

Neurotrophin-4 is a target-derived neurotrophic factor for neurons of the trigeminal ganglion

Carlos F. Ibáñez¹, Patrik Ernfors^{1,*}, Tõnis Timmusk¹, Nancy Y. Ip², Ernest Arenas¹, George D. Yancopoulos² and Håkan Persson¹

¹Department of Medical Chemistry II, Laboratory of Molecular Neurobiology, Karolinska Institute, Stockholm S-10401, Sweden

²Regeneron Pharmaceuticals Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA

*Present address: Whitehead Institute for Biomedical Research, Nine Cambridge Center and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142, USA

SUMMARY

The cellular localization of mRNA for neurotrophin-4 (NT-4), a novel neurotrophic factor, in the developing whisker follicles and skin of the embryonic rat is demonstrated by *in situ* hybridization. Levels of NT-4 mRNA in the whisker pad decrease between embryonic day 13 (E13) and E20, correlating in time with the onset of naturally occurring neuronal death in the innervating trigeminal ganglion. In addition to NT-4, brain-derived neurotrophic factor (BDNF) mRNA is also shown to be expressed in the rat embryonic whisker follicles although in a different cellular localization, which combined with previous data on the expression of NGF and NT-3 mRNAs, shows that all four neurotrophins are expressed during development of this structure. NT-4 protein is shown to elicit neurite outgrowth from explanted embryonic trigeminal ganglia and to promote neuronal survival of dissociated trigeminal ganglion

neurons when cultured during the phase of cell death. NT-4 and NT-3 mainly support different neuronal subpopulations, whereas some NT-4-responsive cells appear to respond also to NGF and BDNF. Analysis of mRNAs for members of the Trk family of neurotrophin receptors in neurons rescued by different neurotrophins demonstrates the presence of distinct neuronal subpopulations that respond to specific combinations of these factors. Based on these results we propose that NT-4, together with the other three neurotrophins, orchestrate the innervation of the different structures of the developing whisker pad by the trigeminal ganglion, acting as target-derived neurotrophic factors for different subpopulations of trigeminal ganglion neurons.

Key words: nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, *in situ* hybridization, development

INTRODUCTION

During development of the vertebrate nervous system, a given target field is able to support the survival of only a limited number of neurons, superfluous neurons being lost during a period of cell death that begins shortly after the arrival of axons in the target areas. The neurons that reach maturity are thought to have successfully competed for a target-derived, retrogradely transported neurotrophic factor supplied in limited amounts by the target fields (Hamburger and Oppenheim, 1982; Oppenheim, 1985; Barde, 1989). The molecular basis for such an interaction has been established from work on the protein nerve growth factor (NGF), a well characterized neurotrophic factor that supports the survival of sympathetic and neural crest-derived sensory neurons in the peripheral nervous system (PNS) and cholinergic basal forebrain neurons in the central nervous system (CNS; Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980; Whittemore and Seiger, 1987; Thoenen et al.,

1987). When administered during the period of neural cell death, NGF can prevent the loss of sympathetic and sensory neurons *in vivo* (Thoenen and Barde, 1980; Hamburger et al., 1981) and, conversely, the presence of anti-NGF antiserum during development greatly increases the loss of these neurons (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980; Ruit et al., 1992). NGF is delivered from the target by retrograde axonal transport to the cell bodies of sympathetic, sensory and basal forebrain neurons (Stöckel and Thoenen, 1975; Johnson et al., 1978; Schwab et al., 1979). NGF synthesis in developing skin begins with the arrival of the first sensory nerve fibres (Davies et al., 1987), and in adult peripheral tissues, the levels of both NGF mRNA and protein correlate with the extent of sympathetic innervation (Heumann et al., 1984; Shelton and Reichardt, 1984).

NGF belongs to a family of structurally and functionally related molecules, collectively known as neurotrophins, which includes three other members, brain-

derived neurotrophic factor (BDNF; Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (NT-3; Hohn et al., 1990; Maisonpierre et al., 1990b; Rosenthal et al., 1990; Ernfors et al., 1990) and neurotrophin-4 (NT-4; Hallböök et al., 1991; Ip et al., 1992), also known as neurotrophin-5 (Berkemeier et al., 1991). All neurotrophins display both overlapping and specific sets of neurotrophic activities on peripheral and central neurons. Thus, for example, the survival of embryonic neural crest-derived sensory neurons has been shown to be supported, in various proportions, by all neurotrophins (Thoenen and Barde, 1980; Lindsay et al., 1985; Davies et al., 1986b; Hohn et al., 1990; Rosenthal et al., 1990; Hallböök et al., 1991; Ip et al., 1992). However, survival of embryonic sympathetic neurons is only supported by NGF, whereas placode-derived sensory neurons are supported by BDNF, NT-3 and, to a lesser extent, NT-4, but not by NGF (Lindsay et al., 1985; Davies et al., 1986b; Hohn et al., 1990; Hallböök et al., 1991 and unpublished results). Additive effects on the survival of a given neuronal population have also been observed using combinations of neurotrophins, arguing for the idea that they may be supporting different subpopulations of cells within a given ganglion (Lindsay et al., 1985; Hohn et al., 1990). Neurotrophins can also influence populations of neurons from the CNS. Thus, for example, both NGF and BDNF have been shown to increase choline acetyltransferase activity in cultures of neurons from the basal forebrain (Honegger and Lenoir, 1982; Martínez et al., 1987; Alderson et al., 1990), whereas BDNF, but not NGF, has been shown to increase survival and tyrosine hydroxylase activity in cultures of neurons from the substantia nigra (Hyman et al., 1991; Knusel et al., 1991). Recently, trophic effects on cultures of noradrenergic neurons from the locus coeruleus could be demonstrated for NT-3 and NT-4, providing the first evidence of neurotrophic influences in this brain region (Friedman et al., 1993).

The expression patterns of NGF, BDNF and NT-3 during development and in the adult have been deduced from studies on the cellular localization of their corresponding mRNAs by *in situ* hybridization (see for example Ernfors et al., 1990; Phillips et al., 1990; Friedman et al., 1991a; Ernfors et al., 1992). A developmentally regulated expression has been shown for all the neurotrophins in many different tissues, and brain regions and sites of synthesis consistent with both target-derived and local modes of action have been described (Friedman et al., 1991a; Ernfors et al., 1992; Ernfors and Persson, 1991; Maisonpierre et al., 1990a). In contrast, no information is available on the sites of synthesis or possible functions of the recently isolated mammalian NT-4. We report here on the cellular localization and developmental regulation of NT-4 mRNA in cells of the developing skin and hair follicles of the embryonic rat whisker pad, targets for sensory neurons of the trigeminal ganglion. We show that NT-4 supports the survival of these neurons in culture during the phase of naturally occurring cell death, supporting the argument that this protein is a target-derived neurotrophic factor for neurons of the developing trigeminal ganglion.

MATERIALS AND METHODS

In situ hybridization

48-mer oligonucleotides complementary to rat NT-4 mRNA (nucleotides 423-470 of the published DNA sequence (Ip et al., 1992)) or to rat BDNF (Ernfors et al., 1990) were labeled at the 3' end with ^{-35}S -dATP using terminal deoxyribonucleotidyl transferase (IBI, New Haven, CT) to a specific activity of approximately 1×10^9 cts/minute per μg and subsequently purified on a Nensorb column (Du Pont, Wilmington, DE) prior to use. Rat embryos, staged by measuring the crown-rump length, were collected from pregnant Sprague-Dawley rats. Coronal and sagittal sections ($14 \mu\text{m}$) were cut from fresh-frozen embryos at -20°C on a cryostat (Leitz, FRG). The sections were thawed onto slides pretreated with poly-L-lysine ($50 \mu\text{g}/\text{ml}$) and frozen at -20°C until hybridization. Hybridization was performed as previously described (Ernfors et al., 1992). After hybridization, the sections were dried and subsequently dipped in Kodak NTB-3 photo emulsion (diluted 1:1 in water), exposed for 7 weeks at -20°C , developed, fixed, and counterstained with cresyl violet. The NT-4 oligonucleotide did not label regions that in parallel sections were strongly labeled by oligonucleotides specific for NGF, BDNF or NT-3, therefore demonstrating the specificity of the NT-4 mRNA labeling. As a negative control, an unrelated oligonucleotide of similar length and G+C content was used for hybridization of adjacent sections.

RNAse protection analysis

Polyadenylated (poly(A) $^{+}$) RNA was prepared from the indicated tissues as previously described (Hallböök et al., 1991). RNAse protection assays were performed with RPAII Ribonuclease Protection Assay Kit (Ambion, Austin, Texas). NT-4 cRNA probe (350 bases) was synthesized by *in vitro* transcription from DNA fragments coding for the mature NGF, BDNF, NT-3 or NT-4, subcloned in pBS-KS $^{+}$ (Stratagene, La Jolla, CA) in the presence of ^{-32}P CTP and T3 RNA polymerase. The probe was hybridized to $10 \mu\text{g}$ of the indicated poly(A) $^{+}$ RNAs, and the nuclease protection assay was performed as described by the manufacturer. Protected cRNA fragments were separated on 4% polyacrylamide gels under denaturing conditions and the gels were exposed to X-ray film. Sequence reactions were used in parallel as size markers.

Assays of neurite outgrowth and neuronal survival

Recombinant NGF and NT-4 (in the range of 1-30 ng/ml) were assayed for stimulation of neurite outgrowth from explanted E15 rat trigeminal ganglia cultured in collagen gels as described by Ebendal (1989). For assays of neuronal survival, dissociated neurons from the rat E14 trigeminal ganglion were preplated on plastic for 2 hours and then cultured in 96-well plates coated with poly-L-ornithine and laminin at a density of 2,800 cells/well. Purified NGF, BDNF, NT-3 and NT-4 were added either alone or in different combinations at 30 ng/ml. Neuronal survival was determined after 36 hours by phase contrast microscopy, scoring the number of surviving neurons in the entire well.

RNA analysis of embryonic trigeminal ganglion explants

RNA was prepared from rat embryonic trigeminal ganglia (removed at day 14 of embryonic development) immediately after explantation, or after 24 hours incubation in the presence of various neurotrophins on polylysine/laminin coated plates. Northern analysis, performed as previously described (Ip et al., 1992), uti-

lized randomly labeled DNA fragments derived from the kinase-encoding domains of *trk*, *trkB* and *trkC* cDNAs.

RESULTS

Cellular localization of NT-4 mRNA in the embryonic rat whisker pad

In situ hybridization was used to study the cellular localization of NT-4 mRNA expression during development of the rat embryo. In general, labeling for NT-4 mRNA throughout the embryo was low, indicating low levels of expression of this neurotrophin. However, higher levels of expression were observed in the developing whisker follicles of E16 and E18 rats (Fig. 1A). The labeling was concentrated within the follicles and in the mesenchyme adjacent to the surface epithelium (presumed dermis), lower levels were also seen over the surface epithelium itself. At higher magnification, labeling appeared over outer layers of the epidermally derived follicle proper (i.e. external root sheath) as well as in layers in close proximity to the vibrissa (i.e. internal root sheath; Fig. 1B,C). Distinct labeling

was also seen over the surface epithelium and adjacent mesenchyme of the head (Fig. 1D,E).

Developmental regulation of NT-4 mRNA expression in the embryonic rat whisker pad

The expression pattern observed for NT-4 mRNA in the whisker pad comprised one of the major peripheral target fields of the trigeminal ganglion. We therefore investigated whether the expression of NT-4 mRNA in the whisker pad is developmentally regulated during the phase of cell death of trigeminal ganglion neurons (Davies and Lumsden, 1984; Davies et al., 1987). For this purpose, polyadenylated RNA from E13, E16 and E20 rat whisker pad was analysed for the presence of NT-4 mRNA using an RNase protection assay. The level of NT-4 mRNA in the whisker pad was maximal at E13, after which it decreased 2.5-fold and 4-fold in the E16 and E20 whisker pads, respectively (Fig. 2). Interestingly, the levels of NT-4 mRNA in the E15 pons and medulla, including the central targets of trigeminal ganglion neurons, as well as in E16 total brain, were substantially lower (more than 50-fold) than those seen in the whisker pad (Fig. 2).

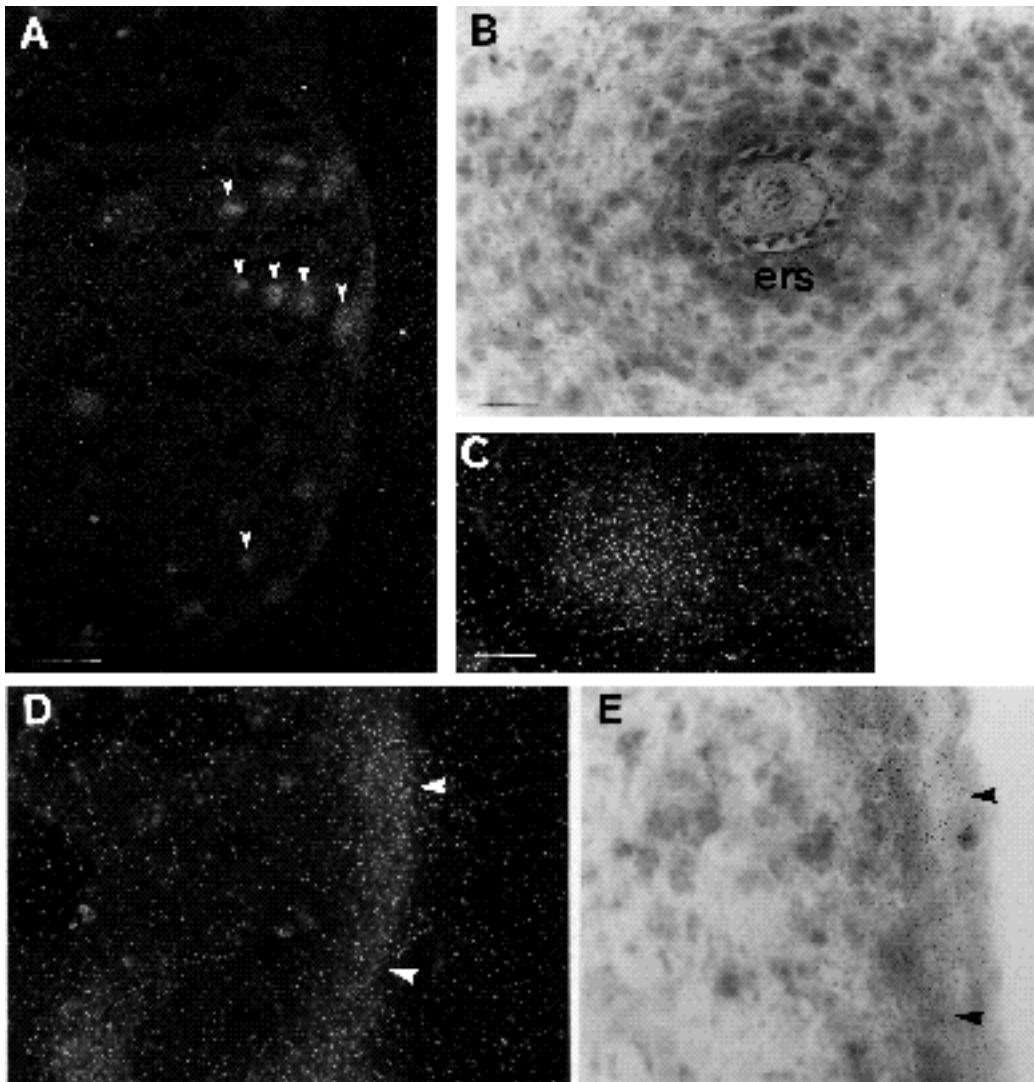


Fig. 1. Cellular localization of rat NT-4 mRNA in the rat embryo by in situ hybridization. (A) Dark-field photomicrograph of a section through the E16 rat whisker pad hybridized with an oligonucleotide specific for NT-4 mRNA. Arrowheads point at labeled follicles. (B) Bright-field high magnification of a E18 follicle revealing NT-4 mRNA labeling in both external and internal layers of the epithelial root sheath (ers) of the follicle. (C) Dark-field view of B. (D) Dark-field photomicrograph of a section through the E18 rat head epithelium hybridized with an oligonucleotide specific for NT-4 mRNA. Note intense labeling over the developing skin (arrowheads). (E) Bright-field view of D. Scale bar in A, 200 μ m; B and E, 20 μ m; C and D, 40 μ m.

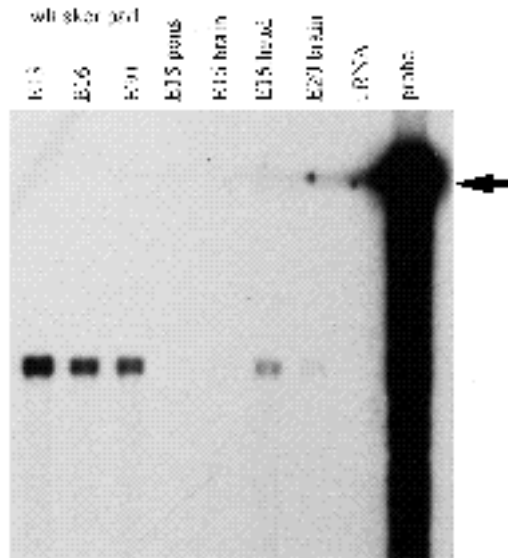


Fig. 2. RNase protection analysis of NT-4 mRNA from peripheral and central developing targets of the trigeminal ganglion. 10 μ g of poly(A)⁺ RNA from the indicated tissues and ages was processed as indicated in Materials and Methods. Shown in the figure is an autoradiogram of an overnight exposure, three days of exposure was needed to detect NT-4 mRNA in the E15 pons (which also contains the medulla) and E16 brain samples. tRNA was used as a negative control. Arrow points at full length unprotected probe.

Expression of NGF, BDNF and NT-3 mRNA in the developing rat whisker pad

The developmental expression of the other neurotrophin mRNAs, NGF, BDNF and NT-3 mRNA was also investigated in the embryonic rat whisker pad. Polyadenylated RNA from E13, E16 and E18 rat whisker pad was analysed for the presence of NGF, BDNF and NT-3 mRNA using an RNase protection assay. All mRNA were expressed in the developing whisker pad at the three time points examined. As in the case of NT-4, the level of expression was highest at E13 and progressively decreased at E16 and E18 (Fig. 3A).

The expression of NGF and NT-3 mRNA in the embryonic rat whisker pad has previously been described, with a different cellular localization than the one described here for NT-4 mRNA (Bandtlow et al., 1987; Davies et al., 1987; Ernfors et al., 1992). We therefore investigated the cellular localization of BDNF mRNA in this structure by in situ hybridization. Interestingly, BDNF mRNA expression in the developing whisker pad demonstrated yet another distribution when compared to the other neurotrophins. Labeling for BDNF mRNA appeared over mesenchyme surrounding the follicles but not in the epidermally derived follicle proper (Fig. 3B) or in the adjacent developing skin (not shown). A control probe of similar length and G+C content failed to label this region (Fig. 3C).

Neurotrophic activities of NT-4 protein on trigeminal ganglion neurons

The cellular localization and developmental regulation of NT-4 mRNA expression in the rat whisker pad opened up

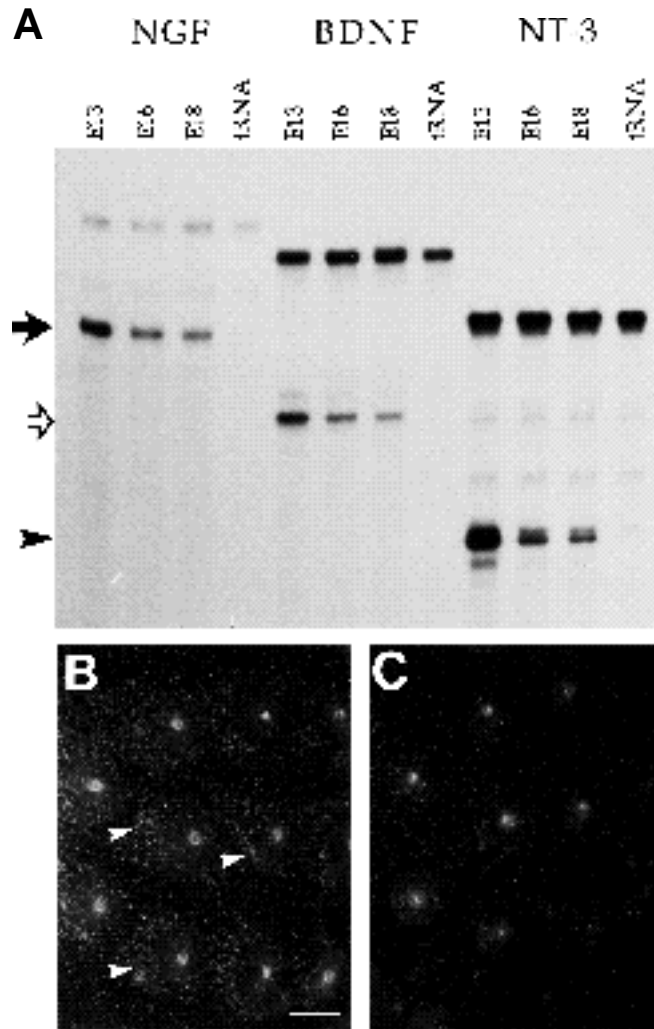


Fig. 3. Expression of NGF, BDNF and NT-3 mRNAs in the developing rat whisker pad. (A) RNase protection analysis of NGF, BDNF and NT-3 mRNAs in the developing rat whisker pad. 10 μ g of poly(A)⁺ RNA from the indicated ages was processed as described in Materials and Methods. tRNA was used as negative control. The solid arrow, open arrow and arrowhead indicate RNAs protected with the NGF, BDNF and NT-3 specific probes, respectively. (B) Dark-field photomicrograph of a section through the E18 rat whisker pad hybridized with an oligonucleotide specific for BDNF mRNA. Arrowheads point at labeled mesenchyme surrounding the follicles. (C) Dark-field photomicrograph of a section, adjacent to the one shown in B, hybridized with a control oligonucleotide. Note that in B and C the central part of the developing follicles, although devoid of labeling, reflects light under dark-field illumination. Scale bar for B and C, 100 μ m.

the possibility that this neurotrophin could act as a target-derived neurotrophic factor for the innervating neurons of the trigeminal ganglion. We therefore examined the ability of this protein to stimulate neurite outgrowth and neuronal survival of rat trigeminal ganglion neurons in culture. Serial dilutions of recombinant rat NT-4 protein were tested for stimulation of neurite outgrowth from explanted E15 rat trigeminal ganglia. Dilutions of recombinant rat NGF, a well known neurotrophic factor for these neurons, and con-

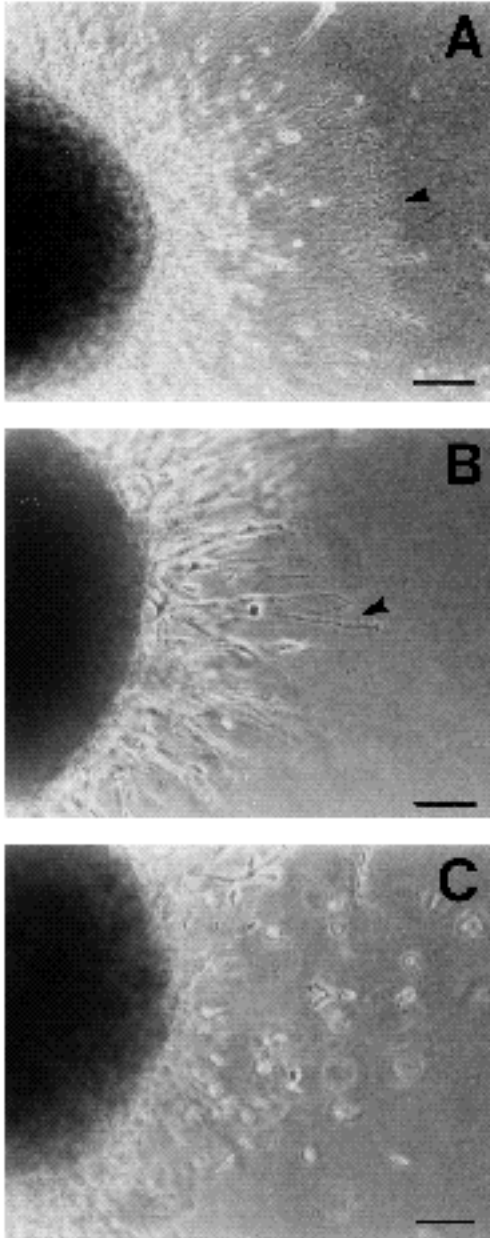


Fig. 4. Stimulation of neurite outgrowth from the E15 rat trigeminal ganglia by NT-4. Explants of E15 trigeminal ganglia were incubated in collagen gels for 48 hours in the presence of 2 ng/ml recombinant NGF (A), 2 ng/ml recombinant NT-4 (B) or in media from mock-transfected cells (C). Neurite outgrowth (arrowheads) was stimulated by NGF and NT-4 but not by control medium. Scale bar in A, 0.2 mm and in B and C, 0.1 mm.

control media were used as positive and negative controls, respectively. As expected, NGF stimulated a massive neurite outgrowth from the explanted trigeminal ganglia after 48 hours in culture (Fig. 4A). In contrast, NT-4 stimulated a moderate but consistent neurite outgrowth (Fig. 4B). No neurite outgrowth was observed in ganglia cultured in the presence of control medium (Fig. 4C).

Next, dissociated neurons from the E14 rat trigeminal ganglion were tested for survival after 2 days in culture in

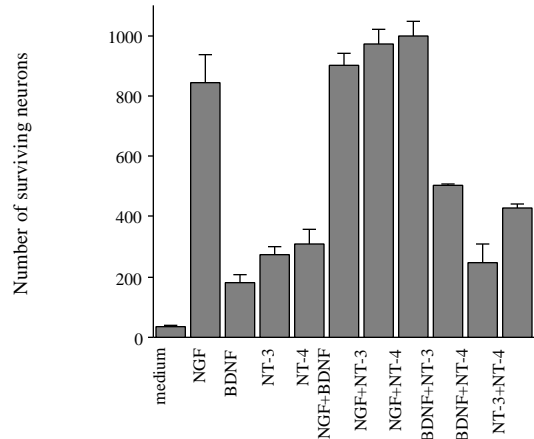


Fig. 5. Neuronal survival of dissociated E14 rat trigeminal ganglion neurons with different neurotrophins. Dissociated neurons (2,800 cells per well) were incubated in medium alone (control), or in medium containing saturating amounts (30 ng/ml) of purified NGF, BDNF, NT-3 or NT-4, alone or in different combinations. Cell survival was determined in the entire well after incubation for 36-48 hours. Results are presented as the number of surviving neurons per well \pm s.d.

the presence of saturating amounts (30 ng/ml) of purified human NT-4 protein. Purified NGF, BDNF and NT-3, which are also expressed in the developing whisker pad (Davies et al., 1987; Ernfors et al., 1992, and this study), were also tested. Less than 1% of the cells survived in media that was not supplemented with neurotrophic factors (Fig. 5). However, 15% of the plated neurons could be rescued by addition of NT-4 to the culture medium (Fig. 5), while NGF, BDNF and NT-3 rescued 35%, 12% and 7% of the neurons, respectively (Fig. 5).

Neurotrophins support both distinct and overlapping neuronal subpopulations in the trigeminal ganglion

The additive effects of neurotrophins in survival of trigeminal neurons were then tested, to investigate whether different neurotrophins could support different subpopulations of trigeminal neurons. All double combinations of the four purified factors were tested. BDNF and NT-3 showed almost complete additive effects ($P < 0.001$; Fig. 5), while NT-4 showed partial but significant additive effects with NGF ($P < 0.01$) and NT-3 ($P < 0.05$). Although NT-4 was able to rescue more neurons than BDNF, these two factors did not show additive effects, indicating that they may be acting on overlapping subpopulations of cells.

Next, we investigated whether the responses to the different factors could be due to the existence of specific subsets of receptor-bearing neurons. For that purpose, the content of *trkB* and *trkC* mRNAs in neurons of the developing trigeminal ganglion rescued by different neurotrophins was analyzed by northern blotting. In agreement with previous *in situ* hybridization data (Ernfors et al., 1992), both *trkB* and *trkC* mRNAs were detected in explanted trigeminal ganglia (Fig. 6). In explanted ganglia maintained in culture for 24 hours, *trkB* mRNA was detected in ganglia treated with NGF, BDNF and NT-4, but it was absent in NT-3

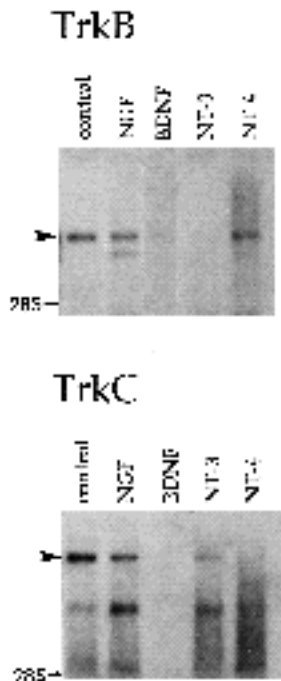


Fig. 6. Different neurotrophins rescue both distinct and partially overlapping subsets of receptor-bearing neurons. Northern blotting analysis of RNA prepared from embryonic rat trigeminal ganglia immediately post-explantation (control) or after 24 hours treatment with 50 ng/ml of the indicated neurotrophins. Top and bottom panels are probed with *trkB* and *trkC* specific probes, respectively. Arrowheads indicate *trkB* and *trkC* mRNAs, respectively, the position of 28S ribosomal RNA is also indicated.

treated ganglia (Fig. 6). In contrast, *trkC* transcripts were absent in BDNF or NT-4 treated ganglia, but they were detected in ganglia treated with NGF and NT-3 (Fig. 6).

DISCUSSION

In this report we describe the expression and developmental regulation of NT-4 mRNA in the embryonic rat whisker pad, a target of the trigeminal ganglion. NT-4 mRNA expression was also detected in the surface epithelium and adjacent mesenchyme of the developing skin of the head. In addition, a sensitive RNase protection assay allowed the detection of low levels of NT-4 mRNA in many peripheral tissues and brain regions (Timmusk et al., unpublished data). We also demonstrate that NT-4 can stimulate moderate neurite outgrowth from explanted trigeminal ganglia and that it can promote survival of dissociated trigeminal ganglion neurons when cultured during the phase of naturally occurring cell death. Our results represent the first attempts to characterize the expression and function of this novel neurotrophin in the mammalian nervous system. The pattern of expression of NT-4 mRNA in the target field of trigeminal ganglion neurons, the observation that NT-4 mRNA levels in the whisker pad decrease while these neurons are in the phase of cell death and the functional properties of the NT-4 protein on these neurons imply that NT-4 is a target-derived neurotrophic factor for neurons of the trigeminal ganglion.

NGF, BDNF and NT-3 mRNA are also expressed in the rat whisker pad (Bandtlow et al., 1987; Davies et al., 1987; Ernfors et al., 1992 and this study). Davies et al. have demonstrated that NGF mRNA and protein peak in the

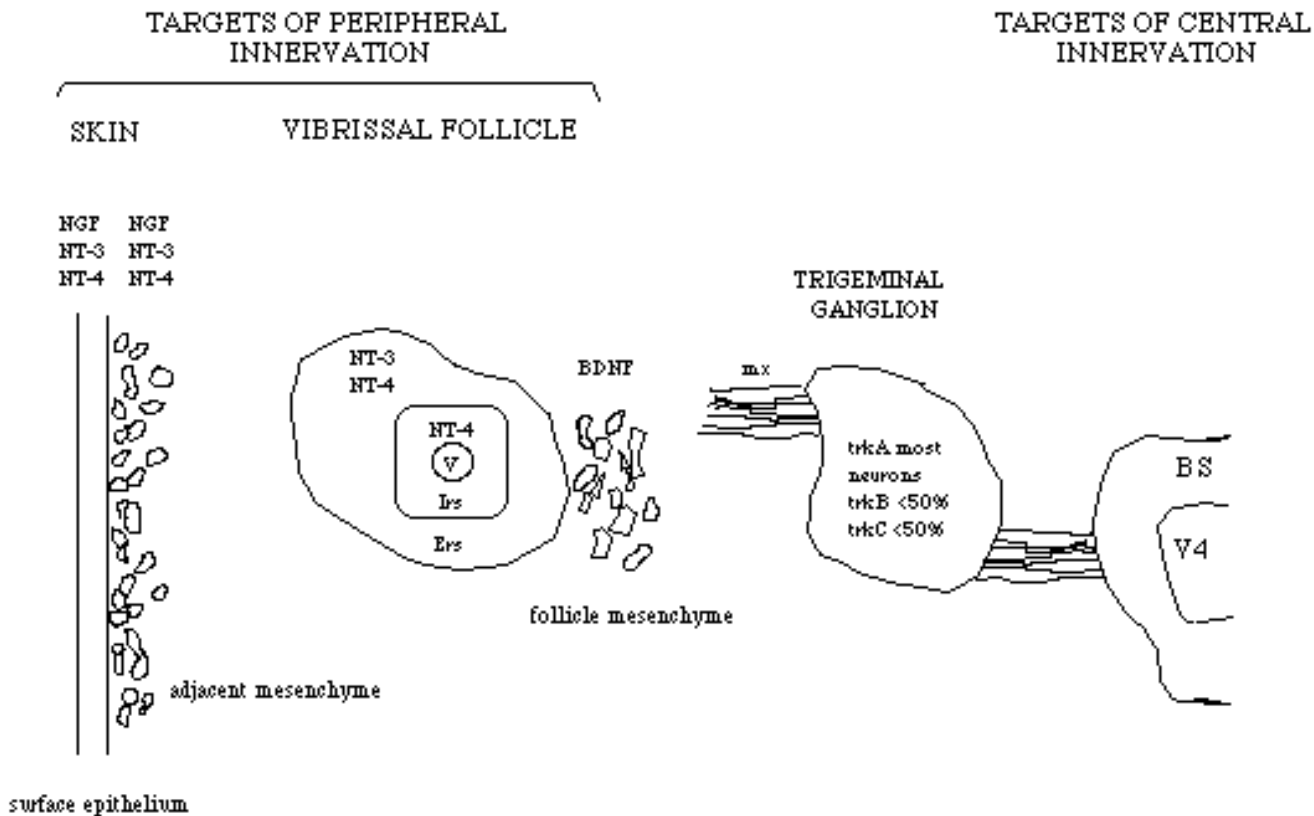


Fig. 7. Schematic representation of the localization of neurotrophin mRNAs in the peripheral targets of the trigeminal ganglion. BS, brain stem; Ers, external root sheath; Irs, internal root sheath; mx, maxillary process of the trigeminal ganglion; V, vibrissae; V4, fourth ventricle.

maxillary process/whisker pad of the mouse at embryonic day 12.5, shortly after the arrival of the first innervating sensory neurons from the trigeminal ganglion (Davies et al., 1987). After E13, both the local concentration of NGF mRNA and protein decreased, correlating in time with the onset of neuronal death in the trigeminal ganglion (Davies et al., 1987). Similarly, we also found a decrease in the amount of NT-4 mRNA in this target field after E13. BDNF has also been shown to support survival of trigeminal ganglion neurons (Davies et al., 1986a; Davies et al., 1986b). The high level of BDNF mRNA detected in the pons and medulla of newborn and adult rats (Ernfors et al., 1990; Maisonpierre et al., 1990a; Friedman et al., 1991b) has provided support to the notion that BDNF provides neurotrophic support to trigeminal sensory neurons from their target in the CNS. However, we demonstrate here that BDNF mRNA is also expressed in the developing rat whisker pad, suggesting that this factor could also play a trophic role from the peripheral target. In addition, the expression of NT-3 mRNA has recently been described in the developing whisker pad of the rat, specifically in the external layers of the epithelial root sheath of the follicles (Ernfors et al., 1992) and, as shown here, NT-3 is also able to support survival of trigeminal neurons in culture. Therefore, all neurotrophins appear to provide neurotrophic support to trigeminal ganglion neurons from their peripheral targets in the whisker pad: NGF, NT-3 and NT-4 from the developing skin, NT-3 and NT-4 from the developing follicles and BDNF from the mesenchyme surrounding the follicles. Within the follicle proper, both NT-3 and NT-4 mRNA are expressed in the outer layers of the follicle, whereas only NT-4 appear to be expressed in the internal layers in close contact with the hair (Fig. 7).

A detailed study of the innervation of mammalian whiskers by neurons of the trigeminal ganglion has revealed a complex pattern with morphologically distinct fibres terminating either deeply or superficially in different layers of the follicle proper as well as in the dermally derived surrounding sinus (Rice et al., 1986). However, the trigeminal ganglion is in its turn a complex structure with neurons arising from different embryonic origins. In birds, the neural crest-derived and placode-derived neurons of this ganglion have been subdivided and shown to have different neurotrophic requirements. NGF has been shown to support the neural crest-derived neurons but not the placode-derived ones, which in turn can be supported by BDNF (Davies and Lindsay, 1984; Davies et al., 1986b). In this study, we demonstrate that the four neurotrophins support both distinct and overlapping subsets of neurons within the rat trigeminal ganglion. Additive or partially additive effects were observed with combinations of BDNF and NT-3, NT-4 and NT-3 and NGF and NT-4, suggesting that subsets of neurons exist that respond to some but not all neurotrophins.

The observation that members of the Trk family of tyrosine kinase receptors mediate signal transduction in response to specific neurotrophins (Kaplan et al., 1991; Klein et al., 1991a,b; Soppet et al., 1991; Squinto et al., 1991; Lambelle et al., 1991; Ip et al., 1992) has provided an alternative way to make predictions about the neurotrophic requirements of subpopulations of neurons by studying at the type of Trk receptor they express. In situ

hybridization studies have shown that most of the developing neurons in the rat trigeminal ganglion express high levels of *trkA* mRNA (Ernfors et al., 1992), which codes for a signal transducing receptor for NGF. However, only a subpopulation of neurons express *trkC* mRNA, which codes for a receptor for NT-3, and an even smaller subpopulation express *trkB* mRNA, encoding a receptor for BDNF and NT-4 (Ernfors et al., 1992). This observation correlates with the result from our in vitro survival assay showing that NGF was more effective in supporting survival of trigeminal ganglion neurons than NT-3, NT-4 and BDNF. The discovery that different Trk receptors mediate the response to different neurotrophins has also provided a set of markers that can be used to distinguish cells responsive to one neurotrophin from those responding to others. In the present study we used DNA probes for the different Trk mRNAs to further characterize the neurons that respond to the different neurotrophins. This allowed us to identify subsets of neurons responsive to specific combinations of neurotrophins, although some overlap is evident. Thus, for example, NT-4 responsive neurons are $TrkA^+$, $TrkB^+$, $TrkC^-$, whereas NT-3 responsive neurons are $TrkA^+$, $TrkB^-$, $TrkC^+$. Taken together, our results suggest that in the embryonic rat whisker pad the specificity for sensory innervation by different neurons of the trigeminal ganglion may be controlled by the expression of a set of neurotrophic factors displaying both partially overlapping and specific expression patterns in the target field.

Interestingly, NT-4 was significantly ($P < 0.01$) more effective than BDNF in supporting trigeminal neurons, despite the fact that these two factors are equally effective in evoking responses in either NIH3T3 or PC12 cells expressing TrkB receptors (Ip et al., in press). Thus, there may be physiologically important ways in which TrkB receptors can distinguish between these two neurotrophins in certain neuronal cells, or there may be yet another Trk receptor that displays higher specificity for NT-4 as opposed to BDNF. In any event, the number of *trkB* and *trkC* mRNA expressing cells in the trigeminal ganglion decreases between E13 and E16, possibly due to a loss of cells during the phase of neuronal death (Ernfors et al., 1992). In this case, the surviving neurons may have successfully competed for BDNF, NT-3 and NT-4 protein produced by target fields of these neurons like, as show here, the rat whisker pad.

The first and second author (C. F. I. and P. E.) contributed equally to the results reported in this manuscript. We thank Dr Jan Arvidson, Department of Anatomy, Karolinska Institute, Stockholm, for help with dissections of embryonic rat trigeminal ganglia and Ms Annika Kylberg and Dr Ted Ebendal, Department of Developmental Biology, Biomedical Center, Uppsala, for advice on dissections of embryonic chick trigeminal ganglia used in preliminary experiments. Technical assistance was given by Ms Mona Gullmert. Financial support was obtained from the Swedish Natural Science Research Council, The Swedish Medical Research Council (B93-13X-10368-01A), US grants (University of Colorado) AG04418, NS09199, The Bank of Sweden Tercentenary Foundation, Konung Gustav V:s and Drottning Victorias Stiftelse, Gertrude och Ivar Philipsons Stiftelse, Fredrik och Ingrid Thuring's Stiftelse, The Swedish Board for Technical Development, Association Française Contre les Myopathies, The Swedish Royal Academy of Sciences, Erik och Edith Fernströms Stiftelse, Tore

Nilssons Foundation and funds from the Karolinska Institute. C. F. I. and P. E. were supported by the Swedish Medical Research Council.

REFERENCES

- Alderson, R. F., Alterman, A. L., Barde, Y.-B. and Lindsay, R. M. (1990). Brain-derived neurotrophic factor increases survival and differentiated functions of rat septal cholinergic neurons in culture. *Neuron* **5**, 297-306.
- Bandtlow, C. E., Heumann, R., Schwab, M. E. and Thoenen, H. (1987). Cellular localization of nerve growth factor synthesis by in situ hybridization. *EMBO J.* **6**, 891-899.
- Barde, Y.-A. (1989). Trophic factors and survival. *Neuron* **2**, 1525-1534.
- Barde, Y.-A., Edgar, D. and Thoenen, H. (1982). Purification of a new neurotrophic factor from mammalian brain. *EMBO J.* **1**, 549-553.
- Berkemeier, L., Winslow, J., Kaplan, D., Nicolics, K., Goeddel, D. and Rosenthal, A. (1991). Neurotrophin-5: A novel neurotrophic factor that activates trk and trkB. *Neuron* **7**, 857-866.
- Davies, A. M., Bandtlow, C., Heumann, R., Korsching, S., Rohrer, H. and Thoenen, H. (1987). Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. *Nature* **326**, 353-8.
- Davies, A. M. and Lindsay, R. M. (1984). Neural crest-derived and cranial sensory neurons are equally sensitive to NGF but differ in their response to tissue extracts. *Dev. Brain Res.* **14**, 121-127.
- Davies, A. M. and Lumsden, A. G. S. (1984). Influence of nerve growth factor on developing dorso-medial and ventrolateral neuros of chick and mouse trigeminal ganglia. *Int. J. Dev. Neurosci.* **1**, 171-177.
- Davies, A. M., Thoenen, H. and Barde, Y.-A. (1986a). Different factors from the central nervous system and periphery regulate the survival of sensory neurons. *Nature* **319**, 497-499.
- Davies, A. M., Thoenen, H. and Barde, Y.-A. (1986b). The response of chick sensory neurons to brain-derived neurotrophic factor. *J. Neurosci.* **6**, 1897-1904.
- Ernfors, P., Ibáñez, C. F., Ebendal, T., Olson, L. and Persson, H. (1990). Molecular cloning and neurotrophic activities of a protein with structural similarities to -nerve growth factor: developmental and topographical expression in the brain. *Proc. Natl. Acad. Sci. USA* **87**, 5454-5458.
- Ernfors, P., Merlio, J.-P. and Persson, H. (1992). Cells expressing mRNA for neurotrophins and their receptors during embryonic rat development. *Eur. J. Neurosci.* **4**, 1140-1158.
- Ernfors, P. and Persson, H. (1991). Developmentally regulated expression of HDNF/NT-3 mRNA in rat spinal cord motoneurons and detection of BDNF mRNA in embryonic dorsal root ganglion. *Eur. J. Neurosci.* **3**, 953-961.
- Ernfors, P., Wetmore, C., Olson, L. and Persson, H. (1990). Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. *Neuron* **5**, 511-526.
- Friedman, W., Ernfors, P. and Persson, H. (1991a). Transient and persistent expression of NT-3/HDNF mRNA in the rat brain during postnatal development. *J. Neurosci.* **11**, 1577-1584.
- Friedman, W., Olson, L. and Persson, H. (1991b). Cells that express brain-derived neurotrophic factor mRNA in the developing postnatal rat brain. *Eur. J. Neurosci.* **3**, 688-697.
- Friedman, W., Ibáñez, C. F., Hallböök, F., Persson, H., Cain, L. D., Dreyfus, C. F., and Black, I. B. (1993). Differential actions of neurotrophins in the locus coeruleus and basal forebrain. *Exp. Neurol.* (in press).
- Hallböök, F., Ibáñez, C. F. and Persson, H. (1991). Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. *Neuron* **6**, 845-858.
- Hamburger, V., Bruno-Bechtold, J. K. and Yip, J. W. (1981). Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. *J. Neurosci.* **1**, 60-71.
- Hamburger, V. and Oppenheim, R. W. (1982). Naturally occurring neuronal death in vertebrates. *Neurosci. Commun.* **1**, 39-55.
- Heumann, R., Korsching, S., Scott, J. and Thoenen, H. (1984). Relationship between levels of nerve growth factor (NGF) and its messenger RNA in sympathetic ganglia and peripheral target tissues. *EMBO J.* **3**, 3183-3189.
- Hohn, A., Leibrock, J., Bailey, K. and Barde, Y.-A. (1990). Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature* **344**, 339-341.
- Honegger, P. and Lenoir, D. (1982). Nerve growth factor (NGF) stimulation of cholinergic telencephalic neurons in aggregating cell cultures. *Brain Res.* **255**, 229-38.
- Hyman, C., Hofer, M., Barde, Y.-A., Juhasz, Yancopoulos, Squinto, S. and Lindsay, R. (1991). BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. *Nature* **350**, 230-232.
- Ip, N. Y., Ibáñez, C. F., Nye, S. H., McClain, J., Jones, P. F., Gies, D. R., Belluscio, L., Le Beau, M. M., Espinosa III, R., Squinto, S. P., Persson, H. and Yancopoulos, G. (1992). Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution and receptor specificity. *Proc. Natl. Acad. Sci. USA* **89**, 3060-3064.
- Ip, N. Y., Stitt, T. N., Tapley, P., Klein, R., Glass, D. J., Fandl, J., Greene, L. A., Barbacid, M. and Yancopoulos, G. D. (1993). Similarities and differences in the way neurotrophins interact with the Trks in neuronal and non-neuronal cells. *Neuron* (in press).
- Johnson, E. J., Andres, R. Y. and Bradshaw, R. A. (1978). Characterization of the retrograde transport of nerve growth factor (NGF) with high specific activity: 125I-NGF. *Brain Res.* **150**, 319-31.
- Kaplan, D., Martin-Zanca, D. and Parada, L. (1991). Tyrosine phosphorylation and tyrosine kinase activity of the *trk* proto-oncogene product induced by NGF. *Nature* **350**, 158-160.
- Klein, R., Jing, S., Nanduri, V., O'Rourke, E. and Barbacid, M. (1991a). The *trk* proto-oncogene encodes a receptor for nerve growth factor. *Cell* **65**, 189-197.
- Klein, R., Nanduri, V., Jing, S., Lambelle, F., Tapley, P., Bryant, S., Cordon-Cardo, C., Jones, K., Reichardt, L. and Barbacid, M. (1991b). The *trkB* tyrosine kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. *Cell* **66**, 395-403.
- Knusel, B., Winslow, J., Rosenthal, A., Burton, L., Seid, D., Nicolics, K. and Hefti, F. (1991). Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. *Proc. Natl. Acad. Sci. USA* **88**, 961-965.
- Lambelle, F., Klein, R. and Barbacid, M. (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., Lottspeich, A. H., Hofer, M., Hengerer, B., Masiakowski, P., Thoenen, H. and Barde, Y.-A. (1989). Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* **341**, 149-52.
- Levi-Montalcini, R. and Angeletti, P. (1968). Nerve growth factor. *Physiol. Rev.* **48**, 534-569.
- Lindsay, R. M., Thoenen, H. and Barde, Y.-A. (1985). Placode and neural crest-derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor. *Dev. Biol.* **112**, 319-328.
- Maisonpierre, P. C., Belluscio, L., Friedman, B., Alderson, R. F., Wiegand, S. J., Furth, M. E., Lindsay, R. M. and Yancopoulos, G. D. (1990a). NT-3, BDNF, and NGF in the developing rat nervous system: Parallel as well as reciprocal patterns of expression. *Neuron* **5**, 501-509.
- Maisonpierre, P. C., Belluscio, L., Squinto, S., Ip, N. Y., Furth, M. E., Lindsay, R. M. and Yancopoulos, G. D. (1990b). Neurotrophin-3: A neurotrophic factor related to NGF and BDNF. *Science* **247**, 1446-1451.
- Martínez, H. J., Dreyfus, C. F., Jonakait, G. M. and Black, I. B. (1987). Nerve growth factor selectively increases cholinergic markers but not neuropeptides in rat basal forebrain in culture. *Brain Res.* **412**, 295-301.
- Oppenheim, R. W. (1985). Naturally occurring cell death during neuronal development. *Trends Neurosci.* **8**, 487-493.
- Phillips, H. S., Hains, J. M., Laramée, G. R., Rosenthal, A. and Winslow, J. W. (1990). Widespread expression of BDNF but not NT-3 by target areas of basal forebrain cholinergic neurons. *Science* **250**, 290-294.
- Rice, F., Mance, A. and Munger, B. (1986). A comparative light microscopic analysis of the sensory innervation of the mystacial pad. I. Innervation of vibrissal follicle-sinus complexes. *J. Comp. Neurol.* **252**, 154-174.
- Rosenthal, A., Goeddel, D. V., Nguyen, T., Lewis, M., Shih, A., Laramée, G. R., Nicolics, K. and Winslow, J. W. (1990). Primary structure and biological activity of a novel human neurotrophic factor. *Neuron* **4**, 767-773.
- Ruit, K. G., Elliott, J. L., Osborne, P. A., Yan, Q. and Snider, W. D. (1992). Selective dependence of mammalian dorsal root ganglion neurons on nerve growth factor during embryonic development. *Neuron* **8**, 573-587.

- Schwab, M. E., Otten, U., Agid, Y. and Thoenen, H.** (1979). Nerve growth factor (NGF) in the rat CNS: absence of specific retrograde axonal transport and tyrosine hydroxylase induction in locus coeruleus and substantia nigra. *Brain Res.* **168**, 473-483.
- Shelton, D. J. and Reichardt, L. F.** (1984). Expression of the nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. *Proc. Natl. Acad. Sci. USA* **81**, 7951-7955.
- Soppet, D., Escandon, E., Maragos, J., Middlemas, D., Reid, S., Blair, J., Burton, L., Stanton, B., Kaplan, D., Hunter, T., Nikolics, K. and Parada, L.** (1991). The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the *trkB* tyrosine kinase receptor. *Cell* **65**, 895-903.
- Squinto, S., Stitt, T., Aldrich, T., Davis, S., Bianco, S., Radziejewski, C., Glass, D., Masiakowski, P., Furth, M., Valenzuela, D., DiStefano, P. and Yancopoulos, G.** (1991). *trkB* encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. *Cell* **65**, 885-893.
- Stöckel, K. and Thoenen, H.** (1975). Retrograde axonal transport of nerve growth factor: specificity and biological importance. *Brain Res.* **85**, 337-341.
- Toenen, H., Bandtlow, C. and Heumann, R.** (1987). The physiological function of nerve growth factor in the central nervous system: comparison with the periphery. *Rev. Physiol. Biochem. Pharmacol.* **109**, 145-78.
- Toenen, H. and Barde, Y. A.** (1980). Physiology of nerve growth factor. *Physiol. Rev.* **60**, 1284-1325.
- Whittemore, S. R. and Seiger, A.** (1987). The expression, localization and functional significance of beta-nerve growth factor in the central nervous system. *Brain Res.* **434**, 439-64.

(Accepted 4 January 1993)