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Ontogenetic Expression of Trk Neurotrophin Receptors in the Chick Auditory System

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ABSTRACT

Neurotrophins and their cognate receptors are critical to normal nervous system development. Trk receptors are high-affinity receptors for nerve-growth factor (trkA), brain-derived neurotrophic factor and neurotrophin-4/5 (trkB), and neurotrophin-3 (trkC). We examine the expression of these three neurotrophin tyrosine kinase receptors in the chick auditory system throughout most of development. Trks were localized in the auditory brainstem, the cochlear ganglion, and the basilar papilla of chicks from embryonic (E) day 5 to E21, by using antibodies and standard immunocytochemical methods. TrkB mRNA was localized in brainstem nuclei by in situ hybridization.TrkB and trkC are highly expressed in the embryonic auditory brainstem, and their patterns of expression are both spatially and temporally dynamic. During early brainstem development, trkB and trkC are localized in the neuronal cell bodies and in the surrounding neuropil of nucleus magnocellularis (NM) and nucleus laminaris (NL). During later development, trkC is expressed in the cell bodies of NM and NL, whereas trkB is expressed in the nerve calyces surrounding NM neurons and in the ventral, but not the dorsal, dendrites of NL. In the periphery, trkB and trkC are located in the cochlear ganglion neurons and in peripheral fibers innervating the basilar papilla and synapsing at the base of hair cells.The protracted expression of trks seen in our materials is consistent with the hypothesis that the neurotrophins/tyrosine kinase receptors play one or several roles in the development of auditory circuitry. In particular, the polarized expression of trkB in NL is coincident with refinement of NM terminal arborizations on NL. J. Comp. Neurol. 413:271–288, 1999.

Indexing terms: cellular protein sorting; nucleus laminaris; nucleus magnocellularis; basilar papilla; brain-derived neurotrophin factor; neurotrophin-3

Classically, neurotrophins and their cognate receptors were considered mediators of target innervation and selective neuronal survival (Levi-Montalcini and Angeletti, 1968). Since the discovery of the first neurotrophin, nerve growth factor (NGF), three other neurotrophins (brain-derived neurotrophic factor (BDNF), neurotrophin-4/5 (NT-4/5), and neurotrophin-3 (NT-3) have been discovered, and our understanding of neurotrophins has broadened sufficiently such that we are beginning to identify the numerous aspects of neuronal development, maintenance, and repair that neurotrophins can mediate. These varied aspects include cell differentiation (reviewed in Chao, 1992; Lachyankar et al., 1997), cell survival (reviewed in Levi-Montalcini, 1987), axonal growth and morphology (Segal et al., 1995; San José et al., 1997), dendritic morphology (Cohen-Cory and Fraser, 1994, 1995; McAllister et al., 1995, 1997), synaptic efficacy (Patterson et al., 1996; Suen

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et al., 1997), ion channel expression (Jimènez et al., 1997), neuronal plasticity (Lindholm et al., 1994), and cell death (Carter et al., 1996; Frade et al., 1996; Bredesen and Rabizadeh, 1997; for review: Frade and Barde, 1998). Moreover, the presence of neurotrophins and their receptors has been demonstrated in the adult brain (Friedman et al., 1991; Wu et al., 1996; Conner et al., 1997; Yan et al., 1997), suggesting that neurotrophins are necessary both for the initiation and for the maintenance of synaptic circuitry.

Studies of developing sensory systems have suggested a ubiquitous involvement of neurotrophins in the establishment of the intricate patterns of connectivity that underlie sensory function. Neurotrophins and their transmembrane protein tyrosine kinase receptors (trkA: NGF; trkB: BDNF and NT-4/5; trkC: NT-3) have been observed in the developing olfactory (Holcomb et al., 1995; Roskams et al., 1996), gustatory (Nosrat et al., 1996, 1997; Fritzsch et al., 1997b; Zhang et al., 1997), somatosensory (Nosrat et al., 1997), and visual (Allendoerfer et al., 1994; Cohen-Cory and Fraser, 1994, 1995; Lauterborn et al., 1994; von Bartheld et al., 1996a,b; Cabelli et al., 1997) systems. Studies of the auditory system have demonstrated the presence of neurotrophins and trks in amphibians (Don et al., 1997), avians (Bernd and Represa, 1989; Represa et al., 1991; von Bartheld et al., 1991; Represa et al., 1993; Bernd et al., 1994; Hallböök and Fritzsch, 1997; Pirvola et al., 1997), and mammals (Després et al., 1991; von Bartheld et al., 1991; Pirvola et al., 1992, 1994; Ylikoski et al., 1993; Schecterson and Bothwell, 1994; Wheeler et al., 1994; Hafidi et al., 1996; Knipper et al., 1996; Vàzquez et al., 1996; Wiechers et al., 1999). Furthermore, the exigency of neurotrophins and trks in the stabilization of inner ear innervation of mammals has been established (Ernfors et al., 1994, 1995; Shimmang et al., 1995; Fritzsch et al., 1997a; and for review, see Fritzsch et al., 1997c).

In contrast to the abundance of studies describing the expression and the biological significance of neurotrophins and their associated receptors in the mammalian auditory system, fewer studies to date have addressed these same questions in the avian auditory system. The present study examines the distribution of the three trk receptors in the cochlear ganglion, basilar papilla (cochlea), and auditory brainstem nuclei of the chick during embryonic development. We describe the dynamic changes in the patterns of trk protein expression throughout most of the embryonic period and correlate trk expression with established ontogenetic events.

MATERIALS AND METHODS

Tissue preparation

Fertilized White Leghorn chicken (*Gallus domesticus*) eggs were purchased from H & N International (Redmond, WA). Eggs were incubated at 37–38°C and a relative 80% humidity. Over 150 embryos were used for this study. The Animal Care and Use Committee of the University of Washington approved all procedures described below.

The expression of trk receptors in White Leghorn chickens was followed from embryonic day 5 (E5) until just prior to hatching (E21). Embryonic age was determined by staging according to Hamburger and Hamilton (1951) and is expressed in embryonic days. All data reported were confirmed on a minimum of four animals at each age reported. For tissue processing, the embryo was removed from the egg, decapitated, and the tissue (brainstem or temporal bone encasing the cochlear ganglion and basilar papilla) was quickly dissected in Hank's buffered salt solution (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD). The tissue was immersion-fixed overnight in methacarn fixative (60% methanol, 30% chloroform, and 10% glacial acetic acid) in preparation for immunocytochemical or histological processing. In some cases, the tissue was immersion-fixed with 4% paraformaldehyde. In both the hindbrain and the periphery, the methacarn fixation increased the overall intensity of staining while equally preserving the anatomical structure of the tissue. Paraformaldehyde-fixed tissue was rinsed in phosphate-buffered saline (PBS, 0.01 M, pH 7.4). The methacarn-fixed tissue was rehydrated through a series of decreasing concentrations of methanol, then rinsed in PBS. Hindbrain tissue was embedded in agar and sectioned on a vibratome into three alternating sets of 50 µm sections. Initially, in each case, these three sets were simultaneously processed for trkA, trkB, and trkC immunoreactivity. In later experiments, tissue was not processed for trkA immunoreactivity. Instead, the third set was reserved for Nissl staining with thionin. The isolated temporal bone was cryoprotected in 30% sucrose in PBS until it sank. The tissue was flash-frozen in dry ice-cooled heptane, covered in OCT compound (Sakura Finetek USA Inc., Torrance, CA), cryostat-sectioned at 14 µm, and thaw-mounted onto chrome alum-subbed slides, again generating three sets of alternating sections. The sections were stored at −20°C until processed.

Trk antibodies

The trk antibodies used in this study were kindly provided by Drs. Frances Lefcort (Montana State University, Bozeman, MT) and Louis Reichardt (Howard Hughes Medical Institute, University of California, San Francisco, CA). The antibodies are rabbit polyclonal antibodies specifically directed against the entire extracellular domain of trkA, trkB, or trkC chick tyrosine kinase neurotrophin receptors. They have been previously characterized (Lefcort et al., 1996; Oakley et al., 1997).

Immunocytochemistry

Standard immunocytochemical methods were used for immunolabeling trk receptors in the chick inner ear and auditory brainstem. The brain tissue was processed as free-floating sections in multiwell plates. The temporal bone tissue was processed on slides because it was thaw-mounted during the cutting procedure. Although the protocol for both was similar, longer incubation times for the thicker brain tissue were used; these times are detailed below. All steps were performed at room temperature, with the exception of the primary antibody incubation.

To quench endogenous peroxidase activity, the sections were initially incubated for 1.5 hours in Tris-buffered saline (TBS: 25 mM Tris, pH 7.4, 150 mM NaCl) containing 3% hydrogen peroxide and 10% methanol. The sections were rinsed in a series of 25 mM Tris buffer washes, then preincubated for 3 hours in a blocking solution to prevent nonspecific IgG binding (TBS, 0.03% Triton-X-100, 10% normal goat serum [Vector Laboratories, Burlingame, CA], and 0.1% bovine serum albumin [Sigma, St. Louis, MO]). The sections were subsequently incubated overnight in primary antibody (1:3,000) at 4°C. Negative control sections were incubated in the same solution with the pri-

mary antibody omitted. Sections were rinsed in a series of buffer washes, then incubated in biotinylated goat anti-rabbit secondary antibody (Vector; 1:200) for 4 hours. After a series of washes in buffer, tissue was incubated for 1.5 hours in ABC solution from a Vectastain Elite ABC-peroxidase kit (Vector) in order to amplify the immunoreactivity. The tissue was rinsed in several 50 mM Tris/HCl buffer washes (pH 7.6), then antibody labeling was visualized by using diaminobenzidine tetrahydrochloride (DAB) as the chromagen (0.05% DAB, 0.003% hydrogen peroxide, 50 mM Tris). After DAB processing, sections were washed in a series of 50 mM Tris washes and given a final rinse in distilled water. The brain sections were mounted onto chrome alum-subbed slides and allowed to dry overnight. All tissue sections were dehydrated in a series of increasing ethanols and xylene and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ).

Cryostat sections through the dorsal root ganglia (DRG) of an E8 chick were routinely included in the immunolabeling experiments as positive controls. In each case, we found restricted patterns of trk protein expression in the DRG similar to that described previously (Lefcort et al., 1996; Oakley et al., 1997).

TrkB digoxigenin-labeled RNA probes

TrkB full-length cDNA cloned into pBluescript was generously provided by Drs. Kristin Boeshore and Thomas Large (Case Western University, Cleveland, OH). The plasmids were linearized and cRNA probes synthesized using T_3 and T_7 RNA polymerase. To enhance probe penetration into the tissue sections, probes were hydrolyzed to an average of 250 bp in length.

In situ hybridization

E12 embryos were removed from the egg, decapitated, and the brainstem was quickly dissected in cold PBS with 2 mM EGTA; 400 µm coronal sections generated with an "egg-slicer" guillotine (Katz, 1987) were fixed in 4% formaldehyde in PBS/2 mM EGTA for 2 hours. Sections were washed in PBS containing 0.1% Tween 20 (PTW), then in 50% MeOH/PTW, and then stored in 100% MeOH at -70°C until processed. The in situ hybridization protocol we followed was based on Henrique et al. (1995). All steps were performed at room temperature unless otherwise noted. The sections were rehydrated through a methanol series, rinsed in PTW, and treated with 10 µg/ml proteinase K in PTW for 15–20 minutes. The sections were then washed in PTW and postfixed for 20 minutes in PTW containing 4% formaldehyde and 0.1% glutaraldehyde. Following several washes in PTW, the sections were rinsed in 1:1 hybridization mix/PTW. The hybridization mix contains 50% formamide, 1.3× saline/sodium citrate buffer, 5 mM EDTA, 50 µg/ml yeast RNA, 0.2% Tween-20, 0.5% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), 100 µg/ml heparin. Sections were rinsed in hybridization mix, then incubated in fresh hybridization mix for 1 hour at 65°C. After 1 hour, prewarmed hybridization mix containing the digoxigenin-labeled RNA probe was added and the tissue was incubated overnight at 65°C. The tissue was washed for 1 hour in several changes of prewarmed hybridization mix, washed for 10 minutes in prewarmed 1:1 hybridization mix/ MABT buffer (100 mM maleic acid disodium salt, 150 mM NaCl, 0.1% Tween-20, pH 7.5), and finally, washed in MABT alone. The sections were then incubated for 1 hour in MABT with 2% Boehringer blocking reagent (BBR: Boehringer Mannheim Biochemicals Inc., Indianapolis, IN) added. In a second blocking step, sections were incubated for 1 hour in 20% heat-treated goat serum in MABT/2% BBR. The tissue was incubated overnight at 4°C in an anti-digoxigenin antibody (Boehringer Mannheim) diluted 1/2,000 in MABT/2% BBR/20% serum. The next day, three postantibody rinses were followed by three 1-hour washes in MABT. The tissue was then rinsed twice in NTMT buffer (5 M NaCl, 2 M Tris HCl, pH 9.5, 2 M $MgCl_2$, 10% Tween-20) before incubating the tissue in the color reagent containing 3-nitro blue tetrazolium salt, 5-bromo-4-chloro-3-indolyl phosphate, and levamisole in NTMT. The sections were allowed to react at room temperature for up to 24 hours, then washed several times in PTW. The tissue was refixed in 4% formalin/PTW for 2 hours, rinsed several times in PTW, and stored at 4°C in PTW/0.1% sodium azide.

Analysis and imaging

The tissue was visualized under light microscopy on a Leitz Aristoplan microscope by using standard brightfield and Nomarski optics. Sections were photographed with Velvia color slide film (Fuji Photo Film Co., Ltd., Tokyo, Japan). Digital photomicrographs were generated by scanning the slides into Adobe Photoshop 4.0 (Adobe, Mountain View, CA) with a Nikon LS-1000 film scanner. Digitized Photoshop images were enhanced for contrast and we attempted to make parallel enhancement to plates that were compared within the same figure. Images were printed with a dye-sublimation Phaser printer (Tektronix, Beaverton, OR).

RESULTS

The specificity of the polyclonal antibodies for the chick trk neurotrophin receptors has been demonstrated previously using several approaches (see Lefcort et al., 1996; Oakley et al., 1997). In the present studies, we routinely ran both positive and negative controls to assay the specificity of the trk antibodies (see Fig. 1). Beyond these controls, however, the spatially and temporally distinct patterns of immunolabeling of each trk lend confidence to the specificity of the antibodies.

Trk protein immunolabeling is present in the auditory system of the chick throughout the embryonic period we assessed and into posthatch development, though the pattern of labeling differs remarkably among receptors, among tissues, and across ages. In general, trkB (the primary receptor for brain-derived neurotrophic factor and neurotrophin 4/5) and trkC (the primary receptor for neurotrophin-3) immunolabeling is prevalent at all ages both peripherally and centrally. In contrast, trkA (the primary receptor for nerve growth factor) immunolabeling is distinctly absent in all tissues at the ages studied.

Trk immunolabeling in the cochlear ganglion and basilar papilla

E5– 8. The peripheral processes of cochlear ganglion cells (afferent fibers) invade the sensory epithelium (the basilar papilla) predominantly during the period between E5 to E7 (Whitehead and Morest, 1985a). TrkA immunolabel is not detectable in the auditory periphery at this age (Fig. 2A), nor in successively older animals. TrkB staining in the cochlear ganglion is very light and is distributed throughout the neurons of the cochlear ganglion (Fig. 2B).

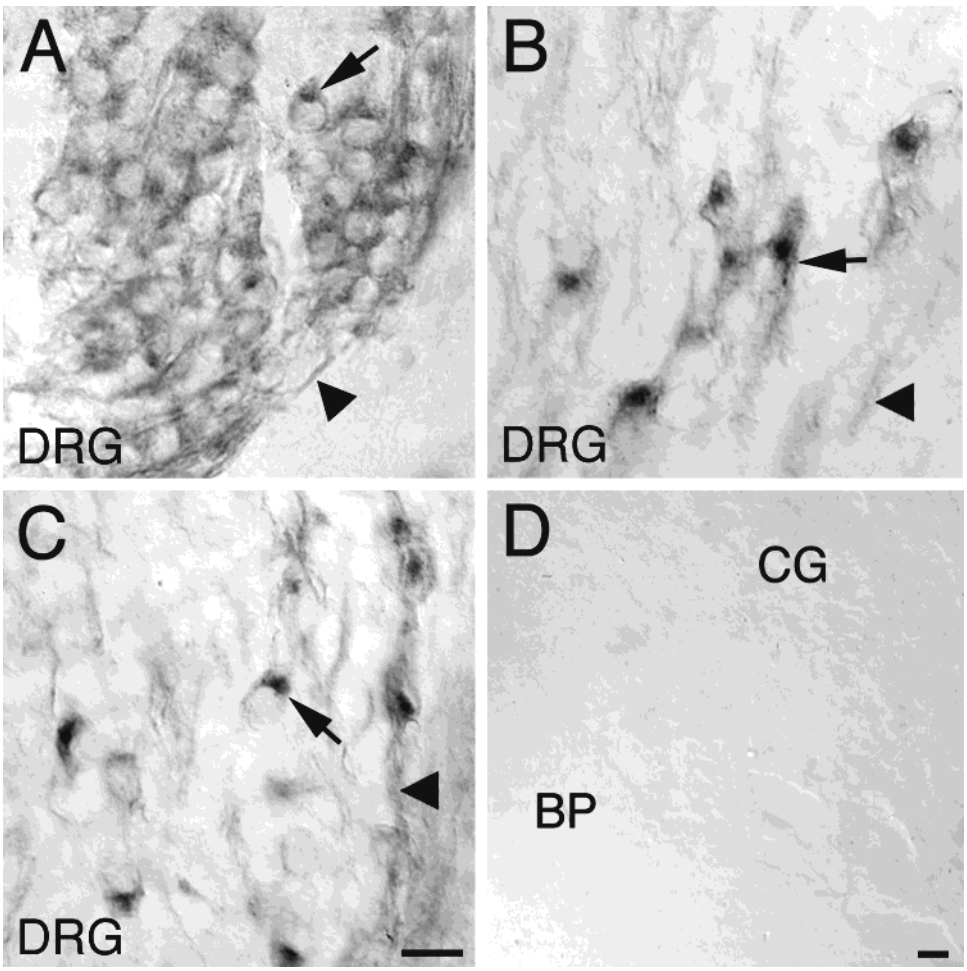

Fig. 1. Positive and negative controls for trk immunolabeling. Antibodies to chick trkA (**A**), trkB (**B**), and trkC (**C**) show patterns of staining in transverse sections of the embryonic day (E)6 dorsal root ganglion (DRG) that are similar to those described previously. TrkA and trkB antibodies label neurons (arrows) and fibers (arrowheads) in the lateral portion of the DRG, whereas trkA antibodies label neurons and fibers throughout the ganglion. In negative controls (**D**) in which primary antibody is omitted from the reaction, no staining is present in transverse sections of the basilar papilla (BP) or the cochlear ganglion (CG). Photomicrographs were taken under Nomarski optics. Scale bars = 10 μm.

In addition, there is light immunolabeling along the superior edge of the basilar papilla (arrow, Fig. 2B), which is the portion of the sensory epithelium that will be occupied primarily by tall hair cells once hair cell differentiation has taken place (Fischer, 1992). This light labeling appears fibrous, and therefore, we suggest that it is present in a handful of trkB-labeled fibers that have penetrated the basilar papilla at its superior edge. These trkB-labeled fibers course radially through the basilar papilla toward the lumenal edge of the epithelium. In contrast, trkC appears to be the most strongly expressed high-affinity neurotrophin tyrosine kinase receptor in the

Fig. 2. Trk immunolabeling in the cochlear ganglion (CG; stippled outline) and the basilar papilla (BP) of the embryonic chick. A–C: Embryonic day (E)6.5. Sections through the midapical region of the BP. **A:** Absence of trkA labeling in the auditory periphery. **B:** TrkB immunolabel is light in the CG (stippled outline), and it is virtually absent from the BP, with the exception of a few labeled fibers invading the epithelium at its superior edge and extending to the lumenal surface (arrow). **C:** TrkC labeling is strong in the CG (stippled outline) and very light in the BP. D,E: E7–8. Sections through the midregion of the BP. **D:** Light trkB immunolabel is found in fibers throughout the CG and the BP (arrow). **E:** Strong trkC labeling is present in fibers and/or cells throughout the CG and the BP (arrow). TrkC-labeled fibers also congregate at the basal lamina of the BP (arrowhead). Note that in both D and E that the trk-positive fibers invading the epithelium do so along the entire inferior/superior extent of the BP (compare to Fig. 1B). F–I: E10. Sections through the midregion of the BP at a higher magnification than A–E. **F:** TrkB labeling in the CG is light in neuronal cell bodies (arrow). **G:** TrkC labeling in the CG is strong in neuronal cell bodies (arrow). **H:** TrkB labeling in the BP is pronounced in the region directly below the hair cells (arrow) and light throughout the rest of the epithelium. Cells lining the scala tympani are also labeled (arrowhead). **I:** TrkC labeling in the BP is very strong below the hair cells (arrow) and light throughout the rest of the epithelium. J,K: Posthatch day 7. Sections through the CG. **J:** TrkB labeling in the mature CG is virtually absent. Arrow points to an unlabeled neuronal cell body. **K:** TrkC labeling in neurons of the mature CG is highly pronounced (arrow). Photomicrographs were taken under Nomarski optics. Scale bars = 30 μm in A–E; 15 μm in F–I; 10 μm in J, K.

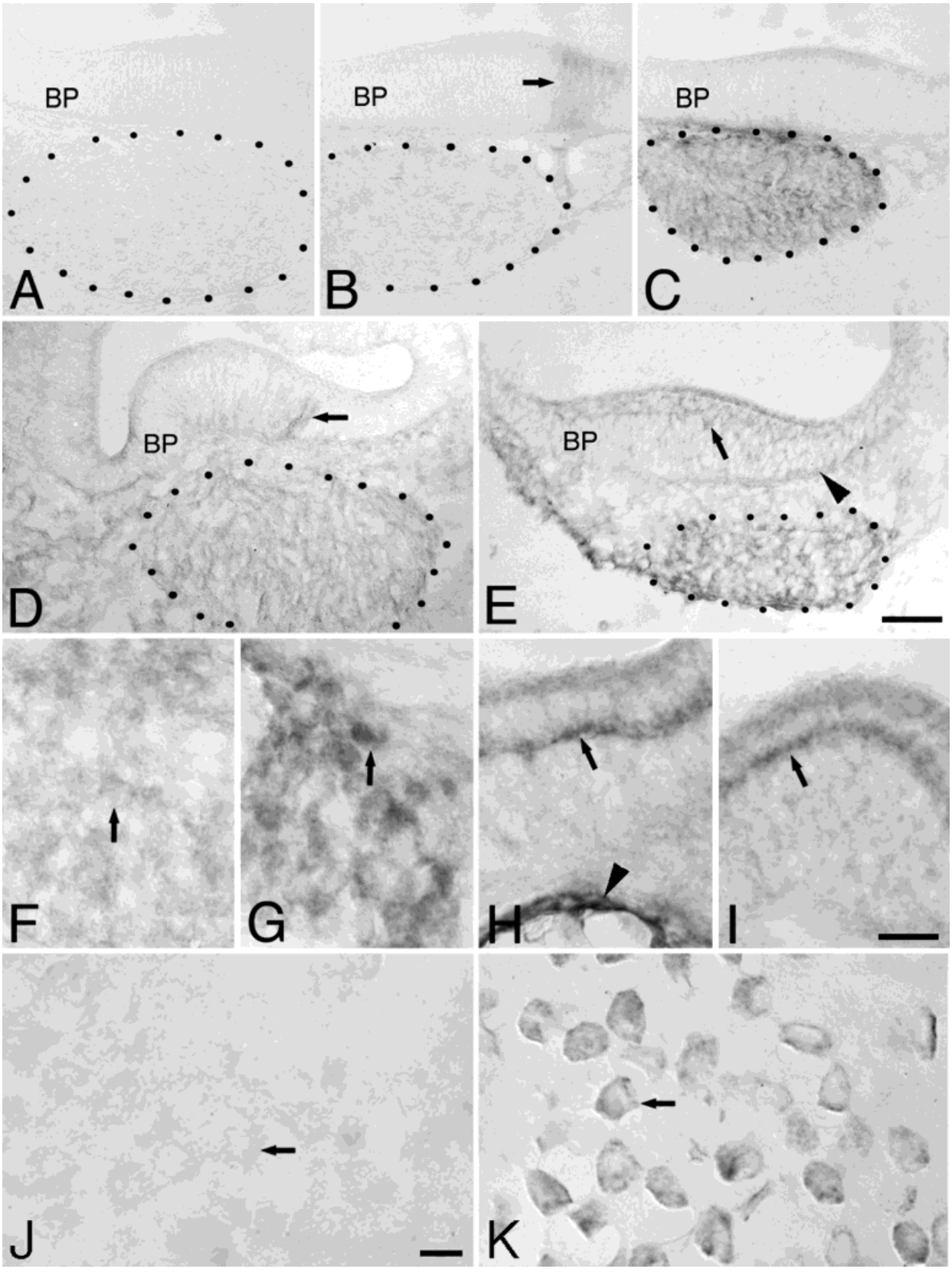

Figure 2

developing auditory periphery, and this pattern is already evident in the E6.5 chick. TrkC immunolabel is abundant in the cochlear ganglion (Fig. 2C). At this age, heavy trkC immunolabel is distributed in and around the neurons of the cochlear ganglion, suggesting that it is present within neural cell bodies as well as the neural processes of the ganglion. Although the cochlear ganglion lies in close apposition to the sensory epithelium at this age, the heavy trkC labeling does not appear to extend into the epithelium. Rather, there is light immunolabel within the epithelium that may be due to its presence in the immature epithelial cells or the neural processes, or both.

By E8, efferent projections from the brainstem begin invading the basilar papilla (Fritzsch et al., 1993), and cells within the basilar papilla have begun to differentiate into either sensory (hair) cells or support cells (Cotanche and Sulik, 1984; Fermin and Cohen, 1984; Whitehead and Morest, 1985b; Molea et al., 1999). Figure 2D and E depicts typical trkB and trkC staining, respectively, in the E8 auditory periphery. The patterns of trkB and trkC immunolabel at E8 are similar to those seen at E6.5, with some notable exceptions. The distribution of trkB label in cochlear ganglion neurons is consistent with that seen previously, i.e., it is somatic and is distributed uniformly throughout the ganglion, though the signal at E8 appears to be slightly darker. The distribution of trkB label in the basilar papilla, however, is quite different at E8 than that seen earlier. It is still fibrous, but it is distributed throughout the superior/inferior axis of the sensory epithelium (Fig. 2D). Some labeled fibers appear to span the entire width of the basilar papilla, whereas others stop short of the lumen.

At E8, trkC patterns of staining in the auditory periphery are similar to patterns of trkB staining, with some exceptions. Figure 2E demonstrates the trkC staining typical of the cochlear ganglion and basilar papilla. TrkC staining is pronounced in neurons throughout the extent of the cochlear ganglion, and it appears more intense than the trkB staining. In contrast to staining seen at E6.5, there appears to be less extrasomatic labeling in the ganglion at this time. In the basilar papilla, trkC immunolabel appears more pronounced and more specific than that seen in younger tissue. TrkC labeling is present in neural fibers within the epithelium and in processes near the basal lamina. In addition, many trkC-labeled fibers extend radially through the epithelium; some emerge at the lumenal edge, whereas others stop short of the lumen. Like the trkB-labeled fibers, trkC-labeled fibers are distributed along the entire superior/inferior axis of the papilla. Some trkC labeling appears to be in the hair cells or support cells of the basilar papilla as well as in the neural fibers.

We should note that despite our close scrutiny, any gradients in trkB or trkC labeling in the cochlear ganglion or in the basilar papilla (i.e., along the inferior/superior or the proximal/distal axes) have escaped our detection. Careful analyses of older embryos have similarly resulted in a lack of staining gradients within labeled structures. The single exception to this is the appearance of the handful of trkB-labeled fibers confined to the superior edge of the basilar papilla at E6.5 mentioned previously (and shown in Fig. 2B). At E8, trkB- and trkC-labeled fibers can be seen entering and leaving the ganglion in sagittal sections (data not shown). No trkB labeling was present in the ventral region of the medulla, where the somata of the efferent tracts are located (Whitehead and Morest, 1981; Fritzsch et al., 1993), nor in efferent fibers emanating from that region (data not shown). Therefore, we believe that the trkB-labeled fibers in the receptor epithelium are afferent fibers derived from cochlear ganglion rather than efferent fibers.

E9–10. By E9–10, synapses between cochlear ganglion cells and hair cells in the basilar papilla have begun to form (Rebillard and Pujol, 1983; Whitehead and Morest, 1985a) and hair cell differentiation is still underway (Cotanche and Sulik, 1984; Whitehead and Morest, 1985b). Figure 2F–I illustrates the distribution of trkB and trkC immunolabeling in the auditory periphery of the chick at E9–10. In the cochlear ganglion, the general patterns of trkB (Fig. 2F) and trkC (Fig. 2G) staining resemble those seen at E8. The trkC labeling remains considerably heavier than the trkB labeling. However, there is a marked difference in the distribution of the trk-labeled fibers within the basilar papilla at this time. Both trkB (Fig. 2H) and trkC (Fig. 2I) immunolabeling is localized to a horizontal stripe near the lumenal surface of the epithelium. At this point in development, the cells within the receptor epithelium have begun segregating into two layers, with newly differentiated hair cells tightly packed in an upper layer, and support cells loosely organized in a lower layer (Whitehead and Morest, 1985b). Thus, the trkB and trkC labeling in the basilar papilla appears to be present in neural fibers that have coalesced at the base of the newly differentiated hair cells, but the label may also be localized within the hair cells themselves.

We noted strong trkB immunolabeling in cells lining the scala tympani that was highly reproducible (Fig. 2H). This staining was not seen in negative controls in which the primary antibody was omitted. Although it is possible that this labeling actually represents detection of the trkB antigen, it is more plausible that: (1) these cells have a noncovalent affinity for the primary antibody; or (2) they contain an antigen that resembles trkB and this labeling represents cross-reactivity.

Posthatch. In the posthatch chick, trk labeling in the basilar papilla has disappeared (data not shown). In the cochlear ganglion, trkB labeling has abated as well (Fig. 2J). However, trkC labeling in the ganglion cell bodies (Fig. 2K) persists at levels comparable to those seen in younger animals (see Fig. 2C, E, G). Our analyses indicate that this mature trk labeling pattern is achieved by E14 in all tissues of the auditory periphery. Although a considerable portion of ontogenetic development has occurred in the chick auditory periphery by E11–12, synapse formation (afferent and efferent) continues in the basilar papilla until around E17 or so (Rebillard and Pujol, 1983; Fermin and Cohen, 1984; Whitehead and Morest, 1985a), and central cochlear ganglion terminations only begin to condense into their characteristic calyces at E13–15 (Jhaveri and Morest, 1982). Thus, it appears that both trkB and trkC may be essential for developmental events occurring in the chick auditory periphery during the first two-thirds of development, whereas trk C alone may play a role in events occurring during the final week in ovo and in the posthatch animal.

Trk Immunolabeling in the auditory brainstem

E5–8. The anlage of the auditory brainstem nuclei in the chick first appears as a C-shaped structure at the

lateral edge of the brainstem, where the three principal nuclei, nucleus magnocellularis (NM), nucleus laminaris (NL), and nucleus angularis (NA), are indistinguishable from one another. Immunolabeling for the neurotrophin tyrosine kinase trkB and trkC receptors is visible in the anlage of the auditory brainstem as early as the anlage can be clearly identified (E6–7). In contrast, trkA immunolabel is not apparent in the auditory brainstem anlage, nor is it apparent in the auditory brainstem nuclei of successively older animals. This absence of trkA in the central auditory system (at least at lower levels) is commensurate to the absence of trkA noted peripherally. Our observations suggest that trkA does not participate at all in the embryonic development of the peripheral or brainstem auditory system in chick.

At E7–8, the distribution and intensity of trkB and trkC immunostaining in the auditory brainstem anlage are indistinguishable from one another. The staining is moderate and diffuse, appearing somewhat "hazy," as illustrated by Figure 3A and B. Trk-labeled fibers are not apparent either within or surrounding the labeled anlage. Although diffuse, trkB and trkC immunolabel is distributed uniformly throughout the anlage, both in the mediolateral and dorsoventral extents of the structure, as illustrated in the coronal sections in Figure 3A and B, and throughout the rostrocaudal extent. Although the label is specific, trkB and trkC immunolabel in the auditory anlage is not remarkably different than that found in the surrounding brainstem structures. That is, trkB and trkC labeling is eminent throughout many of the hindbrain structures at more or less equal intensities at this age.

E9–10. As the surrounding brainstem is developing and expanding in all three dimensions, the relative positions of the auditory brainstem nuclei shift medially within the brainstem. By E9–10, NM, NL, and NA are easily distinguished in Nissl-stained tissue or in tissue stained by Bodian's protargol method (Rubel et al., 1976; Jhaveri and Morest, 1982; Young and Rubel, 1986). At this time, the refinement of NM axonal terminations on NL has begun (Young and Rubel, 1986). Trk immunolabel in the E10 auditory brainstem nuclei is moderate to heavy and somewhat diffuse, as illustrated in Figure 3C–H. TrkB and trkC immunolabeling patterns are indistinguishable from one another in the E10 brainstem, just as in the E8 tissue (Fig. 3A and B). In NM, trkB, and trkC immunolabel is located in the neuronal cell bodies throughout NM and in the neuropil surrounding NM proper (Fig. 3C–F). It is likely that the hazy appearance of trkB and trkC immunostaining in and around NM at this age is due to the profusion of somatic processes (or dendrites) that characterize NM from about E7 to E11 and which will eventually retract (Jackson and Parks, 1982; Jhaveri and Morest, 1982; Young and Rubel, 1986). In NL, trkB and trkC immunolabel is located most densely in the dorsal and ventral neuropil. Little immunolabel is apparent in the neuronal cell bodies of NL, which may be due to the rather scant amount of somatic cytoplasm present at this stage of development. The labeling found in the dorsal and in the ventral neuropil of NL appears symmetrical at this age (Fig. 3C, D, G, and H). In nucleus angularis, trkB and trkC label at this stage of embryonic development is moderately intense and is distributed throughout the extent of the nucleus (not shown). Typical of the entire region, trkB and trkC staining patterns in nucleus angularis are indistinguishable from each other in E9–10 animals. We are unable to detect gradients in trkB or trkC immunolabel in any of the auditory brainstem nuclei along the mediolateral or rostrocaudal axes at this age, nor can we detect them in successively older embryonic chicks.

E11–12. The first time physiological responses can be observed in NM and NL is at about E11 (Jackson et al., 1982). In parallel, NM and NL neurons begin to undergo a period of exuberant growth of dendrites (Jackson and Parks, 1982; Jhaveri and Morest, 1982; Smith, 1981) and a period of dramatic cell death in NL (Rubel et al., 1976). Whereas both the intensity and the distribution of trkB and trkC immunolabel are remarkably similar in animals prior to E11, from E11 onward, the distributions of trkB and trkC in the auditory brainstem are markedly distinct.

By far, the most striking change from earlier timepoints is the pattern of trkB labeling now observed in NL. Figure 4A illustrates this unique pattern in an E12 embryo. At this time, trkB label is predominantly confined to the neuropil, as seen in younger animals. However, in stark contrast to that seen at younger ages, the trkB label in E11–12 animals (and in older embryos) is uniquely polarized. TrkB immunolabel is apparent only (or predominantly) in the ventral neuropil of NL (Fig. 4A). TrkB immunolabel is not apparent in the dorsal neuropil of NL, nor is it abundant in the neuronal cell bodies of NL. This result is more evident at higher magnification, where unlabeled NL neurons appear to be perched upon a shelf of trkB label in the ventral neuropil (Fig. 5). This pattern will be discussed in more detail in a following section.

TrkB immunolabeling in NM is distributed throughout the nucleus proper (Fig. 4A). Although the "hazy" appearance of label is still predominant, often the trkB label appears to be condensed around the periphery of the neuronal cell bodies, i.e., perisomatically, and less condensed within NM cells. At this age, the auditory nerve endings terminating on NM form immature, highly arborized endings (Jhaveri and Morest, 1982; Whitehead and Morest, 1985a), rather than the nerve calyces characteristic of older animals. Thus, the heterogeneous trkB label in NM suggests that trkB is located in the auditory nerve endings contacting NM cells rather than in the NM cells themselves.

TrkC immunolabeling in NM, in contrast, is becoming increasingly confined to the neuronal cell bodies of NM, as illustrated in Figure 4B. The trkC label in the cell bodies is strong, whereas the trkC labeling in the surrounding NM neuropil is becoming light and diffuse. In NL, trkC immunolabeling is quite robust, equal in intensity to that found in NM. As illustrated in Figure 4B, it is now located in both the cell bodies of NL neurons and in the neuropil, where before it had been located almost exclusively in the neuropil. In some caudal sections through NL, light to moderate trkC labeling is observed in the NL dendrites.

>E12. The last one-third of embryonic development is characterized by several prominent events: the concomitant retraction of NM dendrites (Jackson and Parks, 1982; Jhaveri and Morest, 1982; Young and Rubel, 1986) with the formation of true auditory nerve "calyces" surrounding NM cell bodies on which they terminate (Jhaveri and Morest, 1982); the continued refinement of NM axonal arborizations on NL (Young and Rubel, 1986); the stabilization of dendrite number and length and of cell number in NL (Rubel et al., 1976; Smith and Rubel, 1979; Smith, 1981); and the development of acoustically driven responses (Saunders et al., 1973, 1974; Jackson and Rubel,

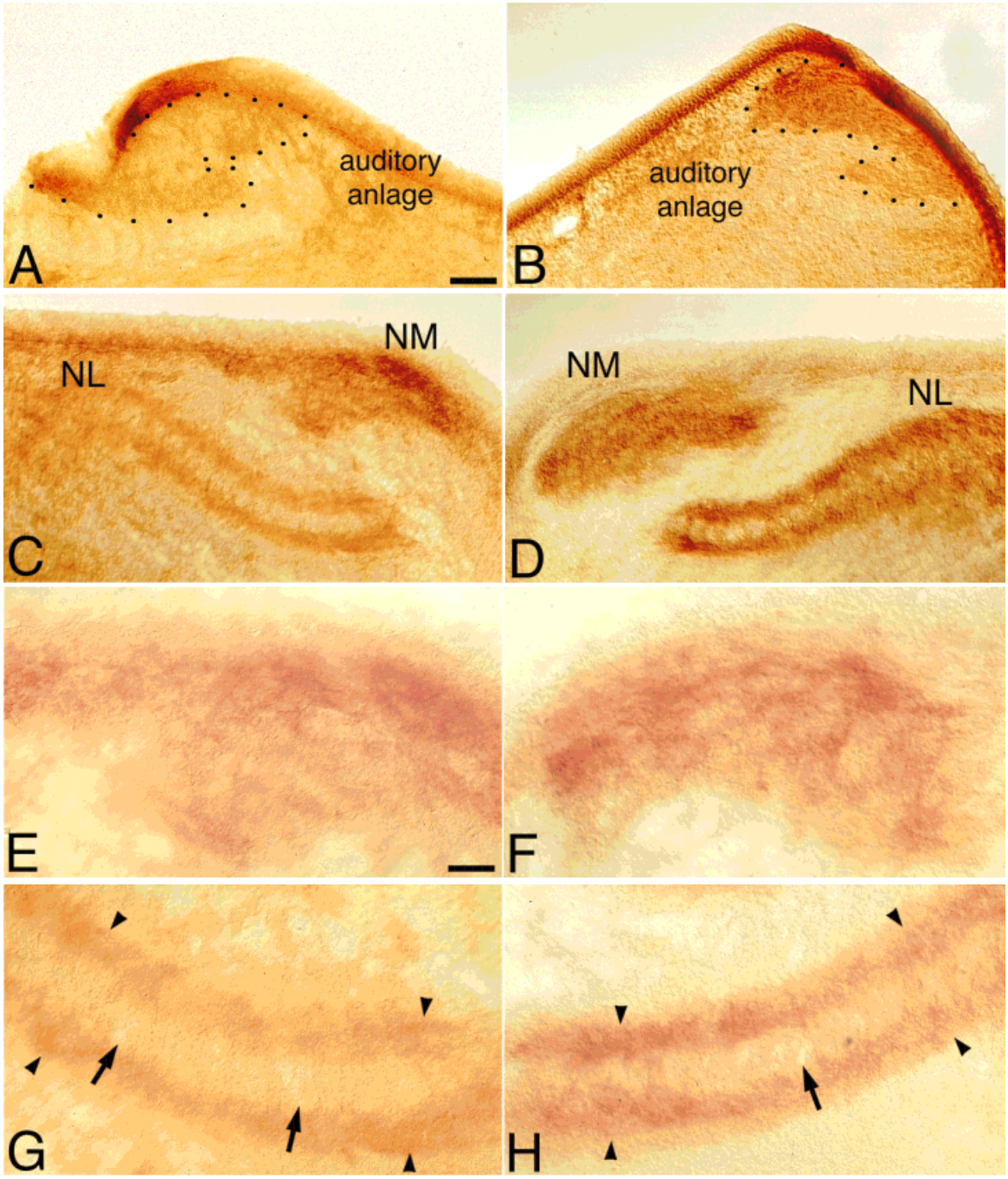

Fig. 3. TrkB (left panels) and trkC (right panels) immunolabeling in 50 µm coronal sections through the chick auditory brainstem at embryonic day (E)8 (A, B) and E10 (C–H). In all images, dorsal is up. In left panel images (A, C, E, G), medial is to the right. In right panel images (B, D, F, H), medial is to the left. **A,B:** E8. Paired low-magnification images showing moderate and diffuse trkB (A) and trkC (B) labeling throughout the auditory anlage (stippled outline). **C,D:** E10. Paired low-magnification images showing strong trkB (C) and trkC (D) labeling in nucleus magnocellularis (NM) and nucleus laminaris (NL). **E,F:** Paired high-magnification images showing the strong trkB (E) and trkC (F) staining in NM in E10 chicks. The immunostain is dispersed throughout the NM neuronal cell bodies and the surrounding neuropil, giving the stained NM nucleus its "hazy" appearance. **G,H:** Paired high-magnification images showing the strong trkB and trkC staining in NL in E10 chicks. TrkB and trkC immunolabeling is found in the dorsal and ventral neuropil (arrowheads) sandwiching the lightly labeled or unlabeled NL cell bodies (arrows). A–H: Note the strong similarity in trkB and trkC labeling patterns in the NM and NL at these ages (compare to the distinct trkB and trkC labeling patterns seen in older embryos, as illustrated in Fig. 6). Photomicrographs taken under Nomarski optics. Scale bars = 50 µm in A–D; 20 µm in E–H.

1978; Rebillard and Rubel, 1981). Figure 6 illustrates the intense trkB and trkC immunolabeling observed in the auditory brainstem nuclei in these older embryos. This result indicates that trkB and trkC label in central auditory brainstem structures is consistently robust throughout embryonic development. The divergent patterns of

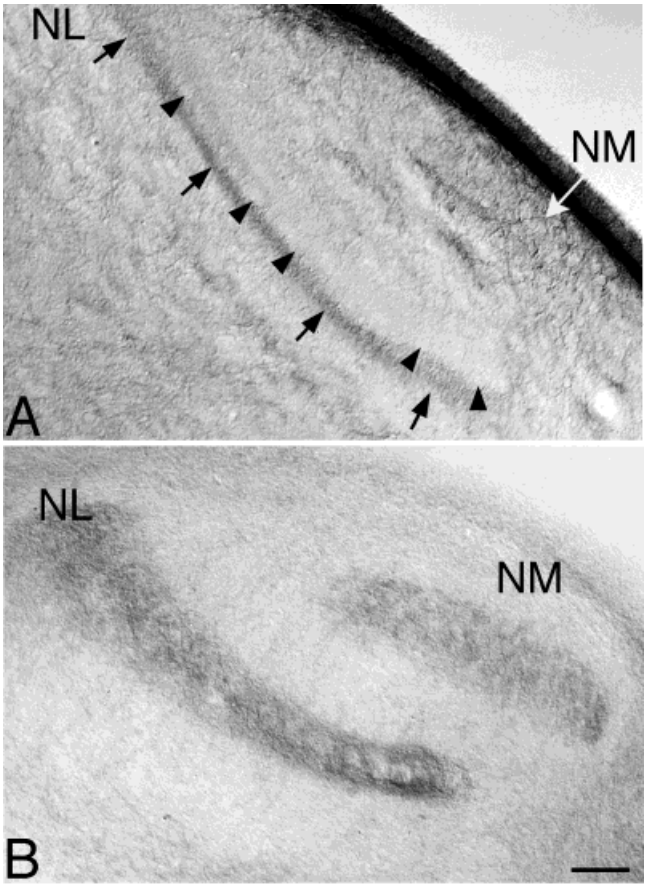

Fig. 4. Distinct patterns of trkB and trkC immunolabeling in 50 μm coronal sections through nucleus magnocellularis (NM) and nucleus laminaris (NL) of an embryonic day (E)12 chick. **A:** Polarized expression of trkB immunolabeling in NL. TrkB immunostain is confined to the ventral neuropil of NL (arrows). TrkB immunolabel is not found in the dorsal neuropil, nor is it found in the NL cell bodies (arrowheads). This unique pattern of trkB expression is first evident in an E11–12 chick and it is maintained throughout embryonic development. **B:** TrkC immunolabeling in NM and NL is found in cell bodies and in limited regions of the surrounding neuropil. Photomicrographs were taken under Nomarski optics. Scale bar = 20 μm.

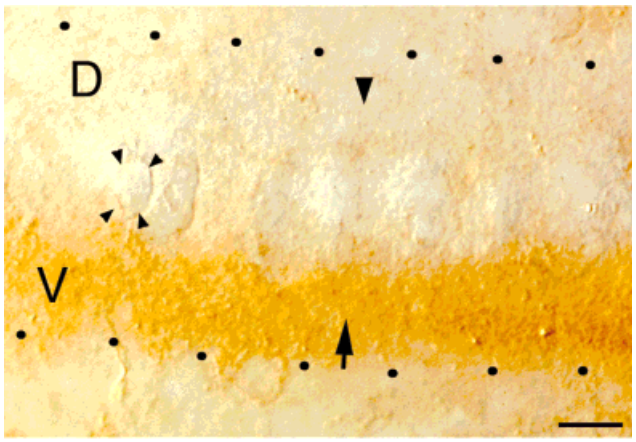

Fig. 5. TrkB immunolabel in nucleus laminaris (NL) in an embryonic day (E)12 embryo shown at high magnification. Stippled lines denote the extent of the dorsal (D) and ventral (V) NL neuropil. Note the stripe of trkB label in the ventral neuropil (arrow) which is offset by the lamina of unlabeled NL somata just above. Small arrowheads demarcate one NL soma. In contrast, the faint labeling of the dorsal NL neuropil (large arrowhead) makes it difficult to distinguish its boundaries. Photomicrograph was taken under Nomarski optics. Scale bar = 10 μm.

trkB and trkC label in the auditory brainstem in embryos older than E12 resemble those first observed in E11–12 animals. TrkC label in NM and NL is now confined solely to the neuronal cell bodies in both nuclei (Fig. 6B, D, F, and H), except in occasional sections through the caudal extent of NL where light label may be observed in the neuropil as well (not shown). TrkC label in nucleus angularis is also robust and somatic (not shown). This pattern of trkC staining in NM, NL, and NA persists in the posthatch chick.

Figure 6C illustrates the distinct perisomatic trkB immunolabeling found in NM in chicks older than E12; the adendritic cell bodies of NM are clearly unlabeled, whereas distinct rings of label encircle the neurons. Thus, we believe that the trkB staining is not in the NM neurons themselves, but rather in the auditory nerve endings which form nerve calyces around NM somata on which they terminate. A similar pattern was noted above in E11–12 chicks, but the pattern is more prominent in older animals, probably due to the maturation of the nerve calyx (Jhaveri and Morest, 1982). TrkB immunolabeling in NL continues to be confined primarily to the ventral neuropil, as shown in Figure 6E. This remarkable polarized expression of trkB, first observed in E11–12, does not appear to result from a rostrocaudal developmental gradient of trkB expression. If it did, we might expect to see trkB label confined strictly to the dorsal neuropil at later stages of development. We should note that in some cases, trkB immunolabeling can appear almost equally intense in the dorsal and the ventral NL neuropil in sections through the caudal extent of the nucleus (Fig. 6G). We have never, however, observed trkB labeling in the dorsal neuropil alone, regardless of the age of the embryo or the rostrocaudal position within the nucleus.

TrkB in situ hybridization

Our immunocytochemistry studies demonstrate a unique pattern of trkB expression in the ventral neuropil of NL during the final 8–9 days of embryogenesis (see Figs. 4A; 5; 6A, E). Nucleus laminaris cells, homologous to mammalian medial superior olive cells, have dendrites that extend from opposite sides of the cell body and that receive segregated but essentially identical inputs from the two ears (see Rubel and Parks, 1988). The question that arises is whether the trkB protein observed in the ventral NL neuropil is presynaptic (located in the terminal arborizations from the contralateral NM which terminate solely on the ventral side of NL cells, but not in ipsilateral, dorsally located terminals from the same NM neurons) or postsynaptic (located in the ventral NL dendrites, but not the symmetrical dorsal dendrites of the same cells), or both. To address this question, we localized trkB mRNA in the E12–13 chick hindbrain by means of in situ hybridization. Embryos of this age are well within the period where we see the polarized trkB immunolabeling.

Figure 7 illustrates typical results from our in situ hybridization studies. Little or no signal was ever observed in NM neurons (Fig. 7A). Occasionally, very lightly labeled cells were seen in NM, but we are not convinced that this signal was ever above background. In contrast, trkB mRNA is abundant in NL neurons, as shown in Figure 7B.

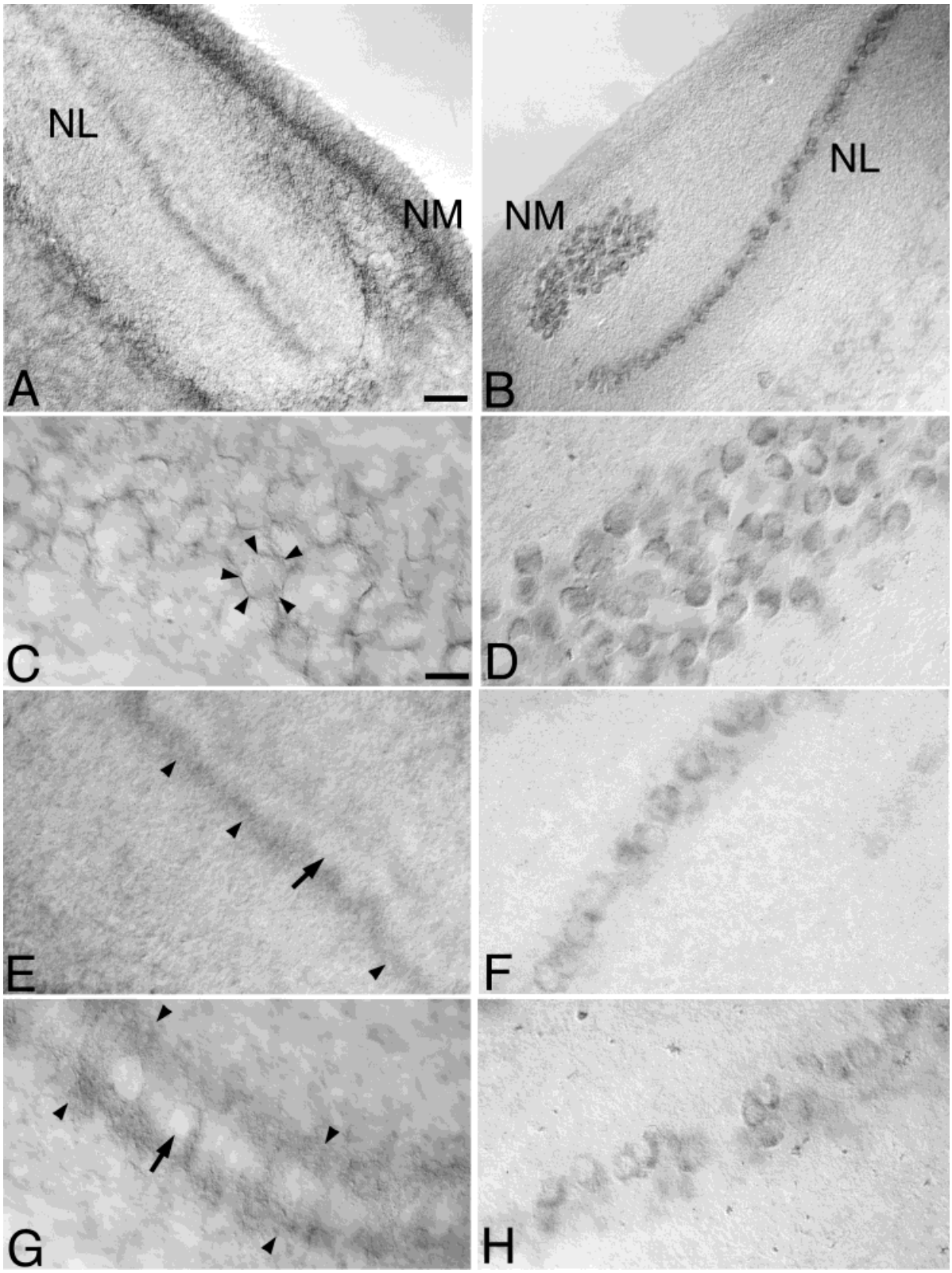

Fig. 6. Pattern of late embryonic trkB (left panels) and trkC (right panels) immunolabeling in 50 µm coronal sections through the chick auditory brainstem. In all images, dorsal is up. In left panel images (A, C, E, G), medial is to the right. In right panel images (B, D, F, H), medial is to the left. **A, B:** Paired low-magnification digital micrographs through nucleus magnocellularis (NM) and nucleus laminaris (NL) in an embryonic day (E)18 (A) or E16 (B) chick. Note the distinct and complementary trkB and trkC labeling patterns at these ages. **C:** High magnification of trkB labeling in NM. The strong label forms a ring around NM neuronal cell bodies (arrowheads), indicating that labeling is in the afferent nerve calyces rather than in NM cell bodies (compare to D). **D:** High magnification of trkC in NM showing the strong somatic label seen throughout NM. **E:** Polarized trkB labeling in rostral NL. TrkB labeling is located exclusively in the ventral NL neuropil (arrowheads). TrkB labeling is not found in the dorsal neuropil. NL cell bodies (arrow) are largely unstained. Polarized expression of trkB in NL first appears at E11–12 (see Figs. 4A, 5). **F:** Strong trkC labeling in rostral NL is confined to the cell bodies. **G:** TrkB labeling in NL at a level more caudal to that in E. At this level, trkB label is occasionally found both in the ventral and in the dorsal NL neuropil (arrowheads), but not in the cell bodies (arrow). **H:** TrkC labeling in NL at a caudal level similar to that in G. Labeling in NL is somatic, as in NM (D), leaving the surrounding neuropil unstained. Photomicrographs were taken under Nomarski optics. Scale bars = 50 µm in A,B; 20 µm in C–H.

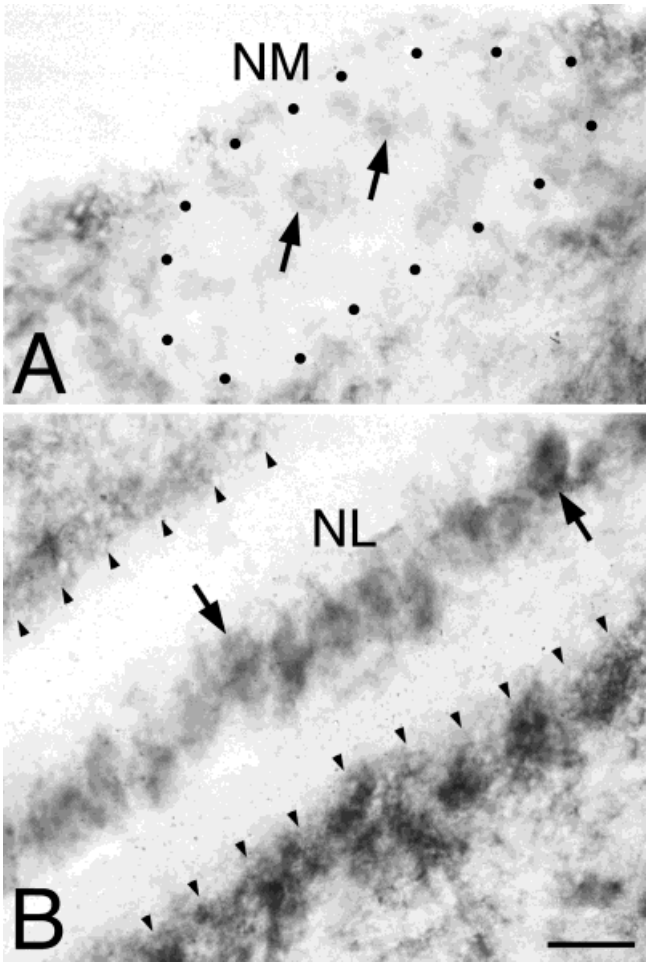

Fig. 7. TrkB mRNA in a 100 μm coronal slice through the chick hindbrain. **A:** Neurons in nucleus magnocellularis (NM; stippled outline) appear to be virtually unstained, except for an occasional lightly labeled cell (arrows). These cells do not appear to be above background. **B:** Neurons in nucleus laminaris (NL) display abundant levels of trkB mRNA. Two NL cells are denoted by arrows. Note that glia located in the glial-rich zones bordering the NL neuropil (arrowheads) are labeled. The stronger label in the ventral glia is atypical. In most sections, the trkB signal in glial somata circumscribing dorsal and ventral NL neuropil is equivalent. Scale bar = 30 μm.

The trkB labeling is very pronounced, particularly in comparison to the completely unstained cell-free zone surrounding NL cell bodies. TrkB mRNA is also found in the glial somata that border the NL neuropil. In Figure 7B, an intense trkB signal is seen in the glia rich zone ventral to the NL neuropil. We examined numerous sections and found there to be no reliable intensity differences in trkB signal in glia either dorsal or ventral to NL. In most sections, the staining appears to be equivalent. In sum, these results suggest that the trkB receptors in the ventral NL neuropil that we observed in our immunocytochemical studies are located in the postsynaptic NL dendrites and not in the presynaptic NM terminal arborizations.

DISCUSSION

The involvement of neurotrophins and their cognate receptors in establishing the intricate patterns of connectivity within the nervous system is well established. The fact that only a handful of neurotrophins and their associated receptors have emerged amidst intense study suggests that the intricacy in the neuronal networks arises from equally intricate and subtle distributions of these neurotrophins both alone and in combination throughout the developing nervous system.

In this study, we analyzed the temporal and spatial expression of trk neurotrophin tyrosine kinase receptors during embryonic development of the inner ear and auditory brainstem nuclei of the chick. We demonstrate the distinct yet partially overlapping patterns of expression for trkB and trkC proteins throughout the period studied (E5–21), as summarized in Figure 8. The protracted trkB and trkC expression demonstrated in the present study suggests that brain-derived neurotrophic factor/trkB and neurotrophin-3/trkC signalling may be instrumental in a range of events including axon guidance, synaptogenesis, cell death, and even cell maintenance. Our results further suggest that trkA and its associated neurotrophin, nerve growth factor, are not involved despite previous studies demonstrating the presence of receptors previously termed "nerve growth factor receptors" in the chick inner ear and cochlear ganglion (Bernd and Represa, 1989; Represa et al., 1991; von Bartheld et al., 1991).

Overlapping expression of neurotrophins/trks in avian auditory structures

Studies of neurotrophin expression and function in the mammalian inner ear have established the predominance of NT-3 and its associated trkC receptor in the cochlea and the predominance of BDNF and its associated trkB receptor in the vestibular organs (Pirvola et al., 1992, 1994; Ernfors et al., 1995; Fritzsch et al., 1997a; and reviewed by Fritzsch et al., 1997c). We and others provide evidence that this dichotomous expression of BDNF/trkB and NT-3/trkC characteristic of the mammalian inner ear is not shared in birds. Taken together, studies of the avian auditory system indicate that both BDNF and NT-3 and their associated receptors may play active roles in the development, survival, and maintenance of auditory structures. In addition to the concomitant trkB and trkC protein expression in the cochlea and cochlear ganglion demonstrated by the present study, previous studies of the avian auditory periphery have documented the presence of BDNF (Hallböök et al., 1993; Hallböök and Fritzsch, 1997; Pirvola et al., 1997), NT-3 (Pirvola et al., 1997), and trkB (Hallböök and Fritzsch, 1997; Pirvola et al., 1997) mRNA in the basilar papilla and BDNF (Bernd et al., 1994), NT-3 (Bernd et al., 1994), trkB (Déchant et al., 1993; Bernd et al., 1994; Hallböök and Fritzsch, 1997; Pirvola et al., 1997), and trkC (Bernd et al., 1994; Pirvola et al., 1997) mRNA in the cochlear ganglion. Furthermore, in vitro studies of the developing cochlear ganglion have shown active effects of BDNF (Avila et al., 1993; Represa et al., 1993; Hossain et al., 1997; Pirvola et al., 1997; Sokolowski, 1997) and NT-3 (Avila et al., 1993; Represa et al., 1993; Jimènez et al., 1997; Pirvola et al., 1997; San José et al., 1997; Sokolowski, 1997) on cell proliferation, cell survival, and neurite outgrowth. Our study is the first to demonstrate neurotrophin tyrosine kinase receptors in the central auditory system of birds; we find a pattern of spatial and temporal overlap of trkB and trkC expression in auditory brainstem nuclei that is even more pronounced and pro-

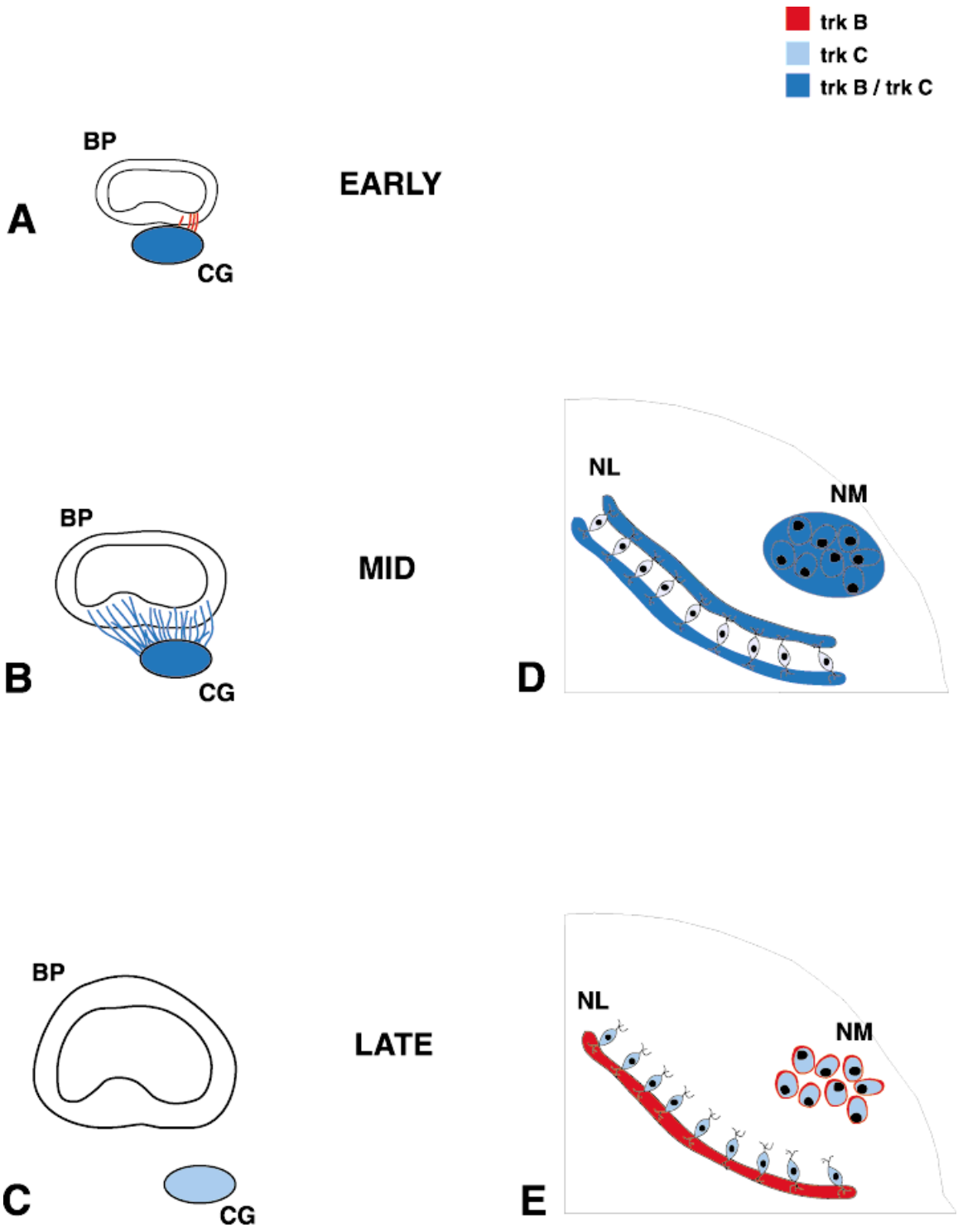

Fig. 8. Expression patterns of trk (high-affinity neurotrophin tyrosine kinase) receptors in the avian auditory periphery (**A–C**) and auditory brainstem nuclei (**D,E**) during embryonic development. Early, Mid, and Late periods correspond roughly to embryonic days E6.5, E7–11, and E11–20. TrkB (brain-derived neurotrophic factor) and trkC (neurotrophin-3) receptors are expressed throughout embryonic development. Note the spatial overlap of trkB and trkC expression at earlier stages of embryogenesis (A,B,D) as compared to the unique patterns of expression found at later stages (C,E). TrkA (nerve growth factor) receptors are not expressed in the auditory system during the period we studied (E5–20). BP, basilar papilla; CG, cochlear ganglion; NL, nucleus laminaris; NM, nucleus magnocellularis.

tracted than in the periphery. As yet, it is unknown whether the overlap in trk expression in central auditory structures is accompanied by an overlap in neurotrophin expression as it is in the periphery.

Previous studies of neurotrophin receptors in the developing avian auditory system

NGF receptors. The present study describes the general lack of trkA receptors, receptors with specific affinity for the neurotrophin NGF, in chick in any of the auditory structures at any embryonic stage examined. Several previous studies of nerve growth factor receptor expression in avians had demonstrated the presence of putative NGF receptors during auditory ontogeny. In situ hybridization studies by von Bartheld et al. (1991) demonstrated the presence of low-affinity nerve growth factor receptors (which they termed NGFR) in the chick otocyst epithelium beginning at E3. These receptors were observed in the cochlear ganglion at moderate levels from E4 to E13 and at lower levels from E13 to posthatch. The NGF receptors were not observed in the cochlea at any age (von Bartheld et al., 1991). The NGF receptors were not observed either in the auditory brainstem (von Bartheld et al., personal communication). In other studies, NGFRs were noted in the otic vesicle in another avian species, the quail (Bernd and Represa, 1989; Represa et al., 1991). In these studies, radioactively labeled NGF was used to localize putative nerve growth factor receptors. These studies found NGF receptors in the medial half of the otic vesicle (Bernd and Represa, 1989) and in the cochlear portion of the cochleovestibular ganglion (Bernd and Represa, 1989; Represa et al., 1991). Our current understanding of neurotrophin receptors lends insight to the fact that these studies were, in fact, characterizing the expression and distribution of p75 receptors, receptors which have equal affinities for all neurotrophins (Rodriguez-Tébar et al., 1990; Squinto et al., 1991), rather than characterizing that of trkA receptors, receptors with specific affinity for NGF. At the time of these studies, trk receptors, neurotrophin receptors activated differentially by different neurotrophins, were just being discovered (reviewed in Bothwell, 1995). The p75 receptors lack the tyrosine-kinase signalling domain and may facilitate or compete with trk receptors (e.g., Davies et al., 1993; Hantzopoulos et al., 1994; Chao and Hempstead, 1995; Kaplan and Miller, 1997; and see Ibañez, 1998 for a review of the distinctions between trk and p75 receptors). In sum, our finding trkB and trkC receptors in the cochlear ganglion and the inner ear suggests that BDNF and NT-3 are released in the vicinity and are available to bind to local trk and p75 receptors. Thus, our results are not inconsistent with previous literature, but rather they pose the intriguing question as to what the combined expression of p75 receptors and trkB and trkC receptors may be effecting in the cochlear ganglion that is not occurring elsewhere through this mechanism.

NT-3 receptors. In an initial study of neurotrophin-3 in the chick, Hallböök et al. (1993) reported that NT-3 mRNA is not found in the chick otic vesicle during embryonic days 4–6. Because we find clear evidence for trkC receptors in the acoustic ganglion when we first look (at E6), it appears that trkC receptors are present before NT-3 is available to bind them. However, it is possible that the expression of NT-3 turns on rapidly just around the time that we first see trkC receptors in the ganglion. In support of this, Pirvola et al. (1997) first note NT-3 mRNA labeling in the basilar papilla at E6, though labeling appears weak and by E12 it is absent. Interestingly, neurotrophin-3 mRNA is found in the quail cochlear ganglion by E4, and it is still present at E7 and E10 (Bernd et al., 1994). In an early study of trkC expression in the developing chick, Williams et al. (1993) reported the lack of trkC mRNA in the chick cochlear ganglion at E3, E9, and E19. We and others have ample data that suggest that trkC is expressed in the avian cochlear ganglion from E5 onward. Besides the present study, Pirvola et al. (1997) describes robust levels of trkC mRNA in the cochlear ganglion of younger chicks (E5–8), with reduced levels in increasingly older animals. Likewise, Bernd et al. (1994) describes trkC mRNA expression in the cochlear ganglion of the quail at stages 26, 31, and 36 (~E5, E7, and E10 in the chick). In addition, the in vitro effects of NT-3 on cultured ganglion neurons of the chick have been demonstrated repeatedly (Avila et al., 1993; Represa et al., 1993; Jimènez et al., 1997; San José et al., 1997). It is possible that the discrepant results of Williams et al. (1993) arise because trkC mRNA levels in the developing cochlear ganglion fall below the detection limit of the oligonucleotide probe used in their study. Finally, we are intrigued by the decreases in trkC mRNA signal reported by Pirvola et al. (1997) because trkC expression in the cochlear ganglion, as demonstrated by immunocytochemistry, appears to remain robust throughout embryogenesis and into the posthatch period. Again, we propose that perhaps the trkC mRNA levels in older embryos fall below the detection limit of the in situ hybridization technique used. As a final note, we add that it is puzzling that trkC receptors are expressed in the cochlear ganglion throughout embryonic development and even into the posthatch period, whereas peripheral levels of NT-3 in the chick appear to abate during midembryogenesis (Pirvola et al., 1997) or are entirely undetectable (Hallböök et al., 1993). The presence of trkC receptors in the absence of the only ligand known to signal through them suggests that a ligand as yet unidentified may initiate trkC signalling. Because the pattern of NT-3 distribution in the brain is not known, it is also possible that cochlear ganglion cells in older embryos maintain trkC receptors because they are enjoying NT-3 support from central sources (e.g., nucleus magnocellularis and nucleus angularis).

BDNF receptors. Prior studies have localized trkB mRNA in the cochlear ganglion and basilar papilla of the embryonic chick (Déchant et al., 1993; Hallböök and Fritzsch, 1997; Pirvola et al., 1997) and quail (Bernd et al., 1994). An early study of BDNF expression during avian ontogeny localized BDNF mRNA in the cochlear ganglion (Bernd et al., 1994), whereas more recent studies have failed to see BDNF mRNA in the cochlear ganglion (Hallböök and Fritzsch, 1997; Pirvola et al., 1997), but have noted BDNF mRNA in hair cells of the basilar papilla (Pirvola et al., 1997). In vitro studies have reported effects of BDNF on cochlear ganglion cells in culture (Avila et al., 1993; Represa et al., 1993; Hossain et al., 1997; Pirvola et al., 1997; Sokolowski et al., 1997). The present results describe trkB receptors in the afferent fibers of the basilar papilla, but not in the hair cells or support cells of the sensory epithelium. Interestingly, light levels of trkB mRNA have been reported in some non-neuronal cells (tympanic border cells, support cells, and hyaline cells) of the E18 basilar papilla (Pirvola et al., 1997). These cells were labeled lightly when the hybridization probe used in

the studies encoded the extracellular domain of the trkB receptor. Other studies in which the hybridization probe encoded the functional tyrosine kinase domain yielded results in which trkB mRNA label was not found in the basilar papilla, but rather was confined to the cochlear ganglion (Pirvola et al., 1997). The trkB antibody used in the present study was directed against the extracellular domain, so we might have expected to see some cells in the basilar papilla labeled as Pirvola et al. (1997) described. We did not. It is possible that the truncated trkB message in the non-neuronal cells is of such low levels that the level of trkB receptor expression is little to none and therefore escaped our detection. We note that one report of trkB expression in the mammalian auditory periphery describes trkB immunoreactivity in sensory hair cells in the rat during the period of synaptic innervation (Knipper et al., 1996). A more puzzling finding is that Pirvola et al. (1997) continue to see high levels of trkB mRNA in the neurons of the cochlear ganglion in the mature basilar papilla, whereas we find that trkB immunolabeling in the cochlear ganglion abates after E12. There is no obvious reason for the discrepant results from these two studies. We can only posit that trkB message in the cochlear ganglion is not translated into trkB protein after E12.

Lack of gradients in ontogenetic expression of trks within individual auditory structures

A common feature of a developing tissue is an orderly progression of development that proceeds along an identifiable anatomical axis. In the embryonic chick basilar papilla, for instance, hair cell differentiation occurs in a distal (apical) to proximal (basal) direction (Cotanche and Sulik, 1984), whereas synaptogenesis proceeds in the opposite (proximal to distal) direction (Fermin and Cohen, 1984). As Cotanche and Sulik (1984) observe, this anomaly implies that hair cells differentiate independently and that newly differentiated hair cells may themselves guide developing afferent nerve processes by providing target sites for the formation of synapses. In the brainstem, development proceeds along the tonotopic axis, from rostromedial to caudolateral (for review, see Rubel and Parks, 1988). Some of these processes take up to a week (or one-third of embryonic development) to complete. Thus, marked differences within the basilar papilla itself, or within neurons in nucleus magnocellularis or nucleus laminaris, exist at any given time during ontogeny. It seemed likely, then, that we would find spatial/temporal gradients or waves of neurotrophin receptor expression within individual tissues. For instance, we might have expected to see a proximal to distal wave of neurotrophin receptor expression in the fibers invading the basilar papilla that would accompany the wave of synaptogenesis. However, despite efforts to identify developmental gradients of neurotrophin receptor expression within individual auditory tissues, we observed none, with the exception of the small set of lightly labeled trkB fibers found in the superior basilar papilla which preceded the robust fiber labeling found throughout the papilla 1 day later.

Interestingly, Pirvola et al. (1997) report a gradient of BDNF mRNA expression during a short window of development. They find BDNF mRNA levels in the E12 basilar papilla are more intense in the distal end than those in the proximal end. By E16, this spatial gradient of BDNF mRNA expression is not apparent— the BDNF mRNA signal is equally robust along the distal-proximal and the superior-inferior axes. Because we never saw this sort of spatial/temporal gradient in trkB expression, it appears that the graded expression of BDNF is not paralleled by expression of its cognate receptor trkB.

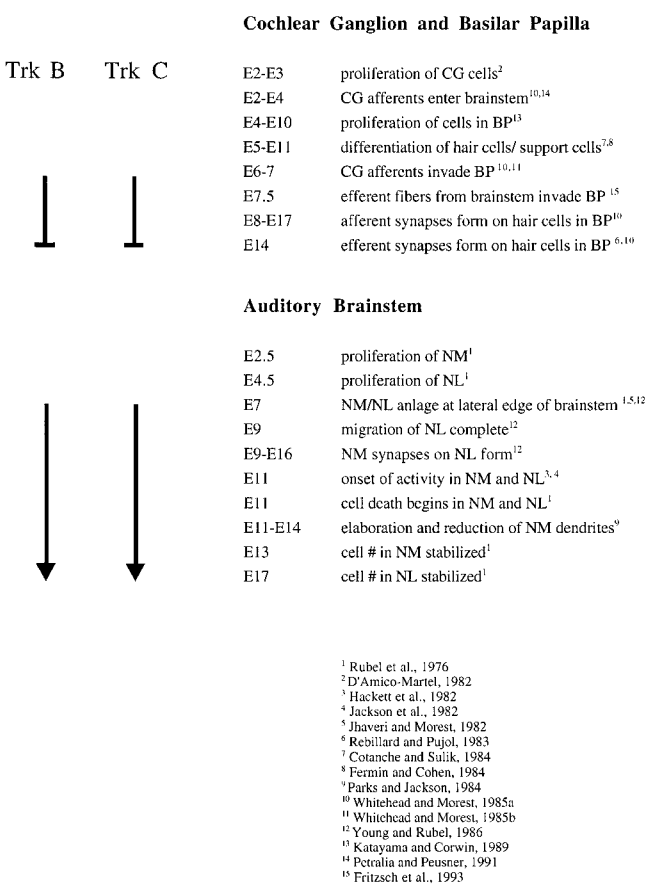

Fig 9. TrkB and trkC expression coincides with multiple events characterizing the embryonic formation of the chick auditory system. Note the temporally restricted pattern of trkB and C expression in the developing basilar papilla and cochlear ganglion. The period of expression corresponds roughly to the second week of in ovo development, when afferent and efferent fibers are penetrating the sensory epithelium and forming synapses on newly differentiated hair cells. In contrast, note the protracted pattern of trkB and C expression in the hindbrain nuclei. TrkB and C expression in nucleus magnocellularis (NM) and nucleus laminaris (NL) is evident as early as the auditory anlage can be identified. TrkB and trkC receptors persist as NM and NL neurons progress through multiple morphological stages even into postnatal development. BP, basilar papilla; CG, cochlear ganglion.

Patterns of trk expression and other developmental events

The expression patterns of the high-affinity receptors for BDNF / NT-4/5 (trkB) and for NT-3 (trkC) in the basilar papilla and auditory brainstem overlap with a number of developmental events. Some of these events are illustrated in Figure 9. In the basilar papilla, the expression of these receptors falls within a discrete developmental period. The onset of trk expression in the basilar papilla occurs as both afferent and efferent fibers begin to penetrate the receptor epithelium and several days before the differentiation of hair cells and support cells within the papilla. Trk expression in the basilar papilla ends before synaptogenesis in

the receptor epithelium is complete (Whitehead and Morest, 1985b). These findings suggest that the trk receptors and their associated neurotrophins may play a role in the early synaptogenesis in the basilar papilla. In addition, the sustained levels of trkC within the cochlear ganglion throughout ontogeny prompts us to believe that the neurons in the cochlear ganglion enjoy ongoing trophic support by NT-3 secreted from central sources. We note that we are not the first to describe differential expression of neurotrophin receptors in neuronal somata and their peripheral and central projections. von Bartheld et al. (1991) demonstrated that the early p75 expression in cell bodies of cochlear ganglion neurons is lost despite the maintained p75 expression in the peripheral processes of the cochlear nerve and the maintained expression of p75 mRNA in the ganglion cell bodies. More recently, Lefcort et al. (unpublished observations) have observed the dynamic ontogenetic distribution of trks in the dorsal root ganglion. Early in embryonic development, trkA, trkB, and trkC are found in dorsal root ganglion somata and in peripheral and central fibers. As embryogenesis proceeds, trkB and trkC expression is lost in the peripheral axons but is maintained in the cell bodies and the central projections of the dorsal root ganglion neurons. Curiously, trkA expression is maintained in the peripheral processes of the chick dorsal root ganglion even during later stages of embryogenesis. Perturbation experiments may lend insight to these common findings.

In the auditory brainstem nuclei, trk receptor expression persists throughout embryonic development and into the posthatch period. TrkB and trkC expression begins after terminal mitosis of NM and NL but before the migration of NL is complete. The high expression of trkB and trkC in the auditory brainstem nuclei is maintained throughout embryonic development, overlapping the period of synaptogenesis and the period of cell death in NM and NL (reviewed in Rubel and Parks, 1988). It remains to be investigated what role(s) the trks may play during brainstem development. Information about the source(s) and availability of BDNF and NT-3 in developing brainstem structures is likely to be useful. The prolonged expression of trkB and trkC in the auditory hindbrain suggests that the neurotrophins and their cognate receptors may be in part responsible for a number of different developmental processes, including cell migration, cell survival, and cell maintenance. One specific hypothesis is discussed below.

Functional implications: Role of trkB in development of nucleus laminaris

Perhaps the most intriguing finding in this study is the asymmetrical distribution of trkB- immunoreactive tissue in NL from E11 onward. To our knowledge, this study is the first to describe a polarized expression of receptors within individual neurons such as found in NL, where trkB is confined to the ventral, and not the dorsal, dendrites. Previous studies have described restricted tissue expression patterns of another subfamily of receptor protein tyrosine kinases, the Eph receptors, in early development. For example, EphA4 (Sek-1), EphB2 (Nuk), and EphB3 (Sek-4) are concentrated in rhombomeres 3 and 5 of the developing mouse hindbrain (Nieto, 1992; Becker et al., 1994; Henkemeyer et al., 1996; Theil et al., 1998), whereas EphA2 (Eck) is observed predominantly in rhombomere 4 (Ganju et al., 1994; Ruiz and Robertson, 1994). These studies suggest that these receptor tyrosine kinases may be involved in cell-cell interactions guiding early hindbrain development, particularly in distinctive aspects of pattern formation. More akin to the present findings, EphB2 (Cek5) has been shown to exhibit a polarized expression along the dorsal-ventral axis of the chicken retina throughout ontogeny (Holash and Pasquale, 1995). This represents the first signal transduction molecule found to exhibit the polarized pattern of expression predicted for proteins guiding the specificity of retinotectal projections.

In the present study, the polarized distribution of trkB receptors within the ventral neuropil of nucleus laminaris prompted us to perform in situ hybridization experiments in order to ascertain which of the auditory brainstem nuclei is making the trkB protein. The results from these in situ hybridization studies suggest that the trkB message is made in the cell bodies of nucleus laminaris and then the trkB mRNA or trkB protein is selectively transported to the ventral and not to the dorsal dendrites. A recent study of the subcellular localization of neurotrophins and their cognate receptors described an activity-dependent modulation of trkB mRNA targeting and protein accumulation in dendrites of hippocampal neurons (Tongiorgi et al., 1997).

The mechanism for selective transport of trkB mRNA or protein to the ventral dendrites of nucleus laminaris cells is unknown. Whatever the mechanism, the polarized expression of trkB in NL is intriguing to us in view of the binaural circuitry in nucleus laminaris and the development of highly stereotyped dendritic architecture of this nucleus (Smith and Rubel, 1979; Smith, 1981). In the normal animal, the ipsilateral and contralateral NM terminations on NL are strictly segregated from the earliest time they can be identified in development (Benes et al., 1977; Parks and Rubel, 1975; Young and Rubel, 1983, 1986). Ipsilateral projections terminate on the dorsal dendrites, whereas contralateral projections terminate on the ventral dendrites. Furthermore, the contralateral input to the ventral dendrites forms a "delay line," whereas the dorsal (ipsilateral) input does not (Young and Rubel, 1983). The precision of this "delay line" circuitry, which is topographically organized, is essential for proper encoding of sound source location (Jeffress, 1948; Colburn and Durlach, 1978; Overholt et al., 1992). One question that has persisted in developmental studies of the chick auditory brainstem concerns the molecular signal or signals that underlie the development and maintenance of such stereotyped circuitry. The polarized expression of trkB receptors in NL dendrites is coincident with refinement of NM terminal arborizations on NL (Young and Rubel, 1986), although expression persists past the major period of refinement.

As a working hypothesis, we speculate that trkB receptors on the ventral dendrites of NL cells may provide the local guidance cues necessary for proper innervation by contralateral NM fibers. One possibility is that trkB signalling in the ventral nucleus laminaris dendrites stimulates the maturation and synaptogenesis of contralateral connections, thus preventing an axonal "overshoot" through the NL cell line by contralateral NM fibers. There are several recent reports that lend credence to this idea. BDNF, NT-3, and other guidance molecules have been demonstrated to modulate the pathfinding behaviors of growth cones (Song et al., 1997). The turning behaviors

elicited depend upon differences in cAMP-dependent activity. Moreover, localized sources of nerve growth factor have been shown to initiate axon collateral sprouting in dorsal root ganglion neurons (Gallo and Letourneau, 1998) which is trkA activation-dependent (Gallo et al., 1997). Growth cone responses to collapsin-1 also seem to be dependent upon trk receptor activation (Tuttle and O'Leary, 1998). Finally, studies have demonstrated that trkB and trkC signalling is necessary for normal development of axonal arborizations and synaptic densities in the hippocampus (Martínez et al., 1998).

Despite the conventional tenet that neurotrophins reside in the target tissues, a number of recent discoveries have demonstrated instead that anterograde transport of neurotrophins (specifically BDNF) may actually play the major role in systems in the peripheral (Tonra et al., 1998) and central (von Bartheld et al., 1996a; Zhou and Rush, 1996; Altar et al., 1997; Conner et al., 1997) nervous systems. Studies have demonstrated axonal accumulation of BDNF within vesicular compartments of cortical nerve terminals (Fawcett et al., 1997) and the presence of trkB and trkC in the postsynaptic region of synaptic profiles and within the cytoplasm of dendritic processes in mammalian auditory brainstem neurons (Hafidi et al., 1996). Finally, functional trkB receptors have been demonstrated in the postsynaptic density of hippocampal neurons (Wu et al., 1996) and the release of BDNF in neural tissue has been induced by depolarization (Androutsellis-Theotokis et al., 1996). Current studies are directed toward testing the involvement of trkB in the polarization of these ipsilateral and contralateral NM inputs to NL.

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