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Original Article

Distribution of BDNF and *trk*B mRNA in the otic region of 3.5 and 4.5 day chick embryos as revealed with a combination of *in situ* hybridization and tract tracing.

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ABSTRACT We have used a recently developed technique which combines fluorescent tract tracing and *in situ* hybridization to study co-localization of neurotrophin mRNA and neurotrophin receptor mRNA expression simultaneously with the pattern of innervation in the developing chick ear. Efferent and afferent fibers from the VII/VIIIth cranial nerves were retrogradely and anterogradely filled with Dextran amines conjugated to Texas red and the brain stem was incubated for 2 hours in tissue culture medium. The tissue was subsequently fixed, sectioned frozen, mounted and subjected to *in situ* hybridization analysis using probes for brain-derived neurotrophic factor (BDNF) and its tyrosine kinase receptor, *trk*B. The results show that afferent and efferent fibers to the ear innervate areas of the developing otocyst which express BDNF mRNA. We also found that neurons in the stato-acoustic ganglion express high levels of *trk*B mRNA whereas the subset of facial motor neurons that is efferent to the ear only had no or very low levels of *trk*B mRNA. From our results we conclude that chicken otic efferent fibers preferentially project to areas with BDNF mRNA expression. The very low levels of *trk*B mRNA in these motor neurons compared to afferent neurons innervating the same region suggest that other factors, perhaps co-expressed with BDNF, may support efferents. A possible involvement of afferents in guiding efferents to specific areas of the ear is suggested.

KEY WORDS: *avian, development, efferents, inner ear, neurotrophin.*

Introduction

In rodents, brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) are produced in the developing sensory epithelia of the otic vesicle and the appropriate tyrosine kinase receptors for these two neurotrophins (TrkB and TrkC, respectively) are expressed in the stato-acoustic (otic) ganglion cells innervating the sensory epithelia of the otocyst. These neurotrophins and their receptors as well as nerve growth factor (NGF) and its tyrosine kinase receptor, TrkA, have been well characterized in rodents by *in situ* hybridization (Ernfors *et al.*, 1992; Pirvola *et al.*, 1992, 1994; Schecterson and Bothwell, 1994; Wheeler *et al.*, 1994). Targeted deletions of BDNF and NT-3 or their receptors cause loss of vestibular and/or cochlear ganglion cells (Klein *et al.*, 1993; Ernfors *et al.*, 1994a,b, 1995; Fariñas *et al.*, 1994; Jones *et al.*, 1994; Schimmang *et al.*, 1995) and a concomitant change in the pattern of innervation (Fritzsch *et al.*, 1995; Bianchi *et al.*, 1996; Fritzsch, 1996). Moreover, it appears that the pairs BDNF/NT-3 and *trk*B/*trk*C are essential for the survival of all inner ear ganglion cells: Mice with targeted deletions of the genes for either both neurotrophins or both neurotrophin receptors show a complete loss of inner ear ganglion cells (Ernfors *et al.*, 1995; Fritzsch *et al.*, 1995, 1997). Taken together, BDNF and NT-3 are essential for rodent inner ear development and NGF plays a less pronounced role except for the autonomic innervation (reviewed in Fritzsch *et al.*, 1997).

Much less is known in the developing avian ear with respect to the expression of the neurotrophins and their receptors, and the reports are somewhat contradictory. For example, while one report describes expression of *trk*B mRNA in all otic ganglion cells from embryonic day 4.5 (E4.5) until E14 (Dechant *et al.*, 1993) using *in situ* hybridization, another report could not detect *trk*B mRNA in the stato-acoustic ganglion of similar aged quail embryos employing PCR technique (Bernd *et al.*, 1994). This could however be a

Abbreviations used in this paper: BDNF,brain-derived neurotrophic factor; E4.5,embryonic day 4.5; NGF, nerve growth factor; NT-3, neurotrophin-3; otic, stato-acoustic; TDA, texas red conjugated Dextran amines.

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species difference between chick and quail. One of the factors characterized in mammals, BDNF, is specifically expressed in certain sensory cell primordia of the developing ear at E4 but was not detected in the developing cochlear duct (Hallböök *et al.*, 1993). Lack of BDNF expression in the avian cochlea tends to agree with the prominent role of NT-3 in mice ganglion cell survival (Fariñas *et al.*, 1994; Fritzsch, 1996). While no targeted gene deletions are available to substantiate the role of the neurotrophins in chicken ear development, existing *in* vitro data employing co-cultivation of the otocyst with otic ganglion cells indicate a neurotrophic role of the ear. However, there is no agreement as to the nature of the actual neurotrophin performing this task (Ard *et al.*, 1985; Bianchi and Cohan, 1993) and there is a possibility that different factors may be involved at different times.

Conflicting data have been reported about the role of neurotrophins for developing facial motor neurons : Lack of *trk*B, but not of BDNF alone (Ernfors *et al.*, 1994a; Jones *et al.*, 1994), was claimed to cause loss of facial motor neurons (Klein *et al.*, 1993). This data was consistent with earlier reports that suggest BDNF promotes facial motor neuron survival (Oppenheim *et al.*, 1992; Sendtner *et al.*, 1992). However, more recent data in mice indicate no loss of facial motor neurons in BDNF/Neurotrophin-4 double knockout mice, the two ligands that bind with high affinity to *trk*B (Conover *et al.*, 1995). This issue is relevant for the development of otic innervation because recent evidence suggests that the anlage of the facial motor complex also provides another set of motor neurons which do not end on mesodermally derived muscle fibers (facial branchial motor neurons) or neural crest derived parasympathetic ganglia (facial visceral motor neurons) but instead end at the placodally derived ear (otic efferents; (Fritzsch and Nichols, 1993; Fritzsch *et al.*, 1993b; Fritzsch, 1996). Whether there is a direct effect on the numbers of these efferent cells in BDNF or *trk*B null mutant mice ($BDNF^{-/-}$ and $trkB^{-/-}$) was not assessed in recent reports despite an apparent change in the pattern of efferent innervation (Ernfors *et al.*, 1995; Fritzsch *et al.*, 1995, 1997; Schimmang *et al.*, 1995;).

In order to shed light on these conflicting reports we examine here the expression of mRNA's for both BDNF and its receptor, *trk*B in the developing otocyst, the otic ganglion, otic efferents and facial motor neurons of 3.5 and 4.5 day old chicken (E3.5, E4.5). We chose this age because previous work has shown that the last sensory epithelium to form, the cochlea, begins to form at E3 and becomes distinct at E5 (Knowlton, 1967; Cohen and Cotanche, 1992), afferents penetrate with their neurites the otic wall between E3.5 to E7 (Whitehead and Morest, 1985; Hemond and Morest, 1991) and otic efferents disperse along the differentiating sensory epithelia between E3.5 to E4.5 (Fritzsch *et al.*, 1993b). If path finding is dependent on the neurotrophin BDNF both afferents and efferents should express the receptors for this neurotrophin during this period. Employing a newly developed technique that allows for simultaneous tracing of efferent fibers with fluorescent dyes and *in situ* hybridization in embryos (Fritzsch and Hallböök, 1996) we investigated whether efferent and afferent fibers reach the ear where BDNF mRNA is expressed and if the efferent and afferent neurons express *trk*B mRNA. Our data show that afferents and efferents reach indeed the otocyst at areas of BDNF expression. We also show high levels of *trk*B mRNA expression in otic ganglia but only no or very low levels of *trk*B mRNA expression in otic efferents and facial motor neurons at this time of development.

Results

We will subdivide this section into three parts, dealing with (a) the double labeling technique which combines fluorescent tracing using Texas red conjugated Dextran amines (TDA) and oligonucleotide based *in situ* hybridization histochemistry, (b) the labeling of efferent fibers from the brain stem in conjunction with the *in situ* hybridization for BDNF and *trk*B mRNA's (c) labeling of afferents and efferents after VII/VIIIth nerve application in conjunction with *in situ* hybridization analysis for *trk*B mRNA. Also we obtained rather similar results at both ages, neither the fillings with the dextrans nor the *in situ* hybridization signal was at E3.5 as obvious as at E4.5 owing to the presence of only few fibers and the low level of mRNA. Moreover the axons are essentially reflecting their choices for pathway selection by their trajectory (Fritzsch *et al.*, 1993b). For simplicity we will therefore base our results on the E4.5 embryos and present here only this material. Effects found in E3.5 embryos will be mentioned where appropriate, in particular where they differ.

Double labeling technique

The TDA signal was compared before and after the *in situ* hybridization procedure and was found to be equally intense in the hybridized sections as in the ones without hybridization. Figure 1 shows intense staining in the facial nerve after *in situ* hybridization analysis. In fact, using the Vectashield mounting medium we could keep the slides after viewing and photographing them for over a year without any appreciable loss of the fluorescence signal. However, after even longer storage the background fluorescence tended to increase. The signal for the *in situ* hybridization was only marginally weaker compared to freshly sectioned controls of the same age (data not shown) but showed no difference in distribution. The difference in handling was two hours of incubation as tissue fragments in medium and this could result in increased mRNA degradation. However, the pattern of expression was the same in the double labeled sections as in the controls and largely agreed to earlier published BDNF mRNA patterns in the ear (Hallböök *et al.*, 1993). These controls were also employed to test that no unspecific hybridization was introduced by the tracing procedure. On both materials, subjected to tracing before sectioning and the freshly sectioned specimen, labeling could be competed out with unlabeled oligonucleotides whereas an unrelated oligonucleotide did not affect the labeling. In addition, the different probes served as controls for unspecific hybridization to each other, which makes it unnecessary to include non-specific oligonucleotides as controls.

Anterograde labeling of efferent fibers from the brain stem

Cutting through the basal plate at rhombomeres 4 and 5 labeled all the efferent fibers of the facial motor neurons and the otic efferents as previously described (Fritzsch *et al.*, 1993a). The facial motor axons and otic efferent fibers left the brain stem through the facial root (Figs. 1, 5A). Within the facial complex the otic efferent fibers formed two distinct bundles, one entering the posterior and the other the anterior ramus of the octaval nerve (Fig. 1). Fibers of the anterior (inferior) ramus ran rather dispersed along the ventral

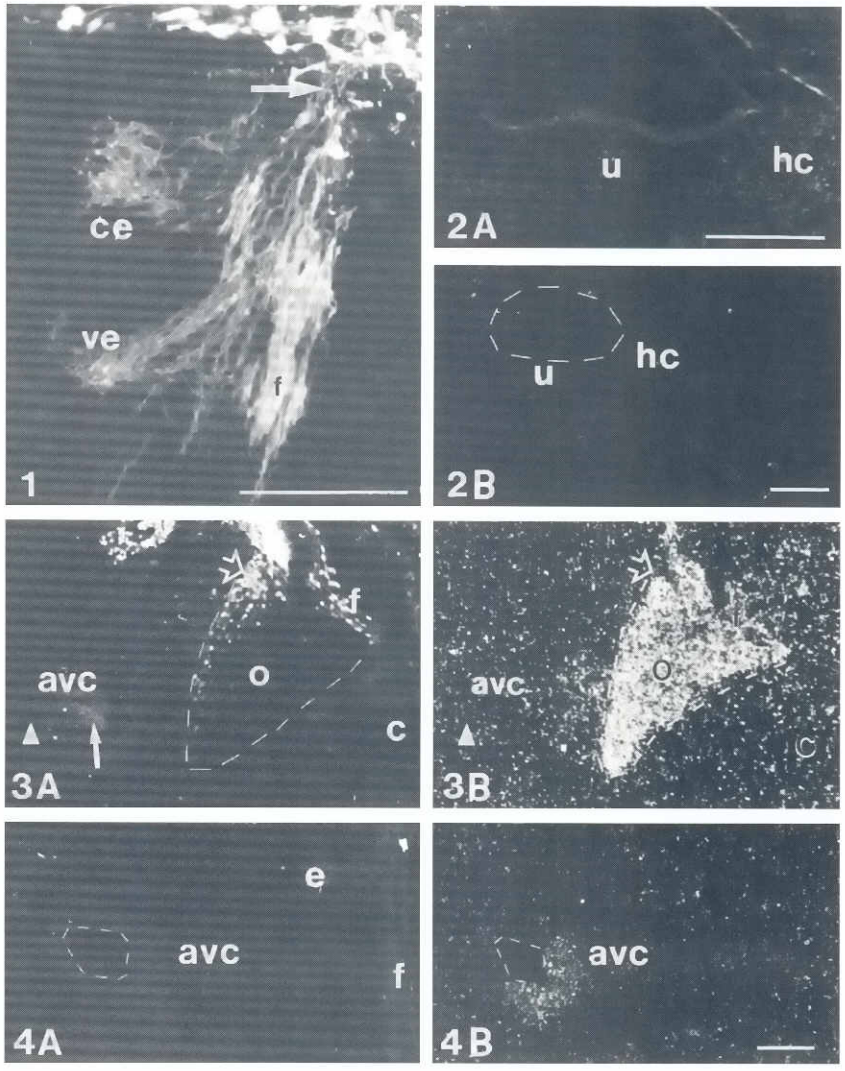

Fig. 1. TDA tracing of facial motor neurons and otic efferents. *Epifluorescence image of a section subjected to in situ hybridization analysis using a BDNF probe after TDA filling of efferents. Fibers efferent to the brain such as the branchial- and visceral motor fibers of the facial nerve (f) and the efferents to the ear are seen exiting the brain (arrow). Further distal otic efferents segregate as two distinct fascicles at the vestibular root (ve) and the cochlear root (ce). The quality of TDA tracer remains unaffected by the in situ hybridization procedure. Bar, 100 μm.*

Fig. 2. BDNF expression in the inner ear. *In situ hybridization analysis using* **(A)** *an anitsense oligonucleotide probe for BDNF and* **(B)** *a control for the BDNF labeling. Note the BDNF labeling at the horizontal canal (hc) and utricle (u) in A. The control shown in B is done by the ability to outcompete the labeling with a 100 times excess of unlabeled BDNF oligonucleotide. The dashed line indicate the inner ear lumen. Bars, 100 μm.*

Fig. 3. Anterograde TDA tracing of otic efferents and *trk*B mRNA expression. *Matching pair of epifluorescence and dark field images. The figure shows in* **(A)** *the distribution of otic and facial ganglion cells retrogradely filled through the application of TDA to the VII/VIIIth nerve root as well as anterogradely filled otic efferents (open arrow) In* **(B)**, *strong in situ hybridization labeling for TM-trkB is shown over the otic ganglion (O) but less in situ labeling is shown over the facial root ganglion (f). The area of the otic efferent bundle is marked by a reduced signal (open arrow). Note that many more postmitotic ganglion cells apparently express TM-trkB then are labeled with the dye* **(A,B)**. *Differentiated afferents have already invaded (arrow) the future sensory epithelia of the anterior vertical canal (avc). The arrow head indicates inner ear lumen and the dashed line outline the vestibular ganglion. c indicates cochlea. Bar as in 4B.*

Fig. 4. BDNF expression in sensory epithelium. *Matching pair of epifluorescence* **(A)** *and dark field* **(B)** *images showing the distribution of otic efferent fibers (e) and facial motor axons (f) and BDNF in situ signal over the sensory epithelium of the anterior vertical canal (avc). Note that the BDNF signal is restricted to the future sensory epithelium and absent in the lumen which is outlined by the dashed line. Bar in 3B, 100 μm, also for 3A,B and 4A.*

margin of the nerve to reach the utricle, anterior vertical and horizontal canal. A few efferent fibers were found next to the anlage of the sensory epithelia, which were characterized by a thickening of the epithelia. A few fibers penetrated through the basal lamina surrounding the otic vesicle and entered the otic epithelium. While similar in overall distribution, efferents did not extend as far in the E3.5 embryos and none entered the otic epithelium, as previously described (Fritzsch *et al.*, 1993a; Fritzsch, 1996).

In situ hybridization with the BDNF probe revealed a detectable signal over areas of all future vestibular sensory epithelia (Figs. 2A, 4B, 6A,B). A similar but even weaker signal was detected at E3.5 (data not shown). The ganglia and the surrounding periotic mesenchyme showed no signal above background, i.e., they were less than two times the grain density. Controls with excess of unlabeled BDNF oligonucleotide did not reveal any labeling above background in these areas at all (Fig. 2B). Thus, the efferent fibers extend to areas of the otic vesicle with expression of BDNF mRNA at the time efferents and afferents start to invade the future sensory epithelia (Hemond and Morest, 1991; Fritzsch *et al.*, 1993a; Fritzsch, 1996).

Efferent fibers in the posterior ramus form a tight bundle (Fig. 6A,C). This bundle approaches the ventromedial aspect of the posterior ramus where it abuts against the developing saccular and cochlear epithelium. Typically, efferent fibers could not be traced much further owing to the limited diffusion time used in this study (Fritzsch *et al.*, 1993a). While no fibers were seen entering sensory epithelia, the efferent fiber bundle was adjacent to the epithelia showing an elevated BDNF signal within the future saccule and cochlea (Fig. 6A-D). It was not clear whether there was a detectable signal over the just forming cochlea duct at E3.5. Again, no signal was detected with the control for BDNF hybridization.

Retrograde and anterograde filling of afferents and efferents

Severing the facial/otic nerve complex between the brain and the ganglia resulted in anterograde filling of otic efferent fibers and facial motor neuron axons, retrograde filling of facial motor neurons

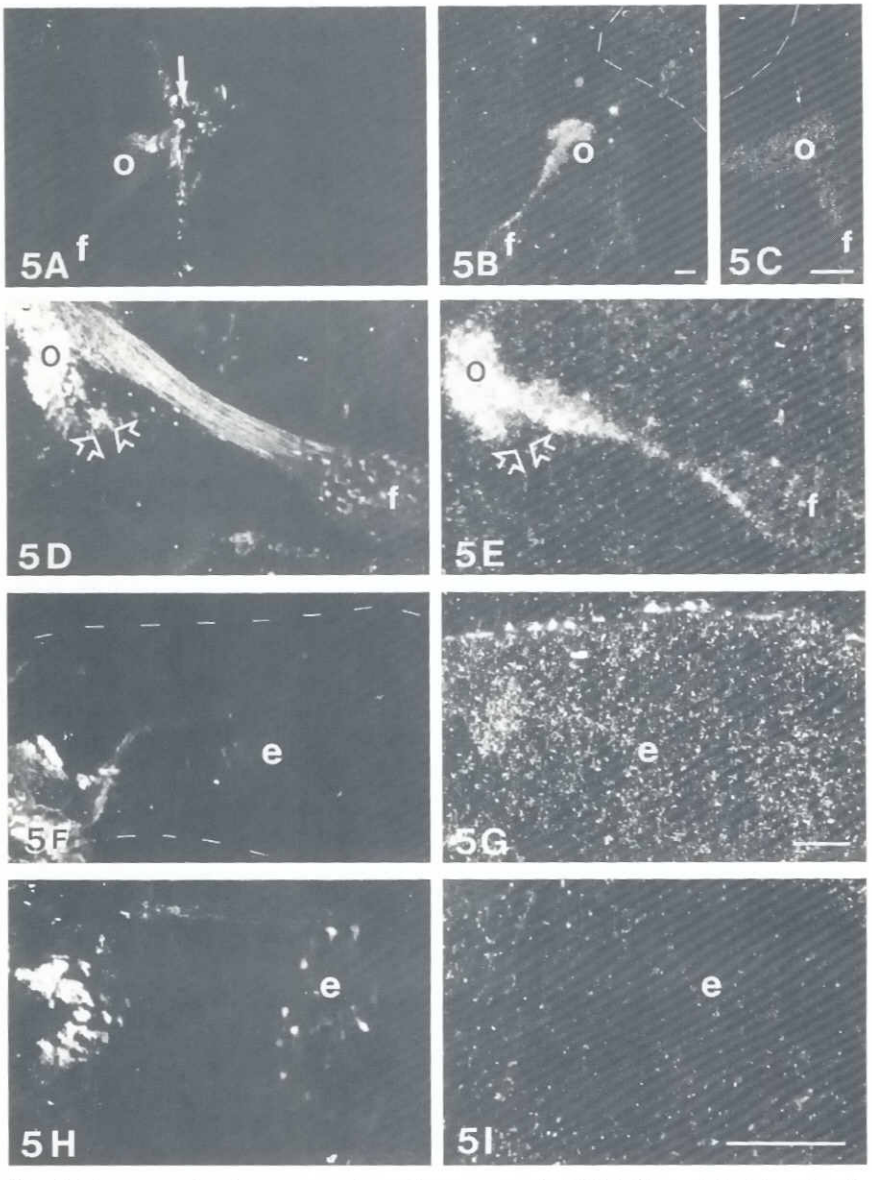

Fig. 5. Transganglionic, retrograde and anterograde TDA filling of facial and otic efferents and cell bodies in comparison with labeling for *trk*B mRNA expression. *These figures show low power* **(A,B)** *and higher power* **(C-I)** *images of otic (o) and facial (f) ganglion cells labeled transganglionically with TDA* **(A,D,F,H)** *and hybridized with either the kin-trkB* **(B,C,I)** *or TM-trkB* **(E,G)** *probes. Otic efferents (the open arrows,* **D***) are labeled anterogradely and facial motor neurons and otic efferents (e,* **F-I***) are labeled retrogradely. The trkB in situ hybridization signal obtained with both the kin- and the TM-trkB probes are strongest over the otic ganglion (o,* **B,E***). The hind brain (outlined by a dashed line,* **F-I***) is above background and so is the geniculate facial ganglion (f,* **D,E***). No apparent accumulation of either TM-trkB* **(G)** *or kin-trkB signal* **(I)** *can be seen over facial motor neurons or the otic efferent cells. However, the brain is above background with the TM-trkB probe and thus some small amount of trkB mRNA may be expressed in these cells. Open arrows indicate the efferent fiber bundle. Bars, 100 μm in all panels.*

and otic efferent cells, and transganglionic filling of facial ganglion cells (both the proximal, neural crest derived root ganglion and the distal, placodally derived geniculate ganglion; (Webb and Noden, 1993) and otic ganglion cells (Figs. 3A, 5A,D). Clearly, all ganglia had many more neurons when filled with the dye (Figs. 3A, 6E,F). Within the VIIIth nerve the efferent fibers formed a distinct fascicle that ran in exactly the same position as the fibers found after an application to the brain stem (Figs. 4A, 6C). Retrogradely filled facial motor neurons and otic efferent cells were both labeled (Fig. 5F-H) but not yet completely segregated on the ipsilateral side of the brain.

These preparations were hybridized either with a probe specific for mRNA encoding the catalytic *trk*B receptors (kin-*trk*B; Fig. 5C,I) or with a probe directed to a part of the mRNA encoding the transmembrane region (TM-*trk*B; Figs. 3B, 5B,E,G, 6F) that will recognize presumably all mRNA's of *trk*B. Additional slides were hybridized with controls for kin-*trk*B and TM-*trk*B mRNA.

No kin-*trk*B signal was detected in the otic epithelium (data not shown) and the brain showed very low or no levels of kin-*trk*B signal (Fig. 5C,I). In fact the signal with both kin-*trk*B and the TM-*trk*B probes at the otic ganglia was several fold higher than anywhere else (Figs. 3B, 5B,C,E). Whether the signal is exclusively in the placodally derived otic ganglion cells or, in addition, in the neural crest derived (Webb and Noden, 1993) Schwann cells is not clear, but its low levels in other nearby ganglia in which the Schwann cells are equally derived from neural crest suggest that the *trk*B signal is associated with otic ganglion neurons. Owing to this high level of kin-*trk*B signal within the otic ganglion cells the efferent bundle, surrounded by the same Schwann cells as the otic ganglion processes, stood out as the only area of the otic ganglion without *in situ* hybridization signal for *trk*B (Figs. 3A,B, 5D,E, 6E,F). This is in agreement with the concept of no or very low levels of mRNA in axons. No preferential *trk*B signal was found either over the retrogradely filled facial motor neurons or otic efferent cells (Fig. 5G,I). However, given that the entire brain showed a *trk*B mRNA signal of at least twice the background (Fig. 5G), there is a possibility that these cells may possess some truncated TrkB receptors, although the level of mRNA is substantially lower than in the otic ganglion cells.

Similar labeling patterns were found after hybridization with the TM-*trk*B and kin-*trk*B probes with the single exception of the cochlea. Here we detected a TM-*trk*B signal above background and almost as strong as above the otic ganglion (Fig. 6F). Both controls for TM-*trk*B (Fig. 13) and kin-*trk*B probes hybridization showed only a random distribution of silver grains.

Discussion

The otic ganglion is derived from the developing otocyst but has to extend its afferents back to the otocyst to innervate the various sensory epithelia. While transplantations as early as E3 have

shown that the otocyst can develop independent of innervation *in vitro* (Ard *et al.*, 1985) or when grafted (Swanson *et al.*, 1990), it appears that survival of otic ganglion cells is influenced by the presence of the ear (van de Water *et al.*, 1992; Bianchi and Cohan, 1993 for review). Moreover, the distal processes of the otic ganglion appear to be attracted towards the differentiating sensory epithelia (Bianchi and Cohan, 1991; Hemond and Morest, 1992) which may also provide trophic support in the form of NGF, BDNF and NT-3 (Hallböök *et al.*, 1993; Bernd *et al.*, 1994). The suggestion that BDNF may play a role in trophic support of the early stage otic ganglia was previously rejected based on *in vitro* assays (Davies *et al.*, 1986) but was later suggested again for chicken (Vogel, 1992; Avila *et al.*, 1993) and mice (Staecker *et al.*, 1995). In contrast, no evidence favoring a single neurotrophic factor or various combinations was found in *in vitro* studies on differentiating chicken otic ganglia at embryonic day 5 (Bianchi and Cohan, 1993). It was suggested that different aspects of neurite outgrowth and ganglion cell survival may be regulated by different factors or various combinations at different stages of development, a suggestion that agrees with recent data on temporal changes in neurotrophin expressions.

Whatever the solution for these divergent *in vitro* data may ultimately be, there is clearly expression of the *trk*B mRNA in the ganglion of rats (Ernfors *et al.*, 1992; Pirvola *et al.*, 1994; Schecterson and Bothwell, 1994) and chicken (Dechant *et al.*, 1993); this study (Figs. 3B, 5B,C,E, 6F) and expression of BDNF mRNA in the target (Pirvola *et al.*, 1992; Hallböök *et al.*, 1993; Figs. 2, 4B, 6B,D). However, one study showed no evidence for the presence of *trk*B in otic ganglia of quail employing polymerase chain reaction techniques (Bernd *et al.*, 1994). It is unclear why this later study failed to show this neurotrophin receptor as it temporally overlaps with one study in chicken that demonstrates the presence of *trk*B mRNA by *in situ* hybridization (Dechant *et al.*, 1993) and our study. However, species specific expression could account for the differences.

Otic ganglion cells become postmitotic between 2-5 days (D'Amico-Martel, 1982). Thus, some ganglion cells have already differentiated and have started to enter with their processes the otic epithelia (Whitehead and Morest, 1985; Hemond and Morest, 1991; Fritzsch *et al.*, 1993b) at the stages we examined. Our data suggest that *trk*B mRNA expression occurs in postmitotic ganglion cells even before they develop an axon. We base this conclusion on the fact that many ganglion cells remained consistently unlabeled with the Dextran tracer after transection of the proximal VIIIth nerve but showed strong *trk*B mRNA signal (Figs. 3A,B, 6E,F). Absence of labeling for *trk*B mRNA around the otic efferent bundle argues against the possibility that otic Schwann cells may be the only source expressing *trk*B mRNA. The early presence of *trk*B mRNA in postmitotic otic ganglion cells and the results showing that BDNF elaborates axonal growth (Cohen-Cory and Fraser, 1994) and guides axons (Panni *et al.*, 1994; Snider, 1994) suggest that BDNF (possibly in conjunction with NT-3) may have a neurotropic effect on otic ganglion cells guiding the fibers to the target areas. Evidence in other cranial ganglia also strongly supports the presence of TrkB in ganglia but not in Schwann cells (Hallböök *et al.*, 1995). While BDNF could be a major player in this early action according to its own expression pattern and the pattern of expression of its receptor, other neurotrophic factors (e.g., NT-3) and their receptors (*trk*C) as well as ciliary neurotrophic factor (Bianchi and Cohan, 1993; Staecker *et al.*, 1995) need to be characterized further to limit the possible candidates. In fact, mice lacking NT-3 or *trk*C apparently have many fewer cochlear ganglion cells (Bianchi and Cohan, 1993; Fariñas *et al.*, 1994; Ernfors *et al.*, 1995; Hallböök *et al.*, 1995; Schimmang *et al.*, 1995; Staecker *et al.*,

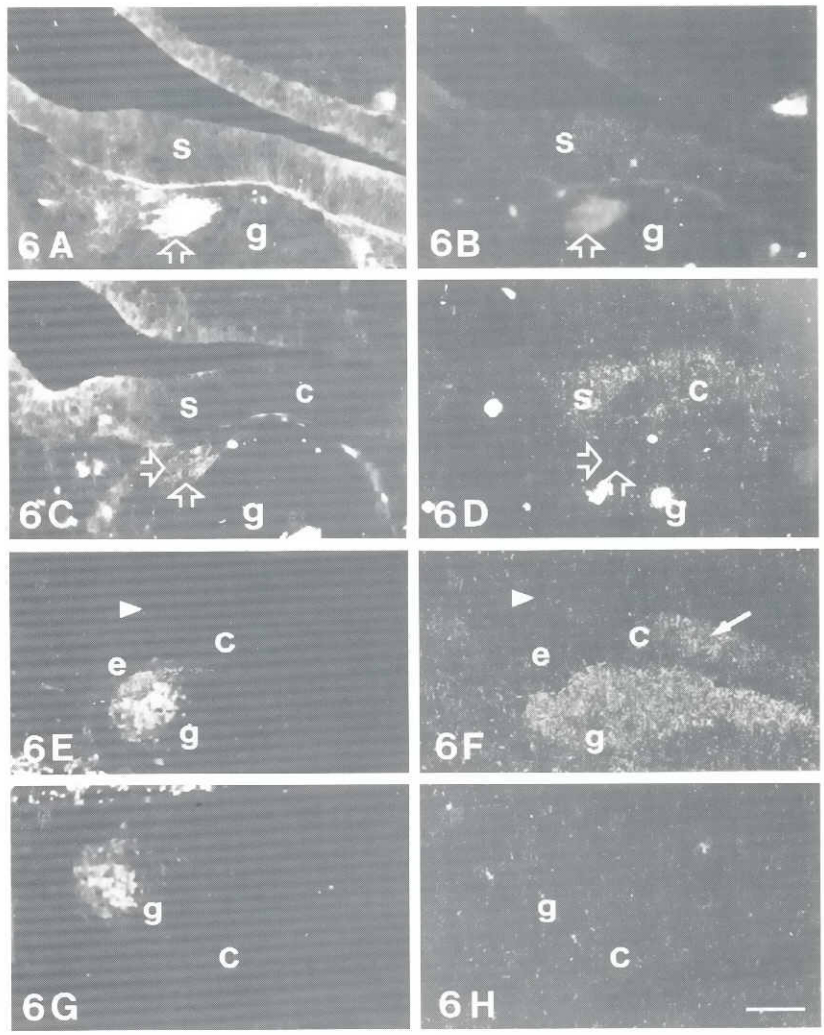

Fig. 6. BDNF and *trk*B mRNA expression. *Matching epifluorescent images* **(A,C,E,G)** *and in situ hybridization images* **(B,D,F,H)** *show the distribution of otic efferents (open arrows,* **A-D***) and otic ganglion cells (g) near the saccule (s) and the future cochlear epithelium (c). The otic efferents are located closest to the future sensory epithelia, which are in part labeled with the BDNF probe* **(B)** *and TM-trkB probe (arrow,* **F***). Strong TM-trkB in situ hybridization analysis signal is seen over the otic ganglion (g) except for the notch which carries the efferent fibers (e, demarcated with a dashed line,* **E,F***). No specific signal was obtained over the otic ganglion (g) or the cochlear sensory epithelium (c) using the control for the trkB probe hybridization* **(G,H)**. *Arrowheads* **(E,F)** *indicate the similar structures in matching images. Bar, 100 μm in all panels.*

1995) and have a reduced innervation density at the base of the cochlea (Fritzsch *et al.*, 1995; Fritzsch, 1996).

A neurotrophic action for BDNF is supported by the data in BDNF knockout mice based on the absence or reduction of fibers in vestibular sensory epithelia (Ernfors *et al.*, 1994b, 1995; Bianchi *et al.*, 1996). These data on BDNF and *trk*B knockout mice (Fritzsch *et al.*, 1995) provide the strongest evidence thus far for a possible neurotrophic action of BDNF for the otic ganglion cells in mice. However, the suggested neurotropic role of BDNF is questionable based on most recent evidence in knockout mice which show that initial fiber outgrowth of afferents and efferents appears to be independent of this neurotrophin and its receptor (Ernfors *et al.*, 1995; Bianchi *et al.*, 1996).

Several isoforms of the TrkB receptor (kinase associated catalytic and truncated forms) have been postulated in mammals on the basis of cDNA sequences and immunoprecipitation (Klein *et al.*, 1990; Middlemas *et al.*, 1991). Interestingly, in the cochlea the truncated receptor expression appears to overlap with the region that expresses BDNF (Fig. 6B,D,F) and the mRNA encoding the P75 low affinity neurotrophin receptor (Hallböök *et al.*, 1990, 1993).

In fact, tissue that express *trk*B mRNA encoding only truncated TrkB receptors have been identified before (Klein *et al.*, 1990; Middlemas *et al.*, 1991) Middlemas, 1991 #2379; Beck, 1994 #465 and these receptors can bind BDNF with similar affinity as the catalytic *trk*B receptors. Expression of the P75 low affinity neurotrophin receptor together with Trk receptors is abundant and these receptors have been suggested to collaborate in the binding of neurotrophins (Hempstead *et al.*, 1991); Hantzopoulos, 1994 #2089; Mahadeo, 1994 #2336. We have found that mRNA for BDNF and the truncated *trk*B receptor are regionally co-expressed in the developing epithelium (Ylikoski *et al.*, 1993; Biffo *et al.*, 1995), and it is likely that the receptors bind the BDNF and may compete with catalytic receptors on the innervating sensory fibers for available BDNF.

The BDNF and *trk*B knockout mice clearly show that BDNF and its receptor play a major role for the survival of vestibular ganglion cells. However, their role for facial motor neurons is less clear-cut. We could not detect any elevated *trk*B signal at the facial motor neurons or the octaval efferent cells, which was above the signal in the brain (Fig. 5G,I). These low levels of *trk*B mRNA expression in chick appear to be in conflict with the initial report showing reduction of facial motor neurons in the *trk*B knockout mice (Klein *et al.*, 1993). Thus, while otic efferent axons as well as otic afferent processes project to the BDNF signal areas (Fig. 6A,C), the level of *trk*B mRNA expression is well below that of the otic ganglion cells. Nevertheless, we cannot exclude the possibility that even this low level of *trk*B mRNA expression is sufficient for the translation of enough *trk*B receptors to support facial motor neurons and otic efferents. The possibility that not BDNF but other neurotrophic factors such as neurotrophin-4 (Snider, 1994; Silos-Santiago *et al.*, 1995), may play a more important role in the survival of facial motor neurons and otic efferents was recently put in question (Minichiello and Klein, 1996) or dismissed (Conover *et al.*, 1995). The latter evidence would agree with the comparatively low level of *trk*B mRNA expression found over facial motor neurons in our hands. The evidence for motoneuron support by TrkB is thus not very compelling and further studies are needed.

Given that the efferents reach to and invade these same areas of the otocyst at the same time as otic afferents, efferent fibers may simply be guided along early differentiating otic afferents (Figs. 4A, 7A. 12A) to reach the otocyst and may use other trophic factors like the glial cell-line derived factor (Henderson *et al.*, 1994) or other growth factors (van de Water *et al.*, 1992; Yin *et al.*, 1994) for their support. Recent data in *trk*B$^{-/-}$ mice are consistent with the latter suggestion (Fritzsch *et al.*, 1995, 1997). Further studies using the techniques developed for this study but examining the distribution of other neurotrophins and their receptors in the otic region are needed to clarify this issue.

Materials and Methods

We used 35 chickens, *Gallus domesticus*, for this study. The fertilized eggs were incubated and at 3.5 days (N=7) and 4.5 days (N=28) the egg shell was opened, the embryos were dissected and the head was placed in cold Neurobasic (Gibco) medium and staged according to (Hamburger and Hamilton, 1951). The VII/VIIIth nerve (9 cases) or the rhombomeres 4/5 of the hind brain (26 cases) were exposed, cut and re-crystallized 3 kDa Dextran amines conjugated to Texas red (TDA; Molecular Probes) was applied for 5-10 sec (Fritzsch, 1993). The surgical cut of a given structure (i.e., a nerve or a rhombomere) and the shortness of the Dextran application were the factors that determined the specificity of the labeling. The dissected hind brain area with the ear attached was incubated for 2 h at 22° C and subsequently fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Length of fixation (up to several months) did not play any apparent role as long as it was longer than 30 min. The fixed tissue was cryoprotected (30% sucrose) and cut at a freezing microtome. All sections were 15 μm thick transverse frozen sections of 3.5 and 4.5 day old chicken embryos (Hamburger and Hamilton stages 24-28) in which TDA has been applied to a longitudinal cut into the basal plate of rhombomeres 4 and 5 or the cut VII/VIIIth nerve. Sections were collected on a poly-L-lysine coated slide and *in situ* hybridization was performed. Care was taken to avoid any contact with bare hands to minimize contamination with RNAses as described (Fritzsch and Hallböök, 1996).

In situ hybridization was performed using synthetic single stranded oligonucleotide probes complementary to the chicken BDNF and *trk*B mRNAs as described previously (Dechant *et al.*, 1993; Hallböök *et al.*, 1993). The oligonucleotides were labeled at their 3'-end with a-[^{35}S] dATP using terminal deoxyribonucleotidyl transferase (Promega, WI) to a specific activity of 1.5×10^9 counts per minute/μg and purified on a Nensorb column (Dupont, DE) prior to use. The oligonucleotide complementary to chicken BDNF mRNA corresponds to amino acids 61-77 of the BDNF protein.

For analysis of *trk*B mRNA expression two probes were used. One is directed to the part of the mRNA encoding the transmembrane region (TM-*trk*B) and corresponds to amino acids 429 to 444 of the chicken TrkB receptor (Dechant *et al.*, 1993). The other is directed to the tyrosine kinase domain (kin-*trk*B) corresponding to amino acids 696 to 713. *In situ* hybridization was performed at 42°C for 15 h in a humidified chamber with 150 μl of hybridization cocktail containing 1×10^6 counts per minute of respective probe. The hybridization cocktail contained 50% formamide, 4 x standard saline citrate, 10% Dextran sulfate, 0.5 mg/ml yeast tRNA, 0.06 dithiothreitol and 0.1 mg/ml sonicated salmon sperm DNA. After hybridization, the slides were rinsed and first washed 4x15 min in 0.5 x standard saline citrate at 55°C and dehydrated in ethanol and left to air-dry and then dipped in Kodak NTB-2 photographic emulsion diluted 1:1 in 0.6 M ammonium acetate. Exposure time was 9 weeks at 4°C. The specificity of the hybridization was controlled for using a 100 times excess of non-labeled oligonucleotide to compete out the hybridization signal from the corresponding labeled probe on the sections. All labeling could be competed out with excess oligonucleotides. The three probes served as controls for each other for unspecific hybridization. Furthermore, some areas within the sections are known to contain mRNA for BDNF and *trk*B and were internal positive controls during the evaluation of the double labeling procedure.

The sections were then cover slipped with Vectashield (Vector Laboratories) mounting medium, viewed and photographed with differential interference contrast, dark field (for the *in situ* hybridization analysis signal,

silver grains) and epifluorescence (for the Texas red signal). Images were analyzed for silver grain density using the Image Pro software on grabbed images. Only grain densities which were at least twice the background density were accepted as a signal. Images were de convoluted using the Vaytek software and aligned images taken with DIC, DF or EF were combined into single color images to show the critically assess the alignment of structures and labeling.

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