. A few labeled fibers (arrow) approach the organ. The organ proper shows no immunoreactivity. IHC, inner hair cell; IPC, inner pillar cell; OPC, outer pillar cell; L, spiral limbus. Scale bars: 20 μm (A–D); 50 μm (E).
Water, 1988; Hauger et al. 1989), but the relevant trophic molecule(s) have not been identified.

Recent studies implicated functions for the trophic molecule nerve growth factor (NGF) in the developing inner ear (Finn et al. 1987; Raivich et al. 1987; Després et al. 1988, 1991; Yan and Johnson, 1988; Bernd and Represa, 1989; Hallböök et al. 1990; Lefebvre et al. 1990; Mayer et al. 1990; Van De Water et al. 1991). The developmental distribution of NGF receptors (NGFRs) in the early rat inner ear has not been investigated, in particular at the stage of putative NGF function (E12–13, Lefebvre et al. 1990) and the localization of NGFRs and their developmental expression is controversial in the avian otocyst (Raivich et al. 1987; Bernd and Represa, 1989; Hallböök et al. 1990; Represa et al. 1991). We investigated the distribution of NGFR expression in the developing inner ear of chicks and rats to determine which tissues and cell types may be regulated by NGF or NGF-like molecules. The riboprobe and the antibody that we used to characterize NGFR expression identifies a 75×10^3 M_r protein, the 'low-affinity NGFR'. This protein appears to be a common component of the receptor for all three neurotrophins (NGF, BDNF, NT-3), whereas specificity for each neurotrophin may be determined by association with a second component, presumably a member of the trk proto-oncogene family of tyrosine protein kinase receptors (Hempstead et al. 1991; Bothwell, 1991). Therefore, it is possible that the NGF-like molecules BDNF and NT-3 bind to the NGFR whose expression we describe (Rodriguez-Tebar et al. 1990; Hohn et al. 1990; Maisonpierre et al. 1990).

**Early differentiation of the otocyst**

Experimental studies of amphibian otocysts showed that the otic epithelium becomes polarized and determined during the otic pit stage (E2–2.75 in the chick) (Harrison, 1936, 1945; Hall, 1939, 1941; Yntema, 1955). Early otocyst epithelial cells have been investigated in chick embryos with various techniques. These cells appear homogeneous before E4, at both the ultrastructural (Ginzberg and Gilula 1979, 1980; Whitehead and Morest, 1985b) and lightmicroscopic levels (Richardson et al. 1987; Obata and Tanaka, 1988; Alvarez and Navascués, 1990; cf. also Raphael et al. 1987, for mammalian otocysts). The heterogeneous distribution of NGFR mRNA and NGFR in the early otocyst is unmistakable in our material (Figs 2, 7A,B).

Information about the normal distribution and the developmental expression of growth factor receptors in the otocyst will be useful to elucidate functions of prospective growth factors in otocyst differentiation under experimental conditions (cf. Represa et al. 1988; Swanson et al. 1990). Expression of NGFR mRNA in the chick otic epithelium (E3–4.5) precedes by several days the differential expression of cell adhesion molecules (after E5, Richardson et al. 1987; Raphael et al. 1988) and related proteins (Obata and Tanaka, 1988) as well as gamma-aminobutyric acid (after E6, von Bartheld, 1990). Besides the NGFR gene, two other genes appear to be differentially expressed in the early otic epithelium: the fibroblast growth factor-related proto-oncogene int-2 is expressed in presumptive sensory regions of the early mouse otocyst (Wilkinson et al. 1988, 1989), and preliminary experiments from our laboratory suggest that the epidermal growth factor (EGF) receptor gene is expressed in the E3–4 chick otocyst in a pattern that is in part complementary to that of the NGFR gene (C.S. von Bartheld, unpublished). The similarity of the mosaic pattern of NGFR expression in chick and rat otocysts (Figs 1, 2 and 7) may indicate that this pattern is evolutionarily conserved.

An important question that cannot yet be answered is whether or not NGFR mRNA and NGFRs are expressed in early otic epithelial cells that generate specific cell types or specific structures of the inner ear. The anterior–posterior NGFR expression in the otocyst (Fig. 1B) resembles the early anterior–posterior invagination pattern (Kuratani et al. 1988, their Fig. 5b). Unfortunately, the fates of distinct portions of the early otocyst are not clear. Sensory epithelia are often believed to derive exclusively from the ventromedial portion of the otocyst (Norris, 1892; Fuchs, 1923; Guareschi, 1930; Knowlton, 1967; Orr, 1975). However, fate mapping studies indicate that this is not the case; dorsoposterior portions of the otic epithelium also contribute to the formation of sensory epithelia (Kaan, 1926; Li et al. 1978; Noden, 1984). Detailed cell lineage studies of the otocyst are required before NGFR expression can be correlated with prospective sensory regions of the inner ear. It is clear, however, that there is no or very little expression of NGFR mRNA in the ventromedial portion of the chick otocyst (present study, cf. Hallböök et al. 1990) which gives rise to the acoustic ganglion and part of the utricle (Noden, 1984), nor is NGFR mRNA expressed in cells migrating towards the acoustic ganglion. Expression of NGFR mRNA has ceased in sensory epithelia when the first hair cells have differentiated sufficiently to be identified. It cannot be ruled out that NGF or NGF-like molecules may affect hair cell precursors prior to hair cell differentiation.

**Alternating phases of NGFR mRNA expression in the otic mesenchyme and epithelium**

Periepithelial mesenchyme and epithelia of the inner ear show a conspicuous alternation and reciprocal pattern of NGFR mRNA expression in the chick embryo. Following initial NGFR mRNA expression in the mesenchyme at E2–3, expression ceases in the mesenchyme from E4–6. At this time, epithelial expression becomes pronounced, followed by a decrease of expression in the epithelium and a marked increase of NGFR mRNA expression in the mesenchyme surrounding the epithelia. NGF-like immunoreactivity has been reported in developing bony tissue of the chick embryo (Frenkel et al. 1990). Are NGF or NGF-like molecules involved in epithelial/mesenchymal induction? The otocyst induces chondrogenesis in periotic mesenchyme tissue (McPhee and Van De Water, 1986). Generally, growth factors other than
NGF have been implicated in interactions between mesenchymal and epithelial tissues, e.g. TGF beta (Lehnert and Akhurst, 1988). The function of mesenchymal NGFR expression surrounding epithelial tissue is not clear, but this phenomenon may have a general significance because it is seen in a variety of mesenchymal tissues bordering epithelial structures in avian and mammalian embryos (M. Bothwell, unpublished). It has been suggested that NGFR may be expressed to prevent spread or diffusion of secreted NGF (Taniuchi et al. 1986; Wyatt et al. 1990).

**NGF and NGFRs in sensory epithelia**

Little is known about the cells that produce NGF or NGF-like molecules in the developing inner ear. Lefebvre et al. (1990) demonstrated NGF or NGF-like activity in homogenates of E12–13 rat otocysts, but not in otocysts from later stages of development. In agreement with these data, our laboratory obtained preliminary immunocytochemical evidence for NGF-like immunocytochemistry in early (E9–11) rat otocysts (S. Patterson, unpublished). NGF-like proteins have been localized to hair cells in the organ of Corti of newborn rats by immunocytochemistry (Després et al. 1988), and it was suggested that release of NGF may be important for afferent and efferent innervation of hair cells. Preliminary work in our laboratory indicates that cells in the early inner ear sensory epithelium of mouse express BDNF and NT-3 mRNA but not NGF mRNA (L. Schecterson and M. Bothwell, unpublished).

Developing hair cells may release neurotrophins during afferent innervation, and these growth factors may provide trophic and trophic support to ingrowing peripheral processes from acoustic ganglia (Lefebvre et al. 1990; Van De Water et al. 1991). NGF-expressing pillar cells are located between the row of inner and outer hair cells (Fig. 8C; cf. Kikuchi and Hilding, 1965; Angelborg and Engström, 1972). If inner hair cells and outer hair cells release two different neurotrophins, one may speculate that NGFR on pillar cells could assist in keeping the two factors separated. This may help ingrowing nerve fibers to distinguish the two cell types and to develop their final mode of innervation (Lenoir et al. 1980).

Recent studies of NGF-binding concluded that NGFR are expressed on developing hair cells in the quail inner ear, with maximal levels of expression in the cochlear duct at E10, and that hair cells may be responsive to or dependent on NGF (Represa et al. 1991). Our in situ hybridization results in the chick embryo, however, suggest that NGF expression in the second week of incubation is restricted to the innervating eighth nerve fibers, and that NGFR are not expressed in hair cells when they undergo final mitosis (at E6–9 in the chicken cochlea; Cohen and Fermin, 1978; Katayama and Corwin, 1989). Our data do not exclude the possibility that precursors of hair cells may express NGFR and that these cells may respond to NGF. Hair cell regeneration in the avian cochlea has recently been demonstrated (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). Since NGF can act as a mitogen in the quail otocyst (Represa and Bernd, 1989), we tested the hypothesis that NGF or NGFR may be involved in hair cell regeneration, e.g. by an up-regulation or re-expression of NGFR in stem cells and precursor cells of regenerating hair cells. Preliminary results do not show any indication of increased expression of NGFR in the organ of Corti in P10–P14 chicks following either noise or drug damage of the hair cells (C.S. von Bartheld, E.C. Österle, and E.W Rubel, unpublished).

**NGFR expression in acoustic ganglia and nerve fibers**

From in vitro studies, it is known that the peripheral as well as the central targets provide trophic support for acoustic ganglion cells (Ard et al. 1985; Zhou and Van De Water, 1987; Hauger et al. 1989). The nature of the hypothetical trophic molecule(s) is not known. The eighth nerve ganglion cells are generally believed not to respond to NGF (Ard, 1982; Pearson et al. 1983; Davies and Lindsay, 1985; Lindsay et al. 1985), and NGFR label of the eighth nerve and acoustic ganglion has been associated with low-affinity receptors on Schwann cells (Yan and Johnson, 1988). Recently, Lefebvre and coworkers provided evidence that NGF has a trophic effect on early rat acoustic ganglion cells in culture (Lefebvre et al. 1990; Van De Water et al. 1991), but other investigators implicated NGFR with the development of the efferent innervation of the organ of Corti rather than the afferent system (Després et al. 1991). Our data provide unequivocal evidence for the expression of NGFR in early acoustic ganglia as well as in later stages when eighth nerve fibers enter the sensory epithelia of the inner ear endorgans. The developmental loss of NGFR immunoreactivity in cell bodies of the rat cochlear ganglion during the third week of gestation is in remarkable contrast to the maintained expression of NGFR mRNA in these cells (Fig. 8F) and the heavy immunolabel in peripheral processes of the cochlear nerve (Fig. 8A, B). These data strongly suggest that receptor proteins can be differentially distributed in different compartments within neurons; therefore, the lack of immunoreactivity in one cellular compartment does not exclude the possibility that expression of the NGFR protein has occurred in another compartment of the same cell.

The acoustic ganglia of both avian and mammalian embryos show substantial levels of NGFR expression (present study; Raivich et al. 1987; Yan and Johnson, 1988; Bernd and Represa, 1989; Hallböök et al. 1990; Mayer et al. 1990; Van De Water et al. 1991). Information about the possible significance of growth factors can be obtained by comparing the timing of growth factor and receptor expression with the timing of target innervation (Wyatt et al. 1990). In chick and rat, NGFR expression commences early (at E4 and E11, respectively). Does the onset of NGFR expression in the acoustic ganglia correlate with the onset of peripheral or central target innervation? The arrival of acoustic nerve fibers in their targets is not clear in
rodent embryos (Lenoir et al. 1980; Mbiene et al. 1988), but this issue has been investigated extensively in chick embryos.

The onset of innervation (fiber ingrowth) in the avian cochlea was described unequivocally (E4–5; Whitehead and Morest, 1985a,b; Hemond and Morest, 1986), but the numerous attempts to determine the onset of afferent vestibular innervation rendered conflicting results, ranging from E2.75 to E6 in chick embryos: E2.75: (Knowlton, 1967); E3: (van Campenhout, 1937); E3–4: (Proctor and Proctor, 1967); after E3.5: (Whitehead and Morest, 1985); E4: (Baumann, 1947; Vazquez-Nin and Sotelo, 1968); after E4: (Ramon y Cajal, 1929/1960; Obata and Tanaka, 1988); E4.5: (Ginzberg and Gilula, 1979, 1980); E6: (Visintini and Levi-Montalcini, 1939; Friedmann, 1969). In the present study, by using the fluorescent dyes Dil and DiO, we found that the first peripheral processes of the eighth nerve penetrate the otic epithelium between E4 and E4.5. The onset of NGFR mRNA expression in the acoustic ganglion (E4) coincides with the onset of peripheral target innervation. Ingrowth of central acoustic nerve processes into and within the CNS, on the other hand, is substantial between E2.5 and E3.5 (see Results) and precedes NGFR mRNA expression in the acoustic ganglion (E4). The timing of NGFR mRNA expression in the acoustic ganglion of chick embryos is consistent with a chemotactic/trophic function of NGF in peripheral targets and is similar to the increase of NGFR expression during target innervation in trigeminal nerve development (Wyatt et al. 1990).

In many areas of the nervous system, Schwann cells express NGFRs (Zimmermann and Sutter, 1983) which may be involved in the development of neural pathways (Johnson et al. 1988), in particular by regulating cell adhesion molecules (Seilheimer and Schachner, 1987). With both in situ hybridization and immunocytochemistry, we found it difficult to determine in the early embryo if the NGFR label is in Schwann cells or neuronal cells or both (cf. Yan and Johnson, 1988). In the E17 rat embryo, strong NGFR expression in the peripheral nerve contrasts with lack of NGFR label on many ganglion cells and Schwann cell bodies. The significance of the reappearance or maintenance of NGFRs in vestibular ganglia (Koh et al. 1989; present study) and in a small subpopulation of cochlear nerve fibers in the adult rat remains to be determined. NGF or NGF-like molecules may serve a multitude of functions for distinct aspects of inner ear development and maintenance. We hope that our study will provide a useful anatomical framework for future studies on functions of NGF or NGF-like molecules in the development of the inner ear.

We thank Lorraine Gibbs and Phyllis Harbor for excellent technical assistance, and Margaret Byers, Glen MacDonald, and Mutsumi Taiji for technical advice. We also thank Soo Borson for assistance with the adult rat experiments, and Moses Chao for the rat NGF receptor cDNA and Charles Chandler who provided the hybridoma (192) producing the monoclonal antibody against the rat NGF receptor. We are indebted to Paul Schwartz and Janet Clardy for their photographic expertise. Leslyann Schecterson shared unpublished results, and Elizabeth Oesterle made valuable comments on the manuscript. Our research was supported by NIH grants DC 00019 and NS 08990 (C.S.v.B.), GM 07108 (S.L.P.), GM 07270 (J.G.H.), HL 43397 (M.B., E.F.W.), NS 23343 (M.B.), and DC 00395 (E.W.R.).

References


central nervous system. Effect of colchicine and correlation with the cholinergic system. II. Brainstem, cerebellum and spinal cord. Neuroscience 34, 89–110.


(Accepted 26 June 1991)