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Brain-derived neurotrophic factor and neurotrophin 3 mRNAs in the peripheral target fields of developing inner ear ganglia

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ABSTRACT **_In situ_ hybridization was used to study the site and timing of the expression of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 5 (NT-5) mRNAs in the developing inner ear of the rat. In the sensory epithelia, the levels of NGF and NT-5 mRNAs were below the detection limit. NT-3 and BDNF mRNAs were expressed in the otic vesicle in overlapping but also in distinct regions. Later in development, NT-3 transcripts were localized to the differentiating sensory and supporting cells of the auditory organ and vestibular maculae. In these sensory epithelia, the intensity of NT-3 mRNA expression decreased in parallel with maturation. The expression of BDNF mRNA was restricted to the sensory cells of both the auditory and vestibular organs, including ampullary cristae. In bioassays, BDNF and NT-3, but not NGF, at physiological concentrations induced neurite outgrowth from the statoacoustic ganglion explants. These results demonstrate that NT-3 and BDNF, rather than NGF and NT-5, are the primary neurotrophins present in the target fields of the cochlear and vestibular neurons. Expression of NT-3 and BDNF mRNAs in the otic vesicle before and during the ingrowth of neurites from the statoacoustic ganglion suggests that NT-3 or BDNF or both may serve as chemoattractants for the early nerve fibers. The results also suggest that these neurotrophins have a role in later development of the cochlear and vestibular neurons.**

Development of the vertebrate inner ear from the otic placode into cochlear and vestibular sensory organs, including their respective ganglia, is a complex process which requires the action of several factors regulating proliferation and differentiation (1). By synthesizing controlled quantities and types of neurotrophic factors, peripheral and central targets of sensory systems can selectively support appropriate numbers and kinds of neurons that innervate them (2).

In avian and rat embryos, low-affinity nerve growth factor (NGF) receptor ($gp75^{LNGFR}$) and its mRNA have been localized to the otic placode and otic vesicle, to nonsensory cells of the developing organ of Corti, and to developing inner ear ganglia (3–5). Binding studies have demonstrated the presence of NGF receptors in the statoacoustic ganglion (SAG) and its target, the otic vesicle (6–8). In addition, an otic conditioned medium- and NGF-induced neurite outgrowth from the SAG has been shown *in vitro* (9, 10). These data suggest that NGF exerts some action on the developing inner ear. However, to date the NGF family of factors, the neurotrophins, includes five members: NGF (11), brain-derived neurotrophic factor (BDNF) (12, 13), neurotrophin 3 (NT-3) (14–17), neurotrophin 4 (NT-4) (18), and neurotrophin 5 (NT-5) (19), sharing almost 60% amino acid identity. All neurotrophins (not shown for NT-5) bind to $gp75^{LNGFR}$ with low-affinity kinetics (18, 20). Furthermore, the members of the Trk family of protein-tyrosine kinase receptors likewise bind neurotrophins with low and high affinity (21–23). The individual neurotrophins exert similar functional effects on overlapping but also on distinct neuronal populations (24). In addition, there is evidence that they have regulatory roles in non-neuronal tissues as well (25, 26). In principle, all neurotrophins could regulate the development of the inner ear. We have used *in situ* hybridization to determine the spatiotemporal expression pattern of NGF, BDNF, NT-3, and NT-5 mRNAs in the cochlea and vestibular organs of embryonic and early postnatal rats.

MATERIALS AND METHODS

Animals and Tissues. Sprague–Dawley rats were mated overnight and the following day was taken as day 0 of embryogenesis (E0). Whole embryos at E9, E11, and E13 and inner ears dissected at E15, E17, E18, and E21 were immersed overnight in 4% paraformaldehyde. On postnatal days 7 and 9 (P7 and P9), inner ears of rats were fixed by perilymphatic perfusion with 4% paraformaldehyde.

Ganglion Explant Cultures. SAGs were dissected from E11 and E12 rats and cultured in three-dimensional collagen matrix (27). Care was taken to remove the neighboring geniculate ganglion. Recombinant BDNF and NT-3 (generously provided by Prof. Y.-A. Barde, Department of Neurochemistry, Max-Planck Institute for Psychiatry, Munich, Germany), chromatographically purified βNGF (Institute of Physiology, Minsk, Byelorussian Republic), and 2.5S NGF (Collaborative Research) were added at 5 ng/ml (if not stated otherwise). The explants were photographed with an Olympus inverted microscope.

Preparation of Complementary RNA (cRNA) Probes. Rat NT-3, NT-5, BDNF, and NGF cDNAs were prepared by polymerase chain reaction and blunt-end cloned into the *Sma* I site of pGEM-4Z (Promega). NT-3-specific cRNA was synthesized from a 187-base-pair (bp) cDNA fragment encompassing positions 145–331 of rat preproNT-3 mRNA (16). The rat NT-5 cDNA fragment of 256 bp corresponds to the rat full-length cDNA (19) at positions 295–550. The rat BDNF-specific cDNA fragment of 299 bp corresponds to the pig full-length BDNF cDNA (13), which is 95% identical to nucleotides 414–712 of the rat BDNF cDNA (unpublished data). Rat NGF cRNA was synthesized from a 434-bp cDNA fragment corresponding to rat preproNGF mRNA at 283–716 (28). ^{35}S-labeled single-stranded sense and antisense cRNA probes were prepared (29) by using ^{35}S-labeled UTP (≥1200 Ci/mmol, Amersham; 1 Ci = 37 GBq) and an appropriate SP6 or T7 transcription system (Promega). The probes were used at a final concentration of 20,000 cpm/μl.

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Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-*n*, neurotrophin *n*; $gp75^{LNGFR}$, low-affinity NGF receptor; SAG, statoacoustic ganglion; E*n*, embryonic day *n*; P*n*, postnatal day *n*.

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***In Situ* Hybridization.** Paraffin-embedded sections of whole embryos were cut sagittally, and cochleas midmodiolarly, to a thickness of 5 μm. *In situ* hybridization was performed as described (29). For autoradiography, the slides were dipped in Kodak NTB-2 nuclear track emulsion and exposed at 4°C for 12–14 days. After development, the slides were counterstained with hematoxylin and mounted with Permount (Fisher Scientific). Antisense and parallel control sense sections were examined under dark- and brightfield illumination with a Leitz Axiophot microscope.

RESULTS

Ganglion Explant Cultures. The ability of the E11 and E12 SAG to respond to the neurotrophic factors by neurite outgrowth was assayed in explant cultures in three-dimensional collagen matrix. BDNF (Fig. 1*a*) and NT-3 (Fig. 1*b*) at either 1 or 5 ng/ml induced neurite outgrowth, the effect of BDNF being more pronounced. With βNGF (Fig. 1*c*) and 2.5S NGF, applied at 0.1–200 ng/ml, and in non-treated SAGs (Fig. 1*d*) no neurites were observed. All three neurotrophic factors evoked a dense neuritic halo from E8 chick dorsal root ganglia (data not shown).

***In Situ* Hybridization.** *NT-3 mRNA.* NT-3 transcripts were not found in the otic placode at E9, but in the otic vesicle, at E10–E11, NT-3 mRNA was strongly expressed in its ventral half, particularly in the area adjacent to the SAG, which itself was unlabeled (Fig. 2 *a* and *b*). At E13, a high level of NT-3 mRNA was detected in the cochlear outgrowth (Fig. 2 *c* and *d*) and in the utricular and saccular maculae of the otocyst.

From the early stages of cochlear coiling, beginning at about E15, NT-3 mRNA was strongly expressed in the developing cochlear sensory epithelium (Fig. 2 *e* and *f*). Around birth the maturation gradient from basal to apical coil of the cochlea was reflected in the level of NT-3 mRNA expression. It was more intense in the immature organ of Corti of the apical coil (Fig. 2 *g* and *h*) than in the more developed basal coil (Fig. 2 *i* and *j*). At E21, NT-3 transcripts in the basal coil were localized to the differentiating inner and outer hair cells and surrounding supporting cells (Fig. 2 *i* and *j*). In the basal coil at P7–P9, the inner hair cells still showed intense NT-3 mRNA expression (Fig. 2 *k* and *l*).

At E15–18, a strong NT-3 mRNA hybridization signal was detected in the differentiating vestibular sensory epithelia, including basal cells, of the saccular and utricular maculae (Fig. 2 *e*, *f*, *m*, and *n*). Here too, the quantity of NT-3 transcripts decreased with maturation. By P7–P9, this labeling had almost disappeared. Notably, the ampullary cristae were unlabeled for NT-3 mRNA throughout development.

NGF and NT-5 mRNAs. NGF (Fig. 2 *o* and *p*) and NT-5 (data not shown) transcripts were not detected by *in situ* hybridization in the sensory epithelial targets of the inner ear ganglia. NGF mRNA was weakly expressed in restricted regions of the nonsensory epithelia of the developing auditory and vestibular organs.

BDNF mRNA. Low levels of BDNF transcripts were found at E11 in restricted regions of both the ventral and dorsal halves of the otic vesicle (Fig. 3 *a* and *b*). By E13, this labeling, now more intense, was localized to the vestibular sensory patches and to the cochlear outgrowth of the otocyst.

At E15–E18, the BDNF signal was restricted to the region of presumptive sensory cells (Fig. 3 *c* and *d*). Around birth and during the first postnatal week BDNF mRNA was localized to the inner and outer hair cells of the organ of Corti (Fig. 3 *e* and *f*). Distinct from NT-3 transcripts, BDNF mRNA was not found in the supporting cells of the cochlear sensory epithelium.

Throughout the study period BDNF mRNA was strongly expressed in the sensory epithelium of the sacculus (Fig. 3 *c* and *d*) and utriculus (Fig. 3 *g* and *h*) and, in contrast to NT-3 mRNA, in the ampullary cristae as well (Fig. 3 *i* and *j*). Also, in contrast to NT-3 mRNA, the basal cells of the vestibular organs did not express BDNF mRNA (Fig. 3 *g*, *h*, *i*, and *j*).

Sense controls for BDNF and NT-3 mRNAs were negative (data not shown). The expression patterns of BDNF and NT-3 mRNAs in the late embryonic inner ear are shown in Fig. 4.

DISCUSSION

The sensory epithelia of the auditory organ (organ of Corti) and vestibular organs (saccular and utricular maculae and the three ampullary cristae) form restricted and well-defined target fields for the cochlear (spiral) and vestibular ganglia. Cochlear and vestibular neurons have a peripheral process that synapses on the hair cells (sensory cells) and a central process that terminates in the brainstem nuclei.

Our *in situ* hybridization results show that NT-3 and BDNF, rather than NGF and NT-5, are the predominant neurotrophins present in the target fields of the developing inner ear ganglia of the rat. This is consonant with the evidence that NGF supports survival of sensory neurons derived from the neural crest, but not of those of placodal origin (30, 31), whereas BDNF and NT-3 promote survival of sensory neurons of both of these origins (14–17, 32). Most, if not all, SAG neurons originate from the otic placode (33).

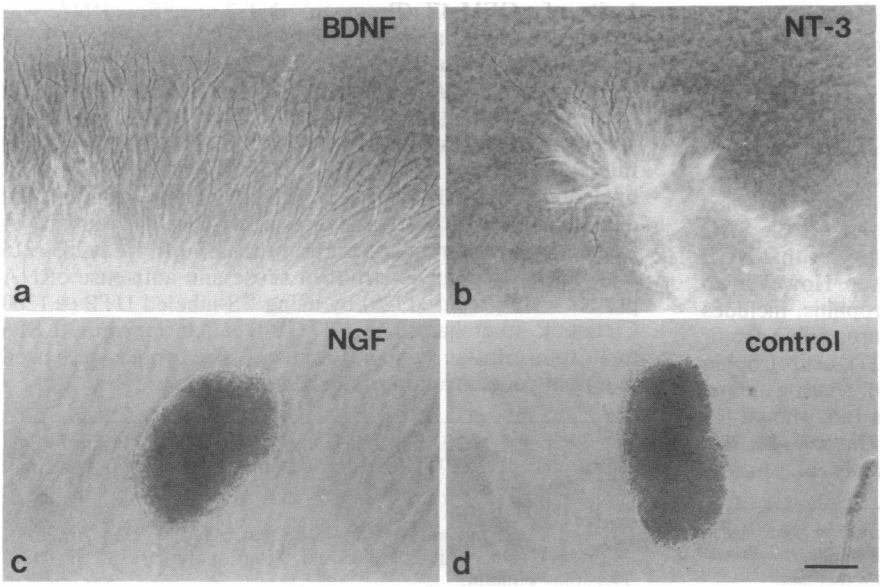

FIG. 1. E11 SAG explants cultured in three-dimensional collagen matrix in the presence or absence of neurotrophic factors. (*a*) A dense neuritic halo was induced by BDNF. (*b*) NT-3 enhanced moderate neurite outgrowth. (*c*) No neurites were induced by βNGF. (*d*) Control explants lacked neurites. [Bar = 50 μm (*a* and *b*) or 80 μm (*c* and *d*).]

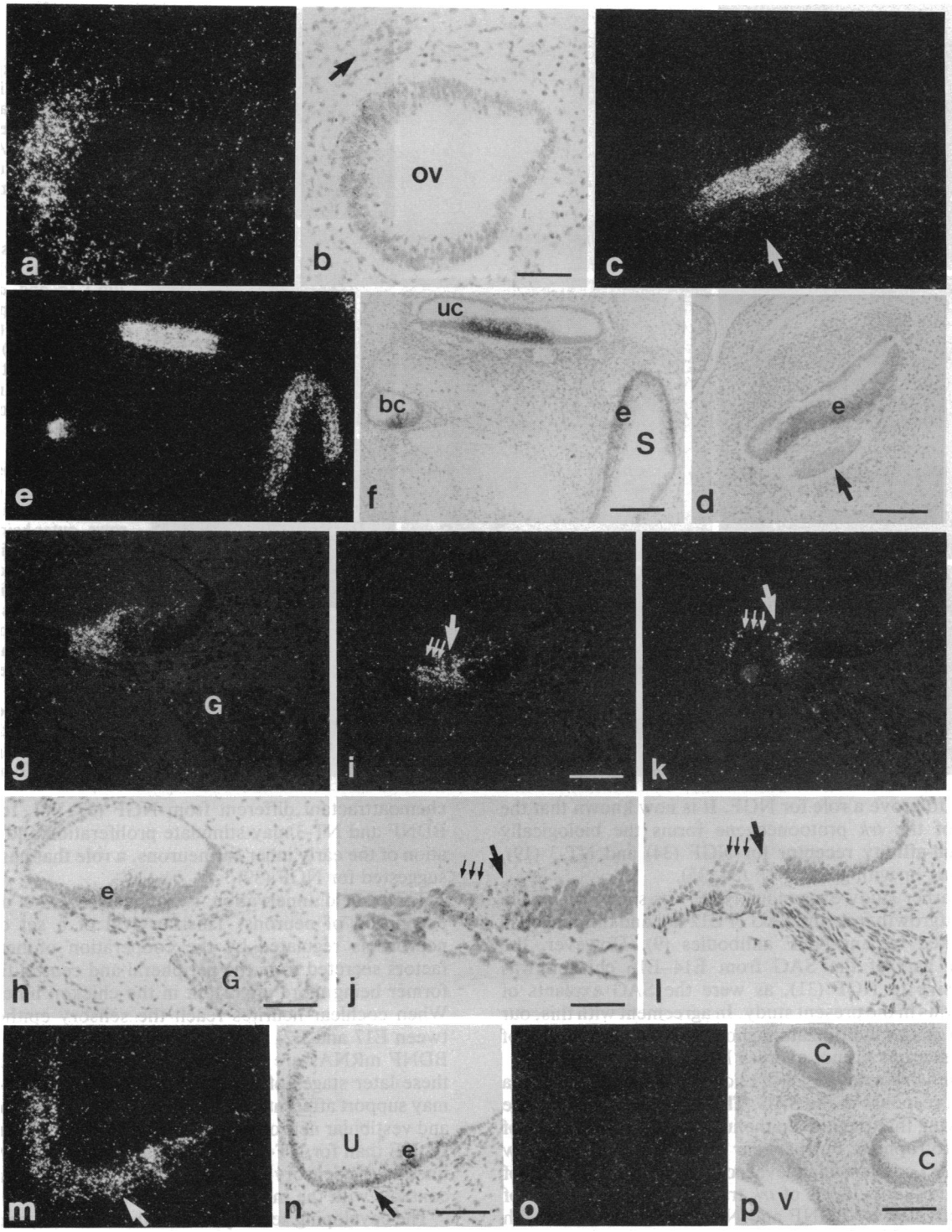

FIG. 2. Expression of NT-3 and NGF mRNAs in the developing inner ear. Sections were hybridized with the antisense cRNA probes and photographed under darkfield and brightfield illumination. (*a* and *b*) Sagittal section through the otic vesicle at E11. Ventral half of the vesicle is labeled. SAG (arrow) is unlabeled. (Bar = 60 μm.) (*c* and *d*) Sagittal section through the otocyst at E13. Strong hybridization is seen in the cochlear outgrowth but not in the spiral ganglion (arrow). (Bar = 60 μm.) (*e* and *f*) A midmodiolar section through the cochlea at E17 shows intense NT-3 mRNA expression in the sensory epithelia of the two cochlear coils and in the saccular macula. (Bar = 120 μm.) (*g* and *h*) At E21, the hybridization signal in the sensory epithelium of the apical coil is strong. (Bar = 70 μm.) (*i* and *j*) At E21, the signal in the basal coil is lower compared with the apical coil. Small arrows, the three rows of outer hair cells; large arrow, the row of inner hair cells. [Bar = 70 μm (*i*) or 30 μm (*j*).] (*k* and *l*) In the basal coil at P7, NT-3 mRNA is expressed in the hair cells and supporting cells (arrows as in *i* and *j*). (Magnification as in *g* and *h*.) (*m* and *n*) At E18, the sensory epithelium of the utricular macula, including the basal cells (arrow), is labeled. (Bar = 60 μm.) (*o* and *p*) At E16, cochlear and vestibular epithelia are unlabeled when hybridized with the NGF antisense probe. (Bar = 120 μm.) ov, otic vesicle; e, sensory epithelium; bc, basal coil; uc, upper coil; G, spiral ganglion; S, sacculus; U, utriculus; c, cochlea; v, vestibular part.

A role for NGF in inner ear development has been established from the demonstration of $gp75^{LNGFR}$ and its mRNA in the innervating neurons and epithelial structures (3–5). However, $gp75^{LNGFR}$ binds NGF, BDNF, NT-3, and NT-4 with low affinity that is not sufficient to mediate signal transduction (18, 20). Thus, the mere presence of $gp75^{LNGFR}$ is not

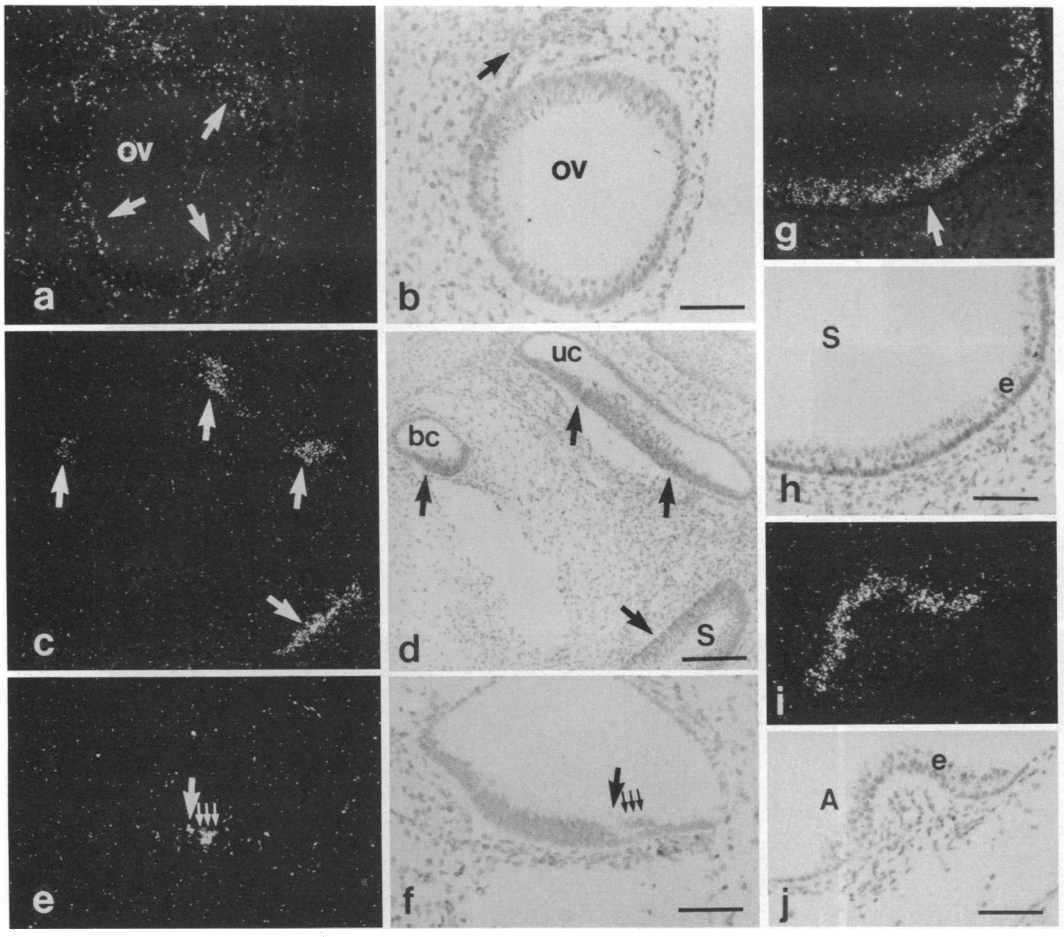

FIG. 3. Expression of BDNF mRNA in the developing inner ear. Sections were hybridized with the antisense cRNA probe and photographed under darkfield and brightfield illumination. (*a* and *b*) At E11, BDNF mRNA (arrows) is weakly expressed in the otic vesicle (ov). (Bar = 60 μm.) (*c* and *d*) At E17, the region of presumptive sensory cells (arrows) of the upper (uc) and basal (bc) coils of the cochlea and saccular macula (S) shows expression of BDNF mRNA. (Bar = 120 μm.) (*e* and *f*) At E21, the hybridization signal is restricted to the sensory cells of the organ of Corti (small arrows, outer hair cells; large arrow, inner hair cell). (Bar = 70 μm.) (*g* and *h*) At E21, the sensory epithelium (e) of the sacculus (S) shows strong expression of BDNF mRNA. Note that the basal cell layer is negative (arrow). (Bar = 60 μm.) (*i* and *j*) At P7, the sensory epithelium (e) of the ampulla (A) shows a high level of expression. (Bar = 60 μm.)

sufficient to prove a role for NGF. It is now known that the product of the *trk* protooncogene forms the biologically active high-affinity receptor for NGF (34) and NT-3 (19), either alone or with $gp75^{LNGFR}$ (34, 35).

In an earlier study exogenous NGF was shown to induce neurite outgrowth from the SAG of E12 rat, and this response was blocked by anti-NGF antibodies (9). However, the vestibular part of the SAG from E14–E16 chicken was unresponsive to NGF (31), as were the SAG explants of E11–E12 rats in the present study. In agreement with this, our data from *in situ* hybridization show that the target fields of the SAG neurons do not express NGF mRNA.

In our *in vitro* assays BDNF elicited a strong, and NT-3 a moderate, response of the SAG. These results agree with the data showing that the otic epithelium contains transcripts of these neurotrophins. Further, our unpublished results show that the SAG contains high levels of *trkB*, moderate levels of *trkC*, and very low levels of *trk* transcripts. The product of *trkB* is activated by BDNF and NT-3, either alone or with $gp75^{LNGFR}$ (21, 22, 35, 36). The product of *trkC* is a functional receptor for NT-3 (23). Taken together, these data indicate that BDNF and NT-3, instead of NGF, are the major neurotrophic factors involved in neuron–sensory cell interactions in the developing inner ear.

NT-3 mRNA was strongly and BDNF mRNA weakly expressed in overlapping but also in distinct regions of the undifferentiated epithelium of the otic vesicle at E10–E11. Notably, NT-3 and BDNF transcripts were found in the otic vesicle before and during the ingrowth of SAG neurites. We speculate that BDNF or NT-3 or both may attract neurites from the SAG to their peripheral targets, the presumptive sensory epithelia of the inner ear. In the mouse trigeminal system, the early maxillary nerve fibers are directed toward their presumptive target field by a specific diffusible chemoattractant different from NGF (37, 38). In addition, BDNF and NT-3 may stimulate proliferation and differentiation of the early inner ear neurons, a role that has also been suggested for NGF (39).

Target-field innervation is followed by the death of a proportion of neurons. This survival of a set of sensory neurons is regulated by the cooperation of neurotrophic factors secreted from the peripheral and central targets, the former being more important in the chicken inner ear (40). When cochlear neurites reach the sensory epithelium, between E17 and E21 in the rat (41), high levels of NT-3 and BDNF mRNAs are expressed in this region. Thus, also at these later stages of development, BDNF or NT-3 or both may support afferent innervation and survival of the cochlear and vestibular neurons. These roles appear more suitable for BDNF than for NT-3 because BDNF transcripts were confined to the sensory cells of the inner ear that form synaptic contacts with the innervating axons.

The developing sensory cells of the inner ear, except those of the ampullary cristae, synthesize both BDNF and NT-3, which may indicate that these neurotrophins act in an additive manner on the same neuronal populations, as shown for the action of NGF and BDNF on neurons of the dorsal root ganglia (32). Interestingly, the ampullary target fields seem to exert neurotrophic actions by BDNF exclusively, although no morphological differences in neuronal populations projecting to different vestibular sensory epithelia have been shown. The high levels of NT-3 transcripts in noninnervated supporting cells of the organ of Corti and vestibular maculae may reflect yet another role for it in these locations, possibly in supporting maturation as has been proposed for NT-3 in several peripheral tissues (42).

After birth both BDNF and NT-3 mRNAs decreased in the sensory epithelia of the inner ear, but they were still present

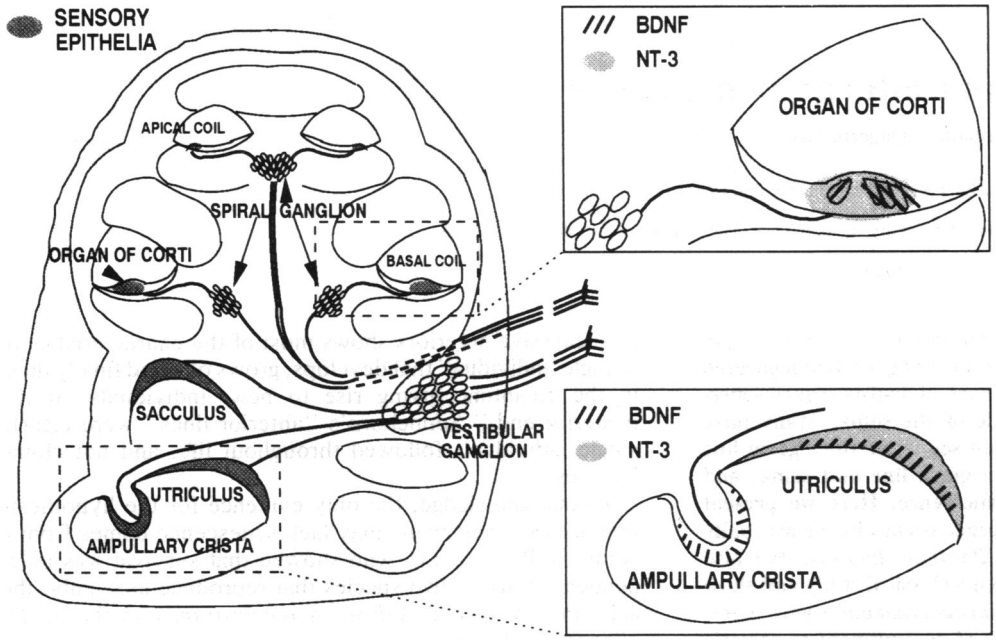

FIG. 4. (*Left*) Schematic representation of the late embryonic inner ear. (*Right*) The organ of Corti (*Upper*) and the utriculus and ampulla (*Lower*), including the expression patterns of BDNF and NT-3 mRNAs.

at P9. At birth, the rat cochlea is still immature, and the first two postnatal weeks are characterized by rapid maturation of the organ of Corti and remodeling of axon connections (43). The efferent innervation develops after birth (43). These processes progress simultaneously in neurons, sensory cells, and nonsensory elements of the organ of Corti. Such a synchronized maturation of neuronal and non-neuronal cells could be regulated by factors which are present in, or have connections with, all three cell types. BDNF and NT-3 and the components of their high-affinity receptors appear to be suitable candidates for this role as well.

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