# Recombinant fish neurotrophin-6 is a heparin-binding glycoprotein: implications for a role in axonal guidance

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Neurotrophin-6 (NT-6) was identified in the teleost fish *Xiphophorus* as a new member of the neurotrophin gene family. NT-6 binds specifically the glycosaminoglycan heparin. In this study NT-6 was expressed in a stably transfected mammalian cell line, and in insect cells via a recombinant baculovirus. It was purified to homogeneity and characterized by MS and N-terminal sequencing. NT-6 from both expression systems was proteolytically processed at one of two protease cleavage motifs and was found to be glycosylated. It supported the survival of embryonic chick sensory neurons; half-maximal survival was observed at 100 ng/ml. Furthermore, NT-6 elicited neurite outgrowth in explanted embryonic dorsal root ganglia. Addition of heparin into the medium did not potentiate the activity of NT-6 in survival assays. However, when a sensory ganglion explant was cultured in a collagen gel matrix assay adjacent to a heparin bead coated with NT-6, neurite outgrowth directed towards the bead was observed. This indicated that NT-6 was slowly released from the heparin bead generating a concentration gradient of NT-6 instrumental for axonal guidance *in vitro*. Thus the interaction of NT-6 with heparin might not be required for the activation of the cellular receptor for NT-6 on responsive cells but rather may serve to control, *in vivo*, the distribution of NT-6.

## INTRODUCTION

The past few years have seen an impressive increase in the number of newly discovered neurotrophic factors and the understanding of their function at the cellular and molecular level. Nerve growth factor (NGF) is the best characterized molecule of the neurotrophin family of neurotrophic factors. It is expressed in small, limiting amounts during development of the nervous system in the peripheral target fields of innervating axons and its inactivation by neutralizing antibodies causes the elimination of NGF-dependent sensory and sympathetic neurons [1,2].

NGF, and the additional members of the neurotrophin protein family discovered more recently, brain-derived neurotrophic factor (BDNF), neurotrophin-3, neurotrophin-4/5 and neurotrophin-6 (NT-6) [3,4], contain N-terminal signal sequences, are synthesized as precursor molecules, and are released after proteolytic cleavage as their mature forms. The mature neurotrophins are basic molecules of approx. 120 amino acid residues that share approx. 50 % sequence identity. They exist as homodimers under normal conditions. The determination of the crystal structure of NGF revealed that hydrophobic residues constitute a dimer interface in the crystal [5]. These residues are mostly conserved among the various neurotrophins, in contrast with surface-exposed domains that differ and are responsible for the specific actions of the different neurotrophins in distinct populations of neurons *in vitro* [6]. Gene knockout experiments have helped to establish the crucial role played by neurotrophins in the formation of specific populations of the peripheral sensory and sympathetic nervous system [7]. There are two classes of cell-surface receptors for the neurotrophins, a low-affinity neurotrophin receptor ($p75^{NTR}$) which binds the neurotrophins with similar affinity, and a family of structurally related tyrosine kinase receptors (Trk) that are able to bind the various neurotrophins differentially and to mediate neuronal cell survival and other biological effects [8–11]. In contrast with mammals, where three Trks have been identified, there are five *trk* genes in zebra fish, whose ligand specificities have yet to be determined [12].

NT-6 contains an additional basic domain of 22 amino acid residues between cysteine residues 2 and 3. The corresponding region in NGF forms a loop on the protein surface [13]. This domain might be responsible for the observed interaction of NT-6 with the glycosaminoglycan heparin that effects the release of NT-6 from the extracellular matrix and surface of producing cells [13]. NT-6 mRNA is expressed as early as stage 13 of embryonic development [13]. At this stage 26 somites have been formed and fibre outgrowth towards the periphery and the establishment of axonal interconnections of several brain regions is in progress [14]. For a characterization of the biological function of NT-6, the small aquarium fishes, such as medaka fish or zebra fish, represent suitable systems [15,16]. Since gene targeting by homologous recombination is not available in any of these lower vertebrate species, alternative methods of interfering with the expression of such signalling molecules will have to be adopted. This requires the availability of recombinant protein.

We have generated both a mammalian cell line and a recombinant baculovirus expressing NT-6 protein to produce an alternative to the vaccinia virus expression system used previously. We have purified NT-6 from the conditioned medium and characterized it with respect to its chemical properties, such as molecular mass, N-terminal sequence and glycosylation. This is the first report of a glycosylated neurotrophin. Characterization of the biological activity of purified NT-6 on sensory neurons confirmed its survival activity and showed that NT-6 induces

Abbreviations used: DRG, dorsal root ganglion(-ia); NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; FGF, fibroblast growth factor; MALDI–TOF-MS, matrix-assisted laser desorption ionization–time-of-flight mass spectrometry; NT-6, neurotrophin-6.

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fibre outgrowth from cultured dorsal root ganglia (DRG). The observed binding of NT-6 to heparin beads might provide a route for the application of NT-6 to embryonic fish.

## MATERIALS AND METHODS

### Plasmid constructions

A 965-bp *Pst*I fragment encoding NT-6 from *Xiphophorus maculatus* [13] was cloned into the plasmid pCMV1, which contains the cytomegalovirus promoter/enhancer and a polyadenylation signal [17]. This fragment was also cloned into the vector pVL1392 (Pharmingen) to construct the baculovirus transfer plasmid used in this study. The orientation of the insert was determined by restriction analysis with *Eco*RI and confirmed by DNA sequencing.

### Cells and virus

Human fetal kidney-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with penicillin, streptomycin and 5% (v/v) fetal-calf serum. The NT-6 plasmid DNA was co-transfected with a plasmid encoding puromycin *N*-acetyltransferase by calcium phosphate precipitation, and selection of stable cell lines was carried out by selection with the antibiotic puromycin [18]. For protein production, cells were grown in rich medium until they were almost confluent, then switched to serum-free medium containing 10 $\mu$g/ml heparin and incubated for 2 days. Several clones were analysed by SDS/PAGE and Western blotting for the ability to produce NT-6 and two clones were used for further studies.

Recombinant baculovirus was obtained by co-transfection of wild-type virus DNA (0.125 $\mu$g; Baculogold; Pharmingen) and plasmid DNA (1 $\mu$g) as calcium phosphate co-precipitate into *Spodoptera frugiperda* (SF-9) cells grown on 3 cm diameter culture dishes (Nunc). After transfection (5 days) the viruses were harvested from the culture medium and plaque-purified. Several plaques were amplified and 3 days post-infection, conditioned medium was harvested, concentrated by precipitation [19] and analysed by SDS/PAGE and Western blotting for the ability to produce NT-6. Positive recombinant virus stock was amplified, and for expression studies, SF-9 monolayers were infected with 5 plaque forming units per cell.

### Protein purification and analysis

The tissue culture medium was clarified of cells and debris by centrifugation and purified in three steps. The conditioned medium (500 ml) was applied, with a peristaltic pump (flow rate 0.5 ml/min), to a heparin–Sepharose column (HiTrap column with 5 ml bed volume; Pharmacia) and the proteinaceous material eluted with PBS plus 1 M NaCl. The eluate (typically 10 ml) was bound to a 4 ml column filled with controlled-pore glass beads (Sigma PG120-200), washed with 10 ml of PBS and the bound protein was eluted with 50% (v/v) acetonitrile containing 100 mM NaCl and 100 mM acetic acid. After concentration by vacuum centrifugation, the material was dissolved in 2 ml of 0.1% (v/v) trifluoroacetic acid (buffer A) and applied at a flow rate of 0.4 ml/min to a reversed-phase C8 column (Aquapore RP-300, 2.1 mm × 220 mm; Applied Biosystems). Elution from the column was achieved with a linear acetonitrile gradient [30–70% (v/v) in buffer A over 50 min] and was monitored by absorbance at 214 nm on a Hewlett Packard HP1090 chromatograph. Protein peaks were collected manually and dried. A quantitative comparison of the individual purification steps by Western blotting revealed good recovery of NT-6 during the purification, except for the C8 reversed-phase column where a significant loss of NT-6 occurred. The typical yield of purified NT-6 from 1 litre of medium was approx. 50 $\mu$g.

Samples were run on reducing 0.1% (w/v) SDS/15% (w/v) polyacrylamide gels [20] and stained with Coomassie Blue. Western blotting was performed as previously described using an antiserum raised against a synthetic peptide corresponding to the N-terminal 15 amino acids of NT-6 [13]. Analysis of glycosylation was performed on NT-6 denatured with $\beta$-mercaptoethanol and SDS and subsequent enzymic treatment with *N*-glycanase-F as detailed by the manufacturer (Boehringer Mannheim). N-terminal sequencing was performed as described by Götz et al. [21]. Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI–TOF-MS) was carried out with a Bruker BIFLEX instrument in the linear mode. The acceleration voltage in the ion source was set to 25.0 kV. The laser source was a VSL-337ND nitrogen laser (Laser Science, Inc.) that provided 337 nm radiation with a spectral bandwidth of 0.1 nm. The desired wavelength was selected and passed through a filter to produce power densities of the desired magnitude. The laser beam was then focused on to the sample-containing surface of the direct insertion probe. For preparing the sample, 0.2 $\mu$l of sinapic acid [1 mg/ml in 50% acetonitrile with 0.05% trifluoroacetic acid] was applied to the probe, followed by 0.2 $\mu$l of the NT-6 solution. Ion detection was accomplished by using a standard dual microchannel plate detector with a post-acceleration of 1.6 kV. The signals coming from the detector were transferred to a Sun computer for further processing of the data. Each spectrum is the sum of five individual readings created with ten laser shots. The flight region was kept at a pressure of $2 \times 10^{-7}$ mbar.

### Biological assays

DRG explants were dissected from chicks (embryonic day 7 or 8). For survival assays cells were dissociated with 0.1% (w/v) trypsin, triturated, and preplated to remove non-neuronal cells, as described earlier [22]. The neurons were cultured in 10 mm diameter plastic tissue culture wells that had been coated with polyornithine (0.5 $\mu$g/ml, overnight) and laminin (10 $\mu$g/ml, 5 h). Cells were plated at a density of approx. 2500 cells/well in Ham's F14 medium containing 10% (v/v) horse serum, penicillin and streptomycin. Cultures were maintained at 37 °C, in a 3% (v/v) $CO_2$ gassed and humidified incubator. The number of surviving neurons was counted 24–36 h later and is given in Figure 5 as a percentage of the initial number of neurons counted 2 h after plating. To evaluate the neurite-outgrowth-promoting activity of NT-6, DRG explants were cultured on plastic coated with polyornithine and laminin, containing 10 $\mu$M cytosine arabinoside. Neurite growth was visualized by phase contrast microscopy. In some experiments, neurites were stained with a rabbit antiserum against neurofilament H (NA1211 in a 1:500 dilution; Affinity Research Products) using a secondary horseradish peroxidase antibody and diaminobenzidine staining. Where indicated, explants were embedded in a three-dimensional collagen gel matrix as described by Lumsden and Davies [23]. Heparin acrylic beads (approx. 100–150 $\mu$m diameter; Sigma H5263) were positioned 100–500 $\mu$m apart from ganglion explants using a graticule with scale division as indicated in the Results section. Each bead had been presoaked for 1 h in a 2 $\mu$l drop of water containing 150–200 ng of NT-6 or mouse NGF, and was subsequently washed by submersion in 5 ml of culture medium.

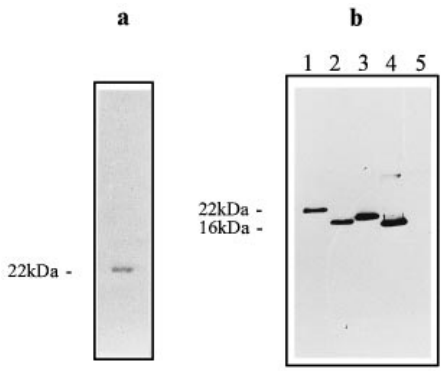


**Figure 1 Recombinant NT-6 is a glycoprotein**

(**a**) Coomassie Blue staining of purified NT-6. (**b**) NT-6 purified from the human fetal kidney-293 cell clone before (lane 1) and after (lane 2) treatment with *N*-glycanase-F, and NT-6 purified from insect cells before (lane 3) and after (lane 4) treatment with *N*-glycanase-F, was separated by electrophoresis, transferred to nitrocellulose and probed with an anti-(NT-6) antiserum. Lane 5, *N*-glycanase only.

## RESULTS

### Biochemical characterization of recombinant NT-6

We previously described the expression of NT-6 using a recombinant vaccinia virus [13]. Here we established derivatives of the human fetal kidney-293 cell line transfected with an expression vector that carried the NT-6 gene encoding a 286-residue precursor protein under the transcriptional control of the cytomegalovirus promoter. When the conditioned medium from the NT-6-expressing line was applied to a heparin–Sepharose column, NT-6 bound to the column and was subsequently eluted with 1 M NaCl (results not shown). The eluate of the heparin column was further purified on controlled-pore glass and reversed-phase HPLC (see the Materials and methods section), as these chromatographic principles have been useful in the purification of other neurotrophins [21,24]. The purified NT-6 migrated in SDS/PAGE as a single band with an apparent molecular mass of 22 kDa (Figures 1a and 1b, lane 1). The NT-6 preparation purified from the conditioned medium of baculovirus-infected insect cells migrated with a molecular mass of 18 kDa on SDS/PAGE (Figure 1b, lane 3).

The molecular mass of mature NT-6 observed in SDS/PAGE and determined by MS (see below) was distinctly higher than that calculated from the translated DNA sequence for fully oxidized NT-6 (15956 Da), which is indicative of a post-translational modification. To determine whether NT-6 was glycosylated, we enzymically treated purified NT-6 before gel electrophoresis with *N*-glycanase-F. As shown in Figure 1(b), the NT-6 from both the mammalian and the insect expression systems shifted to 16 kDa, thereby demonstrating an asparagine-linked glycosylation.

To provide an independent determination of the molecular mass of the purified NT-6 samples, MALDI–TOF-MS was employed. Masses of 22545 Da for NT-6 purified from the human fetal kidney-293 cell line and 18010 Da for NT-6 from insect cells were determined (Figure 2), thereby confirming the molecular-mass determinations of the SDS/PAGE.

The NT-6 precursor protein contains two motifs for proteolytic cleavage (Figure 3). Cleavage of the peptide bond after Arg-66 or Arg-142 would result in a 220- and 144-amino-acid-residue protein respectively. Sequencing of the N-terminus of purified

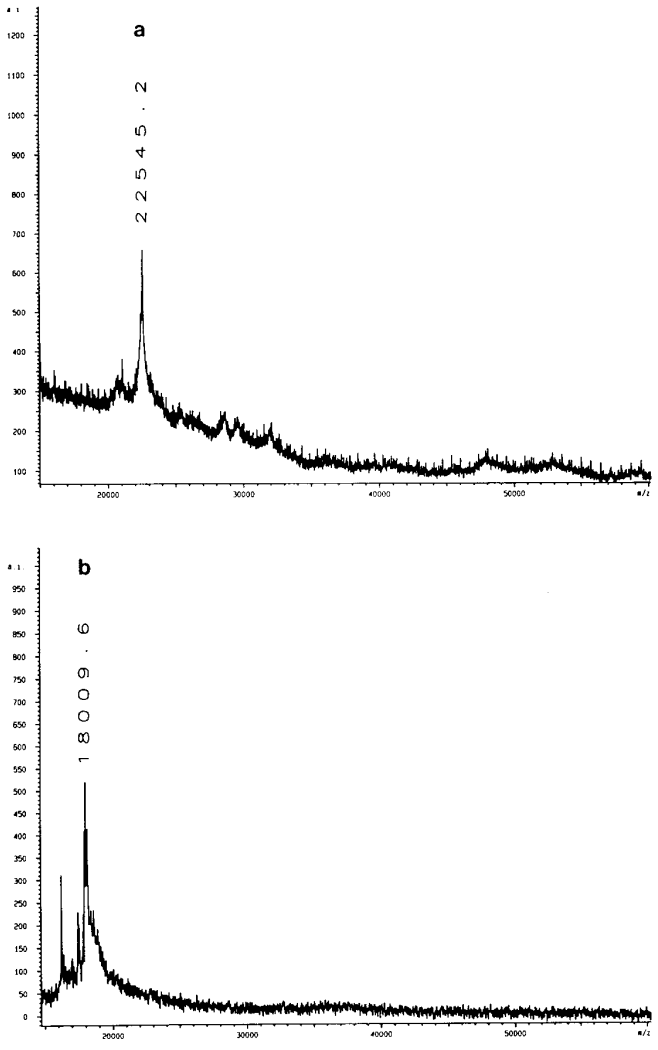


**Figure 2 Molecular mass determination of NT-6 by MS**

MALDI–TOF-MS was carried out with NT-6 purified from mammalian (**a**) and insect (**b**) cells respectively as described in the Materials and methods section.

NT-6 preparations revealed that the cleavage took place between residues Arg-142 and Lys-143, showing that the second of the two possible cleavage sites was used (Figure 3).

### Promotion of neurite outgrowth by NT-6

We began the present study of the biological effect of NT-6 by assessing its neurite outgrowth promoting activity *in vitro* from


**Figure 3 N-terminal amino acid of mature NT-6 is the residue Lys-143 of the precursor**

Partial amino acid sequence of the 286-residue NT-6 precursor translated from the DNA sequence is shown. The sequence of recombinant NT-6 obtained by amino acid sequencing is underlined; the cysteine residue could not be determined. The two basic cleavage motifs are shown in bold and the cleavage site used in human fetal kidney-293 cells is indicated by an arrow.

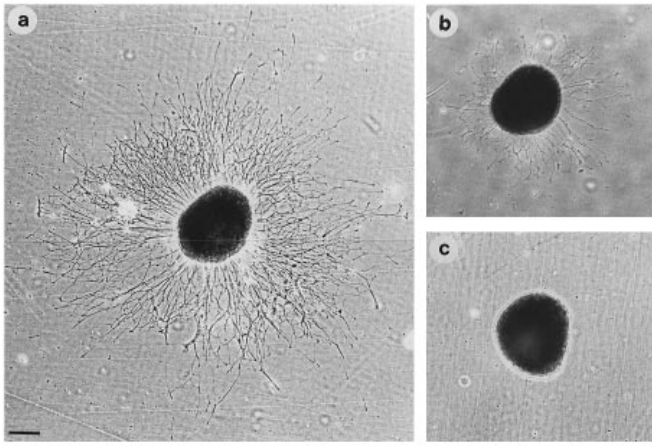


**Figure 4 NT-6 induces neurite outgrowth**

Phase contrast micrographs of DRG explants cultured for 24 h on dishes coated with polyornithine plus laminin in the presence of 250 ng/ml NT-6 (**a**), 125 ng/ml NT-6 (**b**) or without NT-6 (**c**). Pictures shown are representative of ten ganglion assays each, except for (**b**) where neurite outgrowth failed to occur in 50% of the cultures; for details see text. Scale = 200 $\mu$m.

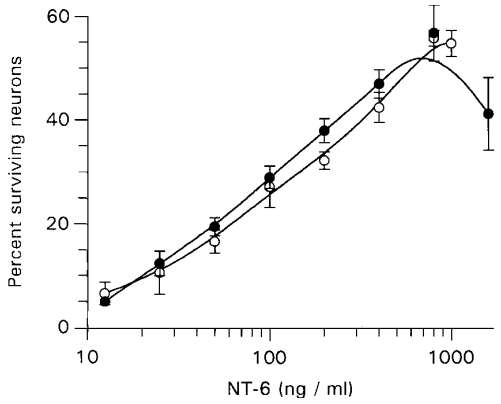


**Figure 5 Dose dependency of neuronal survival promoted by NT-6**

The graph shows the percentage survival of sensory neurons isolated from chick embryonic DRG after 24 h incubation with NT-6 purified from the human fetal kidney-293 cell clone (solid circles) and from baculovirus-infected insect cells (open circles) at concentrations ranging from 12.5 to 1600 ng/ml. Values are means $\pm$ S.E.M. ($n = 6$).

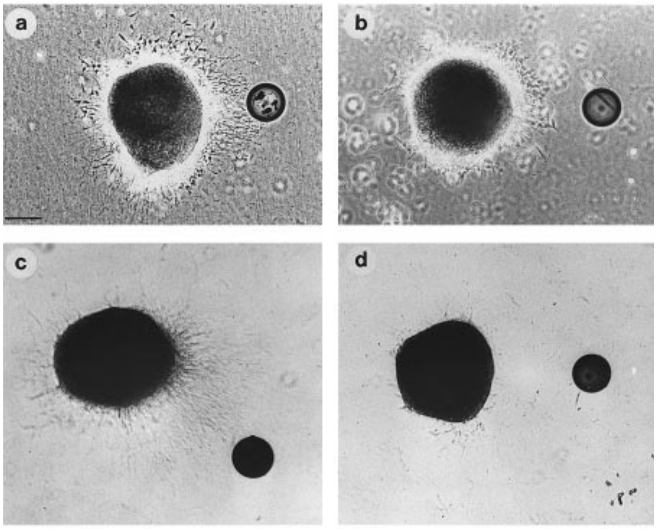


**Figure 6 NT-6-coated heparin beads cause directed neurite outgrowth from DRG**

DRG explants were embedded together with the bead in a three-dimensional collagen matrix and cultured for 24 h. The heparin bead had been presoaked in 2 $\mu$l of solution containing 200 ng of purified NT-6 (**a**) and (**c**), or NGF (**b**) and (**d**). Phase contrast pictures, (**a**) and (**b**), and neurofilament immunostaining photographs, (**c**) and (**d**), are representative of six assays each.

non-dissociated DRG explants. Consistent and robust neurite outgrowth was observed when NT-6 was present at a concentration of 250 ng/ml (Figure 4a) and the density and extent of neurite outgrowth did not increase significantly at 2- or 4-fold higher concentrations of NT-6. At a 2-fold lower concentration of NT-6, outgrowth occurred but was less pronounced (Figure 4b). However, in 50% of the ganglion explant assays performed, no outgrowth was observed at this concentration of NT-6, as was the case when NT-6 was omitted from the culture (Figure 4c).

### Neuronal survival activity of NT-6

We then tested whether NT-6 promoted neuronal survival. Neurons were dissociated from chick DRG, which contain a pure population of sensory neurons that at the time-point of dissection have all sent out one axon to the peripheral target and the other axon into the spinal cord and depend on neurotrophic support for survival. NT-6 was able to promote the survival of approx. 60% of the plated neurons, which is as many neurons as NGF (Figure 5). However, half-maximal survival was observed at 100 ng/ml of NT-6, which is three orders of magnitude greater than the half-maximal survival concentration of NGF. To exclude the possibility that the survival activity was caused by minor contamination by another neurotrophin(s) present in the conditioned medium of the human fetal kidney-293 cell line and co-purifying with NT-6, we also determined the specific activity of NT-6 purified from insect cells, and found no endogenous neurotrophic activity (Figure 5). NT-6 from human fetal kidney-293 cells and insect cells was active in the same concentration range as NT-6 obtained from a vaccinia-virus-based expression system [13]. Our data show that NT-6 promotes the survival and neurite outgrowth of embryonic sensory neurons similar to NGF, but with lower potency.

### Interaction of NT-6 with heparin

In bioassays, heparin did not enhance the biological activity of NT-6 (100 ng/ml) to support neuronal survival at concentrations up to 100 ng/ml. At higher concentrations, heparin had a non-specific detrimental effect on cell attachment that was also observed in experiments with NGF (results not shown). This precluded the investigation of a potential synergistic effect at higher concentrations of heparin. We assessed an alternative possibility of how heparin might modulate the activity of NT-6. Here we determined whether NT-6 coated on to a heparin acrylic bead could elicit neurite outgrowth from a DRG ganglion located next to the bead. Ganglia and beads were submerged in a three-dimensional collagen gel matrix to prevent their dislocation [23]. We observed neurite outgrowth from the ganglion towards the bead at the side of the ganglion proximal to the bead (Figures 6a and 6c). No outgrowth, or only a very weak

outgrowth, was seen on the sides of the ganglion distal and lateral to the bead (Figure 6a and c) and in experiments where the beads had been soaked in NGF or PBS (Figure 6b and d). This observation indicated that a gradient of NT-6 was generated due to the release of NT-6 from the heparin bead that could be sensed by the neurites or their cell bodies.

## DISCUSSION

The characterization of the biochemical properties of NT-6 revealed that it is glycosylated; this is the first report of a glycosylated neurotrophin. The tight band on SDS/PAGE indicates only little heterogeneity of the saccharide structure. The NT-6 structure contains one single motif for N-linked glycosylation (Asn-Lys-Thr) at positions 23–25. The effects of the glycosylation on the biological activity and/or stability remain to be determined. It is unknown whether NT-6 is glycosylated in the fish. It should be possible to characterize the glycosylation of NT-6 expressed in fish cells that apparently will produce sufficient material for such studies upon transient transfection [25].

NT-6 promoted neuronal survival and neurite outgrowth *in vitro*. Several lines of evidence point to the existence of two distinct cellular signal transduction pathways for neuronal survival and neurite outgrowth that can be discerned under experimental conditions. For example, it was shown that an artificial (chimeric) neurotrophic protein could induce neurite outgrowth but not neuronal survival in cultured chick nodose neurons [26]. Conversely, cultured primary peripheral nervous-system neurons that depend on NGF for survival could be rescued from apoptosis by overexpression of the apoptosis-inhibitor protein Bcl-2, but the cells lacked extensive neurite outgrowth [27,28]. In addition, overexpression of Bcl-2 protein in a genetic mouse model with progressive motoneuronopathy, rescued motoneuron cell bodies from death but failed to prevent axonal degeneration [29].

We previously noted the interaction of NT-6 with the glycosaminoglycan heparin [13]. This conclusion was based on the observation that it was necessary to add heparin to producing cells to detect NT-6 in the conditioned medium; another glycosaminoglycan, chondroitin sulphate, could not replace heparin [13]. Here, we use the binding of NT-6 to heparin–Sepharose and its subsequent elution with high salt, in the purification procedure outlined above. The presence of a positively charged 22 amino acid domain in NT-6, that is absent in other neurotrophins, might be responsible for heparin binding. Since this domain is conserved in NT-6 from other species (R. Götz, unpublished results) we sought to investigate the biological significance of this interaction. Fibroblast growth factors (FGFs) are prototypical heparin-binding molecules and their interaction with heparin is essential for an FGF-induced cellular response [30]. Several mechanisms have been proposed for the role of heparin; for example, FGF bound to cell-matrix proteoglycans serves as a reservoir of growth factor that can be released by enzymic degradation of the proteoglycan [31]. Another set of experiments revealed that heparin caused the multimerization of basic FGF and that the FGF–heparin complex induced FGF-receptor dimerization and activation [32]. Interestingly, heparin did not enhance the biological activity of NT-6. However, there might be no need for heparin to cause dimerization of NT-6 because the neurotrophins NGF, BDNF and neurotrophin-3 are already dimeric molecules under normal conditions [33–36], and most of the amino acid residues instrumental for dimer formation [5] are conserved in NT-6.

This report provides experimental evidence that heparin-containing proteoglycans might modulate the activity of NT-6 by reversibly binding to it. The heparin bead experiment indicated that a gradient of NT-6 was generated due to the release of NT-6 from the heparin bead that could be sensed by the neurites or their cell bodies. This finding can be interpreted in at least two ways. Neurons in the explant that were in a position near to the bead might be exposed to an NT-6 concentration that was sufficiently high to promote their survival and neurite outgrowth, in contrast with neurons in positions distal to the bead, which failed to encounter enough NT-6. Alternatively, the gradient of NT-6 could have influenced the direction of neurite growth. Although we included cytosine arabinoside in the medium to inhibit the proliferation of satellite cells, some migrating fibroblast-like cells were observed in the cultures (Figure 6) and they could have influenced indirectly the neurite outgrowth.

It has recently been demonstrated that BDNF plays a role in axonal guidance during the navigation of retinal ganglion cells in the optic tectum [37]. The implication of NT-6 as a molecule capable of guiding neurites *in vitro* remains to be confirmed by assays that more directly evaluate the chemotropic effects of a molecule such as videomicroscopy of isolated neurons [38]. Finally, the distribution of the NT-6 protein *in vivo* remains to be determined, but the *in vitro* evidence presented here is compatible with a guidance role for NT-6.

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