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MINI REVIEW

The conservation of neurotrophic factors during vertebrate evolution

R. Götz* and M. Schartl†

*Max-Planck-Institute for Psychiatry, Department of Neurochemistry, Am Klopferspitz 18A, D-82152 Martinsried, F.R.G.; and †Biozentrum der Universität Würzburg, Abteilung Physiologische Chemie I, Am Hubland, D-97074 Würzburg, F.R.G.

Neurotrophic factors are a family of extracellular ligands that affect the differentiation, survival (by inhibition of apoptosis) and maintenance of function of neuronal cells in vertebrates. The family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). The survival specificities of NGF and BDNF for different classes of chick neurons are maintained from the fish to the mammalian proteins, implying a conserved interaction with neuronal cell surface receptors (of the Trk family). However, the quantitative effect of a fish neurotrophin can differ significantly from that of the mammalian orthologue.

Key words: Neurotrophic factors; Vertebrate evolution.

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Neurotrophins and their Receptors

The development of the vertebrate nervous system is regulated by both intrinsic and extrinsic signals. Around the time of target field innervation, substantial numbers of postmitotic neurons (approximately 40–60%) are eliminated—a phenomenon known as naturally occurring cell death. Surgical manipulation of target tissue influences this nerve cell death: removal of the target reduces the number of surviving neurons without affecting the numbers of neurons initially generated; conversely, grafting extra target tissue enhances neuronal survival. Thus neuronal cell death is not simply the consequence of an intrinsic genetic programme of the neurons themselves, but rather depends on cell–cell interactions. The function of limited neuronal survival is thought to adjust the number of neurons to the requirements of their target fields (reviewed by Oppenheim, 1991).

Correspondence to: R. Götz, Neurologische Klinik, Universität Würzburg, Josef-Schneider Str. 11, D-97080 Würzburg, F.R.G. Tel.: +49-931-201-2535; Fax: +49-931-201-2697.
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The occurrence of dying cells has been noted as a general feature of normal embryonal development in vertebrates (Ernst, 1926). One view is that a cell not only needs a signal from another cell to proliferate, but also a signal for survival (Raff, 1992). The finding that neuronal cell death or apoptosis can be prevented by inhibition of RNA or protein synthesis (Martin *et al.*, 1988; Scott and Davies, 1990) suggests that it depends on the activation of a programme of gene expression in dying cells. Thus, naturally occurring neuronal cell death seems to be a suicide programme "induced" by the reduced availability of a factor required for cell survival. Neurotrophic factors—a family of structurally closely related proteins released by target cells in limited amounts—provide trophic support for which neurons must "compete" in order to survive (reviewed by Levi-Montalcini, 1987; Barde, 1989; Thoenen, 1991).

The prototypical neurotrophic factor or neurotrophin, nerve growth factor (NGF) was the first peptide growth factor to be purified and is probably the best-characterized neuronal

differentiation factor (reviewed by Levi-Montalcini, 1987). The concept of a neurotrophin protein family was established when a survival factor for sensory neurons named brain-derived neurotrophic factor (BDNF) was purified from mammalian brain (Barde *et al.*, 1982) and upon molecular cloning was found to be about 50% identical to NGF (Leibrock *et al.*, 1989). In addition to NGF and BDNF, this family at present includes neurotrophin-3 (NT-3; Ernfors *et al.*, 1990; Hohn *et al.*, 1990; Jones and Reichardt, 1990; Kaisho *et al.*, 1990; Maisonpierre *et al.*, 1990, Rosenthal *et al.*, 1990), neurotrophin-4 (NT-4; Hallböök *et al.*, 1991, Ip *et al.*, 1992), and neurotrophin-5 (Berkemeier *et al.*, 1991).

The first step in the mechanism of action of neurotrophic factors is their interaction with specific receptors on responsive cells. Two types of receptors for NGF on both sensory and sympathetic neurons have been described as high-affinity ($K_d = 10$ pM) and low-affinity ($K_d = 1$ nM; Sutter *et al.*, 1979). The low-affinity NGF receptor p75 has been identified as a 75 kD transmembrane glycoprotein (Johnson *et al.*, 1986; Radeke *et al.*, 1987) that also binds the other neurotrophic factors with similar affinities (Ernfors *et al.*, 1990; Rodriguez-Tébar *et al.*, 1990, 1992; Hallböök *et al.*, 1991); however, doubt still exists about whether this receptor can transduce signals after ligand binding. Transgenic mice bearing a disrupted p75 receptor gene showed no obvious impairment of sympathetic or CNS nerve development but defects in sensory innervation were noted (Lee *et al.*, 1992).

The putative high-affinity receptors for the neurotrophins were found to be 140 kDa integral membrane glycoproteins encoded by members of the *trk* proto-oncogenes (reviewed by Chao, 1992; Meakin *et al.*, 1992; Barbacid, 1993). They consist of an extracellular ligand binding domain and an intracellular tyrosine kinase domain that initiates intracellular signal transduction upon binding of the neurotrophic factor. Importantly, these receptors show specificity for their ligands, e.g. NGF binds only to TrkA, BDNF and NT-4 bind to TrkB and NT-3 binds to TrkC. Several models concerning the signal-transducing receptors in neuronal cells with respect to the combinations of Trk molecules with or without the p75 receptor have been proposed (Chao, 1992; Meakin *et al.*, 1992; Jing *et al.*, 1992; Battleman *et al.*, 1993; Ibáñez *et al.*, 1993b; Barbacid, 1993). Characterization of the signal transdution mechanism of both receptors in neuronal cells remains a considerable task.

The study of neurotrophic proteins and their receptors from lower organisms can be expected to reveal important features of the development of the nervous systems of different organisms. It might also reveal, in combination with other approaches, functionally important sequence motifs. Molecular cloning has demonstrated that the genes for neurotrophic factors exist even in fish (Hallböök *et al.*, 1991; Götz *et al.*, 1992). Furthermore, the proteins encoded by the fish NGF and BDNF genes show survival activity on embryonic chick neurons (Götz *et al.*, 1992). This review will try to summarize the current knowledge of the structural and functional features of vertebrate neurotrophic factors.

Functions of Neurotrophic Factors

All neurotrophins display both overlapping and distinct survival activities on peripheral and

Table 1. Functions of neurotrophic factors

NGF	BDNF	NT-3	NT-4/5
Survival factor for —sensory neurons (from neural crest) —sympathetic neurons	Survival factor for —sensory neurons (from neural crest) —sensory neurons (from placodes)	Survival factor for —sensory neurons (from neural crest) —sensory neurons (from placodes) —sympathetic neurons	Survival factor for —sensory neurons (from neural crest) —sensory neurons (from placodes) —trigeminal neurons
—basal forebrain neurons (during regeneration)	—basal forebrain neurons —motoneurons —neurons of the locus coeruleus —cerebellar granular neurons —dopaminergic substantia nigra neurons —retinal ganglion cells	—basal forebrain neurons —motoneurons —neurons of the locus coeruleus	—basal forebrain neurons —motoneurons —neurons of the locus coeruleus
Differentiation factor for —basal forebrain neurons —monocytes	Differentiation factor for —basal forebrain neurons —motoneurons	Differentiation factor for —basal forebrain neurons —motoneurons —hippocampal neurons Mitogen for —neural crest cells	Differentiation factor for —basal forebrain neurons —motoneurons

central neurons, and a single neuronal class can respond to several different factors (Table 1). The survival function of a neurotrophic factor is assessed by isolation and culture of embryonic neurons in the presence or absence of the candidate molecule. The presence of a neurotrophic factor induces neurite outgrowth (the axons have been injured during the isolation procedure) and prevents the death of the neuronal cells. These actions occur at extremely low concentrations, typically 1–10 pM. The potential role of neurotrophic factors during normal development and during regeneration after nerve transections has also been investigated by the addition of exogenous neurotrophin or its withdrawal using blocking antibodies.

The continued expression of the neurotrophin genes in adult tissues at times where no neuronal death occurs indicates other differentiating actions besides a survival function. Besides acting as target-produced survival molecules for innervating neurons, neurotrophic factors may exert a trophic effect locally (Mobley *et al.*, 1989; Vantini *et al.*, 1989), act in an autocrine mode, i.e. the differentiation factor is synthesized by the neuronal cell itself for proper function and survival (Wright *et al.*, 1992; Miranda *et al.*, 1993; Kokaia *et al.*, 1993), or be a trophic molecule produced by afferent neurons. Furthermore, actions of NGF on non-neuronal cells, e.g. on cells of the immune system, have also been demonstrated (Ehrhard *et al.*, 1993).

NGF promotes the survival of neurons isolated from sympathetic ganglia as well as sensory neurons derived from the neural crest (neurons from dorsal root ganglia and cranial sensory ganglia; Levi-Montalcini and Angeletti, 1963; Davies and Lindsay, 1985). Its function *in vivo* in sensory and sympathetic neuronal development is well established: first, administration of exogenous NGF can almost completely prevent natural nerve cell death (Hamburger *et al.*, 1981); second, the application of function-blocking anti-NGF antibodies results in the elimination of a large proportion of these neurons (Cohen, 1960; Levi-Montalcini and Booker, 1960; Johnson *et al.*, 1980; Ruit *et al.*, 1992). In addition, the limited availability of NGF in the target tissue at the time of innervation of newly formed neurons and the correlation of the amount of NGF with the density of innervation have been demonstrated (Korsching and Thoenen, 1983; Harper and Davies, 1990). In the central nervous system, NGF can induce the expression of choline acetyl transferase in neurons of the basal forebrain (Gnahn *et al.*, 1983) and promote their survival *in vitro* and *in vivo* after nerve transection (Hefti, 1986; Hartikka and Hefti, 1988; Hatanaka *et al.*, 1988). In combination with neurotransmitters, it can promote survival of cerebellar Purkinje cells (Cohen-Cory *et al.*, 1991).

BDNF does not act on sympathetic neurons but is a survival factor for all sensory neurons that are supported by NGF; in addition, it is a survival molecule for neurons that are not supported by NGF, proprioceptive neurons from the trigeminal mesencephalic nucleus and sensory neurons that originate from the ectodermal placodes (nodose ganglion; Lindsay *et al.*, 1985). In the CNS, it acts as a survival factor for a broad spectrum of neuronal cells: basal forebrain neurons (Alderson *et al.*, 1990; Knüsel *et al.*, 1991), motoneurons (Oppenheim *et al.*, 1992; Sendtner *et al.*, 1992; Yan *et al.*, 1992; Henderson *et al.*, 1993; Hughes *et al.*, 1993; Koliatsos *et al.*, 1993), neurons of the locus coeruleus (Friedman *et al.*, 1993), cerebellar granule neurons (Segal *et al.*, 1992), dopaminergic neurons of the substantia nigra (Hyman *et al.*, 1991) and retinal ganglion cells (Johnson *et al.*, 1986; Rodriguez-Tébar *et al.*, 1989). Other functions of BDNF include induction of choline acetyl transferase in basal forebrain neurons (Knüsel *et al.*, 1991), motoneurons (Wong *et al.*, 1993), the potentiation of synaptic activity in frog motoneurons (Lohof *et al.*, 1993) and differentiating effects on neural crest cells and neuronal precursor cells (Sieber-Blum, 1991; Wright *et al.*, 1992). Application of exogenous BDNF to developing quail embryos rescues a significant proportion of sensory neurons from normally occurring death (Hofer and Barde, 1988).

Neurotrophin-3 is a survival factor for sensory neurons of neural crest and neural placode origin (Hohn *et al.*, 1990; Maisonpierre *et al.*, 1990). One paper has reported survival activity on sympathetic neurons (Rosenthal *et al.*, 1990). NT-3 can also support the survival of motoneurons (Henderson *et al.*, 1993; Hughes *et al.*, 1993) and neurons from the locus coeruleus (Friedman *et al.*, 1993). The effect on basal forebrain neurons is controversial (Knüsel *et al.*, 1991; Friedman *et al.*, 1993). It is a mitogen for neural crest cells indicating that the function of neurotrophic factors is not restricted to actions on postmitotic neurons (Kalcheim *et al.*, 1992).

Neurotrophin-4 was first found in a lower vertebrate, *Xenopus laevis* (Hallböök *et al.*, 1991). The mammalian orthologues have been cloned (Ip *et al.*, 1992) and sequence comparisons showed the identity with a neurotrophic molecule identified independently by another group as neurotrophin-5 (Berkemeier *et al.*, 1991). It acts as a survival factor for sensory neurons from neural crest as well as from placodes (Hallböök *et al.*, 1991; Ip *et al.*, 1992; Ibáñez *et al.*, 1993a). In the CNS, it acts as a

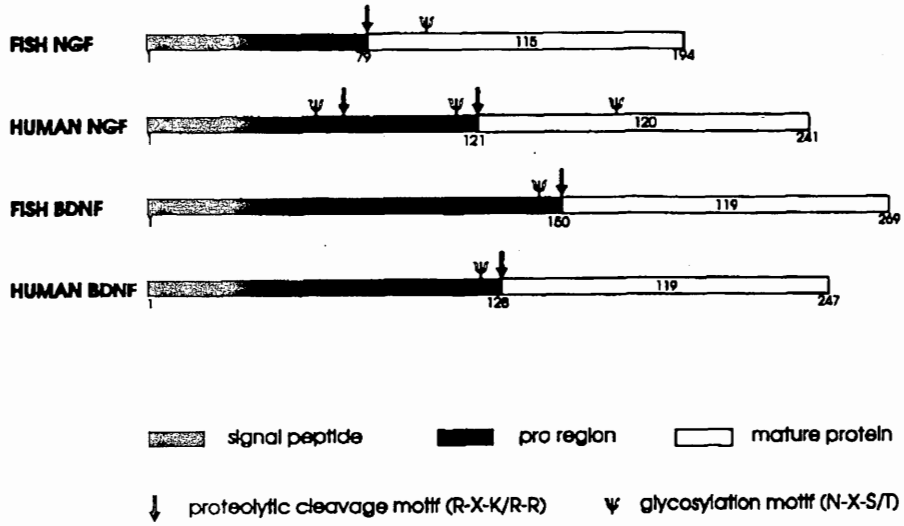

Fig. 1. Schematic representation of the precursor protein structures for fish and human NGF and BDNF. The numbers indicate the length of the precursor and mature proteins, respectively.

survival factor for motoneurons (Henderson *et al.*, 1993; Hughes *et al.*, 1993) and neurons from the basal forebrain and the locus coeruleus (Friedman *et al.*, 1993).

Conservation of Structure

Neurotrophic factors are synthesized as precursor molecules that are approximately twice the size of the mature, biologically active forms. The sequences of the mature neurotrophins are located at the C-terminus of the precursor; this structural organization is conserved from fish to mammals (Fig. 1). The cleavage of the precursor proteins occurs at defined motifs by a subtilisin-like protease (Bresnahan *et al.*, 1990; reviewed by Steiner *et al.*, 1992). The N-terminal signal peptide is followed by a Pro-sequence that is thought to help in the folding of the mature protein: deletion mutagenesis experiments have shown that the Pro-region is instrumental for the folding and secretion of mature neurotrophic proteins in eukaryotic cells (Suter *et al.*, 1991). The functional importance of the Pro-region as an intramolecular chaperon is also suggested by the finding of low biological activity of recombinant proteins when they are produced from gene fragments that cover only the mature coding region (Hu *et al.*, 1988; Negro *et al.*, 1992; Fujimori *et al.*, 1992).

Neurotrophin sequences are known from many vertebrate species (Scott *et al.*, 1983; Ullrich *et al.*, 1983; Ebendal *et al.*, 1986; Meier *et al.*, 1986; Wion *et al.*, 1986; Selby *et al.*, 1987; Fahnestock and Bell, 1988; Whittemore *et al.*, 1988; Schwarz *et al.*, 1989; Kaisho *et al.*, 1990; Maisonpierre *et al.*, 1990; Berkemeier *et al.*, 1991; Hallböök *et al.*, 1991; Carriero *et al.*, 1992; Götz *et al.*, 1992; Maisonpierre *et al.*, 1992) and most of the known complete sequences are aligned in Fig. 2. Important structural and functional points obtained from the elucidation of the X-ray crystal structure of dimeric mouse NGF (McDonald *et al.*, 1991) are highlighted in Fig. 3. The finding that most of the strictly conserved residues in the neurotrophins play a role in the formation and stabilization of the structure of the mouse NGF protomer and the formation of the dimer (which represents the biologically active entity) suggests that all four factors adopt a similar overall conformation. Important features of the three-dimensional structure are the three disulfide bridges that pack closely together (Cys-58-Cys-108 and Cys-68-Cys-110 form a ring through which the Cys-15-Cys-80 disulfide bond passes).

Four extended segments of twisted antiparallel β strands, drawn as arrows in Fig. 3, convey a non-globular, flat structure to the NGF promoter. The β strands are connected by β-hairpin loops, LII and LIII, and a region with three consecutive reverse turns (RT); these domains are variable between the different neurotrophins and are proposed to be responsible for the different neuronal specificities mentioned above. Although the residues believed to be involved in receptor interaction, which are distributed throughout the linear sequence, cluster in the three-dimensional structure, they nonetheless cover about 75% of the molecule (for a detailed discussion, see Ibáñez *et al.*, 1993b). That multiple surface-exposed domains (A, B, LI-LIII, RT in Figs 2 and 3) might determine the specificity of the interaction of neurotrophins with the cell surface receptors is supported by experimental analysis of the binding and

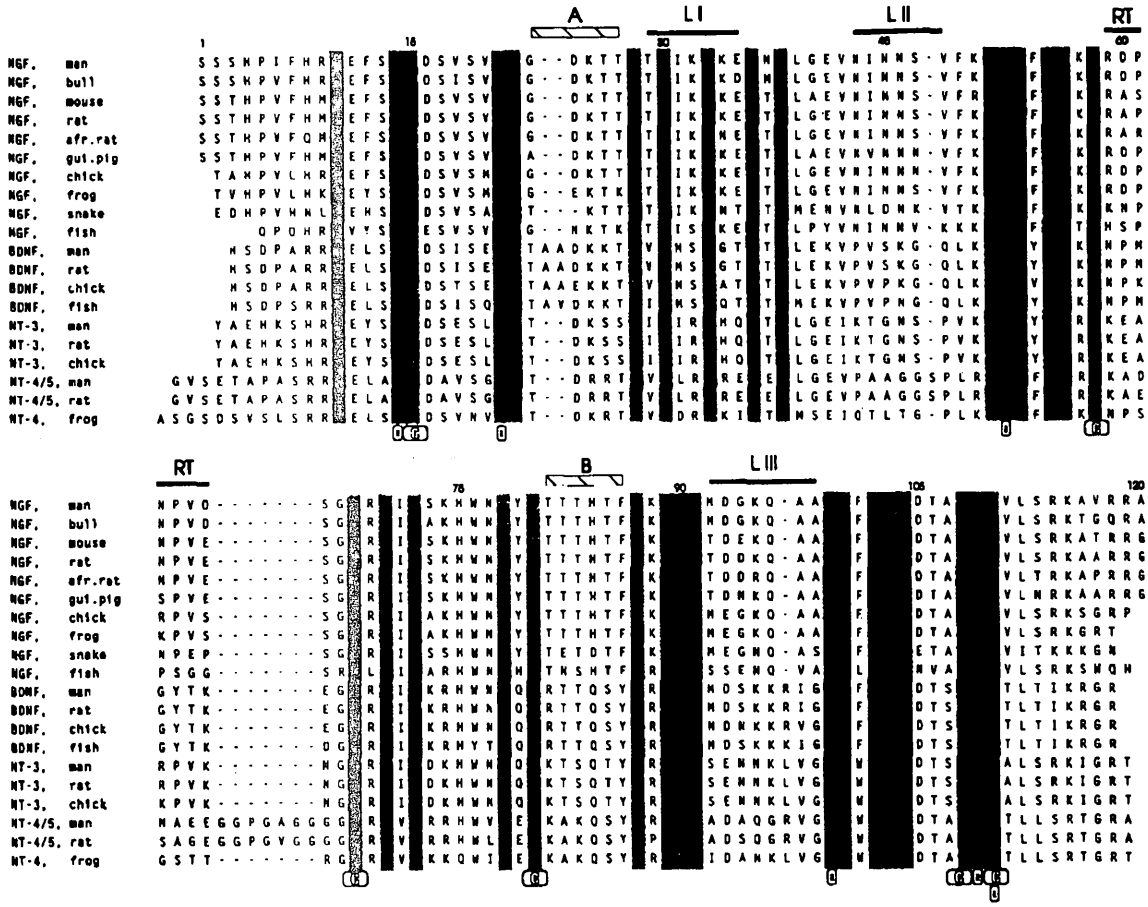

Fig. 2. Sequence alignment of neurotrophins. The numbering relates to human NGF; dashes have been inserted to maintain proper alignment. Conserved residues are shaded. The cysteine residues (□) and residues involved in dimer formation (□) are indicated. L are β-hairpin loops; RT indicates the reverse turn; A and B are surface-exposed residues that are part of the β strand.

biological activities of neurotrophic factor variants (Ibáñez *et al.*, 1991, 1993b; Suter *et al.*, 1992). Mutants with small changes usually retain wild-type function and the exchange of multiple residues or even domains is needed to change binding and survival specificity. The LI β-hairpin loop (not drawn in Fig. 3 for simplicity, but see McDonald *et al.*, 1991) contains residues that are conserved among neurotrophins and it is involved in the interaction

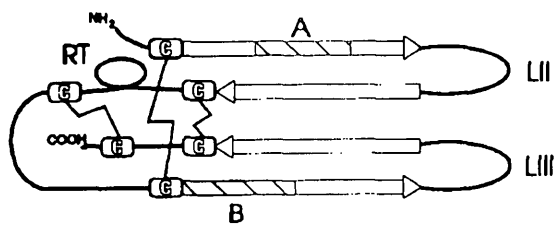

Fig. 3. Simplified representation of the three-dimensional mouse NGF promoter structure. The arrows delineate the extent of the two pairs of antiparallel β strands. L are β-hairpin loops; RT indicates the reverse turn. The LI hairpin loop protrudes from the first β strand; for details see text.

with the p75 neurotrophin receptor (Ibáñez *et al.*, 1992).

Two other differentiation factors, transforming growth factor β2 and platelet-derived growth factor BB show a promoter topology similar to NGF since they also contain six half-cystines and a conserved β strand structure. However, there exist no conserved domains outside of this motif and the modes of dimer formation are very distinct (McDonald and Hendrickson, 1993).

Evolution

The proportions of amino acid residues that are identical between pairs of orthologous and paralogous neurotrophin sequences aligned above were calculated (Table 2). This analysis shows highest conservation for NT-3 followed by BDNF; in salmon, however, NT-3 is less conserved than BDNF (Hallböök *et al.*, 1991). The NGF structure is less well conserved among the different classes of vertebrates. The

Table 2. Amino acid identities in % between pairs of orthologous and paralogous neurotrophins

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
NGF, fish	1		52	65	69	63	63	43	45	45	45	51	51	51	43	41	41
NGF, snake	2	52		68	70	67	67	51	49	48	48	49	49	49	46	45	43
NGF, frog	3	65	68		91	84	85	49	51	47	47	58	58	58	45	44	44
NGF, chick	4	64	70	91		86	88	52	53	53	53	59	60	60	49	48	48
NGF, rat	5	63	67	84	86		92	50	51	50	50	56	55	55	48	51	52
NGF, man	6	63	67	85	88	92		50	52	53	53	56	56	56	50	48	49
BDNF, fish	7	43	51	49	52	50	50		87	90	90	55	56	56	60	53	53
BDNF, chick	8	45	49	51	53	51	52	87		94	94	56	57	57	59	55	55
BDNF, rat	9	45	48	47	53	50	53	90	94		100	56	57	57	61	55	55
BDNF, man	10	45	48	47	53	50	53	90	94	100		56	57	57	61	55	55
NT-3, chick	11	51	49	58	59	56	56	55	56	56	56		99	99	51	52	52
NT-3, rat	12	51	49	58	60	55	56	56	57	57	57	99		100	57	52	52
NT-3, man	13	51	49	58	60	55	56	56	57	57	57	99	100		57	52	52
NT-4, frog	14	43	46	45	49	48	49	50	59	60	60	51	61	61		64	66
NT-4/5, rat	15	41	45	44	48	51	48	53	59	55	55	52	52	52	64		95
NT-4/5, man	16	41	43	44	48	52	49	53	59	55	55	52	52	52	66	95	

evolutionary relationships of the different members of the neurotrophin protein family were analysed by the construction of a phylogenetic tree (Fig. 4) using a protein parsimony algorithm (Felsenstein, 1989).

Using the average number of amino acid substitutions and the time periods of species divergence, the evolutionary rates of amino acid substitutions were calculated (Götz *et al.*, 1992). The gene duplication that gave rise to the BDNF/NGF paralogous genes occurred approximately 600 million years ago when the ancestral vertebrates first appear in the fossil record, earlier than the divergence of the fish lineage but later than the divergence of the arthropods from the phylogenetic tree (Götz *et al.*, 1992). No neurotrophins have yet been found in non-vertebrate species but a gene encoding a receptor tyrosine kinase related to the mammalian *trk*'s has been found in *Drosophila* (Pulido *et al.*, 1992).

The different structural conservation of NGF and BDNF during evolution is also reflected in their different biological activities. Importantly, the neuronal specificities of fish NGF and fish BDNF as compared to their mammalian orthologues have not changed but the potency of fish NGF as a survival factor for chick neuronal cells is nearly two orders of magnitude lower than that of mouse NGF, whereas fish BDNF is as potent as mammalian BDNF (Götz *et al.*, 1992). Since only 71 residues are identical between fish and mouse NGF, probably multiple residue changes between fish and mouse NGF account for the different specific activities. This hypothesis awaits experimental verification.

Role of Neurotrophins in Fish

Fish show a high degree of evolutionary conservation compared to other vertebrates

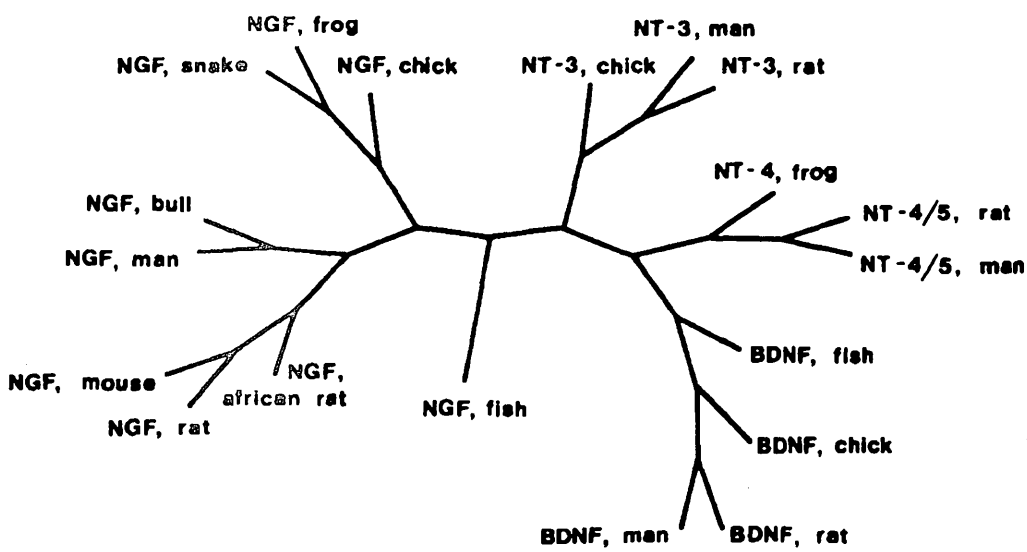

Fig. 4. Deduced phylogeny of the neurotrophins. The phylogentic tree shows the evolutionary relationship of NGF, BDNF, NT-3 and NT-4/5; the tree is rooted to fish NGF.

with respect to gene expression patterns in the developing brain and the establishment of early axonal pathways (reviewed by Kimmel, 1993). Neurogenesis continues well into adult life and the capacity of regeneration in the central nervous system distinguishes fish from higher vertebrate species where neurogenesis in most structures is completed at birth and axons fail to regrow (reviewed by Holder and Clarke, 1988). Natural neuronal death in fish has been described to occur in the fish nervous system (Ernst, 1926) but the number of dying cells as quantitatively studied in the electromotor neurons of the ray *Torpedo* is smaller than in higher vertebrates (Fox and Richardson, 1982). This might be related to the fact that neurons in fish are born continuously and not in a short period in massive numbers as, for example, in the chick; only after lesions, when a massive number of new neurons is produced, is there also a substantial cell death after regeneration of neurons in adult teleost fish (Anderson *et al.*, 1984). However, a specific molecule involved in any of these processes has not been elucidated.

Studies on the potential role of NGF in the development of the peripheral and central nervous system of fish have revealed the presence of an NGF-like activity in the spinal axes of many teleost fish species using a bioassay with embryonal chick sensory neurons (Weis, 1968a). Application of mouse NGF to zebrafish increased the number of cells in sympathetic and sensory ganglia with a concomitant increase in the size of the ganglia; conversly, injection of an antiserum raised against mouse NGF reduced the number of cells in sensory ganglia (Weis, 1968b).

Extracts from goldfish brain contained a considerable amount of NGF-like activity as judged by the neurite outgrowth of an NGF-responsive cell line; the amount of this activity was found to be much higher (nearly 40-fold) than in mouse brain and it was reported that the activity from fish brain could be blocked by a polyclonal antiserum against mouse NGF (Benowitz and Greene, 1979). The potential role of NGF in one well characterized structure of the fish central nervous system capable of functional regeneration—the regeneration of retinal ganglion cells after axotomy—has been investigated. Exogenous NGF can accelerate the regeneration of retinal ganglion cells after a crush of the optic nerve (Yip and Grafstein, 1982); furthermore, the neurite outgrowth from retinal ganglion cells in retina explants is increased and accelerated by the addition of NGF (Turner *et al.*, 1980, 1982). However, since the expected presence of specific NGF receptors on retinal ganglion cells could not be demonstrated (Yip and Johnson, 1983), the mechanism of the NGF action is not understood. The availability of the cloned neurotrophin genes and recombinant fish NGF and BDNF should facilitate further investigations into the physiological roles of neurotrophic factors in fish.

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