

# Neurotrophins and Their Receptors in Rat Peripheral Trigeminal System during Maxillary Nerve Growth

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**Abstract.** We examined the expression of the neurotrophins (NTFs) and their receptor mRNAs in the rat trigeminal ganglion and the first branchial arch before and at the time of maxillary nerve growth. The maxillary nerve appears first at embryonic day (E)10 and reaches the epithelium of the first branchial arch at E12, as revealed by anti-L1 immunohistochemistry. In situ hybridization demonstrates, that at E10–E11, neurotrophin-3 (NT-3) mRNA is expressed mainly in the mesenchyme, but neurotrophin-4 (NT-4) mRNA in the epithelium of the first branchial arch. NGF and brain-derived neurotrophic factor (BDNF) mRNAs start to be expressed in the distal part of the first branchial arch shortly before its innervation by the

maxillary nerve. Trigeminal ganglia strongly express the mRNA of *trkA* at E10 and thereafter. The expression of mRNAs for low-affinity neurotrophin receptor (LANR), *trkB*, and *trkC* in trigeminal ganglia is weak at E10, but increases by E11–E12. NT-3, NT-4, and more prominently BDNF, induce neurite outgrowth from explant cultures of the E10 trigeminal ganglia but no neurites are induced by NGF, despite the expression of *trkA*. By E12, the neuritogenic potency of NGF also appears. The expression of NT-3 and NT-4 and their receptors in the trigeminal system prior to target field innervation suggests that these NTFs have also other functions than being the target-derived trophic factors.

NEUROTROPHINS (NTFs)<sup>1</sup> are the family of related proteins with specific effects on developing nervous system. To date, four NTFs have been characterized. These are NGF (Levi-Montalcini, 1987), brain-derived neurotrophic factor (BDNF) (Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (NT-3) (Ernfors et al., 1990; Hohn et al., 1990; Jones and Reichardt, 1990; Kaisho et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990), and neurotrophin-4 (NT-4) (Hallböök et al., 1991; Ip et al., 1992), also termed neurotrophin-5 (Berke-meier et al., 1991). NTFs are shown to be vitally essential in the development of nervous system at the stage of programmed neuronal death. NTFs, secreted in limiting amounts from relevant peripheral and brain structures are shown to support the survival of sensitive neurons which innervate that particular tissue at the time of programmed neuronal death, thus regulating the number of the innervating neurons (Oppenheim, 1991; Barde, 1989). Such a target-derived neu-

rotrophic role is convincingly established for NGF as a prototypic molecule for the whole family (Levi-Montalcini, 1987; Barde, 1989). Although the survival-promoting abilities of other NTFs are poorly studied *in vivo*, they are shown to have effects similar to those of NGF on overlapping but also distinct neuronal populations *in vitro* (Lindsay et al., 1985; Maisonpierre et al., 1990; Hohn et al., 1990; Rosenthal et al., 1990) suggesting the target-derived neurotrophic function for them as well. This suggestion is supported by simultaneous presence of NTF receptor mRNAs in several neuronal populations and the cognate NTFs in the respective innervated tissues during the time of programmed neuronal death (Schechterson and Bothwell, 1992; Ernfors et al., 1992).

To perform their biological effects, the NTFs must bind to appropriate receptors on the surface of the responsive cells. Binding studies have revealed the presence of high- and low-affinity receptors for NGF, BDNF, and NT-3 on chick embryonic sensory neurons (Sutter et al., 1979; Rodríguez-Tébar and Barde, 1988; Rodríguez-Tébar et al., 1990, 1992). The high-affinity receptors are most probably the members of the *trk* protein tyrosine kinase receptor family: *trkA* (Martin-Zanca et al., 1989), *trkB* (Klein et al., 1989), and

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1. *Abbreviations used in this paper:* BDNF, brain-derived neurotrophic factor; LANR, low-affinity neurotrophin receptor; NTF, neurotrophin.

trkC (Lamballe et al., 1991). Binding of cognate NTFs to their corresponding trk receptors ectopically expressed in fibroblasts induces autophosphorylation of the receptors and transduces the signals. Thus, trkA is a functional receptor for NGF (Klein et al., 1991b; Cordon-Cardo et al., 1991; Jing et al., 1992; Ip et al., 1993), trkB can be functionally activated by both BDNF (Klein et al., 1991a; Squinto et al., 1991; Glass et al., 1991; Ip et al., 1993) and NT-4 (Klein et al., 1992; Ip et al., 1993), whereas NT-3 is a "nonpreferred" ligand for trkB (Glass et al., 1991; Klein et al., 1991a; Squinto et al., 1991; Ip et al., 1993). trkC serves as a functional receptor for NT-3 (Lamballe et al., 1991; Ip et al., 1993). The low-affinity NGF receptor p75 (Johnson et al., 1986; Radeke et al., 1987) is suggested to be designated as low-affinity neurotrophin receptor (LANR) as it binds NGF, BDNF, NT-3, and NT-4 with similar low-affinity kinetics (Rodríguez-Tébar and Barde, 1988; Rodríguez-Tébar et al., 1990, 1992; Hallböök et al., 1991). The LANR is known not to be the signal-transducing receptor and its participation in the formation of the high-affinity NTF receptors is controversial (Hempstead et al., 1991; Klein et al., 1991b; Glass et al., 1991; Jing et al., 1992; Ip et al., 1993). trkB (Klein et al., 1990a; Middlemas et al., 1991) and trkC (Tsoulfas et al., 1993) loci yield multiple mRNA species encoding various receptor isoforms, some of which being catalytic, while some are without the tyrosine kinase domain and thus most probably unable to transduce the signal of the ligand. The truncated trkB transcripts are, however, expressed in several parts of the developing and adult brain of the mouse and rat (Klein et al., 1989, 1990a; Middlemas et al., 1991) suggesting that they are important in the processes not consistent with the neurotrophic concept.

In a particular neuronal population, the programmed death begins at the time the first neurites reach the target tissue (Rohrer et al., 1988; Davies et al., 1987; Vogel and Davies, 1991). Transcripts of trkA and trkB are also reported in the mouse embryos at the earliest stages when the developing neurons are not yet competent to send out neurites (Martin-Zanca et al., 1990; Klein et al., 1990b) suggesting that NTFs may have the functions in the early embryogenesis other than promoting neuronal survival. For example NGF is shown to direct the course of regenerating neurites of its responsive neurons *in vitro* (Gundersen and Barrett, 1980), leading to the hypothesis that *in vivo* it may be a target-derived chemoattractant directing the course of the pioneer neurites during the precise process of neurite pathfinding (Lumsden and Davies, 1983; Placzek et al., 1990). However, the guidance of the primary neurites by target-derived gradients of the NTFs is not demonstrated *in vivo*. Recently, BDNF has been shown to accelerate the maturation of early chick dorsal root ganglion cells *in vitro* (Wright et al., 1992). In general, however, the possible roles of NTFs in early processes of neurogenesis, such as proliferation, commitment, migration, and maturation of neuronal precursors as well as formation of primary neuritic pathways, are poorly studied.

The trigeminal ganglion is a good model to study the effects of NTFs on early neurogenesis because its development is morphologically quite well established (Altman and Bayer, 1982; Davies, 1988; Stainier and Gilbert, 1991) but the factors regulating the development are largely unknown. It has been demonstrated that the first neurites of the maxillary

branch of the mouse trigeminal nerve are directed to the organ of innervation—the whisker pad of the first branchial arch—by a soluble agent secreted from the epithelium (Lumsden and Davies, 1983, 1986). The agent named "trigeminal neurotropic factor," is immunochemically different from NGF, and the expression of NGF is detected in the whisker field at the time when the first neurites contact the cutaneous target (Davies et al., 1987). Correspondingly, the mouse trigeminal ganglion neurons express LANR mRNA at the earliest stages of differentiation, the amount of the transcripts increasing about fivefold when the first axons contact the target field (Davies et al., 1987; Wyatt et al., 1990). The expression of other NTFs and the trk protein tyrosine kinase receptors in the trigeminal system has been studied only at the stages when the neurons have already contacted their targets. Thus, the distal regions of the mouse branchial arches express mRNAs for NGF, BDNF, NT-3, and NT-4 (Davies et al., 1987; Schechterson and Bothwell, 1992; Ernfors et al., 1992; Ibáñez et al., 1993), while expression of mRNAs for LANR, trkA, trkB, and trkC have been reported in mouse and rat trigeminal ganglia (Martin-Zanca et al., 1990; Klein et al., 1990b; Wheeler and Bothwell, 1992; Schechterson and Bothwell, 1992; Ernfors et al., 1992). However, there is no information available about their expression at the early preinnervation stages of trigeminal ganglion development.

As a first step of identifying the roles of the neurotrophins at the initial stages of development of the trigeminal ganglion neurons, we studied the expression of the mRNAs for all known neurotrophins and their receptors in rat preinnervation trigeminal ganglion and in one of its peripheral targets—the first branchial arch. The ability of the developing trigeminal ganglia to send out neurites in response to NTFs was investigated as well. These studies constitute the basis for further functional studies about the role of the NTFs in the preinnervation embryos.

## Materials and Methods

### Construction of Neurotrophin Probes

The cRNA probes for rat NTFs were synthesized from the cDNA fragments (details of the cloning described in Pirvola et al., 1992) encompassing the following nucleotides: 283–716 of rat preproNGF mRNA (Whittemore et al., 1988), 414–712 of rat preproBDNF mRNA (Timmusk et al., 1993), 145–331 of rat preproNT-3 mRNA (Maisonpierre et al., 1990), and 295–550 of rat NT-5 mRNA (Berkemeier et al., 1991).

### Construction of Neurotrophin Receptor Probes

cRNA probes for rat NTF receptors were synthesized from the following regions (details of the cloning described in Ylikoski et al., 1993): nucleotides 343–609 corresponding to human trkA (Martin-Zanca et al., 1989), 562–762 of rat trkC cDNA (Merlio et al., 1992), and nucleotides 230–535 of rat trkB recognizing catalytic as well as truncated forms of the receptor (Middlemas et al., 1991) (pan-trkB probe). To discriminate the full-length (catalytic) and truncated (non-catalytic) forms of trkB, the probes corresponding to nucleotides 1357–1507 of the tyrosine kinase domain of the rat full-length receptor and to nucleotides 1343–1545 of the cytoplasmic domain of the rat trkB-T1 cDNA (absent in the full-length molecule) (Middlemas et al., 1991) were prepared by polymerase chain reaction and cloned into the PCR II vector (Invitrogen Corporation, San Diego, CA). To detect LANR, a cRNA probe was synthesized from a cDNA fragment corresponding to the transmembrane region (nucleotides 853–970) of the rat LANR (Radeke et al., 1987).

## In Situ Hybridizations

Sprague-Dawley rats were mated overnight and the day of the vaginal plug was considered as embryonic day (E)0. Paraffin-embedded serial sections of 5  $\mu$ m thickness were cut sagittally from whole embryos and collected on 3-aminopropyl ethoxysilane-coated (Sigma Immunochemicals, St. Louis, MO) slides. The pretreatment, hybridization, and posttreatment of the sections were carried out as described (Pirvola et al., 1992).

The  $^{35}$ S-UTP-labeled sense and antisense RNA probes were prepared according to Wilkinson and Green (1990) using an appropriate SP6 or T7 transcription system (Promega Corp., Madison, WI) and used at a final concentration of 20,000 cpm/ $\mu$ l. No hybridization signals above the background levels were detected with sense-strand control probes of all NTFs or NTF receptors. Examples of *trkC* and *NGF* sense probe hybridizations are shown (see Fig. 3 *i* and *f*).

## Ganglionic Explant Cultures

Trigeminal ganglia were dissected from E10–E12 rat embryos and embedded into three-dimensional collagen matrix prepared according to Ebendal (1989). Typically, 3–5 ganglia were cultured in 0.5 ml of matrix in 24-well tissue culture plates (Nunc Delta, Denmark). The gels were covered by 0.5 ml of Eagle's Basal Medium (GIBCO BRL, Gaithersburg, MD) containing 1% of heat-inactivated horse serum (PAA-Labor- und Forschungsgesellschaft mbH, Linz, Austria). The collagen gel was prepared into the same medium. Recombinant rat BDNF and NT-3 (a generous gift from Prof. Y.-A. Barde, Max-Planck-Institute for Psychiatry, Munich, Germany) and chromatographically purified mouse  $\beta$ NGF (Institute of Physiology, Minsk, Byelorussian Republic) were added to the culture media. All NTFs induced standard neurite outgrowth from E8 chick dorsal root ganglion explants known to respond to all three factors (Maisonpierre et al., 1990) at 5 ng/ml whereas at 1 ng/ml or below that, virtually no neurites appeared. All three factors were typically applied at 5 ng/ml if not stated otherwise. The explant cultures were incubated at 37°C in the humid atmosphere containing 5% of CO<sub>2</sub>. The cultures were examined after 24 and 48 h.

## Cloning and Expression of Human NT-4

Human gene encoding NT-4 was selected from the human genomic DNA library in EMBL 3 (kindly provided by Dr. R. Allikmets, Institute of Chemical Physics and Biophysics, Estonian Academy of Sciences, Tallinn, Estonia) using the rat NT-4 cDNA probe. An 840-bp *Sau* 3AI fragment containing the coding exon of human NT-4 with 5' noncoding flank of 60 bp and

3' noncoding flank of 20 bp, was cloned into BamHI site of pBluescriptSK<sup>+</sup> (Stratagene, La Jolla, CA) and sequenced. The insert was subcloned into the NotI/XhoI sites of expression vector pMEP4 (Invitrogen Corporation) under inducible metallothionein promoter. COS cells grown to ~70% confluence in DME (GIBCO BRL) containing 10% of heat-inactivated FCS (PAA-Labor- und Forschungsgesellschaft mbH, Linz, Austria) were transfected with 10  $\mu$ g of the expression plasmid per 6-cm cell culture dish by the Lipofectin Reagent (GIBCO BRL) following the manufacturer's instructions. NT-4 expression was induced by 10  $\mu$ M ZnCl<sub>2</sub> in culture medium. The conditioned medium was collected 24 h later from the transfected cells and used in the neurite outgrowth assay on E7–E8 chick dorsal root ganglia and rat trigeminal ganglia as described above. No neurites were obtained by control medium from the transfected but uninduced cells.

## Immunohistochemistry

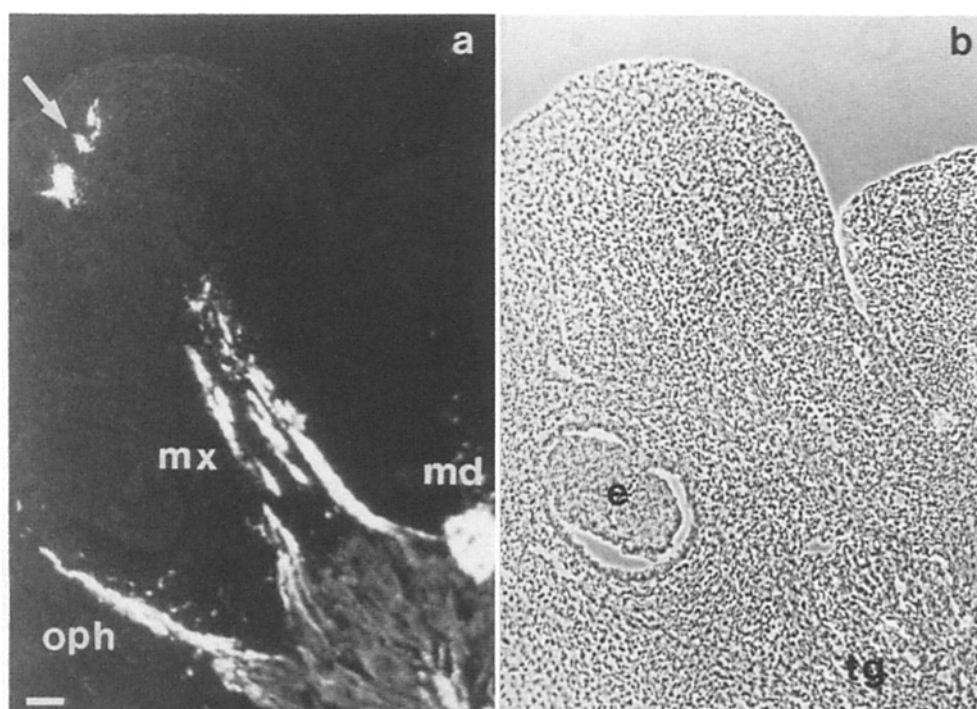
Sagittal cryosections were cut from the fixed E10–E12 rat embryos and immunostained with the affinity-purified rabbit polyclonal antibody to L1 cell adhesion molecule (a kind gift from Prof. M. Schachner, ETH, Zurich, Switzerland) in PBS containing 0.25% Triton X-100 and 1% BSA at +4°C overnight followed by FITC-conjugated donkey anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in the same buffer.

Rat trigeminal ganglia of different ages were cultivated in a thin layer of collagen gel on round coverslips, fixed with 3.5% paraformaldehyde in PBS and indirect immunofluorescence was performed with anti-neurofilament antibody (a kind gift from Prof. I. Virtanen, Department of Anatomy, University of Helsinki, Finland) in PBS containing 0.25% Triton X-100 and 1% BSA at +4°C. The specimens were mounted in DABCO (Sigma Immunochemicals).

## Results

### Development of Maxillary Nerve

The initial stages of the rat maxillary nerve development were studied immunohistochemically using polyclonal antibodies to L1 cell adhesion molecule. The first neurites start to grow into the first branchial arch during E10. At E11, the growing maxillary fibers are in the central region of the first



**Figure 1.** Rat trigeminal ganglion and its peripheral processes at E12. (a) Sagittal section of the rat embryo immunostained for L1 cell adhesion molecule. The maxillary nerve has approached the cutaneous target field (arrow). The corresponding bright-field micrograph of the same section is shown (b). *oph*, ophthalmic nerve; *mx*, maxillary nerve; *md*, mandibular nerve; *e*, eye; *tg*, trigeminal ganglion. Bar, 100  $\mu$ m.

branchial arch and approach their cutaneous target fields at E12 (Fig. 1). Thus, the rat whisker field innervation begins at the late E12.

#### **Expression of mRNAs for Neurotrophins in the First Branchial Arch of the Rat Embryo**

NT-3 transcripts were prominently expressed in the mesenchyme of the whole first branchial arch at early E10, when the trigeminal ganglion was not yet discernible (not shown). The same pattern of expression persisted throughout E10–E11 (Fig. 2, *b* and *c*). By E12, NT-3 mRNA was found mostly in the distal part of the first branchial arch (Fig. 3, *g* and *h*). At every stage studied, the grains were mostly localized to the mesenchyme and the epithelial cells were labeled weakly.

At E10, no NGF transcripts were visible in the first branchial arch (not shown). NGF transcripts appeared first at E11 as a weak labeling of the distal mesenchyme of the first branchial arch, most of the grains localizing in the subepithelial layer (not shown). This labeling clearly increased during the subsequent day (Fig. 3, *d* and *f*). The grains were mostly localized to the mesenchymal cells but the epithelium was also weakly labeled.

BDNF mRNA was not detected in the first branchial arch at E10 or E11 (not shown). At E12, labeling of BDNF mRNA was first seen in the mesenchyme of the distal part of the first branchial arch, the epithelial cells being mostly unlabeled (Fig. 3, *a* and *c*).

Clear labeling of NT-4 mRNA was observed at E10–E12

in the epithelium of several regions of the embryo including that of the first branchial arch, as shown for E11 (Fig. 2 *d*). No labeling of the first branchial arch mesenchyme was detected.

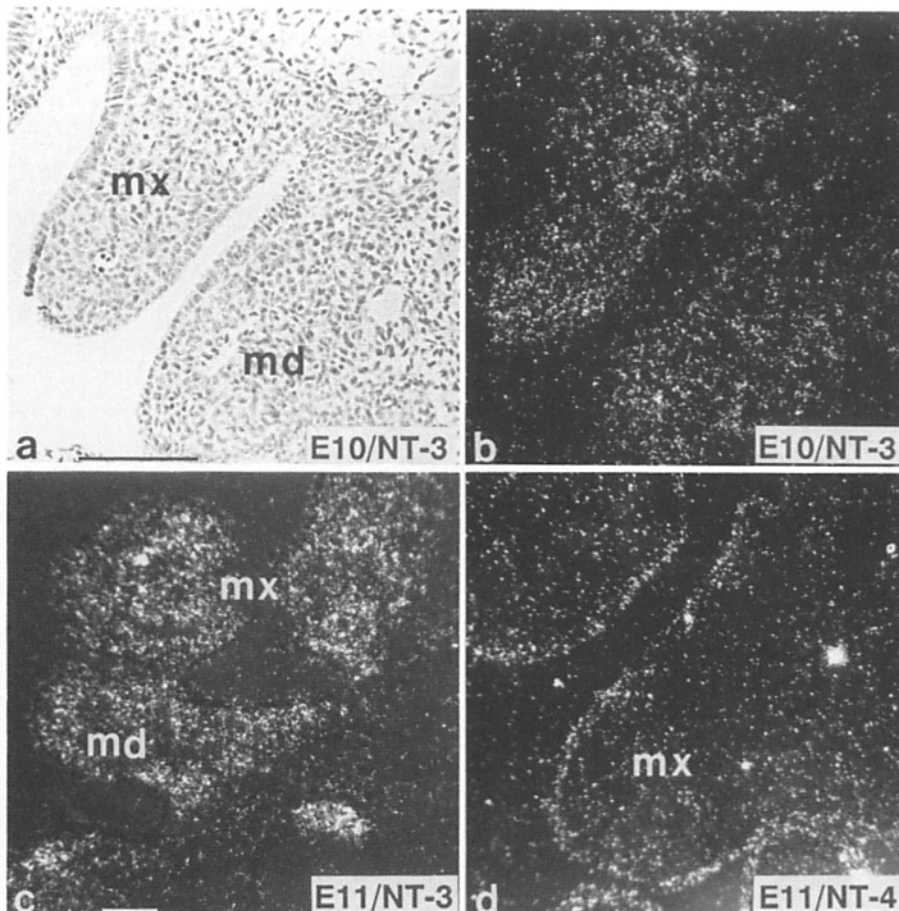
The mRNAs for NGF, BDNF, NT-3, and NT-4 were not detected within the trigeminal ganglia at E10–E12 by in situ hybridization (not shown).

#### **Expression of Neurotrophin Receptor mRNAs in Trigeminal Ganglion of the Rat Embryo**

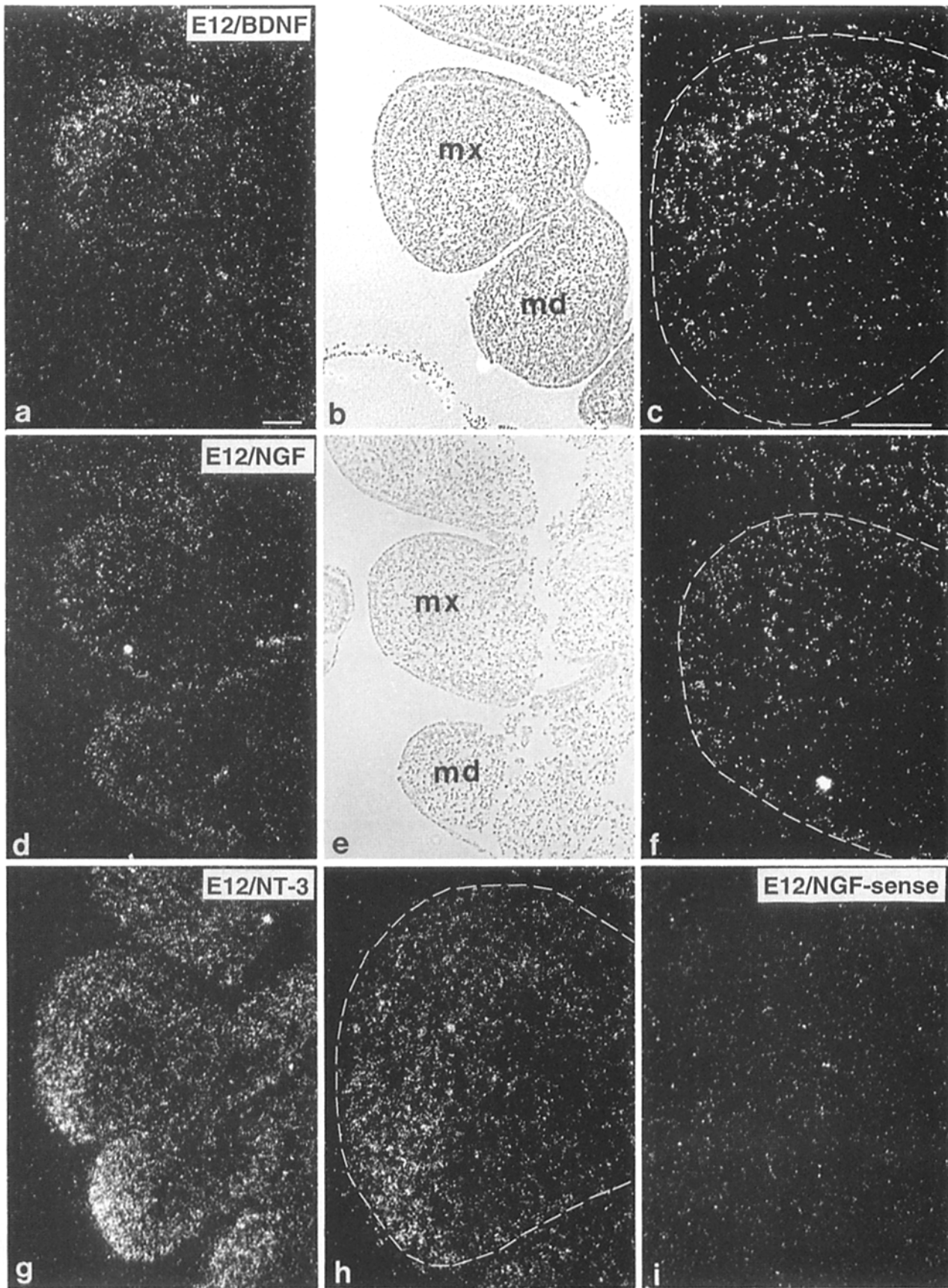
LANR mRNA was weakly expressed in the E10 trigeminal ganglia (Fig. 4 *d*). The expression increased during the following days, as shown for E12 (Fig. 5 *e*). In longitudinal sections of the growing maxillary nerve (but not in the other areas of the first branchial arch) a labeling around the growing nerve and the surrounding mesenchyme was also seen (Figs. 4 *d*, 5 *e*, and 6 *f*). The grains were not seen in the epithelium of the first branchial arch.

Strong expression of *trkA* transcripts was seen in trigeminal ganglia at E10 (Fig. 4 *f*), E11 (not shown) and E12 (Fig. 5 *b*).

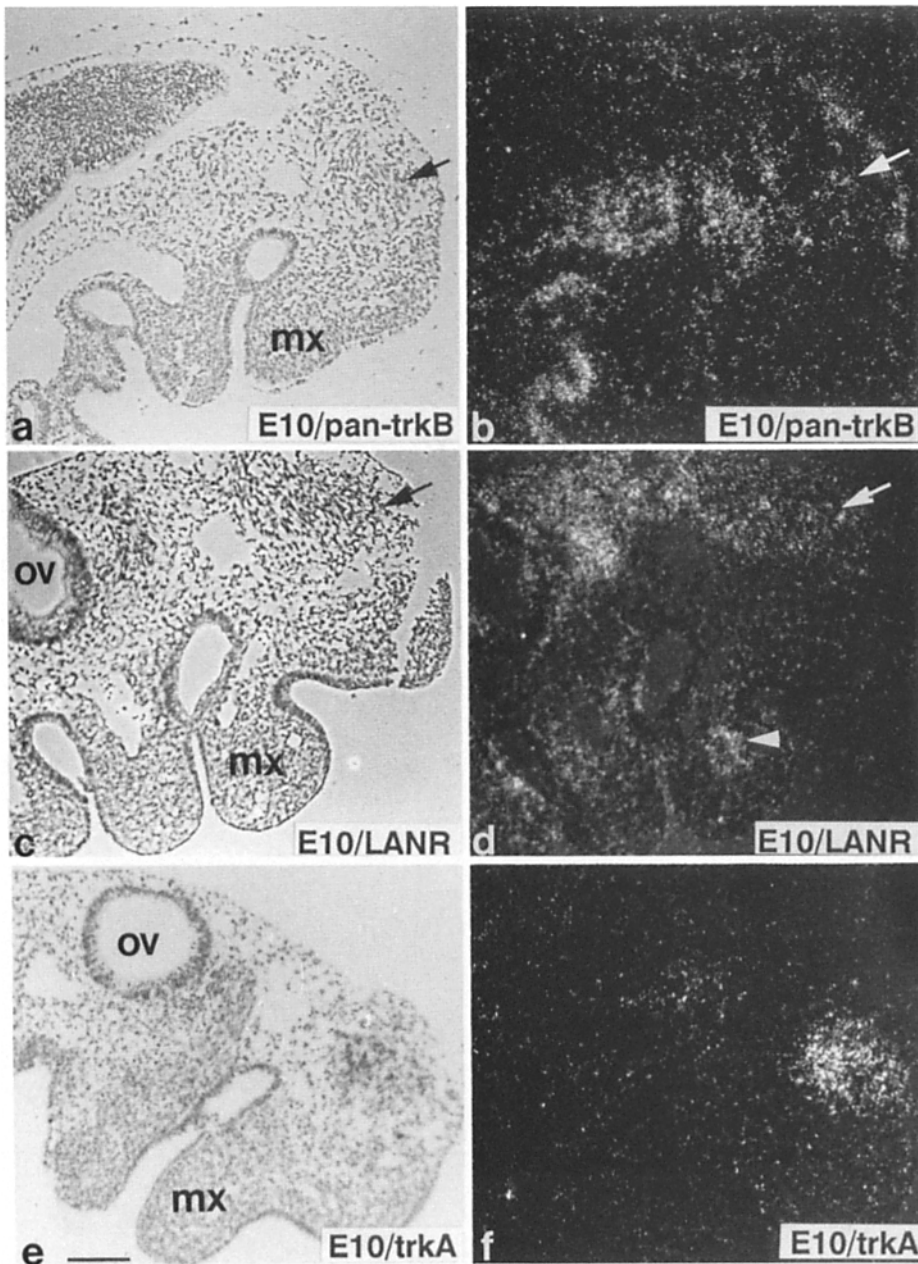
At E10, *trkB* mRNA, detected by a probe from the extracellular part of the receptor (pan-*trkB* probe), was expressed in small groups of cells in the trigeminal ganglion (Fig. 4 *b*). It was also expressed in the mesenchyme of the several regions of the embryo, but not in the first branchial arch (Fig. 4 *b*). At E11 and E12, the transcripts were clearly detected in the trigeminal ganglion (Fig. 5 *c*). Also the



**Figure 2.** Localization of NT-3 and NT-4 mRNAs in the first branchial arch of rat embryo. Sagittal tissue sections of rat embryos of the indicated stage were hybridized with cRNA probes to NT-3 (*b* and *c*) and NT-4 (*d*). Bright-field micrograph *a* is taken from the same section as *b*. *mx*, first branchial arch; *md*, second branchial arch. Bar, 100  $\mu$ m.



**Figure 3.** Expression of NGF, BDNF, and NT-3 mRNAs in the first branchial arch of the E12 rat embryo. Sagittal tissue sections of rat E12 embryos were hybridized with cRNA probes to BDNF (*a*, *b*, and *c*), NGF (*d*, *e*, and *f*), and NT-3 (*g* and *h*). *c*, *f*, and *h* represent higher magnifications of the distal parts of the first branchial arches on *a*, *d*, and *g*, respectively. To facilitate the localization of the grains to the epithelial or mesenchymal components of the first branchial arch, the outer boundaries of the epithelia are shown by dashed line. *i* represents a section from the same embryo as *f* hybridized with sense riboprobe of NGF. *mx*, first branchial arch *md*, second branchial arch. Bar, 100  $\mu$ m.



**Figure 4.** Expression of NTF receptor mRNAs in the E10 rat trigeminal system. Sagittal tissue sections of rat E10 embryos were hybridized with cRNA probes to pan-trkB (*b*), LANR (*d*) and trkA (*f*). The bright-field micrographs *a*, *c*, and *e* correspond to the dark-field micrographs *b*, *d*, and *f*, respectively. trkB transcripts are expressed in several mesenchymal areas, but only small groups of cells are labeled in the trigeminal ganglion (*arrow*). Note the mesenchymal expression of LANR mRNA, including the mesenchyme of the first branchial arch (*arrowhead*). *mx*, first branchial arch; *ov*, otic vesicle. Bar, 100  $\mu$ m.

mesenchyme between the trigeminal ganglion and the otic vesicle remained strongly labeled at these stages (not shown). To distinguish the expression of the full-length (catalytic) and T1-truncated forms of trkB, the E12 sections were hybridized with the corresponding specific probes. The transcripts of the catalytic form of trkB were strongly expressed in the trigeminal ganglion but absent in the neighboring mesenchyme (Fig. 6 *b*). The expression of T1 truncated transcripts of trkB in the trigeminal ganglion was weak but several mesenchymal structures were strongly labeled, as partially shown (Fig. 6 *d*). However, any form of trkB mRNA studied was found neither in the mesenchyme nor in the epithelium of the first branchial arch (Fig. 6, *b* and *d*). trkA and trkC transcripts were also not found in the tissues of the first branchial arch.

Weak but constant trkC mRNA expression was detected

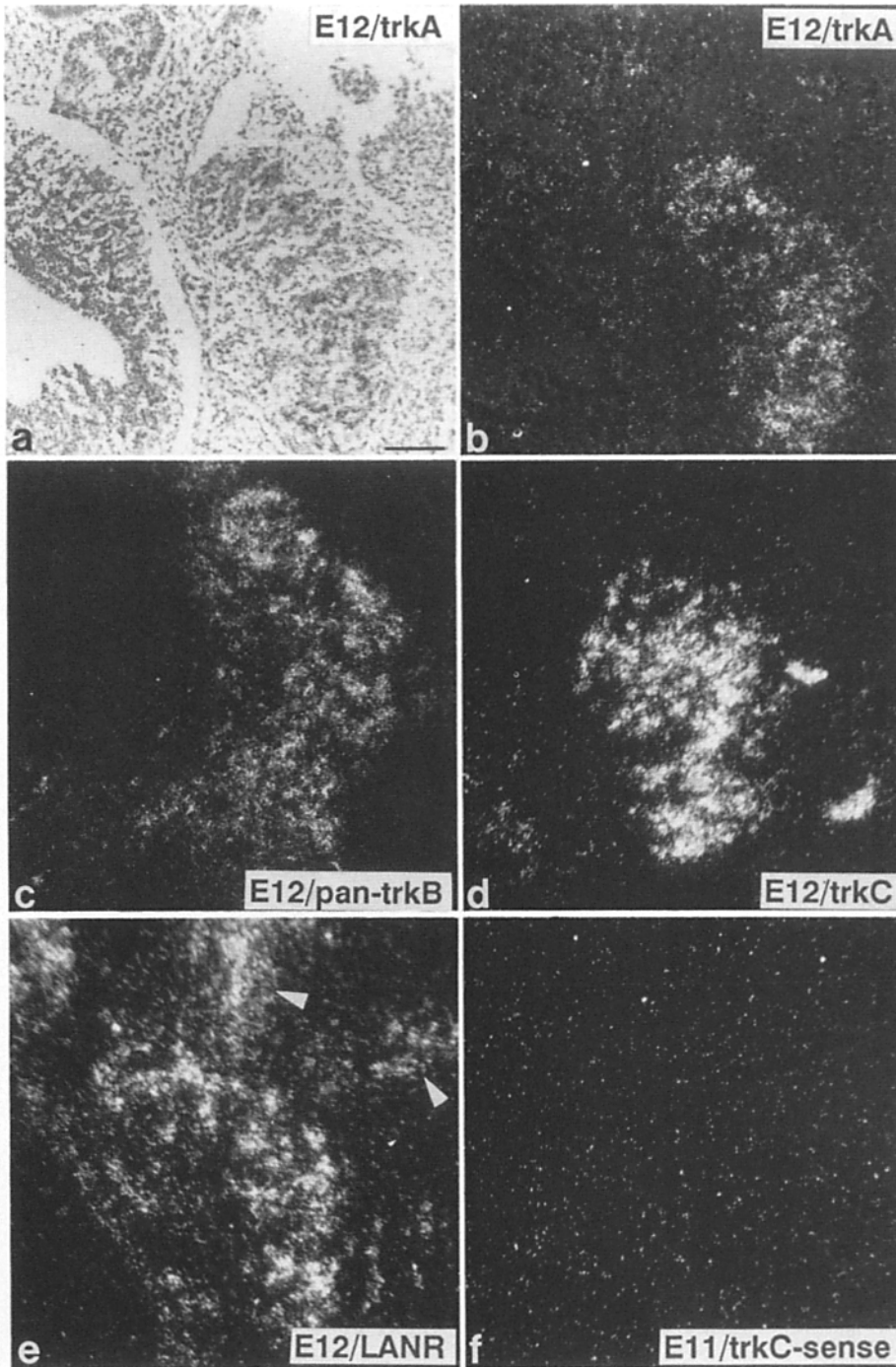
in some cell groups of the trigeminal ganglia at E10 (not shown), but higher levels were detected at E11 (not shown) and E12 (Fig. 5 *d*).

The summary of the expression of the NTFs and their receptors in the developing rat trigeminal system is presented in Table I.

#### **Effect of Exogenous Neurotrophins on Trigeminal Ganglion Explants**

The trigeminal ganglia from E10–E12 rats were explant cultured in three-dimensional collagen matrix and treated with exogenous NGF, BDNF, NT-3, and NT-4. No spontaneous neurite outgrowth was observed from control explants at any stage studied (see Fig. 8 *e*).

NGF induced no neurites from E10 trigeminal ganglia (not shown), although a broad concentration range from 1–200



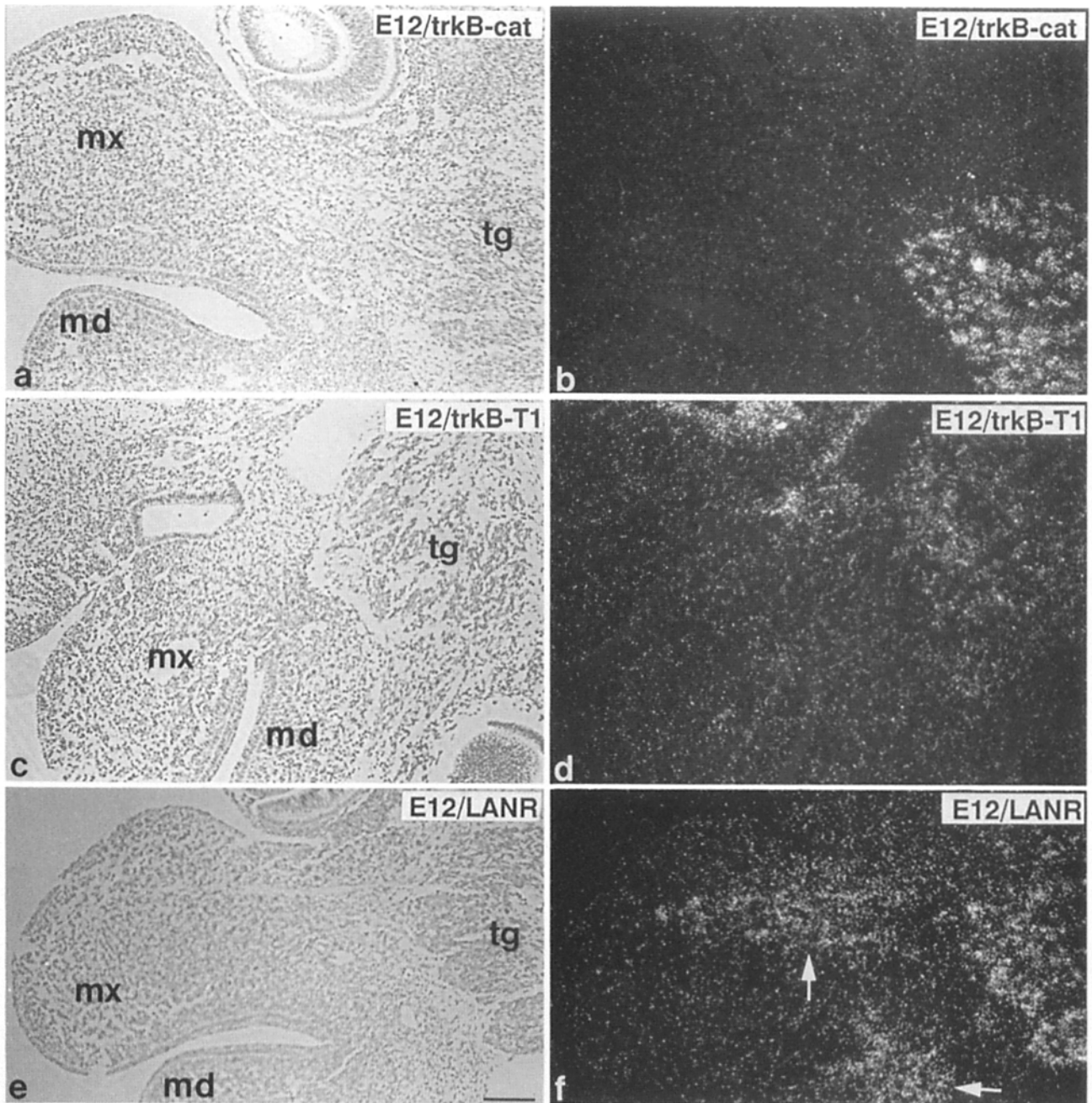
**Figure 5.** Expression of NTF receptor mRNAs in the E12 rat trigeminal ganglion. Sagittal sections of the trigeminal ganglia were hybridized with cRNA probes to *trkA* (*b*), *pan-trkB* (*c*), *trkC* (*d*), and *LANR* (*e*). Bright-field micrograph *a* is taken from the same section as *b*, whereas the sections on *c–e* go through the planes comparable to *a*. A section of E11 rat head hybridized with sense riboprobe of *trkC* is shown on *f*. The maxillary and mandibular nerve area expressing *LANR* mRNA (arrowheads). Bar, 100  $\mu\text{m}$ .

ng/ml was tested (not shown). At E11, first neurites started to emerge (Fig. 7 *a*). In E12 cultures the neurites were clearly visible (Fig. 7 *b*).

BDNF induced a prominent neurite outgrowth from trigeminal ganglia of E10–E12, as shown for E10 and E11 (Fig. 7, *c* and *d*). These neurites were generally long and thin.

NT-3 induced a moderate but constant neurite outgrowth from trigeminal ganglion explants at all stages studied. The processes were morphologically variable, including thin neurites, but also short and thick trunks often covered with nonneuronal cells (Fig. 7, *e* and *g*). The latter ones were seen

neither in nontreated control explants nor in the ganglia treated with NGF or BDNF. The same morphological pattern was induced by NT-3 at 10–20 ng/ml (not shown). The unusual morphology of the many processes induced by NT-3 raises doubts that these are simply formations of the nonneuronal cells migrating out from the ganglia. We therefore stained the ganglionic explants with anti-neurofilament antibodies to reveal the neurites. Neurofilament-positive neurites were clearly visible within every thick trunk induced by NT-3, as shown for E10 and E11 (Fig. 7, *f* and *h*). No neurofilament-positive neurites were seen in the E10 trigemi-



**Figure 6.** Expression of mRNAs for *trkB* and *LANR* in the E12 rat trigeminal system. Sagittal sections of the trigeminal ganglion and first branchial arch were hybridized with cRNA probes of the sequences specific for a catalytic form of *trkB* (*trkB-cat*) (*b*), T1-truncated transcripts of *trkB* (*d*), and *LANR* (*f*). The bright-field micrographs *a*, *c*, and *e* correspond to the dark-field micrographs *b*, *d*, and *f*, respectively. Note the expression of *LANR* mRNA in the region of the maxillary and mandibular nerve (*arrows*), whereas the mRNAs for *trkB*s are not expressed in the first branchial arch (*mx*). The expression of *trkB-T1* transcripts in mesenchymal areas of the head is visible (*d*). *tg*, trigeminal ganglion; *md*, second branchial arch. Bar, 100  $\mu$ m.

nal ganglion explants treated with NGF (not shown) or in the control ganglia (Fig. 8*f*). NT-3 thus affects both neurites and the nonneuronal cells of the early trigeminal ganglion.

The neurites induced by NT-4 from E10–E11 trigeminal ganglia were morphologically similar to those induced by NT-3, as shown for E10 and E11 (Fig. 8, *a* and *c*). Clear neurofilament-positive fascicles were seen in the thick formations covered by the nonneuronal cells, as shown (Fig. 7, *b* and *d*).

## Discussion

By classical neurotrophic theory, the neurons are independent from NTFs at the initial stages of maturation and neurite elaboration. The dependence arises only when the neurites contact the target tissues, i.e., at the beginning of programmed neuronal death (Rohrer et al., 1988; Davies et al., 1987; Vogel and Davies, 1991). We observed by in situ hybridization that NT-3 and NT-4 mRNAs were expressed in



**Table 1. Expression of NTFs and Their Receptors in E10–E12 Rat Trigeminal System**

	E10	E11	E12
<b>Trigeminal ganglion</b>			
trkA	+++	+++	+++
pan-trkB	+	++	+++
trkB-T1	ND	ND	+
trkB-catalytic	ND	ND	+++
trkC	+	++	+++
LANR	+	++	+++
NGF, BDNF, NT-3, NT-4	–	–	–
<b>First branchial arch mesenchyme</b>			
NGF	–	+	+++
BDNF	–	–	+++
NT-3	+++	+++	+++
NT-4	–	–	–
LANR	++	++	++
trkA, trkB, trkC	–	–	–
<b>First branchial arch epithelium</b>			
NGF	–	–	++
BDNF	–	–	–
NT-3	+	+	+
NT-4	++	++	++
LANR, trkA, trkB, trkC	–	–	–

\* Expression in distal, mostly subepithelial mesenchyme.  
Degrees of expression: +, weak; ++, moderate; +++, strong.

the first branchial arch at the early preinnervation stages of trigeminal ganglion development when, according to the neurotrophic theory, their function is not the trophic maintenance of the trigeminal neurons. Although it cannot be finally judged from the present data, we suggest that both these NTFs participate in the differentiation of trigeminal ganglion neurons and formation of primary neuritic pathways.

Expression of NT-4 mRNA in the first branchial arch at E10 already is very consonant with the role of NT-4 as a putative target-derived chemoattractant for the growing maxillary fibers. From the NTFs studied here, only NT-4 met the criteria for the “trigeminal neurotropic factor” postulated by Lumsden and Davies (1983) as its mRNA was clearly expressed in the epithelium but not in the mesenchyme of the first branchial arch at the time of maxillary nerve growth. The mRNA for trkB, encoding the high-affinity receptor for NT-4 (Klein et al., 1992), was expressed in the early trigeminal neurons and NT-4 was able to induce neurites from the E10 trigeminal ganglia. In vivo, the neurites have been described to emerge from the trigeminal ganglion as thick bundles (Stainier and Gilbert, 1991; Moody et al., 1989). The same pattern was also induced by NT-4 from E10 (and also E11 and E12) trigeminal ganglia in our neurite outgrowth assays. However, the inability of the epithelium of the second branchial arch to induce directed neurite outgrowth from preinnervation trigeminal ganglia (Lumsden and Davies, 1986) argues against that proposal, as it expressed NT-4 mRNA in our preparations. Thus, if NT-4, secreted from the epithelium of the first branchial arch, directs the course of trigeminal axons, there must be additional attractive or repulsive factor(s) determining precise regional specificity of the axonal trajectories.

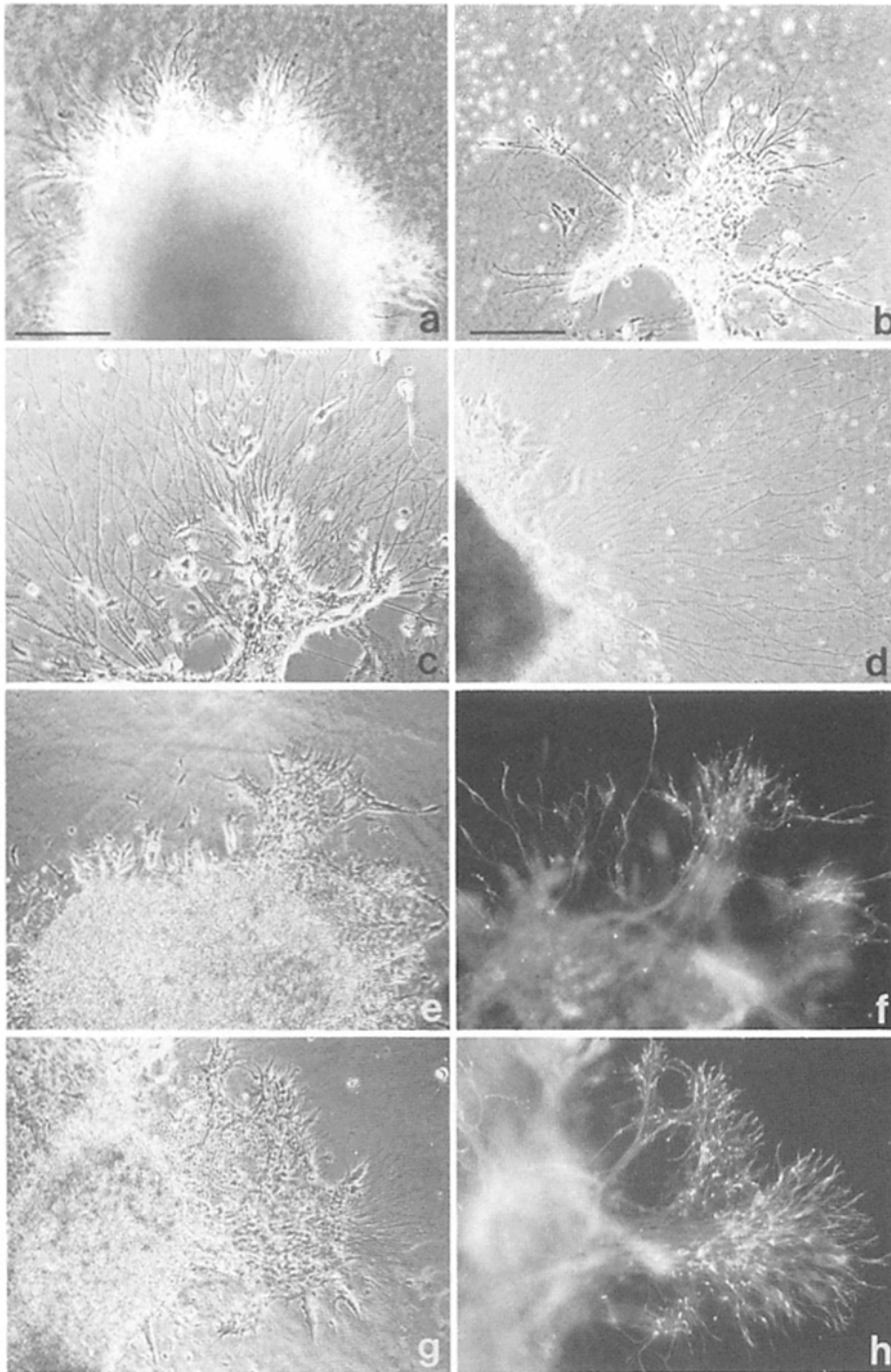
NT-3 seems to have different functions in the preinnervation and postinnervation first branchial arch. At E10–E11, its

mRNA was expressed over the whole mesenchyme of the arch, whereas by E12, its expression remained high in the distal part only. As the distal expression is consistent with the role of NT-3 as a target-derived NTF, the role of NT-3 at the preinnervation stage is more difficult to apprehend. Extensive migration of neural crest cells occurring in the first branchial arch during that time (Serbedzija et al., 1992) and the demonstration that NT-3 affects proliferation of migrating neural crest cells of the trunk (Kalcheim et al., 1992) suggest that NT-3 may affect neural crest cells in the E10–E11 first branchial arch. However, we do not think that this is the case, because trkC mRNA, encoding the high-affinity receptor for NT-3 is not expressed in the first branchial arch mesenchyme. Instead, it was expressed in a subpopulation of the trigeminal ganglion cells. Therefore it seems probable that NT-3, secreted by the neighboring mesenchyme in a paracrine manner may affect the neuronal differentiation and maturation within the trigeminal ganglion.

NT-3 was also able to induce thick fascicles of neurites from the E10 trigeminal ganglia suggesting its participation in the formation of primary axonal pathways. Although it is shown that the epithelium and not the mesenchyme of the first branchial arch directs the neurite outgrowth from mouse trigeminal ganglia in vitro (Lumsden and Davies, 1986), NT-3 may still facilitate the growth of trigeminal axons through the first branchial arch mesenchyme by local interactions with the expanding growth cone. In that context, it is relevant to point out that the mRNA for LANR is expressed in the regions of the first branchial arch mesenchyme penetrated by the growing maxillary nerve. It is tempting to speculate that NT-3 may serve as a bridge linking the LANR on the mesenchymal cells to the NTF receptor on the growth cone of the trigeminal axon, as proposed for NGF in the formation of the sciatic nerve (Johnson et al., 1988). The strong labeling of the nerve itself by LANR mRNA may be due to the formation of the endoneurial tube by Schwann cells which are known to express LANR (Johnson et al., 1988).

Thus, we propose that NT-4, secreted from the epithelium of the first branchial arch, sets the trigeminal ganglion neurites to motion in the right direction (a “coarse” regulation), whereas LANR specifies the “fine” pathway for the moving growth cone, supposedly by the help of NT-3. In that connection, it is interesting to note that the same spatiotemporal expression pattern of NT-3 and LANR mRNAs was observed in the second branchial arch (Figs. 2 *b* and 4 *d*) and the territory of the ophthalmic nerve (not shown). The scheme described here does not exclude the participation of other attractive or repulsive cues in the navigation of trigeminal axons.

During E12 there occurs an important change in the rat first branchial arch, associated with the appearance of NGF and BDNF transcripts and the change in the expression pattern of the transcripts for NT-3 while the NT-4 mRNA expression remains the same. The expression of all four NTF mRNAs in the distal part of first branchial arch at E12 is in good agreement with the data reported by others (Davies et al., 1987; Davies and Lumsden, 1984; Ernsfors et al., 1992; Wheeler and Bothwell, 1992; Schechterson and Bothwell, 1992; Ibáñez et al., 1993) and is obviously associated with the beginning of programmed neuronal death in the trigeminal ganglion. At the same time, the mRNAs for all known

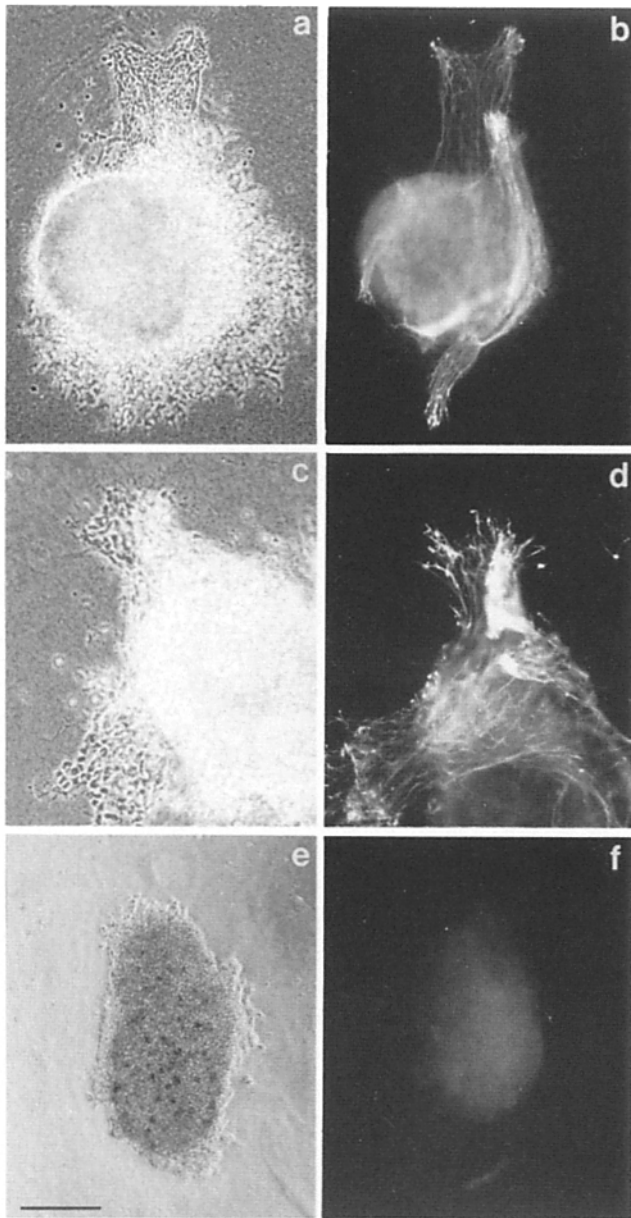


**Figure 7.** Neurite outgrowth from embryonic rat trigeminal ganglion explants. Trigeminal ganglia from rat embryos were cultured in three-dimensional collagen matrix in the presence of NTFs at 5 ng/ml. NGF induced few short neurites at E11 (a) but more neurites were seen at E12 (b). BDNF induced long thin neurites at every stage studied, as exemplified for E10 (c) and E11 (d). Thick fascicles covered with nonneuronal cells as well as short thin neurites were induced by NT-3 at every stage studied as shown for E10 (e) and E12 (g). To reveal that the processes induced by NT-3 contain neurites, the ganglia presented on e and g were stained with anti-neurofilament antibodies as shown on f and h, respectively. Bars, (a) 400  $\mu\text{m}$ ; (b-h) 200  $\mu\text{m}$ .

NTF receptors were observed in the E12 trigeminal ganglion suggesting the existence of neuronal subpopulations with different NTF-specificities within the ganglion.

The neurons of the rat trigeminal ganglion are generated between E10 and E15, with a peak on day E13 (Altman and Bayer, 1982). Although it is not finally established, it seems that both placodal and neural crest precursors populate the ganglion anlage. In mouse, two waves of neurogenesis are described in the trigeminal ganglion (Stainier and Gilbert, 1991). In the first, few sparsely distributed, presumably placode-derived neuroblasts are born and start to differentiate in the very early (E9) trigeminal ganglion. In the second,

$\sim 24$  h later, the majority of neurons are born, presumably from neural crest-derived precursors. These neurons give rise to the maxillary and mandibular projections (Davies and Lumsen, 1984; Stainier and Gilbert, 1991). It seems probable that the groups of cells expressing *trkB* and *trkC* mRNAs observed by us in the E10 trigeminal ganglion mark the neurons generated by the first wave and are therefore able to respond to cognate NTFs by neurite outgrowth. *trkB* and *trkC* can thus be functionally activated in trigeminal neurons at E10 already. The neural crest derived *trkA* mRNA-expressing (Martin-Zanca et al., 1991) precursors remain undifferentiated at that stage and start to differentiate a day later. This



**Figure 8.** Neurite outgrowth from embryonic rat trigeminal ganglion explants. Trigeminal ganglia from rat embryos were cultured in three-dimensional collagen matrix. Conditioned medium from the COS cells transfected with NT-4 cDNA was applied to the ganglia. At all stages studied, NT-4 induced thick short fascicles covered by nonneuronal cells, as represented for E10 (a) and E11 (c). Neurites were revealed by anti-neurofilament immunohistochemistry on the same ganglia as shown on b and d, respectively. No neurites were seen in the E10 control ganglia not treated with NTFs (e). Anti-neurofilament immunohistochemistry of the same ganglion revealed only background staining (f). Bar, 200  $\mu$ m.

can explain the inability of NGF to induce neurite outgrowth from E10 trigeminal ganglion explants, despite the presence of both *trkA* and LANR (this study; Davies et al., 1981). It may well be that the *trkA* mRNA-expressing neural crest-derived precursors remain undifferentiated at the first neurogenesis wave observed in the trigeminal ganglion (Stainier and Gilbert, 1991) and thus unable to respond to NGF, whereas their differentiation at the second wave of neurogen-

esis (E11–E12) accounts for the observed effects of NGF on neurite outgrowth and survival of the trigeminal ganglion neurons (Ebendal and Hedlund, 1975; Davies et al., 1981; Davies and Lumsden, 1984; this study). *trkB*- and *trkC*-expressing neurons may start to differentiate earlier and are thus able to respond to cognate NTF at earlier stages. It may also be that *trkA* mRNA is not translated into protein at E10 and E11. Clearly, techniques different than those used here are required to study the ligand and the role of *trkA* in the preinnervation trigeminal ganglion.

It seems that BDNF is not involved in the processes of early differentiation of trigeminal ganglion neurons, although it induced a massive neurite outgrowth from the E10 trigeminal ganglion, as its mRNA was not detected in the preinnervation trigeminal system by *in situ* hybridization. Most probably BDNF is not a physiologically relevant NTF for the early trigeminal neurons and the observed effects simply refer to the presence of *trkB* whose relevant ligand is NT-4. If this was the case, the early trigeminal neurons are a good example to show that the different *in vivo* availabilities explain how two NTFs with similar *trk*-specificities can operate (Ip et al., 1993). Alternatively, the amounts of BDNF mRNA necessary for the early stages may be too low to be detected by *in situ* hybridization.

The functions of truncated *trk* receptors are not known. It has been proposed that truncated *trk* receptors, while dimerizing with full-length receptors, may change the response of the cell to NTF (Tsoulfas et al., 1993). This may be the case in the trigeminal ganglion, as both full-length and T1-truncated transcripts of *trkB* were expressed there. The expression of mRNA of T1-truncated *trkB* alone in the cranial mesenchyme is more difficult to explain; e.g., its product may participate in the adhesive interactions during cranial mesenchyme migration. Clearly, functional studies are needed to clarify the biological role of truncated *trk* receptors.

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#### References

- Altman, J., and S. Bayer. 1982. Development of the cranial nerve ganglia and related nuclei in the rat. *Adv. Anat. Embryol. Cell Biol.* 74:1–90.
- Barde, Y.-A. 1989. Trophic factors and neuronal survival. *Neuron*. 2: 1525–1534.
- Barde, Y.-A., D. Edgar, and H. Thoenen. 1982. Purification of a new neurotrophic factor from mammalian brain. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:549–553.
- Berkemeier, L. R., J. W. Winslow, D. R. Kaplan, K. Nikolics, D. V. Goeddel, and A. Rosenthal. 1991. Neurotrophin-5: a novel neurotrophic factor that activates *trk* and *trkB*. *Neuron*. 7:857–866.
- Cordon-Cardo, C., P. Tapley, S. Jing, V. Nanduri, E. O'Rourke, F. Lamballe, K. Kovary, R. Klein, K. R. Jones, L. F. Reichardt, and M. Barbacid. 1991. The *trk* tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. *Cell*. 66:173–183.

- Davies, A. M. 1988. The trigeminal system: an advantageous experimental model for studying neuronal development. *Development (Camb.)*. 103 (Suppl.):175-183.
- Davies, A. M., and A. Lumsden. 1984. Relation of target encounter and neuronal death to nerve growth factor responsiveness in the developing mouse trigeminal ganglion. *J. Comp. Neurol.* 223:124-137.
- Davies, A. M., A. G. S. Lumsden, H. C. Slavkin, and G. Burnstock. 1981. Influence of nerve growth factor on the embryonic mouse trigeminal ganglion in culture. *Dev. Neurosci.* 4:150-156.
- Davies, A. M., C. Bandtlow, R. Heumann, S. Korsching, H. Rohrer, and H. Thoenen. 1987. Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. *Nature (Lond.)*. 326:353-358.
- Ebendal, T. 1989. Use of collagen gels to bioassay nerve growth factor activity. In *Nerve Growth Factors*. R. A. Rush, editor. John Wiley and Sons Ltd., Chichester, West Sussex, England. 81-93.
- Edendal, T., and K.-O. Hedlund. 1975. Effects of nerve growth factor on the chick embryo trigeminal ganglion in culture. *Zoon.* 3:33-47.
- Ernfors, P., C. F. Ibáñez, T. Ebendal, L. Olson, and H. Persson. 1990. Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: development and topographical expression in the brain. *Proc. Natl. Acad. Sci. USA*. 87:5454-5458.
- Ernfors, P., J.-P. Merlio, and H. Persson. 1992. Cells expressing mRNA for neurotrophins and their receptors during embryonic rat development. *Eur. J. Neurosci.* 4:1140-1158.
- Glass, D. J., S. H. Nye, P. Hantzopoulos, M. J. Macchi, S. P. Squinto, M. Goldfarb, and G. D. Yancopoulos. 1991. TrkB mediates BDNF/NT-3-dependent survival and proliferation in fibroblasts lacking the low affinity NGF receptor. *Cell*. 66:405-413.
- Gundersen, R. W., and J. N. Barrett. 1980. Characterization of the turning response of dorsal root neurites towards nerve growth factor. *J. Cell Biol.* 87:546-554.
- Hallböök, F., C. F. Ibáñez, and H. Persson. 1991. Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in *Xenopus* ovary. *Neuron*. 6:845-858.
- Hempstead, B. L., D. Martin-Zanca, D. R. Kaplan, L. F. Parada, and M. V. Chao. 1991. High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. *Nature (Lond.)*. 350:678-683.
- Hohn, A., J. Leibrock, K. Bailey, and Y.-A. Barde. 1990. Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature (Lond.)*. 344:339-341.
- Ibáñez, C. F., P. Ernfors, T. Timmusk, N. Y. Ip, E. Arenas, G. D. Yancopoulos, and H. Persson. 1993. Neurotrophin-4 is a target derived neurotrophic factor for neurons of the trigeminal ganglion. *Development (Camb.)*. 117:1345-1353.
- Ip, N. Y., C. F. Ibáñez, S. H. Nye, J. McClain, P. F. Jones, D. R. Gies, L. L. Belluscio, M. M. Le Beau, R. Espinosa III, S. P. Squinto, H. Persson, and G. D. Yancopoulos. 1992. Mammalian neurotrophin-4: Structure, chromosomal localization, tissue distribution, and receptor specificity. *Proc. Natl. Acad. Sci. USA*. 89:3060-3064.
- Ip, N. Y., T. N. Stitt, P. Tapley, R. Klein, D. J. Glass, J. Fandl, L. A. Greene, M. Barbacid, and G. D. Yancopoulos. 1993. Similarities and differences in the way neurotrophins interact with the trk receptors in neuronal and non-neuronal cells. *Neuron*. 10:137-149.
- Jing, S., P. Tapley, and M. Barbacid. 1992. Nerve growth factor mediates signal transduction through trk homodimer receptors. *Neuron*. 9:1067-1079.
- Johnson, D., A. Lanahan, C. R. Buck, A. Seghal, C. Morgan, E. Mercer, M. Bothwell, and M. Chao. 1986. Expression and structure of human NGF receptor. *Cell*. 47:544-554.
- Johnson, E. M., M. Taniuchi, and P. S. DiStefano. 1988. Expression and possible function of nerve growth factor receptors on Schwann cells. *Trends Neurosci.* 11:299-304.
- Jones, K. J., and L. F. Reichardt. 1990. Molecular cloning of a human gene that is a member of the nerve growth factor family. *Proc. Natl. Acad. Sci. USA*. 87:8060-8064.
- Kaisho, Y., K. Yoshimura, and K. Nakahama. 1990. Cloning and expression of a cDNA encoding a novel human neurotrophic factor. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 266:187-191.
- Kalcheim, C., C. Carmeli, and A. Rosenthal. 1992. Neurotrophin 3 is a mitogen for cultured neural crest cells. *Proc. Natl. Acad. Sci. USA*. 89:1661-1665.
- Klein, R., L. F. Parada, F. Moulier, and M. Barbacid. 1989. TrkB, a novel tyrosine kinase receptor expressed during mouse neural development. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3701-3709.
- Klein, R., D. Conway, L. F. Parada, and M. Barbacid. 1990a. The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. *Cell*. 61:647-656.
- Klein, R., D. Martin-Zanca, M. Barbacid, and L. F. Parada. 1990b. Expression of the tyrosine kinase receptor gene trkB is confined to the murine embryonic and adult nervous system. *Development (Camb.)*. 109:845-850.
- Klein, R., V. Nanduri, S. Jing, F. Lamballe, P. Tapley, S. Bryant, C. Cordon-Cardo, K. R. Jones, L. F. Reichardt, and M. Barbacid. 1991a. The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. *Cell*. 66:395-403.
- Klein, R., S. Jing, V. Nanduri, E. O'Rourke, and M. Barbacid. 1991b. The trk proto-oncogene encodes a receptor for nerve growth factor. *Cell*. 65:189-197.
- Klein, R., F. Lamballe, S. Bryant, and M. Barbacid. 1992. The trkB tyrosine protein kinase is a receptor for neurotrophin-4. *Neuron*. 8:947-956.
- Lamballe, F., R. Klein, and M. Barbacid. 1991. trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. *Cell*. 66:967-979.
- Leibrock, J., F. Lottspeich, A. Hohn, M. Hofer, B. Hengerer, P. Masiakowski, H. Thoenen, and Y.-A. Barde. 1989. Molecular cloning and expression of brain-derived neurotrophic factor. *Nature (Lond.)*. 341:149-152.
- Levi-Montalcini, R. 1987. The nerve growth factor: Thirty-five years later. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1145-1154.
- Lindsay, R. M., H. Thoenen, and Y.-A. Barde. 1985. Placode and neural crest-derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor. *Dev. Biol.* 112:319-328.
- Lumsden, A. G. S., and A. M. Davies. 1983. Earliest sensory nerve fibers are guided to peripheral targets by attractants other than nerve growth factor. *Nature (Lond.)*. 306:786-788.
- Lumsden, A. G. S., and A. M. Davies. 1986. Chemotropic effect of specific target epithelium in the developing mammalian nervous system. *Nature (Lond.)*. 323:538-539.
- Maisonpierre, P. C., L. Belluscio, S. Squinto, N. Y. Ip, M. E. Furth, R. M. Lindsay, and G. D. Yancopoulos. 1990. Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. *Science (Wash. DC)*. 247:1446-1451.
- Martin-Zanca, D., R. Oskam, G. Mitra, T. Copeland, and M. Barbacid. 1989. Molecular and biochemical characterization of the human trk proto-oncogene. *Mol. Cell Biol.* 9:24-33.
- Martin-Zanca, D., M. Barbacid, and L. F. Parada. 1990. Expression of the trk proto-oncogene is restricted to sensory cranial and spinal ganglia of neural crest origin in mouse development. *Genes Dev.* 4:683-694.
- Merlio, J.-P., P. Ernfors, M. Jaber, and H. Persson. 1992. Molecular cloning of rat trkC and distribution of cells expressing messenger RNAs for members of the trk family in the rat central nervous system. *Neuroscience*. 51:513-532.
- Middlemas, D. S., R. A. Lindberg, and T. Hunter. 1991. trkB, a neural receptor protein-tyrosine kinase: evidence for a full length and two truncated receptors. *Mol. Cell Biol.* 11:143-153.
- Moody, S. A., M. S. Quigg, and A. Frankfurter. 1989. Development of the peripheral trigeminal system in the chick revealed by an isotype-specific anti-beta-tubulin monoclonal antibody. *J. Comp. Neurol.* 279:567-580.
- Oppenheim, R. W. 1991. Cell death during development of the nervous system. *Annu. Rev. Neurosci.* 14:453-501.
- Pirvola, U., J. Palgi, J. Ylikoski, E. Lehtonen, U. Arumäe, and M. Saarna. 1992. Brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the peripheral target fields of developing inner ear ganglia. *Proc. Natl. Acad. Sci. USA*. 89:9915-9919.
- Placzek, M., M. Tessier-Lavigne, T. Jessell, and J. Dodd. 1990. Orientation of commissural axons in vitro to a floor plate-derived chemoattractant. *Development (Camb.)*. 110:19-30.
- Radeke, M. J., T. P. Misko, C. Hsu, L. A. Herzenberg, and E. M. Shooter. 1987. Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Nature (Lond.)*. 325:593-597.
- Rodríguez-Tébar, A., and Y.-A. Barde. 1988. Binding characteristics of brain-derived neurotrophic factor to its receptors on neurons from the chick embryo. *J. Neurosci.* 8:3337-3342.
- Rodríguez-Tébar, A., G. Dechant, and Y.-A. Barde. 1990. Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron*. 4:487-492.
- Rodríguez-Tébar, A., G. Dechant, R. Götz, and Y.-Z. Barde. 1992. Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:917-922.
- Rohrer, H., R. Heumann, and H. Thoenen. 1988. The synthesis of nerve growth factor (NGF) in developing skin is independent of innervation. *Dev. Biol.* 128:240-244.
- Rosenthal, A., D. V. Goeddel, T. Nguyen, M. Lewis, A. Shih, G. R. Laramée, K. Nikolics, and J. W. Winslow. 1990. Primary structure and biological activity of a novel human neurotrophic factor. *Neuron*. 4:767-773.
- Schechterson, L. C., and M. Bothwell. 1992. Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. *Neuron*. 9:449-463.
- Serbedzija, G. N., M. Bronner-Fraser, and S. E. Fraser. 1992. Vital dye analysis of cranial neural crest cell migration in the mouse embryo. *Development (Camb.)*. 116:297-307.
- Squinto, S. P., T. N. Snitt, T. H. Aldrich, S. Davis, S. M. Bianco, C. Radziejewski, D. J. Glass, P. Masiakowski, M. E. Furth, D. M. Valenzuela, P. S. DiStefano, and G. D. Yancopoulos. 1991. trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. *Cell*. 65:885-893.
- Stainier, D. Y. R., and W. Gilbert. 1991. Neuronal differentiation and maturation in the mouse trigeminal sensory system, in vivo and in vitro. *J. Comp. Neurol.* 311:300-312.
- Suter, A., R. J. Riopelle, R. M. Harris-Warrick, and E. M. Shooter. 1979. Nerve growth factor receptors. Characterization of two distinct classes of

- binding sites on chick embryo sensory ganglia cells. *J. Biol. Chem.* 254: 5972-5982.
- Timmusk, T., K. Palm, M. Metsis, T. Reintam, V. Paalme, M. Saarma, and H. Persson. 1993. Multiple promoters direct tissue-specific expression of rat BDNF gene. *Neuron*. 10:475-489.
- Tsoufas, P., D. Soppet, E. Escandon, L. Tessarollo, J.-L. Mendoza-Ramirez, A. Rosenthal, K. Nicolics, and L. F. Parada. 1993. The rat trkC locus encodes multiple neurogenic receptors that exhibit differential response to neurotrophin-3 in PC12 cells. *Neuron*. 10:1-20.
- Vogel, K. S., and A. M. Davies. 1991. The duration of neurotrophic factor independence in early sensory neurons is matched to the time course of target field innervation. *Neuron*. 7:819-830.
- Wheeler, E., and M. Bothwell. Spatiotemporal patterns of expression of NGF and the low-affinity NGF receptor in rat embryos suggest functional roles in tissue morphogenesis and myogenesis. 1992. *J. Neurosci.* 12:930-945.
- Whittemore, S. R., P. L. Friedman, D. Larhammar, H. Persson, M. Gonzalez-Carvajal, and V. R. Holets. 1988. Rat  $\beta$ -nerve growth factor sequence and site of synthesis in the adult hippocampus. *J. Neurosci. Res.* 20:403-410.
- Wilkinson, D. G., and J. Green. 1990. In *Postimplantation Mammalian Embryos: A Practical Approach*. A. J. Copp and D. L. Cockcroft, editors. IRL Press, Oxford. 155-171.
- Wright, E. M., K. S. Vogel, and A. M. Davies. 1992. Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. *Neuron*. 9:139-150.
- Wyatt, S., E. M. Shooter, and A. M. Davies. 1990. Expression of the NGF receptor gene in sensory neurons and their cutaneous targets prior and during innervation. *Neuron*. 4:421-427.
- Ylikoski, J., U. Pirvola, M. Moshnyakov, J. Palgi, U. Arumäe, and M. Saarma. 1993. Expression patterns of neurotrophin and their receptor mRNAs in the rat inner ear. *Hear. Res.* 65:69-78.