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Expression and binding characteristics of the BDNF receptor chick *trk*B

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SUMMARY

Previous studies using transfected cells have indicated that the mammalian receptor tyrosine kinase *trk*B binds the neurotrophins brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4. However, most studies demonstrating that these neurotrophins prevent the death of embryonic neurons and have specific neuronal receptors have been performed with chick neurons. In order to explore the possibility that *trk*B is the molecular entity representing the high-affinity receptor for brain-derived neurotrophic factor on embryonic chick neurons, we cloned and expressed a chick *trk*B cDNA. In situ hybridisation results indicate that the distribution of *trk*B mRNA in the peripheral nervous system of the developing chick embryo correlates well with the structures known to respond to brain-derived neurotrophic factor. Binding studies performed with a cell line stably transfected with the c*trk*B cDNA indicate a dissociation constant for brain-derived neurotrophic factor of 9.9×10^{-10} M, which is distinctly higher than that found on primary chick sensory neurons (1.5×10^{-11} M). When binding of brain-derived neurotrophic factor was determined in the presence of other neurotrophins, neurotrophin-3 was found efficiently to prevent the binding of brain-derived neurotrophic factor to both the c*trk*B cell line and embryonic sensory neurons. In vitro, neurotrophin-3 at high concentrations completely blocked the survival normally seen with brain-derived neurotrophic factor. Thus, unlike previous cases of receptor occupancy by heterologous neurotrophins (which resulted in agonistic effects), the interaction between the brain-derived neurotrophic factor receptor and neurotrophin-3 on sensory neurons is antagonistic.

Key words: neurotrophin, sensory ganglia, high-affinity receptors, neuronal survival

INTRODUCTION

One of the major biological functions of the neurotrophins is the control of neuronal survival during the phase of naturally occurring cell death (Barde, 1989). Until now, four members of this gene family have been identified: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (for review, see Chao, 1992). While the four neurotrophins have been shown to prevent the death of a variety of embryonic neurons either in vitro or in vivo, the vast majority of these studies have been performed using avian embryonic neurons. Binding studies have also been performed using radiolabelled neurotrophins and neurons isolated from the peripheral nervous system of the chick embryo. With all neurotrophin-responsive neurons studied so far, two distinct binding sites have been identified. The low-affinity binding sites for neurotrophins (K_d of 0.8-2.3×10^{-9} M) are found on neurons and non-neuronal cells and are not ligand specific: for example, binding of labelled NGF to these sites can be prevented by BDNF and unlabelled NGF equally well (Rodríguez-Tébar et al., 1990). The same characteristics have been observed with the p75 low-affinity NGF, or neurotrophin, receptor ($p75^{LANR}$) expressed on fibroblasts (D. Johnson et al., 1986; Radeke et al., 1987), which binds all neurotrophins with similar affinities (Rodríguez-Tébar et al., 1990, 1992; Hallböök et al., 1991). Until now, the exact biological function played by $p75^{LANR}$ is not understood (see Meakin and Shooter, 1992 for review). The high-affinity sites on neurons bind their cognate ligands with a K_d of $1.7\text{-}2.3\times10^{-11}$ M (Sutter et al., 1979; Rodríguez-Tébar and Barde, 1988; Rodríguez-Tébar et al., 1992) and are thought to be the signal transducing elements. In contrast to the low-affinity binding sites, the high-affinity sites are characterised by their ligand specificities (Rodríguez-Tébar et al., 1990, 1992; Dechant et al., 1993).

In addition to their interaction with $p75^{LANR}$, the neurotrophins are now known also to interact with a subfamily of receptor tyrosine kinases known as *trk*-receptors designated *trk*, *trk*B and *trk*C (see Barbacid et al., 1991 for review). Much of the available evidence for these interactions is derived from experiments performed with cell lines and indicate that *trk* interacts with NGF and to a lesser

degree with NT-3 (Cordon-Cardo et al., 1991; Hempstead et al., 1991; Kaplan et al., 1991a,b; Klein et al., 1991a), *trk*B with BDNF, NT-3 and NT-4 (Glass et al., 1991; Klein et al., 1991b; Soppet et al., 1991; Squinto et al., 1991; Berkemeier et al., 1991; Klein et al., 1992; Ip et al., 1993) and *trk*C with NT-3 (Lamballe et al., 1991; Tsoulfas et al., 1993; Valenzuela et al., 1993). These results indicate that, although there is a preferred neurotrophin ligand for each individual *trk* receptor (Ip et al., 1993), there is not a simple one-to-one relationship between receptor and ligand, and that *trk*B shows the most complex pattern of ligand interactions. In all cases investigated so far, binding of a neurotrophin to a recombinant *trk* receptor on fibroblast cell lines is followed by tyrosine phosphorylation of the receptor (Berkemeier et al., 1991; Glass et al., 1991; Kaplan et al., 1991b; Klein et al., 1991a,b; Soppet et al., 1991; Ip et al., 1993; Valenzuela et al., 1993; Tsoulfas et al., 1993). It has been noted, however, that, depending on the cellular context, the degree to which NT-3 interacts with *trk*B can vary markedly, the lesser degree of phosphorylation seen with NT-3 in PC12 cells transfected with *trk*B being thought to result from an increased ligand specificity, compared with fibroblasts (Ip et al., 1993). Several lines of evidence indicate that members of the *trk*-family are very likely to be involved in neuronal high-affinity binding and intracellular signalling following binding. Firstly, the *trk*-family members are the only neurotrophin receptors known to be expressed in neurons with a ligand-specific, intrinsic signal transducing capacity. Secondly, they express a pattern of ligand specificity that is reminiscent of that seen with neuronal high-affinity receptors. Thus, *trk*C, like NT-3 high-affinity receptors, is highly ligand specific (Lamballe et al., 1991; Rodríguez-Tébar et al., 1992; Tsoulfas et al., 1993; Valenzuela et al., 1993), whereas *trk*, like neuronal NGF receptors, does not interact significantly with BDNF, but with NT-3 (Rodríguez-Tébar et al., 1990; Cordon-Cardo et al., 1991; Dechant et al., 1993). Thirdly, the expression patterns of some of the *trk*-family members, as assessed by in situ hybridisation in rodents, are suggestive. Especially *trk* exhibits a convincing correlation between *trk*-expressing and NGF-responsive neurons (Klein et al., 1989, 1990b; Martin-Zanca et al., 1990; Carroll et al., 1992; Holtzman, et al., 1992; Merlio et al., 1992).

In order to gain insight into the signal transduction of neurotrophins on their physiological target cells, it is desirable to study the molecular composition of the neurotrophin-binding sites on embryonic neurons. Given that several populations of BDNF-responsive neurons have been studied in chick and quail embryos (Lindsay et al., 1985; Davies et al., 1986; Kalcheim et al., 1987; Hofer and Barde, 1988; Oppenheim et al., 1992), we were interested in studying by in situ hybridisation the expression of chick *trk*B (c*trk*B) during the development the chick nervous system. Also, as most experiments describing the characteristics of neuronal receptors for neurotrophins have been performed with chick neurons but all *trk*-receptors cloned and characterised in mammalian species only, it was of interest to determine the binding properties of BDNF using c*trk*B expressed in a cell line.

MATERIALS AND METHODS

Cloning and sequencing

The c*trk*B cDNA was cloned from an adult chick brain lambda gt10 cDNA (Couturier et al., 1990) using a rat *trk*B fragment encoding both the transmembrane and tyrosine kinase domain obtained by PCR and corresponding to bp 1120-2434 of the rat sequence (Middlemas et al., 1991). The amplified fragment was cloned, sequenced and radiolabelled using a random hexamer priming kit (Pharmacia-LKB). 9×10^5 phage clones were transferred to nylon membranes (Hybond N, Amersham-Buchler) and the filters hybridised in the following hybridisation mixture: 5× SSC, 5× Denhardt's solution, 40% formamide and the radiolabelled probe (2×10^6 counts/minute/ml) at 38°C for 18 hours. The filters were subsequently washed in 6× SSC, 0.1% SDS at 55°C for 30 minutes. From the primary screening, 11 clones were isolated and the cDNAs from 8 phages were cloned into pBluescript (Stratagene). The sequence of the inserted cDNAs was then determined by dideoxynucleotide sequencing with Taq-Polymerase on a sequencing automate (Type 373A, Applied Biosystems) using various oligonucleotide primers (synthesised on a 381A Applied Biosystems DNA synthesiser) derived from the previously analysed sequence. The sequence reported in Fig. 1 is a result from sequencing on both strands for the entire sequence.

Probe design and preparation

Two probes spanning either the extracellular portion of chick *trk*B (nucleotides 587 to 1298) or approximately 600 bases of the 3′ untranslated region (see Fig. 2) were selected for in situ hybridisation and northern blot analysis. Preliminary in situ experiments run with a riboprobe spanning the entire tyrosine kinase domain of chick *trk*B resulted in a labelling with high background levels; this probe was therefore not employed routinely for in situ hybridisation studies. Riboprobes were prepared by linearising plasmids and transcribing the template in the presence of either ^{32}P-UTP (northern blot analysis) or ^{35}S-UTP (in situ hybridisation) according to standard procedures. Following transcription, the template DNA was removed by DNase A treatment (6 U, 15 minutes at 37°C) and the probe precipitated with 2.5 M ammonium acetate and ethanol. The specific activity of the probes employed was consistently in the range of 5×10^9 counts/minute/μg. A cDNA probe corresponding to the tyrosine kinase domain of *trk*B (nucleotides 1470 to 1856) was also used for determining the *trk*B transcripts coding for a full-length catalytic receptor. This cDNA probe was labelled by random prime labelling in the presence of ^{32}P-dCTP to a specific activity of 4×10^9 counts/minute/μg.

Northern blot analysis

Total RNA was extracted from embryonic chick tissues according to the guanidium thiocyanate phenol-chloroform method (Chomczynski and Sacchi 1987). Subsequently, poly(A)$^+$ enriched RNA was prepared from brain RNA by conventional oligo(dT) chromatography (Sambrook et al., 1989). 5 μg of poly(A)$^+$-enriched brain RNA and 5 μg total RNA from E8 dorsal root ganglia were electrophoresed under denaturing conditions on formaldehyde agarose gel (Sambrook et al., 1989). RNA was transferred to nylon filters by vacuum blotting and UV-fixed. The methylene blue staining patterns of the filters indicated that the amount of poly(A)$^+$ RNA loaded in the different lanes was similar. The northern blots analysed with the riboprobes were prehybridised with 50% formamide, 4× SSC, 4× Denhardt, 1% SDS, 50 μg/ml tRNA for 7 hours at 65°C. Hybridisation was performed overnight at 65°C in the same mixture containing ^{32}P-labelled probe (5×10^6 counts/minute/ml), followed by a 20 minutes wash in the hybridisation mixture at 65°C and two washes of 30 minutes in 0.1% SDS, 0.1× SSC. The northern blots analysed with the cDNA probe were prehybridised with 50% formamide, 7× SSC, 50 mM sodium

phosphate buffer pH 7.4, 5× Denhardt, 0.25% SDS, 100 μg/ml salmon sperm DNA and 40 μg/ml tRNA for 7 hours at 42°C. Hybridisation was performed in the same conditions, overnight with 5×10^6 counts/minute/ml of ^{32}P-labelled probe, followed by a 15 minutes wash at 42°C with 0.1% SDS, 2× SSC and 15 minutes at 55°C with 0.1% SDS, 0.1× SSC. Blots were air dried and exposed to X-ray films (Fuji) with intensifying screens for 2-10 days.

In situ hybridisation

Eggs were collected from embryonic day 3.5 (E3.5) of incubation (or stage 23 of Hamburger and Hamilton, 1951) to day 14. The embryos were either fixed as a whole by immersion (until E7), or, alternatively, the tissues of interest were dissected out and fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The tissues were subsequently cryoprotected in sucrose solutions, frozen in liquid nitrogen cooled isopentane, cut and placed onto polylysine-coated slides as previously described (Biffo et al., 1990).

For in situ hybridisation, the method of Wilkinson et al. (1987) with small variations was used. Following washes and proteinase K digestion (0.3 μg/ml), sections were dehydrated, and the riboprobe was added to a final concentration of 5×10^5 counts/minute per slide (in 100 μl). Hybridisation was performed overnight at 62°C. Stringency washes were performed as previously described (Wilkinson et al., 1987) and included a treatment with RNaseA at 20 μg/ml in 0.5 NaCl, 20 mM Tris-HCl pH 8.0, 2 mM EDTA for 30 minutes at 37°C. Following dehydration sections were exposed to NTB2 (Kodak) emulsion at 4°C for up to 2 weeks, successively developed, counterstained with hematoxylin and mounted.

Neurotrophins

For the preparation of radioligand, recombinant mouse BDNF was produced in a Vaccinia virus expression system as previously described for NT-3 (Götz et al., 1992). In the inhibition assays, BDNF and NT-3 derived from an *E. coli* expression system were used as native ligands (kind gift from R. Lindsay). NGF was isolated from the submandibular glands of adult male mice as described (Suda et al., 1978). Recombinant *Xenopus* NT-4 was purified using the procedure described for NT-3 from the supernatant of A293 cells stably transfected with a *Xenopus* prepro-NT-4 cDNA (Hallböök et al., 1991) cloned in a pCMV expression vector. The specific biological activity of the ligand proteins was analysed in quantitative survival assays using embryonic sensory neurons as described (Lindsay et al., 1985; Götz et al., 1992). Half-maximal survival effects were obtained with concentrations of BDNF, NT-3 and NT-4 of 30-40 pg/ml.

Cell cultures

The human embryonic kidney cell line A293 (Graham et al., 1977) was grown in DMEM (Gibco) containing 10% fetal calf serum (Boehringer Mannhein). A stably transfected cell line expressing chick *trk*B was obtained by transfecting the c*trk*B cDNA (bp 1-2698) subcloned into the *Hin*dIII site of the eucaryotic expression vector Rc/CMV (Stratagene), which drives the expression of inserted DNA from the human cytomegalovirus promoter (Andersson et al., 1989). 20 μg of this expression vector construct (pCMV-c*trk*B) was transfected into A293 cells using the method of Chen and Okayama (1987) together with 1 μg of the plasmid pSV2-pac that confers puromycin resistance to transfected cells (Vara et al., 1986). After 10 days of selection with 1 μg/ml puromycin (Sigma), puromycin-resistant cell clones were subcloned and clonal cultures were further grown in the presence of 1 μg/ml puromycin. Fourteen different clones were analysed for c*trk*B expression by northern blot analysis of total RNA probed against a c*trk*B fragment. RNA blots from all transfected clones, but not from parental A293 cells contained a band of the expected size. The clone with the highest expression level of chick *trk*B mRNA was chosen for further analysis (A293-c*trk*B). For binding assays, the parental A293 and A293-c*trk*B cells were removed from the plastic dishes by gentle trypsinization (0.01% trypsin, 5 minutes) centrifuged and resuspended in Krebs-Ringer-Hepes buffer containing 5 mg/ml BSA and 0.1 mg/ml horse heart cytochromeC (binding buffer, Rodríguez-Tébar and Barde, 1988). The number of living cells was determined using the trypan blue exclusion method and a hemocytometer. Primary sensory neurons from E8 to E10 chick embryos were isolated as described (Rodríguez-Tébar and Barde, 1988). Briefly, the ganglia (dorsal root or nodose ganglion) were dissected and collected in Gey's buffer (Gey and Gey, 1936) on ice. After dissection, the ganglia were treated with 0.02% trypsin for 15 minutes in the same buffer at 37°C. Trypsinization was stopped by the addition of 10% horse serum and the ganglia were dissociated by gentle trituration in a wide-bore glass pipette. The cell suspension was subsequently incubated on culture dishes at 10% CO_2 at 37°C. During this time, non-neuronal cells settle on the plastic dish and the neurons are collected by aspiration of the supernatants. This procedure results in a high enrichment of neurons in the suspension. The supernatant was subsequently centrifuged (1000 ***g***, 5 minutes) and the cell pellet resuspended in binding buffer. The number of cells in the suspension was determined as described above. For the in vitro survival assays, nodose ganglia were dissected from E8 chick embryos as described (Lindsay et al., 1985). The trypsinized ganglia (20 minutes, 0.1% trypsin) were preplated for 3 hours as described above. Neurons were plated on polyornithine-laminin-coated 48-well plates (Costar) at a density of 2000 cells/well in Ham's F14 medium (Gibco) supplemented with 10% horse serum (Boehringer Mannheim). Neuronal survival was determined by counting phase-bright cells with neuronal morphology in duplicate wells after 24 hours.

Radiolabelling of BDNF

Radiolabelling of BDNF was performed using a modification of the lactoperoxidase-labelling protocol described for NT-3 (Rodríguez-Tébar et al., 1992). 10 μg of Vaccinia-derived mouse BDNF were incubated in a sodium phosphate buffer (0.3 M, pH 7.2) for 1 hour on ice in a total volume of 40 μl containing 0.4 μg lactoperoxidase (Sigma), 50 μM H_2O_2 and 1.5 mCi ^{125}I (100 mCi/ml, Amersham). After this incubation, the reaction was stopped by dilution to a final volume of 1 ml followed by an overnight incubation at 4°C. The incorporation of ^{125}I into BDNF was determined by TCA-precipitation. Typically, 88-95% of the total radioactivity were incorporated into the protein resulting in a radiolabelled ligand with a specific activity of 150-200 counts/minute/pg. The biological activity of the radiolabelled material was identical to the native factor in an in vitro survival assay with embryonic sensory neurons (40 pg/ml). The radiolabelled material was found to be stable for at least 2 weeks at 4°C, but was used within 10 days following iodination.

Binding experiments

All binding experiments were performed in suspension at 4°C in a water bath with gentle shaking. 500 μl of cell suspension containing $2.5\text{-}10\times10^5$ cells/ml were incubated in 50 ml plastic tubes (Falcon) either in the absence (total binding) or presence of a 100- to 500-fold excess of native ligand for 45 or 60 minutes (unspecific binding). Subsequently, the radioligand was added and the suspension incubated for a further 60 minutes. Free radioactivity was determined by measuring total activity in the cell suspensions assuming pseudo-first order conditions in the samples. Bound radioactivity was separated from free by rapid centrifugation (3000 ***g***, 1 minute) of 4 samples (100 μl each) in 700 μl plastic tubes (Eppendorf) followed by complete removal of the supernatant. The

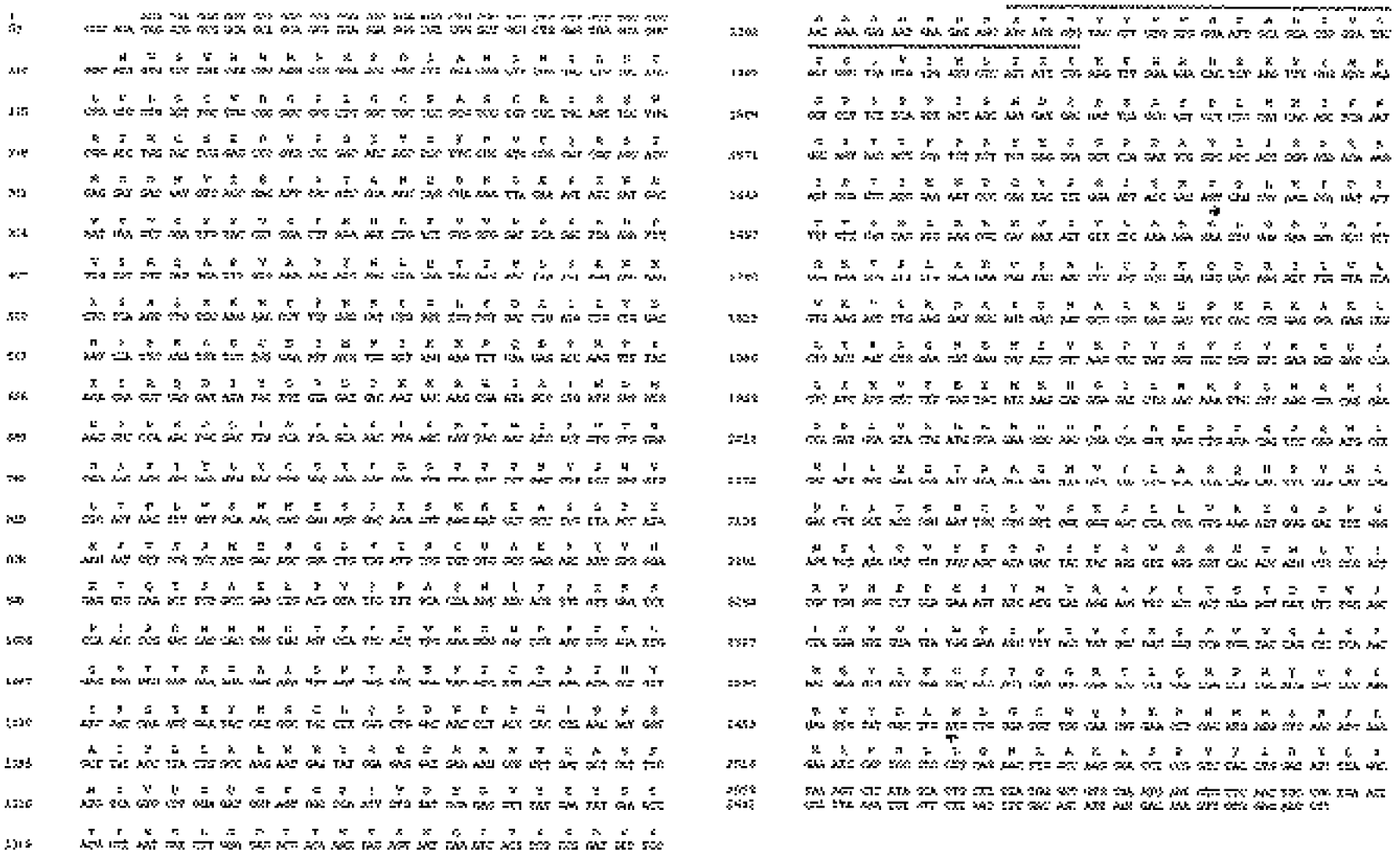

Fig. 1. Nucleotide and protein sequence of chick *trk*B. Nucleotide sequence the first 2699 base pairs (from the 5´ end) of the longest cDNA insert isolated from an adult chick brain library. The sequence contains an open reading frame of 818 amino acids. Computer based analysis of the protein sequence predicted a single membrane spanning domain (=) and a tyrosine kinase domain (start and stop indicated by arrows).

tubes were inverted and air-dried. The bound radioactivity was determined by counting the tips of the tubes in a gamma counter with an efficiency of 78% (Canberra Packard) until the values reached a 1% sigma level. The tetraplicate samples were averaged. All values referring to bound radioligand (fmoles/10^6 cells or percent of binding) represent specific binding determined by subtraction of unspecific binding from total binding. For the steady-state binding experiments, unspecific binding for each individual point of the binding curve was determined. In the inhibition experiments, 100% binding refers to specific binding in the absence of heterologous ligand, 0% binding represents the unspecific binding. The samples in the inhibition experiments including heterologous ligands were incubated in the presence of these ligand for 60 minutes prior to addition of the radioligand. Binding data were analysed using a curve fitting computer program (Grafit, Erythacus Software).

RESULTS

Cloning of a chick *trk*B cDNA

Five independent clones were identified with a probe encoding the tyrosine kinase domain of rat *trk*B. Restriction analysis revealed that these clones contained fragments of the same cDNA. The largest insert had a size of approximately 4 kb. The first 2699 base pairs starting at the 5′-end of the clone were sequenced on both strands (Fig. 1). The sequence contained an open reading frame of 2454 bp. The bases upstream of the first methionine match the consensus sequence for translation initiation sites (Kozak, 1987). The 818 amino acid protein encoded in the open reading frame showed a clear homology to rat *trk*B (76.8% overall identity on amino acid level) and to a lesser degree to human *trk*A and to rat *trk*C (51% and 57.1%). The homology to rat *trk*B is not evenly distributed along the molecule. The putative membrane-spanning domain is the least conserved part of the molecule (45.8% amino acid identity). The extracellular binding domain of the predicted protein was found to be 61.2% identical to its rat homologue, whereas chick and rodent BDNF are 92.4% identical (Isackson et al., 1991). In the intracellular domain, only a small number of amino acids were found to be exchanged between chick and rat (97% identity) indicating that the cDNA encodes the chicken homologue of the mammalian receptor tyrosine kinase *trk*B (chick *trk*B, c*trk*B).

Northern blot analysis

Northern blot analysis with a riboprobe spanning the extracellular domain of chick *trk*B revealed at least 5 transcripts in chick brain (Fig. 2A). Their size was estimated to be 9.0, 8.1, 6.3, 5.0 and 1.6 kb, the 1.6 kb mRNA being more obvious on total RNA samples than on poly(A)$^+$-enriched RNA (data not shown). Analysis of mRNA expression in non-neuronal tissues showed a specific enrichment of the 5.0 kb band in mesenchymal derivatives (data not shown). In contrast, the riboprobe derived from the 3′ untranslated region (3′-probe) of chick *trk*B detected predominantly the 9.0 kb mRNA (Fig. 2C). In order to address the question if the 9.0 kb mRNA detected with the 3′-probe also contained the sequence corresponding to the tyrosine kinase domain of c*trk*B, a blot was hybridised with a cDNA probe spanning this domain (Fig. 2B). Like the 3′-probe, this probe detected the 9.0 kb transcript (compare Fig. 2B and 2C). A transcript of the same size was detected in total RNA prepared from embryonic day 8 dorsal root ganglia neurons (Fig. 2D).

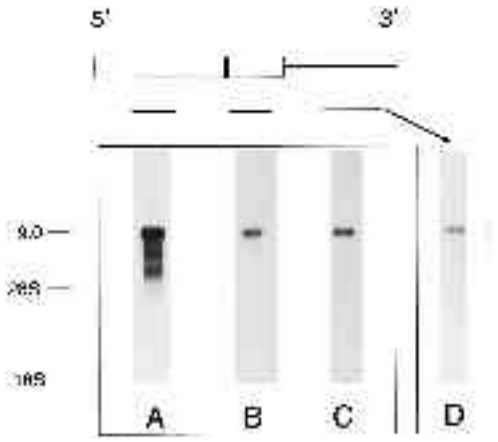

Fig. 2. Northern blot of brain and DRG RNA probed with c*trkB* probes. The chick *trk*B cDNA is divided into 3 domains: the extracellular region, the tyrosine kinase region and the 3′ untranslated region. Probes used for RNA blot analysis were derived from the sequences underlined. Blots A, B and C contains poly(A)$^+$ enriched RNAs from E10 chick brain. Panel D contains total RNA from dorsal root ganglia. A has been hybridised with a cRNA probe derived from the extracellular sequence, B has been probed with a cDNA probe spanning the tyrosine kinase domain and C and D with a riboprobe arising from the 3′ untranslated region. 28S and 18S are the ribosomal markers. Four bands ranging in size from 9.0 kb to 5.0 kb are shown in A. This probe identifies also a 1.6 kb message, not shown in this figure. B and C show that a 9.0 kb transcript can be detected with probes spanning the tyrosine kinase domain or the 3′ untranslated sequence. This band is also present in total RNA from dorsal root ganglia (D)

Table 1. Expression of c*trk*B in the peripheral nervous system of the developing chick embryo

Ganglia	4.0 (24)	4.5	5.0	6.0	8.0	10	12	14
trigeminal, vl (V)	+	+	++	++	++	++	++	++
trigeminal, dm (V)	−	−	−	+	+	+	+	+
geniculate (VII)	−	−	+	+	nd	+	nd	nd
acoustic and vestibular (VIII)	nd	+	++	++	++	++	++	++
superior (IX)	−	−	+	+	+	+	nd	+
jugular (X)	nd	−	nd	−	−	−	nd	nd
petrose (IX)	nd	−	nd	+	+	+	nd	nd
nodose (X)	+	++	++	++	nd	++	nd	nd
dorsal root ganglia	+*	+	++	++	++	++	++	++
sympathetic trunk	−	−	−	−	−	−	−	−
ciliary (III)	nd	−	−	nd	−	−	nd	nd

Experiments were performed using a riboprobe spanning the 3′-untranslated region of the chick *trk*B cDNA.

Abbreviations: nd, not determined; −, no expression; +, moderate expression; ++, strong expression.

The number between parentheses indicates the cranial nerve the ganglion is associated with. Day 4 is stage 24 of Hamburger and Hamilton (1951).

*Expression starts at the mid-thoracic level.

In situ hybridisation

Unless indicated otherwise, the data in the following were

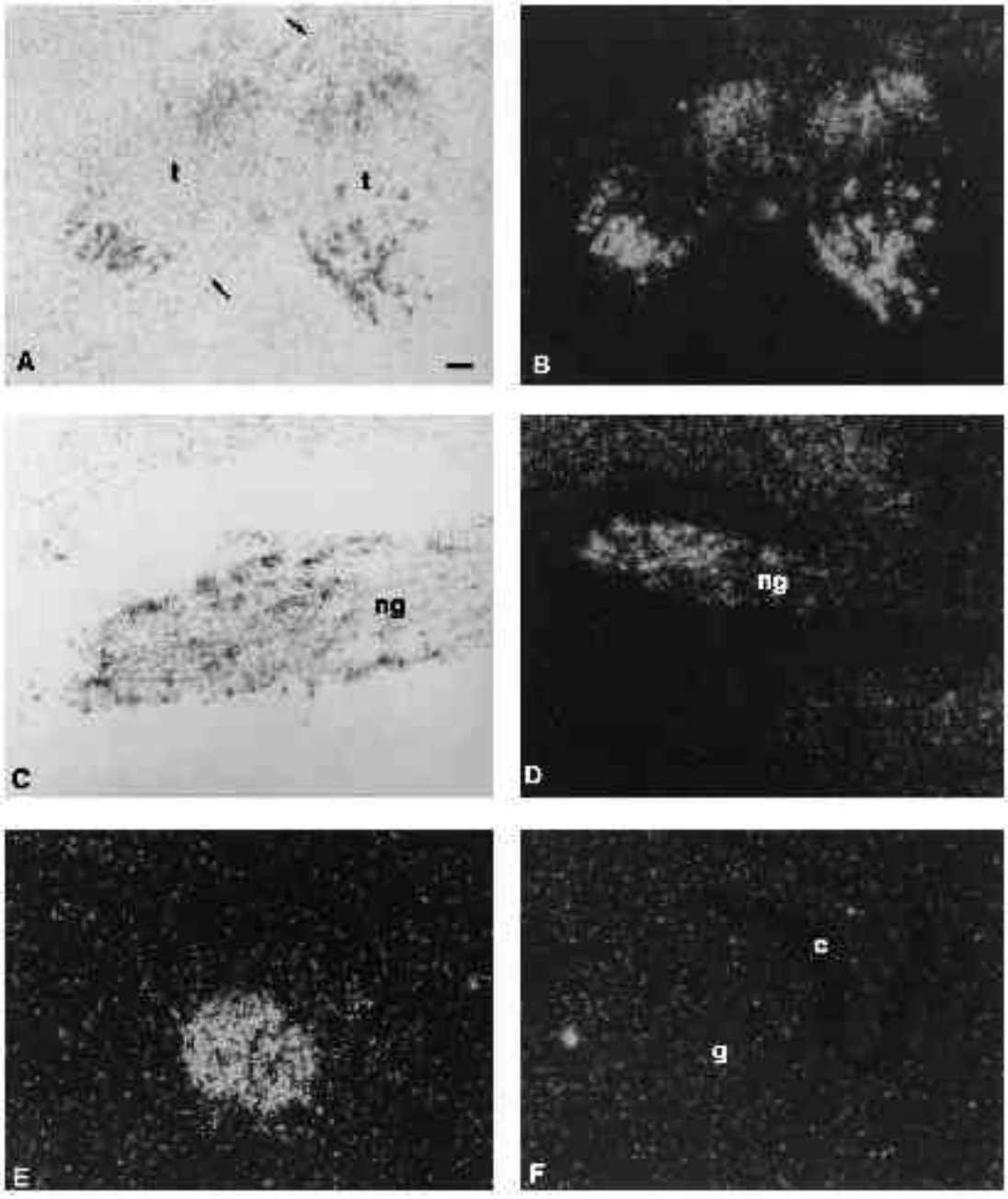

Fig. 3. *trk*B mRNA expression in the trigeminal, nodose and acoustic ganglion. (A,B) Bright- and dark-field images of the E6 trigeminal ganglion (t). Large *trk*B-positive neurons are located in the ventral portion of the ganglion. More diffuse labelling is also visible in the most dorsal part of the ganglion, but no labelling is seen around the trigeminal nerve (arrows) or in the medial portion of the ganglion. (C,D) Bright- and dark-field image of the E4.5 nodose ganglion (ng). C is a higher magnification of D: note that the positive neurons are a subset of the total neuronal population and are scattered throughout the ganglion. (E,F) Dark-field image of the developing (E 6) acoustic ganglion with antisense (E) or sense (F) probe. Abbreviations: c, developing cochlea; g, ganglion. Scale bar shown in A is 65 μm for A, B and D and 32 μm for C, E and F.

obtained using the 3′-probe and are summarized in Table 1 for the peripheral nervous system.

While c*trk*B transcripts were detected early in most sensory ganglia, no expression was detected at any stage in BDNF non-responsive sympathetic and ciliary neurons. Unambiguous expression of chick *trk*B m-RNA was first seen in the developing trigeminal ganglion at E 3.5 (stage 23 of Hamburger and Hamilton, 1951). Subsequently, the levels of *trk*B mRNA increased and at E5, expression was confined to the large neurons in the ventral region of the ganglion. Little or no expression was seen in the early embryo in the dorsal portion of the ganglion. However, at later stages, *trk*B mRNA expression became evident also in the most dorsal part of the trigeminal ganglion although more diffuse and weaker compared with the strongly labelled, large neurons located in the distal most part of the

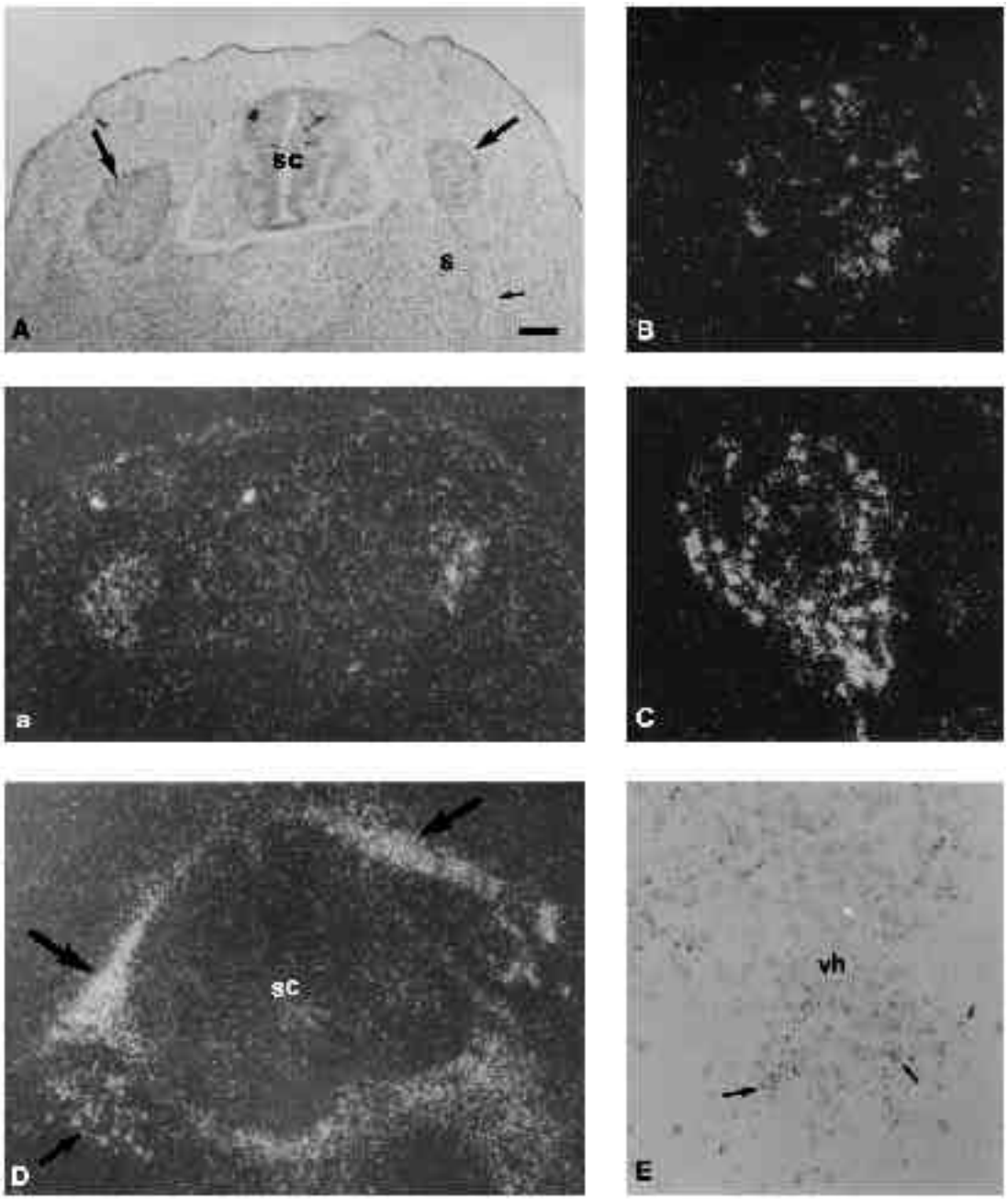

Fig. 4. *trk*B mRNA expression in the spinal cord and in the dorsal root ganglia. Bright- (A) and dark-field (a) images of the dorsal portion of a sagittal section through a E4.5 embryo (the spinal cord, sc, is indicated), showing the expression of *trk*B mRNA as detected with the 3′-probe. Note the labelling in the dorsal root ganglia (large arrows), which is absent from the sympathetic chain (s) and the spinal nerves (small arrow) and from nonneuronal derivatives. B and C show sections of DRG at E6 and E14. (D) Dark-field image of the E6 spinal cord showing the pattern of labelling as detected with the 5′-probe. Note that the DRG are labelled (small arrow) as well a conspicuous ring of mesenchymal cells surrounding the spinal cord (large arrow). (E) Higher magnification of the ventral horn (vh) shows presumptive motoneurons labelled for *trk*B mRNA. Scale bar shown in A is 170 μm for A, a, D, 65 μm for B, C and 32 μm for E.

ganglion. The expression of *trk*B in the dorsal part of the trigeminal ganglion was presumably associated with small sized neurons and/or the satellite cells surrounding them (Fig. 3A,B). In the nodose ganglion, transcripts could also be detected at E4. In contrast to the trigeminal ganglion, the localization of the labelled neurons did not reveal any particular segregation (Fig. 3C,D). About 50% of the neurons were *trk*B positive in some sagittal sections through the ganglion, but *trk*B-positive neurons were always found in close association with cells not expressing *trk*B. The developing acoustic and vestibular ganglion was found to be highly labelled at all the stages examined. In this ganglion, essentially all neurons contained c*trk*B-mRNA (Fig. 3E) as revealed by the antisense 3′-probe (see Fig. 3F for the sense control). The developmental pattern of *trk*B mRNA expression in the dorsal root ganglia (DRG) was examined

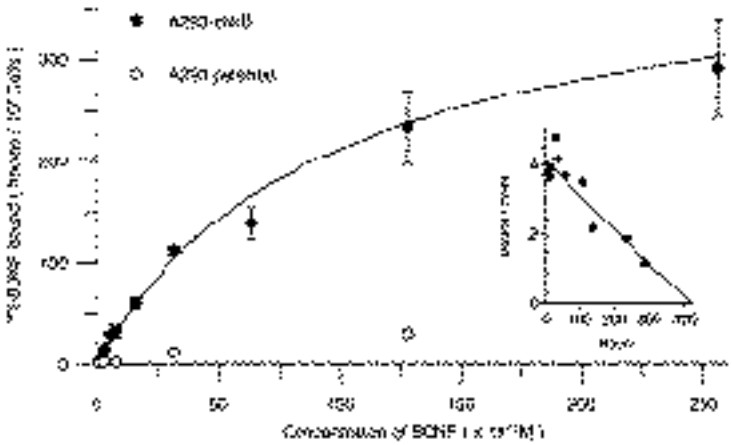

Fig. 5. Binding of ^{125}I-BDNF to A293-ctrkB and A293 parental cells. Cells (400.000 cells/ml for A293-c*trk*B and 480.000 cells/ml for A293) were incubated at 4°C with various concentrations of ligand. The results are mean of 4 determinations±s.d. Linear transformation of the binding data (insert) reveals a K_d of $9.9\pm1.2\times10^{-10}$ M and $2.4\pm0.12\times10^5$ binding sites per cell.

in detail, as these ganglia represent the main source of neurons utilised in both neuronal survival experiments and binding studies. At mid-thoracic level, c*trk*B-transcripts were already detected at E3.5 and a substantial expression was maintained throughout the period studied (Fig. 4A-D). The vast majority of the labelled neurons (about 30% of the total population) was concentrated in the ventral and lateral part of the ganglion and represented mostly large-size neurons (Fig. 4C). c*trk*B-positive cells were identified in the ventral horn of the spinal cord, especially in the areas containing motoneurons, but the levels of expression of c*trk*B mRNA were substantially lower as compared with the sensory ganglia (Fig. 4E). In the retina, no evidence for c*trk*B-positive ganglion cells was found between E6 and E12, although scattered positive cells were detected mainly in the developing inner nuclear layer (not shown). In contrast to the labelling observed with the 3′-probe, that detected with the 5′-probe also revealed non-neuronal structures. However, the neuronal labelling detected with the 5′-probe was completely overlapping with that observed with the 3′-probe. Non-neuronal structures detected with the 5′-probe included restricted sites of the ependyma and various mesenchymal cells, for example those surrounding the spinal cord (Fig. 4D). No such labelling was detected with the 3′-probe.

Binding of BDNF: c*trk*B cell line versus sensory neurons

The binding of radioiodinated BDNF to E8-E9 neurons isolated from DRGs was compared with that to a cell line stably expressing the full-length c*trk*B cDNA described above. In steady-state binding assays, ^{125}I-BDNF was found to bind to A293-c*trk*B cells with a K_d of $9.9\pm1.2\times10^{-10}$ M (Fig. 5). The number of receptors per cell was determined to be $2.4\pm0.1\times10^5$ as calculated by Scatchard transformation of the binding data. Although the A293 parental cells types also appear to bind BDNF at high concentration, no significant binding was detected at low ligand concentrations and no saturation was observed in the range of concentrations of radioligand that we have tested (up to 3×10^{-9} M). We next investigated whether the A293-c*trk*B cell line displays significant numbers of high-affinity binding sites for BDNF. Steady-state binding experiments were performed at low ligand concentrations (below 3.5×10^{-11} M), using sensory neurons as a point of comparison (Fig. 6). At these concentrations no binding to parental A293 cells could be measured (data not shown). High-affinity BDNF-binding sites (3600±500 receptors/cell, K_d $1.5\pm0.5\times10^{-11}$ M) were detected only on sensory neurons. No indication of saturable high-affinity binding was found for A293-c*trk*B cells. Analysis of the binding data shown in Fig. 6 indicates that the specific binding seen with the A293-c*trk*B cells can be attributed to the BDNF-binding sites described in Fig. 5, which are present in very large numbers on these cells (see above). The limit of detection for high-affinity binding sites in this assay was estimated to be about 200 receptors/cell corresponding to about 0.1% of the total receptor population on A293-c*trk*B cells. As neuronal high-affinity sites for BDNF are characterised by a high ligand specificity, we then compared the binding of ^{125}I-BDNF to A293-c*trk*B cells and embryonic DRG neurons in the presence of various concentrations of heterologous neurotrophins. In these experiments, we used ^{125}I-BDNF at a concentration of 3×10^{-11} M as at this low ligand concentration, significant binding is seen with both the c*trk*B cells and sensory neurons (see above). Furthermore, the binding obtained with primary neurons is largely specific for high-affinity receptors because the contribution of the low-affinity binding sites to the total binding at this concentration is small, due to positive cooperativity of the BDNF binding to these sites (see Rodríguez-Tébar et al., 1990 and 1992 for discussion). In these experiments, differences were found between c*trk*B and sensory neurons in all cases tested. Thus, the inhibition of ^{125}I-BDNF binding by NGF was more effective on neurons than on A293-c*trk*B cells: whereas 50% reduction of BDNF binding to DRG neurons was achieved with 2×10^{-8} M NGF (see also Rodríguez-Tébar et al., 1990), no significant reduction was observed for c*trk*B cells throughout the concentration range tested (Fig. 7A). Conversely, while inhibition of BDNF binding to DRG neurons by 50% occurred at 1.1×10^{-9} M NT-3, the same decrease was obtained with an approximately 10-fold lower concentration (1.3×10^{-10} M) on c*trk*B cells (Fig. 7B). *Xenopus* NT-4 resulted in an efficient and complete inhibition of BDNF binding to c*trk*B cells, 50% inhibition being observed at 7.3×10^{-11} M (Fig. 7C). Also with DRG neurons, inhibition by NT-4 was detected already at very low concentrations, but this inhibition of ^{125}I-BDNF binding was incomplete

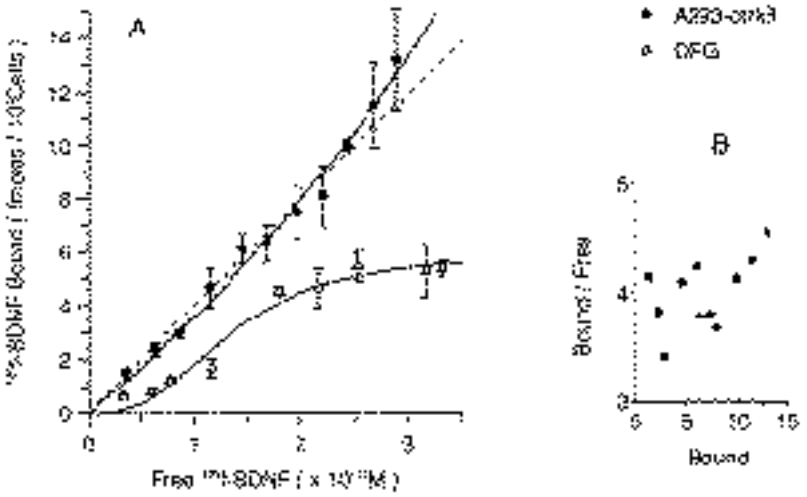

Fig. 6. Binding of ^{125}I-BDNF to DRG neurons and A293-c*trk*B cells. ^{125}I-BDNF in a low concentration range (0.25-3.5×10^{-11} M) was incubated with either transfected A293 cells (330.000 cells/ml) or E8 DRG (400.000 cells/ml). The results are means of quadruple samples±s.d. (A) Saturable high-affinity binding of ^{125}I-BDNF was detected with DRG neurons (K_d 1.5±0.5×10^{-11} M and 3600±500 receptors per cell). No saturable high-affinity binding was obtained with A293-c*trk*B cells. The binding to these cells measured at low concentrations of radioligand (solid line) fits the curve predicted for the low-affinity binding sites on these cells (dashed curve, calculated from the data obtained in Fig. 5). (B) No indication for a saturable binding site was found by Scatchard transformation of the binding data for A293-c*trk*B.

(Fig. 7C). The inhibition curve is best fitted by a 2-site model for BDNF receptors on DRG neurons, in which NT-4 blocks 50% of BDNF binding to the first site at a concentration of 1.1×10^{-10} M. The second BDNF-binding site (representing about 25% of the total number of high-affinity BDNF receptors on E8 DRG neurons) is resistant to inhibition by NT-4.

Neuronal survival response elicited by BDNF in the presence of heterologous neurotrophins

Using cultured neurons isolated from the nodose ganglion of E8 chicks, about 50% of the neurons survive in the presence of BDNF (when used at saturation), whereas NT-3 supports the survival of about 25% of these neurons (Hohn et al., 1990; Götz et al., 1992). The effects of BDNF and NT-3 are additive (Götz et al., 1992; Fig. 8A). In a binding experiment with nodose ganglion neurons (data not shown), NT-3 used at 4×10^{-9} M prevented the binding of ^{125}I-BDNF (3×10^{-11} M) to E8 nodose neurons as observed with the DRG neurons (Fig. 7B). In view of these results indicating that NT-3 at elevated concentrations is able to prevent the binding of BDNF to the high-affinity BDNF receptors on sensory neurons and on A293-c*trk*B cells, we analysed the BDNF-induced survival response of nodose ganglion neurons in the presence of high concentrations of NT-3. At concentrations high enough to completely saturate the high-affinity BDNF receptors with NT-3 (20 μg/ml or 7.7×10^{-7} M, Fig. 8A), NT-3 supported the survival of 28±1% of the neurons. These effects on neuronal survival are not higher than those seen at 1 ng/ml NT-3 (23±4%), indicating that the occupancy of the BDNF receptors by NT-3 does not result in a productive interaction as assessed by neuronal survival. In addition, the effects of BDNF were completely blocked by this high concentration of NT-3 (20 μg/ml). Low concentrations of BDNF (0.1 ng/ml) support the survival of 34±1% of the nodose neurons, and these effects are additive with those of NT-3 when used at 1 ng/ml (65±6%, see Fig. 8A). At 0.1 ng/ml of BDNF in the presence of 20 μg/ml NT-3 only 28±2% of the neurons survived, the same number of neurons that is supported by NT-3 alone (Fig. 8A). This indicates that binding of NT-3 to high-affinity BDNF receptors on nodose ganglion neurons is not sufficient to induce neuronal survival of BDNF-responsive neurons in culture, and that NT-3 interacts in an antagonistic manner with neuronal high-affinity BDNF receptors.

Finally, in view of the binding of NT-4 to c*trk*B and to the majority of BDNF high-affinity binding sites on neurons, we also tested the effects of NT-4 on nodose neurons (Fig. 8B). At concentrations of 1 ng/ml, NT-4 induced the survival of 30±3% of E8 nodose ganglion neurons. The effects of NT-4 were additive to those of NT-3 (51±4% versus 26±1% with NT-3 alone), but not to those of BDNF (44±1% BDNF plus NT-4, all factors at 1 ng/ml). This indicates that both NT-4 and BDNF induce neuronal survival via the same type of receptor, consistent with the results obtained with c*trk*B. However, the effects of BDNF alone (51±4%) were found to be larger than the effects of NT-4 (30±3%) when used at saturating concentrations, suggesting that the effects of NT-4 might be mediated by a subpopulation of high-affinity BDNF receptors.

DISCUSSION

In this study, we isolated a full-length cDNA clone encoding chick *trk*B, currently the most likely functional BDNF receptor candidate. This clone allowed us to study the expression pattern of the chick c*trk*B gene in neuronal populations known to respond to BDNF during development and to examine the binding of ^{125}I-BDNF to c*trk*B-expressing cells in comparison to BDNF-responsive neurons.

Northern blot analysis

Five different mRNAs coding for the extracellular domain of chick *trk*B were revealed by northern blot analysis using a 5′-probe corresponding to this domain. One of these mRNAs (the largest transcript, 9 kb in length) was found to cross-hybridise with a probe spanning the tyrosine kinase domain of chick *trk*B. From this analysis, it appears likely that, in the chick, as observed before in rodents (Klein et al., 1990a; Middlemas et al., 1991), the *trk*B gene encodes for several transcripts, including several lacking the tyrosine kinase catalytic domain. It is worth noting that the shortest mRNA detected in the brain by northern blot analysis is 1.6 kb in length, i.e. substantially shorter than the sequence necessary to code for a full-length receptor. The 9 kb tran-

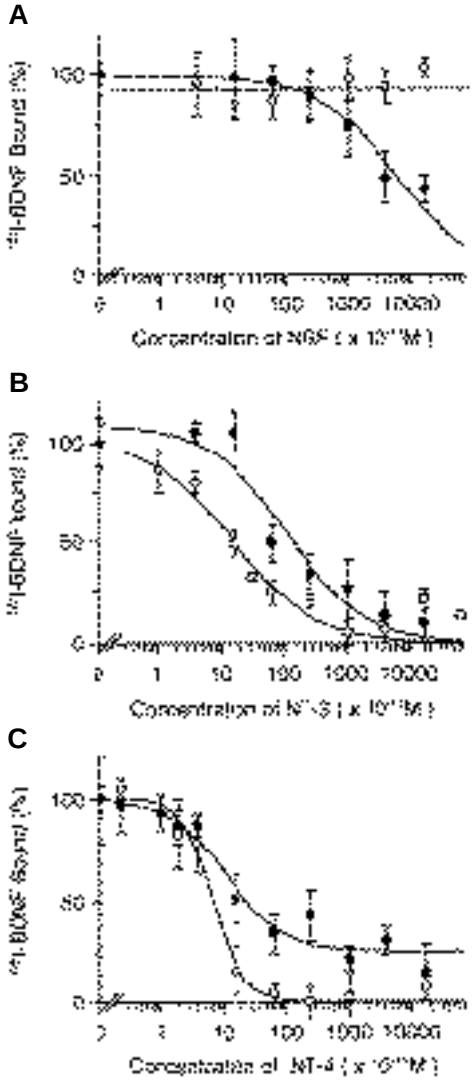

Fig. 7. Inhibition of ^{125}I-BDNF Binding to A293-c*trk*B cells and high-affinity BDNF sites on DRG neurons by heterologous neurotrophins. All experiments were performed at a 3×10^{-11} M concentration of ^{125}I-BDNF. A293-c*trk*B cells and embryonic DRG neurons were preincubated with various concentrations of heterologous ligand for 60 minutes, followed by addition of radioligand for another 60 minutes. For each data point, the bars represent the mean of a tetraplicate determination±s.d.
(A) Inhibition by NGF: binding of radiolabelled BDNF to A293-c*trk*B cells was not significantly inhibited by NGF. An inhibition constant (K_i) of 2×10^{-8} M was found for NGF and DRG neurons.
(B) Inhibition by NT-3: 50% inhibition of ^{125}I-BDNF binding was obtained at 1.3×10^{-10} M NT-3 for A293-c*trk*B and 1.1×10^{-9} M for DRG neurons. (C) Inhibition by NT-4: binding of radioiodinated BDNF to A293-c*trk*B cells could be completely blocked by *Xenopus* NT-4 with a K_i of 7×10^{-11} M. 75% of the BDNF binding to DRG neurons was inhibited with an K_i 1.1×10^{-10} M by NT-4. The remaining 25% of BDNF receptors could not be blocked by the concentrations of NT-4 tested.

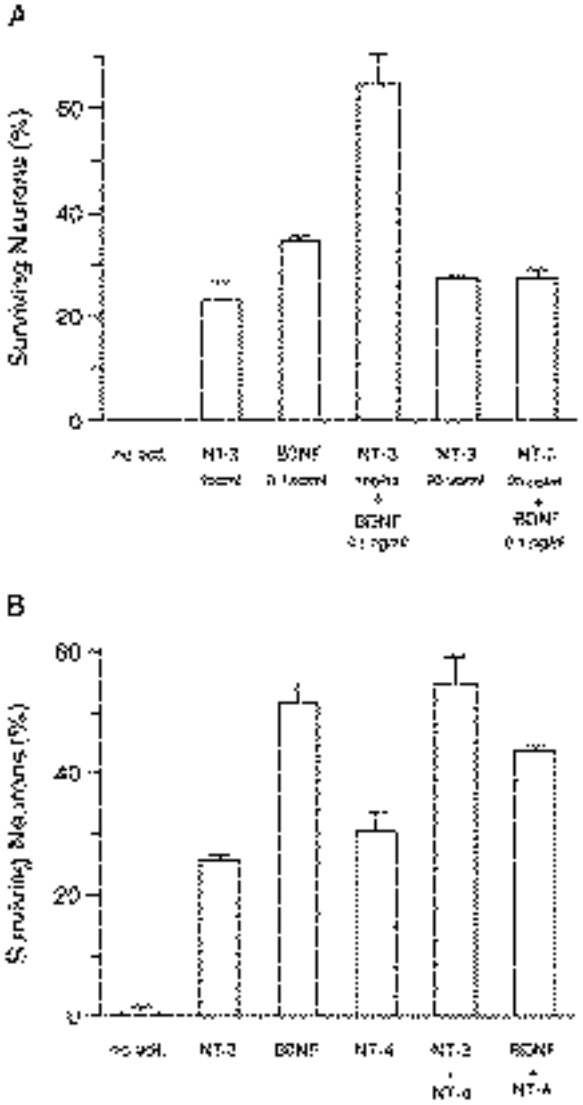

Fig. 8. In vitro survival assay with embryonic nodose ganglion neurons. Neurons prepared from E8 nodose ganglia were plated either in the presence or absence of neurotrophins as indicated. The number of surviving neurons was determined after 24 hours. Results are means of duplicate determinations. (A) NT-3 does not induce the survival of BDNF responsive neurons at concentrations high enough to saturate the high-affinity BDNF receptors.
(B) NT-4 effects on nodose ganglion neurons are smaller than BDNF effects although both effects are not additive. The neurotrophins were added at a final concentration of 1 ng/ml. Higher concentrations did not lead to higher survival.

script was also detected by a 3′-probe corresponding to the non-translated sequence of *trk*B. In situ hybridisation results of experiments performed with this probe showed a much more restricted pattern of labelling as compared to the 5′-probe. In particular, whereas both probes labelled neuronal structures equally well, only the 5′-probe labelled also non-neuronal structures (ependyma, choroid plexus and various mesenchymal derivatives). The combination of the in situ data with the northern blot analysis performed with domain-specific probes leads to the conclusion that a tyrosine kinase containing *trk*B sequence is expressed in neurons, whereas truncated forms are contained in non-neuronal cells, as has been described before for rodents (Klein et al., 1990a; Middlemas et al., 1991). However, our analysis cannot rule out the presence of truncated receptors in neurons as well.

In situ hybridisation

In general, a very good correlation was found between the pattern of expression of *trk*B, particularly in developing peripheral ganglia, and the localization of neurons known to respond to BDNF. Thus, chick *trk*B expression starts very early during embryogenesis, before and during the period of naturally occurring neuronal death in peripheral sensory ganglia. This is consistent with the observation that BDNF plays a role early during gangliogenesis (Kalcheim et al., 1987) and prevents cell death in vivo in DRG and nodose ganglia (Hofer and Barde, 1988). Within the DRG and trigeminal ganglia, the location of *trk*B-positive neurons, found mostly in the larger lateroventral population, is complementary to that of the smaller, mediodorsal neurons, enriched in NGF-responsive neurons (Hamburger et al., 1981; Verge et al., 1992). Complementarity has also been recently reported for the expression of *trk* and *trk*B mRNA in the spinal ganglia of the rat (Carroll et al., 1992). In vitro studies using DRGs and, especially, trigeminal ganglia, have indicated that the survival effects of NGF and BDNF are additive or complementary, suggesting different subpopulations of responsive neurons (Lindsay et al., 1985, Davies et al., 1986). Finally, *trk*B is not expressed at detectable levels in peripheral ganglia such as ciliary and sympathetic ganglia, known not to respond to BDNF (Lindsay et al., 1985). The sympathetic neurons have also been shown to display only low affinity, but not high-affinity BDNF-binding sites (Rodríguez-Tébar and Barde, 1988).

In the CNS, the low levels of *trk*B mRNA seen over the anterior part of the spinal cord fit with recent results indicating that the addition of BDNF during development prevents the death of a portion of spinal motoneurons (Oppenheim et al., 1992). In fact, only one exception to the correlation between BDNF responsiveness and *trk*B expression was noted: the retinal ganglion cells. In vitro, embryonic retinal ganglion cells isolated from the chick (or rat) retinae have been shown to be dependent on BDNF for survival (J. E. Johnson et al., 1986; Rodríguez-Tébar et al., 1989). *trk*B expression in the retinal ganglion cell layer could not be detected in vivo between E6 and E12, although a small number of labelled cells were occasionally seen in the inner nuclear layer. In view of the bulk of our results indicating an excellent correlation between *trk*B expression and BDNF responsiveness, this result is surprising. It should be noted however that while BDNF responsiveness has been observed in vitro, it remains to be demonstrated that BDNF acts on retinal ganglion cells in vivo. If BDNF also acts specifically on retinal ganglion cells in vivo, the possibilities should be considered that this might be through receptors other than the c*trk*B receptor studied here, or that c*trk*B levels of expression substantially below those found on other BDNF-responsive cells are sufficient for a BDNF response.

BDNF receptors on A293-c*trkB* cells versus DRG neurons

As expected from similar studies in rodents (Klein et al., 1991b; Soppet et al., 1991; Squinto et al., 1991), BDNF binds specifically to A293 cells stably transfected with a cDNA encoding c*trk*B. A dissociation constant of 9.9 $\times 10^{-10}$ M was determined, which is very close to that reported for BDNF binding to NIH-3T3 fibroblasts transfected with murine *trk*B, 1.8×10^{-9} M (Soppet et al., 1991). This result indicates that the differences between the affinities of *trk*B receptors expressed on cell lines and BDNF-binding sites on sensory neurons of the chick cannot be simply explained by species differences. No evidence for significant numbers of BDNF receptors on A293-c*trk*B cells comparable to the high-affinity binding sites on sensory neurons was obtained. The number of high-affinity BDNF sites on DRG neurons was higher (about 10-fold) than in two previous studies (Rodríguez-Tébar and Barde, 1988; Rodríguez-Tébar et al., 1990). This difference is likely to result from two reasons: the use of recombinant BDNF (as opposed to BDNF isolated from pig brain before) and lactoperoxidase-induced iodination (rather than the iodinated Bolton-Hunter reagent in previous studies). No differences in affinity or specificity upon challenge with other neurotrophins could be observed. At present, the molecular basis for the increase in the number of ^{125}I-BDNF-binding sites is not clear. Our results thus indicate that the high-affinity binding characteristics of BDNF on peripheral sensory neurons cannot be completely accounted for by c*trk*B expressed in a non-neuronal context. Since the discovery of the *trk*-family members as neurotrophin receptors, much discussion has followed as to whether or not the expression of a *trk*-family member alone can lead to high-affinity receptors, represented, for example, by *trk* homodimers (Jing et al., 1992), or if co-expression of other molecules such as p75^{LANR} are necessary (Battleman et al., 1993). While the emphasis has been placed so far on the discrepancies, it is striking to note that in all studies, the vast majority (90% or more) of the neurotrophin-binding sites formed by recombinant *trk* tyrosine kinases is of the low-affinity type. The question thus arises as to whether low-affinity *trk* receptors also exist in abundance on neurons of the peripheral nervous system, and we feel that the available evidence suggests that this is not the case. While it is clear that these neurons express large numbers of low-affinity neurotrophin receptors (in the order of tens of thousand per neuron), the molecular entity explaining them is much more likely to be p75^{LANR} than any member of the *trk*-family. Firstly, on sensory neurons, low-affinity ^{125}I-NGF binding can be prevented by unlabelled BDNF and NGF equally well (Rodríguez-Tébar et al., 1990), a typical feature of p75^{LANR}, but not of *trk.* Secondly, binding of NGF to low-affinity binding sites on chick sensory neurons (or PC12 cells) is completely prevented by antibodies directed against p75^{LANR} (Weskamp and Reichardt, 1991). The inhibition of BDNF binding by other neurotrophins also revealed quantitative differences between c*trk*B and high-affinity BDNF binding on sensory neurons. Qualitatively, however, the similarities are obvious: BDNF binding to both A293-c*trk*B cells and chick DRG neurons is inhibited best by NT-4 followed by NT-3. NGF did not prevent BDNF binding to A293-c*trk*B cells, even at the very high concentrations used to prevent BDNF binding to sensory neurons (10,000-fold excess). In general, these results are reminiscent of what has been observed with recombinant rodent *trk*B receptors (Klein et al., 1991b; Squinto et al., 1991; Ip et al., 1993). While NT-3 prevents binding of BDNF to both A293-c*trk*B

cells and sensory neurons, a 10-fold higher concentration of NT-3 is needed to prevent BDNF binding to neurons than to A293-c*trk*B cells. This is in line with the findings of Ip et al. (1993), indicating that the cross-linking of BDNF to *trk*B expressed in transfected fibroblast can be prevented by NT-3 much more efficiently than when BDNF is cross-linked to *trk*B on rat cortical neurons. Although the results of previous studies suggested that NT-3 is simply a poorer agonist of *trk*B when the latter is expressed on PC12 cells rather than on fibroblasts (Ip et al., 1993), our study with embryonic neurons reveals that NT-3 acts as an antagonist: when used at concentrations sufficient to prevent the interaction of BDNF with its receptor (either on neurons or on A293-c*trk*B cells), NT-3 completely blocks the survival of BDNF-responsive nodose neurons. It is interesting to note that the interaction of NT-3 with heterologous neurotrophin receptors can be either antagonistic (this study, BDNF receptors) or agonistic: the occupancy of the high-affinity NGF sites on E11 sympathetic neurons by NT-3, which occurs at concentrations similar to those necessary to occupy the BDNF receptors, leads to the survival of these neurons, and not to a blockade of the effects of NGF (Dechant et al., 1993). Thus the interaction of NT-3 on sympathetic neurons with *trk* (the most likely NGF receptor) is agonistic, whereas that of NT-3 with c*trk*B on sensory neurons is antagonistic.

Another indication for differences between BDNF receptors on neurons versus A293-c*trk*B cells was revealed by the inhibition experiments performed with *Xenopus* NT-4. Again, while the A293-c*trk*B cells behave like fibroblasts transfected with rodent *trk*B (50% inhibition of BDNF binding by almost equimolar concentrations of either NT-4 or BDNF, Klein et al., 1992; Ip et al., 1993), the sensory neurons display a small number of BDNF high-affinity binding sites that can be detected in spite of the presence of NT-4. The most likely explanation for this phenomenon is heterogeneity of the high-affinity BDNF receptors on embryonic neurons. Such a heterogeneity in high-affinity neurotrophin-binding sites has already been proposed for NGF and NT-3 (Weskamp and Reichardt, 1991; Rodríguez-Tébar et al., 1993). The incomplete inhibition of BDNF binding obtained with NT-4 is also reflected in the in vitro survival assay with sensory neurons. At saturating concentrations, the effects of NT-4 and BDNF are not additive, but the effects of NT-4 used alone are clearly smaller than those of BDNF. It thus appears that NT-4 is able to recruit only a fraction of the BDNF receptors to induce the survival of sensory neurons.

What could explain the differences in affinity and neurotrophin specificity between c*trk*B and BDNF receptors on peripheral sensory neurons? It is possible that when expressed in such neurons, c*trk*B only exists in dimeric form, thought to have a higher affinity (Jing et al., 1992). Also, other forms of c*trk*B might be found in sensory neurons as a result of subtle alternative-splicing events not detected by northern blot analysis (as has been observed with for example agrin, Ruegg et al., 1992). In this context, it is interesting to note that all *trk*B cDNAs from rodents and chick were so far isolated from the adult CNS, and it remains to be shown that the sequence of the extracellular domain of c*trk*B is identical in CNS and sensory neurons. Other possible mechanisms modifying c*trk*B-binding properties might result from post-translational, neuron-specific modifications of the c*trk*B protein or protein-protein interactions. $p75^{LANR}$ is a natural candidate to suggest as it represents the major source of low-affinity neurotrophin binding on neurons (see above). What is needed to change c*trk*B from a receptor having many characteristics of a high-affinity BDNF receptor on peripheral neurons to one having all of them is an interesting question for the future.

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