Neurotrophic factors: from structure–function studies to designing effective therapeutics

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The development and maintenance of the vertebrate nervous system requires the continuous supply of a number of polypeptide hormones known as neurotrophic factors[1]. The ability of neurotrophic factors to promote the survival of peripheral and central neurones during development and after neuronal damage, has stimulated an interest in these molecules as potential therapeutic agents for the treatment of nerve injuries and neurodegenerative diseases. Understanding the molecular basis of the biological specificity of neurotrophic polypeptides has provided an insight into their mechanisms of action, and allowed the design of derivatives and analogues with specific pharmacological properties.

Neurotrophic-factor research is dominated by the neurotrophins[2,3], a family of structurally and functionally related polypeptides, which includes nerve growth factor (NGF), a prototypic-neurotrophic factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) – also known as neurotrophin-4/5*. This family of molecules has served as the prototype for most of the structure–function studies performed on neurotrophic factors. They are relatively small, homodimeric polypeptides (approximately 120 amino acid residues) with a range of biological activities: they influence the generation, differentiation, survival and regeneration of vertebrate neurones[2,3]. NGF, BDNF, NT-3 and NT-4 are closely structurally related, sharing approximately 50% sequence identity[5]. The regions of sequence similarity and variation are clustered, indicating probable sections of structural and functional importance (Fig. 1). Neurotrophins interact with two classes of receptors on responsive cells: protein tyrosine kinase-type receptors (members of the Trk family), and a smaller receptor [distantly related to the tumour necrosis factor (TNF) and CD40 receptors] containing a short cytoplasmic tail of unknown function – the p75 low-affinity NGF receptor, $p75^{LNGFR}$ (Ref. 9). The four neurotrophins show specific biological activities on peripheral and central neurones[10,11] (Table 1). To some extent, this specificity correlates with their selective interaction with the different members of the Trk family of receptors (Fig. 2). Thus, TrkA, which is a receptor for NGF, has been found in NGF-responsive cells, including sympathetic neurones, small spinal sensory neurones of the dorsal root ganglion (DRG), and basal forebrain cholinergic neurones (Table 1). TrkB is a receptor for BDNF and NT-4, and it is widely expressed in the peripheral and central nervous systems, including nodose ganglion sensory neurones and spinal-cord motor neurones (Table 1). Similarly, TrkC expression has been demonstrated in cells that are responsive to NT-3, including large spinal sensory neurones, motorneurones and the noradrenergic neurones of the locus coeruleus (Table 1). NT-3 is also able to interact, albeit to a lesser extent, with TrkA and TrkB (Refs 9,12) (Fig. 2).

By contrast, all neurotrophins are able to bind with similar affinities to $p75^{LNGFR}$ (Refs 5,13,14). It was originally suggested that this receptor forms part of a functional high-affinity NGF receptor complex, and mediates signal transduction and biological responses to NGF. However, a more recent re-examination of

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*The term neurotrophin-4/5 (NT-4/5) is the result of uncertainties about whether the human neurotrophin NT-5 (Ref. 4) was a species homologue of the NT-4 previously found in *Xenopus* (Ref. 5). The term NT-4/5 was adopted by some laboratories to denote the mammalian form of *Xenopus* NT-4, when it was shown that the differences between the two factors were only due to phylogenetic variation[6,7]. In this review, NT-4 is used to denote all forms of the same factor. Recently, a new member of the neurotrophin family, structurally and functionally related to NGF, was described in the teleost fish *Xiphophorous* and named NT-6 (Ref. 8), despite being only the third neurotrophin identified in this species. As no more than four different neurotrophins have been identified in any given species, current neurotrophin nomenclature does not reflect the size of the family. Indeed, there is no reason to believe that neurotrophins isolated in a given species should be present across all vertebrate groups.

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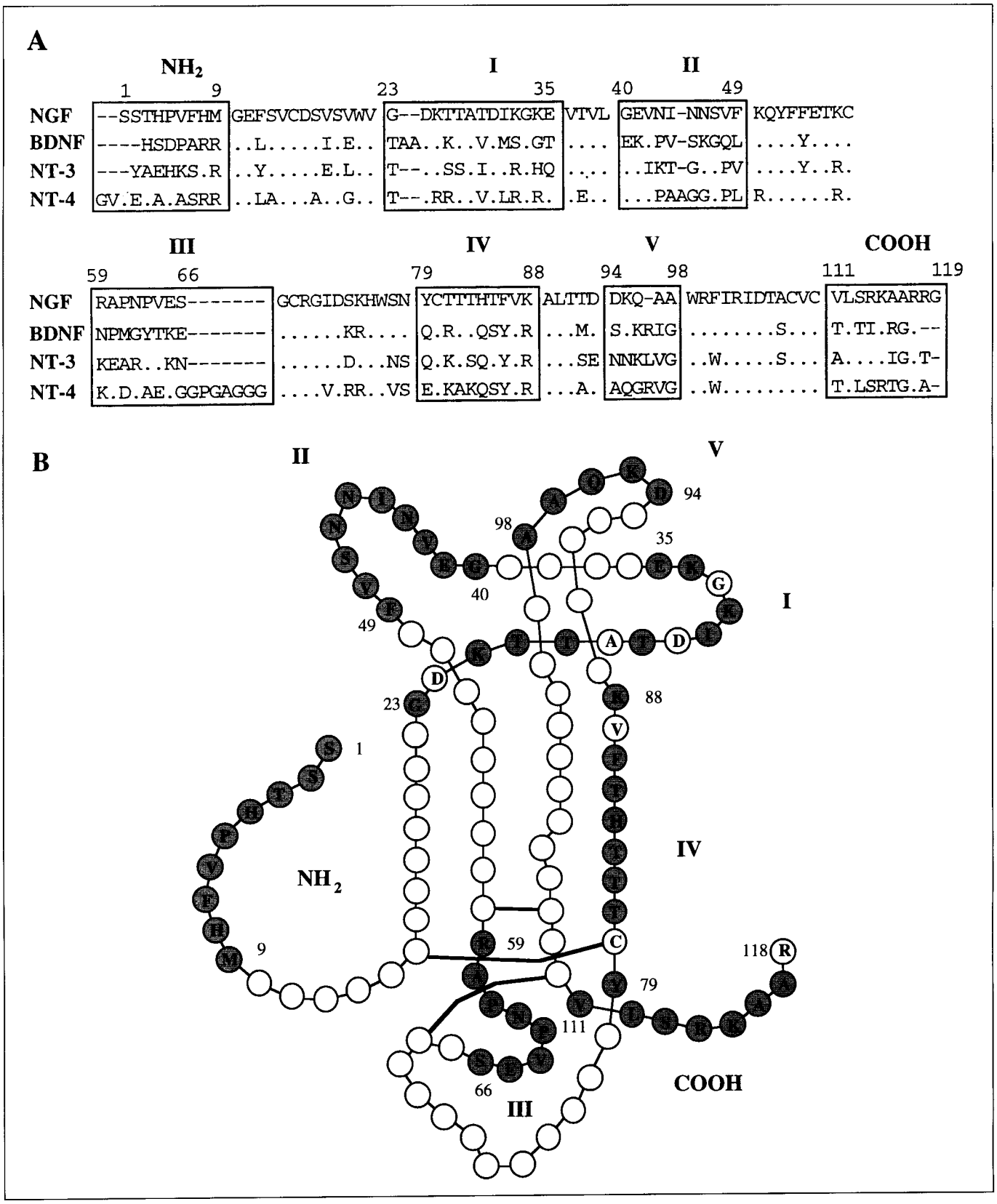

Figure 1

Variable regions of the neurotrophins. **(A)** Alignment of the amino acid sequences (single-letter code) of rat nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). Variable regions are boxed and labelled. Numbers correspond to positions in the NGF sequence. Positions are numbered from the first residue of each neurotrophin. Note that, because of differences in the lengths of the N-termini of the different neurotrophins, homologous positions in different molecules do not have equivalent numbering. Dots indicate identical residues to NGF, and dashes represent gaps introduced for the sake of alignment. **(B)** Schematic representation of the three-dimensional structure of the NGF monomer. Amino acid residues in the different variable regions are indicated by their one-letter code. Variable residues within these domains are shaded. Thick lines represent disulphide bridges.

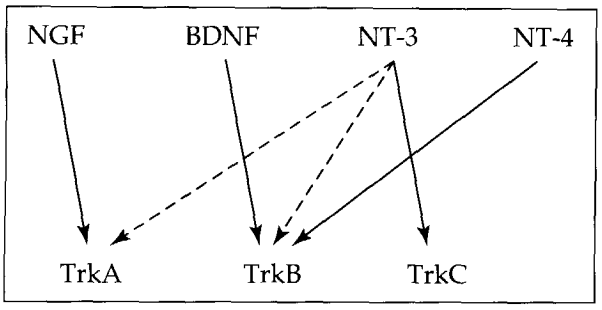

Figure 2
Established interactions between the neurotrophins and members of the Trk family of tyrosine-kinase receptors[12]. TrkA is the receptor for nerve growth factor (NGF), TrkB is the receptor for brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4), whereas TrkC is the preferred receptor for neurotrophin-3 (NT-3) (represented by solid lines). NT-3 can also interact with TrkA and TrkB (broken lines).

the role of $p75^{LNGFR}$ in different systems has revealed important functional aspects of this receptor[15]. Binding and functional assays have indicated that $p75^{LNGFR}$ can cooperate with Trk receptors to increase the affinity of neurotrophin binding and/or signalling efficiency. Such increases might be produced by increasing the local concentration of neurotrophins around Trk receptors[16], by presenting the ligand to Trk receptors in a favourable conformation for binding[17], or by direct signalling through the activation of the sphingomyelin cycle[18].

The three-dimensional structure of NGF has been determined[19], and progress is being made towards determining the crystal structures of BDNF, NT-3 and NT-4. The NGF protomer has an elongated structure, with three pairs of anti-parallel β-strands, connected by three β-hairpin loop structures, in which most of the variable residues of the four neurotrophins are concentrated. A similar arrangement of cysteine bridges, forming a knot at the bottom of the structure, is also found in other growth factors, including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and chorionic gonadotropin[20,21]. Among the important areas to be examined by a study of the structure–function relationships of these molecules are: the role of the constant and variable regions of the neurotrophins in the interaction with their receptors; the identification of residues determining the specificity of binding to, and activation of, different Trk receptors; and the way in which these residues correspond with those involved in binding to the $p75^{LNGFR}$ low-affinity receptor. In recent years, the considerable advance in our understanding of these issues has allowed the engineering of neurotrophin analogues with altered pharmacological properties, which may find important applications in the treatment of brain injury and disease.

Site-directed mutagenesis of neurotrophins: loss-of-function studies

Several strategies have been used to elucidate structure–function relationships in the neurotrophins; these may be broadly classified as loss-of-function and gain-of-function techniques. In the loss-of-function approach, individual residues, or small domains, are either deleted or replaced by small 'permissive' amino acid residues. Alanine is best suited to this approach, because it can adapt to most elements of the secondary structure of proteins, due to its small size and the minimal structural distortion it creates.

Another strategy used in loss-of-function studies, which is only applicable to families of proteins, involves replacing single residues, parts of variable regions, or whole variable regions with homologous sequences from another family member with a different receptor specificity – the homologue-scanning mutagenesis approach. The effects of loss-of-function mutations on the ability of the molecule to bind to its receptors, and to elicit a biological response in target cells, can then be evaluated. Numerous assay systems have been developed for the neurotrophins using both non-neuronal and neuronal cell lines, as well as primary cultures of ganglion explants and dissociated neurones. Receptor binding is typically measured by displacement-binding assays and chemical cross-linking, using primary neurones or fibroblasts ectopically expressing each of the Trks, or $p75^{LNGFR}$. The rat

Table 1. Specificity of neurotrophin action on peripheral and central neurones

Neurotrophin	Responsive peripheral neurones	Responsive central neurones
Nerve growth factor (NGF)	Sympathetic, small-sized (nociceptive?) spinal sensory, trigeminal ganglion	Basal forebrain cholinergic
Brain-derived neurotrophic factor (BDNF)	Medium-sized spinal sensory, vestibular and nodose ganglion, trigeminal ganglion	Retinal ganglion cells, motorneurones, basal forebrain cholinergic, substantia nigra dopaminergic
Neurotrophin-3 (NT-3)	Large-sized (propioceptive?) spinal sensory, nodose ganglion, trigeminal ganglion	Motorneurones, locus coeruleus, noradrenergic, cerebellar granule cells
Neurotrophin-4 (NT-4)	Nodose ganglion, trigeminal ganglion	Motorneurones, substantia nigra, dopaminergic

pheochromocytoma line PC12 has been used for receptor binding as well as for biological assays. This cell line expresses $p75^{LNGFR}$ and TrkA, and responds to NGF by exiting the cell cycle and differentiating into a sympathetic neurone-like cell type. Because each Trk receptor has endogenous tyrosine-kinase activity, activation is usually measured as the level of receptor tyrosine autophosphorylation elicited by exposure to ligand. The biological activity of neurotrophins has traditionally been assessed in survival and neurite extension assays; these use primary cultures of responsive peripheral neurones, typically sympathetic or sensory neurones. Recently, novel bioassay systems have been developed that use the signal emanating from Trk receptors to stimulate the proliferation of fibroblast lines, or the differentiation of PC12 cells carrying reconstituted neurotrophin receptors. Ideally, neurotrophin analogues should be tested in several systems in order to assess fully their potency and specificity.

The loss-of-function approach is likely to identify only those residues that make a substantial contribution to the energy of binding. Therefore, the importance of other residues in the binding interface may be underestimated, especially if the interface is extensive, and has many residues that contribute to receptor binding. This approach has been used to assess the importance of residues in several of the variable regions of the neurotrophins (Fig. 1). It is based on the assumption that these regions are involved in determining the specificity of neurotrophin action, and should be in, or close to, the receptor-binding interface. Despite this prediction, only a few variable residues have been shown to be necessary for receptor binding and biological activity after single point mutation. This suggests that variable residues do not determine biological specificity by making essential contributions to the energy of binding, but by other mechanisms (see below).

Alanine scanning

Alanine scanning of variable region I (residues 23–35) identified the side chain of Ile31 to be important for NGF binding to TrkA, and for biological activity[22,23]. Mutagenesis of individual residues in variable region II (Fig. 1) – a highly variable and exposed-loop region not defined in the NGF crystal structure, and likely to be very mobile – failed to reveal any residues important for NGF binding to TrkA, and for biological activity[23]. A similar result was also obtained after mutagenesis of the homologous region of NT-3 (Ref. 24). However, mutation of either Glu41 or Asn45 in NGF did result in a decrease in binding affinity to TrkA. This also caused a decrease in biological activity in sympathetic neurones when the substitutions were performed in an NGF molecule that had some of its other variable regions replaced by homologous sequences from another neurotrophin[23]. Similarly, mutations in other variable-loop regions have been shown to affect binding and biological activity only when made in a neurotrophin molecule that has previously been modified by other replacements or mutations[23]. Therefore, this approach may be useful for revealing residues that contribute to receptor binding, albeit with low binding energy. This is likely to be true for many of the variable residues identified in gain-of-function experiments (see below), in which a single point mutation in an otherwise wild-type environment produces no loss-of-function.

Homologue scanning

The homologue-scanning approach has been used to show that several variable regions in NGF play a role in binding to, and/or activation of, TrkA. These include loop regions I and V, the N-terminus and region IV of one of the β-strands[23] (Table 2). Results from a recent study suggest that conserved residues in and around this β-strand may be important determinants for binding of NT-3 to the TrkC receptor[24]. A binding surface that runs parallel to the twofold axis, along the side of the NT-3 dimer centered at Arg103, has been characterized using alanine-screening mutagenesis[24]. The low electron density of the N- and C-termini of NGF in structural maps suggests that these terminal regions are both flexible and accessible to solvents. Truncated forms of NGF, which lack the first ten N-terminal amino acid residues, have been generated by proteolytic cleavage[25] and by site-directed mutagenesis[26]; these show reduced binding to TrkA, and reduced biological activity. Homologue scanning has also demonstrated the importance of the NGF N-terminus for TrkA binding[23]. Site-directed mutagenesis appears to show that His4 of NGF provides the N-terminus with the structural properties that are necessary for high-affinity binding and potent TrkA phosphorylation[27]. Due to its flexibility, the N-terminal chain of NGF may function as a docking domain for receptor contact in a similar manner to that proposed for the C-terminus of the insulin B-chain[28]. Substituting NGF sequences into the N-terminus of NT-3 does not affect the function of the molecule[23,24], suggesting that this region may not be equally important in all neurotrophins. Deletion (but not homologue replacement) in the C-terminus of NGF has been shown to affect the ability of the molecule to activate TrkA and to induce differentiation of PC12 cells[23,26]. This indicates that the C-terminus of NGF may be involved in a non-specific interaction with TrkA, possibly through contacts mediated by conserved residues, or by the main amino acid chain.

The observation that all neurotrophins can bind to $p75^{LNGFR}$ has prompted the suggestion that conserved regions in the neurotrophins may be involved in $p75^{LNGFR}$ binding. Paradoxically, alanine-scanning mutagenesis of variable region I demonstrated that the positively charged residues Lys32 and Lys34 are required for the binding of NGF to $p75^{LNGFR}$ (Ref. 22). Although mutation of these residues removed the ability to bind to the low-affinity receptor, it only had minor effects on binding to TrkA, or biological activity, thereby establishing a functional dissociation between the two NGF receptors. This mutant NGF

Table 2. Amino acid residues in the neurotrophins that are important for binding to the Trk family of tyrosine kinase receptors, and/or biological activity, as identified by loss-of-function experiments[a]

Region of neurotrophin examined	Neurotrophins			
	Nerve growth factor	Brain-derived neurotrophic factor	Neurotrophin-3	Neurotrophin-4
N-terminus	1–8 (Refs 23,25,26); His4 (Ref. 22)	–	Arg8, Tyr11 (Ref. 24)	–
Loop region I	23–25, Ile31 (Ref. 23)	–	Thr22 (Ref. 24)	–
Loop region II	45–49, Glu41, Asn45 (Ref. 23)	–	–	–
Loop region III	–	–	–	–
β-strand region IV	79–88 (Ref. 23)	–	Lys80, Gln83 (Ref. 24)	–
Loop region V	94–98 (Ref. 23)	Lys95, Lys96, Arg97 (Ref. 30)	–	–
C-terminus	112–118 (Ref. 26)	–	–	–
Conserved residues	Val21, Arg99, Arg102 (Ref. 54)[b]	–	Glu10, Tyr51, Glu54, Arg56, Arg103 (Ref. 24)	– –

[a] Residue intervals indicate domains identified due to loss-of-function after either deletion, or replacement with homologous sequences from other neurotrophin family members. Single residues (three-letter code) denote positions identified by alanine substitution.
[b] Conserved residues identified in nerve growth factor (NGF) (Ref. 54) correspond to the chicken NGF numbering (equivalent residues in mammalian NGFs are Val22, Arg100, Arg103).
Blank entries (–) denote no residues identified.
Negative results may be inconclusive with regard to the importance of residues or regions and have, therefore, been omitted.

has subsequently been used to show that, although binding to $p75^{LNGFR}$ is not required, it can accelerate the biological actions of NGF in sympathoadrenal progenitor cell lines[29]. More recently, homologous residues in NT-3 and NT-4 that also contain positive charges have been shown to contribute to binding to $p75^{LNGFR}$ (Ref. 30). In NT-4 the loss of $p75^{LNGFR}$ binding affected the potency of the molecule in neuronal cells co-expressing $p75^{LNGFR}$ and TrkB (Ref. 30), suggesting a role for $p75^{LNGFR}$ in regulating biological responsiveness to NT-4. In BDNF, positively charged residues critical for $p75^{LNGFR}$ binding (Lys95, Lys96 and Arg97) were found in a close, but distinct, loop region. Replacement of these residues by Ala also affected the activation of TrkB (Ref. 30), suggesting that $p75^{LNGFR}$ and TrkB have partially overlapping binding sites in BDNF. Although the mutation of positive charges involved in binding to $p75^{LNGFR}$ in the other three neurotrophins does not appear to have major consequences on Trk binding, these residues partially overlap with variable regions contributing to Trk binding specificity (Fig. 3A). Although all four neurotrophins bind $p75^{LNGFR}$ with equal affinity, they appear to differ in their rates of dissociation from this receptor, BDNF being the slowest, at least an order of magnitude slower than NGF (Refs 13,14). Analysis of the electrostatic surface-potentials of the four neurotrophins has revealed similar clusters of positively charged residues in each molecule, but with differences in their precise spatial locations. This supports the increasing evidence that the low-affinity receptor may recognize each of the four ligands as related, but not identical, structures. (For a more comprehensive overview of the interaction of neurotrophins with $p75^{LNGFR}$, see Ref. 30.)

Gain-of-function experiments: chimeric neurotrophins

The gain-of-function approach takes advantage of the similarities and differences among the different members of the neurotrophin family. Because most of the conserved amino acid residues in the neurotrophins play structural roles, all four neurotrophins are likely to have very similar conformations, with individual differences being restricted to the variable regions. The importance of the variable regions in determining the biological specificities of the neurotrophins has been assessed by generating chimeric molecules in which different variable residues are exchanged between different members of the family (Table 3). One of the most striking results of the initial studies with chimeric neurotrophins is the ability of the NGF molecule to retain substantial biological activity despite extensive replacement of its variable sequences with corresponding regions from BDNF (Refs 31,32). The gain of a BDNF-like function in an NGF skeleton (as assessed by the ability to stimulate outgrowth from explants of embryonic nodose ganglia) was achieved after the replacement of

combinations of several variable regions, including loop regions I and V, and region IV of the β-strand[31] (Fig. 1). Subsequently, a more detailed study has identified several individual residues within these regions that allowed activation of the TrkB receptor for BDNF upon exchange into BDNF-like positions in an NGF background[23]. A recent study has reported the occurrence of an NT-3 molecule that acquired BDNF-like activities in TrkB/fibroblasts and PC12/TrkB cells after substitution of Asp15 by Ala (Ref. 24). However, this NT-3 mutant was unable to rescue BDNF-responsive DRG neurones, suggesting that the activity of the mutant on TrkB was restricted to cells over-expressing this receptor. Given that BDNF itself contains Asp at the same position, it is difficult to interpret this result.

Two recent studies have analysed the importance of residues in the highly variable loop region II using the homologue-scanning approach. In one of these, exchanging this variable region between NGF and NT-3, and between NT-3 and NT-4 (but not between NGF and NT-4), resulted in the formation of chimeric molecules that were capable of activating two different Trk receptors; they also displayed broader specificities to responsive neurones[33]. The fact that region II from NGF was unable to confer TrkA binding to NT-4 suggests that this neurotrophin, in contrast to NT-3, may have structural constraints in other regions that prevent the formation of a productive complex with TrkA. However, none of the exchanges resulted in loss-of-function, indicating the tolerance of this domain to sequence variations.

The second study used point mutations to show that residues 43, 44, 45, 48 and 49 from NGF conferred NGF-like biological activities when substituted into NT-3 (Ref. 34). The effects of these replacements on the affinity of binding of the mutant to TrkA and TrkC receptors were not tested in this study. Gain-of-function experiments have demonstrated the ability of the N-terminus of NGF to allow binding and activation of TrkA when substituted into heterologous neurotrophins. A chimeric NT-3, with its N-terminus replaced by corresponding residues from NGF, acquired the ability to bind and activate TrkA, and to elicit outgrowth and survival of embryonic sympathetic neurones[23,24]. This result, indicating that the N-terminus of NGF confers TrkA-like biological specificity, is in agreement with loss-of-function studies that indicated the importance of the N-terminus of NGF for binding to TrkA (see above). A chimeric NGF with the N-terminus from BDNF did not acquire additional specificities[23], supporting the suggestion that the N-terminus may be playing different roles in different neurotrophins.

An alternative approach to generating neurotrophins with extended specificities has been demonstrated by the production of neurotrophin heterodimers[35–37]. The crystal structure of NGF reveals that residues involved in the dimer interface are conserved among different neurotrophins[19], suggesting that dimer interfaces of different neurotrophins may be complementary. Heterodimers between BDNF and NT-3 have been purified and characterized; heterodimers between NGF and BDNF can be formed, but are not stable[37]. BDNF/NT-3 heterodimers are biologically active, and have properties from both factors, although there is some disagreement as to the relative potency of this molecule, compared with the native neurotrophins, in neuronal bioassays[36,37]. An analysis of the biophysical properties of BDNF/NT-3 heterodimers, and their interaction with soluble TrkB and TrkC receptors, has supported earlier mutagenesis data which indicated that the binding interface with Trk receptors in the neurotrophins is made up of residues from both protomers of the neurotrophin dimer[23,38].

A model for neurotrophin–Trk interactions

Summaries of the residues and regions important in neurotrophin–Trk interactions, identified by both loss-of-function and gain-of-function studies, are shown in Table 2 and Table 3, respectively. Although these mutagenesis approaches have not been applied with equal intensity to all the regions of all the neurotrophin molecules, a consensus emerges from the data accumulated over the past five years of study into the structure–function relationships of this family. Amino acid residues contributing to Trk–receptor binding appear to be grouped on one side of the neurotrophin dimer, delineating a continuous surface that extends approximately parallel to the twofold axis, and that contains residues from both protomers. Identical binding surfaces are formed on both sides of the molecule because of this twofold symmetry. At the top of the NGF dimer, amino acid residues in region V of one protomer are in close proximity to residues in region II of the other protomer. This leads to the formation of a patch of residues that stretches across the top of the dimer (Fig. 3). In the middle region of the NGF dimer, this surface is extended by additional contacts provided by residues in loop region I and β-strand region IV of the first protomer (Fig. 3B). The conformation of the N-terminus in NGF is unknown, but it may position along the side of the molecule when binding occurs. Results from extensive alanine-scanning mutagenesis in NT-3 have stressed the importance of the contribution of conserved regions to the energy of binding to TrkC (Ref. 24). The NT-3 binding epitope was found to be dominated by Arg103, with additional contacts grouped in a discrete region along the side of the neurotrophin dimer, including several residues in β-strand region IV (Ref. 24). In NGF, studies of variable residues have shown that Arg103 is located in the binding surface (Fig. 3). This suggests that all neurotrophins make use of the same surface to bind Trk receptors. In addition, most point mutations of variable residues lead to substantial losses of binding affinity to Trk receptors.

Taken together, these data support a model in which conserved residues in the β-strands on the side of the dimer provide the contacts with the highest binding energy. However, variable residues in turns and loop regions along this surface determine biological specificity, either by contributing directly with contacts of

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Region of neurotrophin examined	Neurotrophins			
	Nerve growth factor	Brain-derived neurotrophic factor	Neurotrophin-3	Neurotrophin-4
N-terminus	1–8 (Refs 23,24)	–	–	–
Loop region I	–	26–35 (Ref. 31)	–	–
Loop region II	40–49 (Ref. 33); Asn43, Ile44, Asn45, Val48, Phe49 (Ref. 34)	45–49 (Ref. 31)	39–48 (Ref. 33)	42–53 (Ref. 33)
Loop region III	–	–	–	–
β-strand region IV	–	79–88 (Ref. 23)	–	–
Loop region V	Gln96, Ala97, Ala98 (Ref. 34)	Lys96, Arg97 (Ref. 23)	–	–
C-terminus	–	–	–	–
Conserved residues	–	–	Asp15 (Ref. 24)	–

[a] Residue intervals and single residues (three-letter code) indicate determinants identified by homologue scanning, due to their ability to confer additional specificities when exchanged in a heterologous neurotrophin.
Blank entries (–) denote no residues identified.
Negative results may be inconclusive about the importance of residues or regions and have, therefore, been omitted.

lower energy to cognate receptors or, indirectly, by preventing interaction with inappropriate receptors.

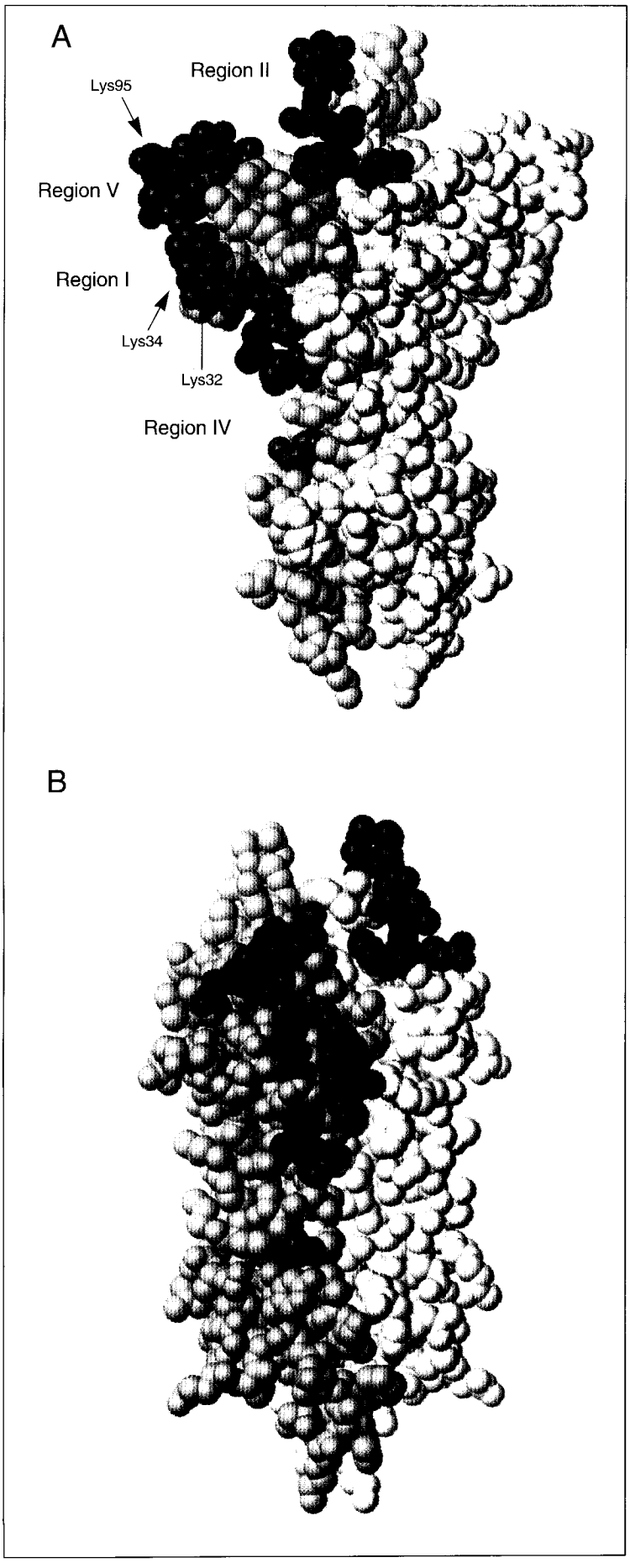
The structural similarities between the neurotrophins suggest that they have evolved from a common ancestor, and have diverged functionally, perhaps by acquiring residues that prevented binding to non-cognate receptors. As has recently been proposed for the evolution of gonadotropins and their receptors[39], this appears to be a general evolutionary mechanism that allows ligand–receptor pairs to be created without requiring the simultaneous development of two new complementary binding sites. Therefore, present-day neurotrophins may share common determinants of binding to different Trk family members, these being represented by conserved residues or the main amino acid chain; their variable regions may have evolved through the acquisition of inhibitory binding determinants that restrict promiscuous ligand–receptor interactions, and allow specificity. As in other families of homologous ligands, neurotrophin–Trk pairs seem to be at different stages in this 'evolutionary' process. This is indicated by the example of different Trks that bind to the same neurotrophin (e.g. TrkA, TrkB and TrkC to NT-3), and different neurotrophins that bind to the same Trk (e.g. BDNF and NT-4 to TrkB) (Ref. 12).

Neurotrophin engineering

The ability of the neurotrophins to prevent neuronal degeneration, and to promote the phenotype of peripheral and central neurones, suggests that they may be valuable therapeutic agents for the treatment of nerve injury and brain disease. *In vivo* evidence for the significance of the neurotrophins includes: their effects on motorneurones[40,41], with implications for the treatment of amyotrophic lateral sclerosis (ALS); their effect on the basal forebrain cholinergic and locus coeruleus noradrenergic neurones[11,42], which degenerate in Alzheimer's disease; their effect on dopaminergic neurones from the substantia nigra[43], the major neuronal population affected in Parkinson's disease; and their effect on peripheral sensory neurones[44,45], providing the possibility of the therapeutic use of neurotrophins for peripheral neuropathies, nerve injury, and to ameliorate the side-effects of anti-tumor therapy.

Why engineer the neurotrophins?

Neurotrophins, like any other polypeptide hormone, have evolved by a long process of mutation and selection that has shaped their specific biological and pharmacological properties to suit the roles they play in animal tissues. In certain circumstances, some of these properties may not be compatible with their use as therapeutic drugs. For example, some of the neurotrophins have been shown to display very poor diffusion in brain tissue, possibly due to interactions with the extracellular matrix, or with truncated receptors[46]. Although limited diffusion may carry physiological advantages, it poses considerable limitations on the use of these neurotrophins as therapeutic drugs. Modifications to the chemical character of the surface of a neurotrophin molecule may greatly influence its diffusion without altering its biological potency. Molecules with reduced binding to a truncated receptor, but that still retain substantial biological activity, may be useful for overcoming the problems of ligand clearance and sequestration, and may help to improve the access of neurotrophins to areas of the brain that are distant from the site of administration. In addition to



physico-chemical properties, the biological specificities of a given neurotrophin may not be suitable for a particular therapeutic application. For example, the variety of neuronal populations affected in numerous neurodegenerative processes is quite complex, each of these populations having different trophic requirements. Therefore, it would be advantageous to have a single molecule that displays the combined trophic specificities required by all the neuronal populations affected by the degenerative process. Conversely, the receptor promiscuity displayed by some of these neurotrophic factors could result in undesired side-effects. In these cases, an analogue with a more restricted range of receptors might be better tolerated.

Structure–function relationships

A knowledge of structure–function relationships would also be useful to help in the design of neurotrophin antagonists and non-peptidic analogues. The fact that high levels of NGF have been shown to produce hyperalgesia in both rodents and humans[47,48] has led to the proposal that NGF antagonists may be useful as analgesics. Also, because peripherally administered neurotrophins do not cross the blood–brain barrier, the search has begun for small non-peptidic molecules capable of activating Trk receptors, or components of their downstream signal transduction pathway. The structures of many oligomeric proteins and protein–protein complexes show them to have generally large and flat interfaces, making the rational design of small-molecule mimics of these surfaces a daunting prospect. However, a recent study on the interaction between human growth hormone with its receptor (for which a crystal structure of the complex is available) has shown that only a small and complementary set of hydrophobic contacts, in an otherwise

Figure 3

(A) Model of the NGF dimer viewed perpendicular to the twofold axis. The first protomer (in a similar orientation to that shown in Fig. 1B) is in white; the second protomer, like the first but rotated 180° around the vertical axis, is in turquoise. Residues from the 'turquoise' protomer that contribute to one TrkA binding site are in purple, and those from the 'white' protomer are in magenta. For simplicity, the symmetrically equivalent residues forming the second binding site are not coloured. Variable regions I, IV and V from the 'turquoise' protomer, and variable region II from the 'white' protomer are indicated. Each protomer begins at Phe12 (shown in yellow in the 'white' protomer). The conformation of the remaining 11 N-terminal residues is unknown, but they may pack parallel to the axis that shows twofold symmetry, and extend the binding surface along the side of the dimer. In the 'turquoise' protomer, Lys32, Lys34 and Lys95, which mediate binding to $p75^{LNGFR}$, are indicated. Note that although these residues only make a minor contribution to TrkA binding, they are also exposed in the binding surface to this receptor. **(B)** Model of the NGF dimer rotated 90° through the twofold axis as compared with (A). Residues contributing to TrkA binding form a continuous surface that extends along the side of the dimer, and that contains elements from both protomers. At the bottom of the molecule, this surface may be extended by the N-terminus. Note that the conserved Arg103 (dark blue), shown to be critical for binding of NT-3 to TrkC (Ref. 24), is also exposed in the NGF binding surface. Images were adapted from Ref. 23.

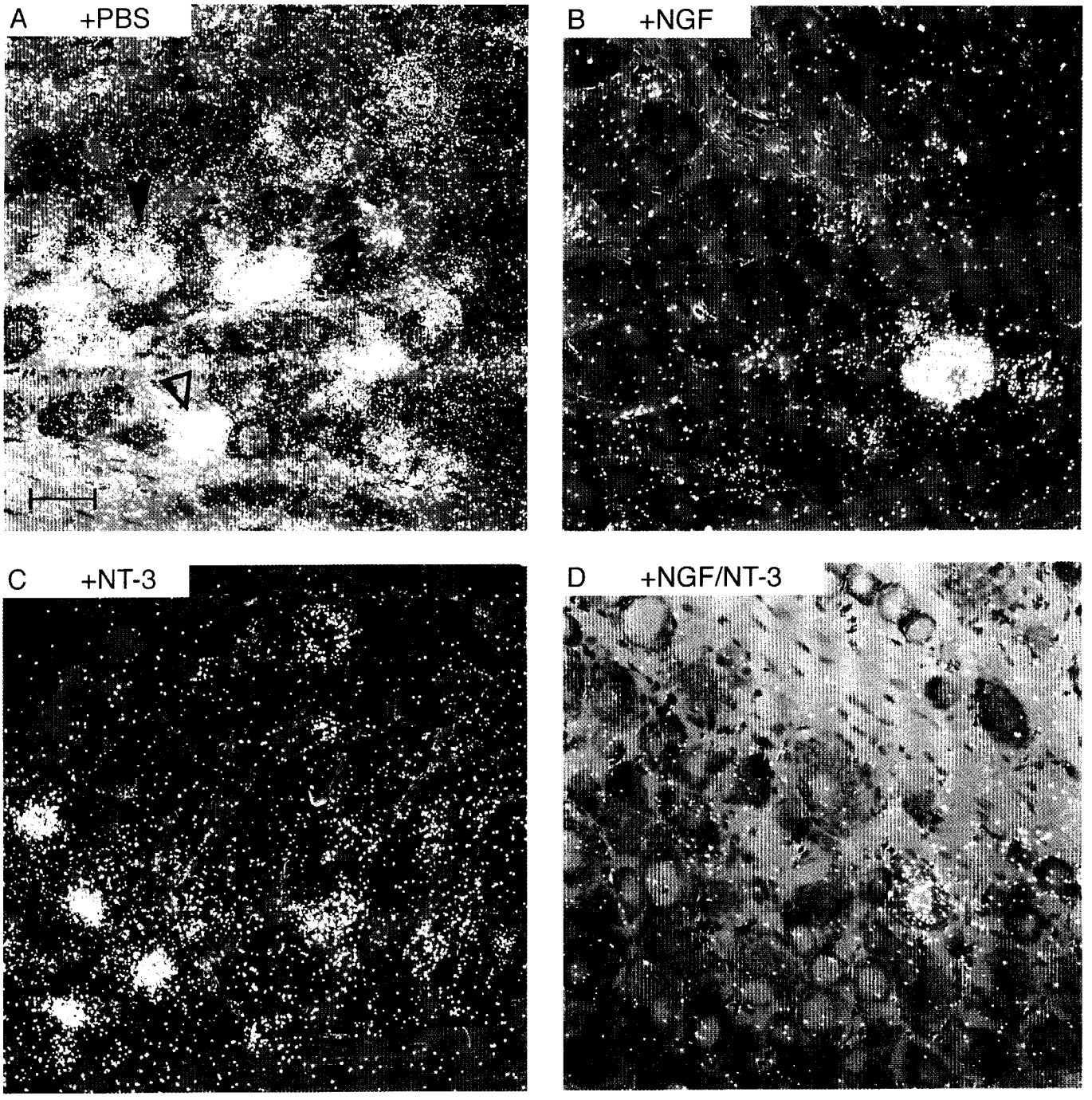


Figure 4

Emulsion autoradiography of rat lumbar dorsal root ganglion (DRG) neurones labelled by retrograde transport, after co-injection of iodinated pan-neurotrophin-1 (PNT-1) with phosphate buffered saline (PBS) **(A)**, or with excess unlabelled nerve growth factor (NGF) **(B)**, unlabelled neurotrophin-3 (NT-3) **(C)**, or unlabelled NGF and NT-3 **(D)**. Note that both small-sized (open arrowheads) and large-sized (solid arrowheads) neurones are labelled by PNT-1. Co-injection with unlabelled NGF competes the labelling from small-sized neurones, while co-injection with unlabelled NT-3 competes the labelling from large-sized neurones. The scale bar represents 100 μm.

extensive interface, accounts for the majority of the free-binding energy[49]. Less-important contact residues surrounding this 'functional epitope' were found to be generally hydrophilic and partially hydrated; the interface resembles a cross-section through a globular protein. If this property is shared by the neurotrophin–Trk interface, the task of designing small neurotrophin analogues might be greatly simplified. The structure–function information obtained by the study of chimeric and mutant neurotrophins has begun to play a part in the rational design of neurotrophin analogues with modified pharmacologic properties.

Multifunctional neurotrophins

An area that has seen great progress in recent years is the engineering of neurotrophin agonists with modified specificities. The observation that the survival and function of several populations of peripheral and

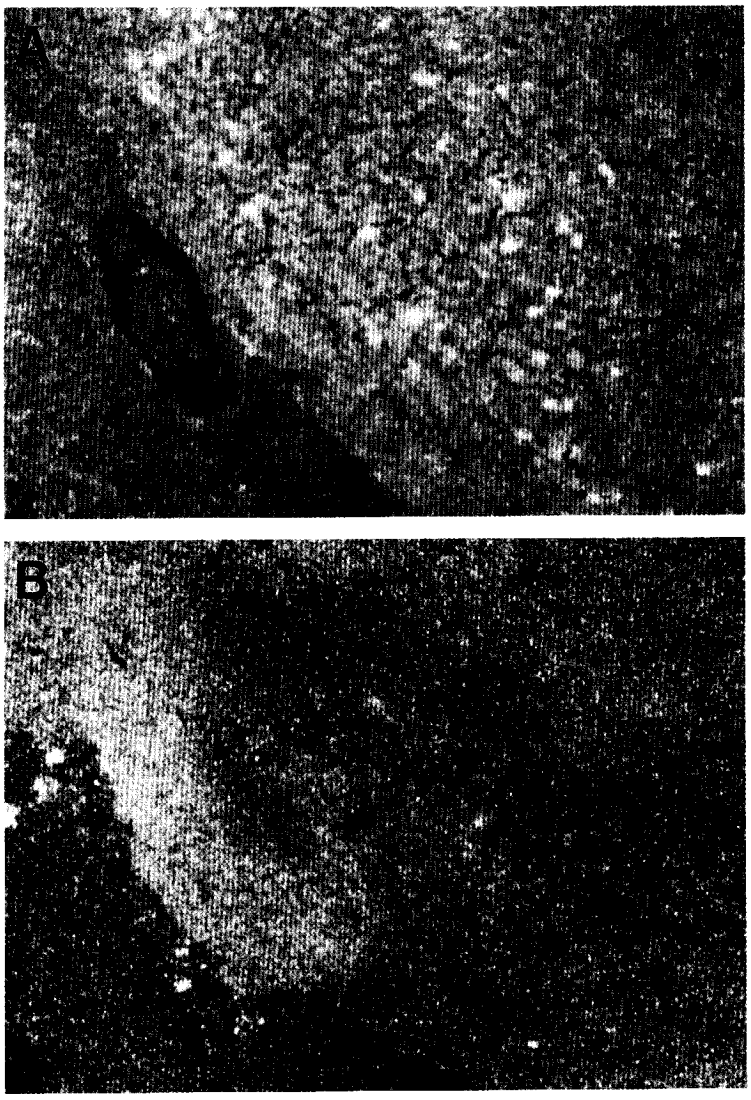


Figure 5

Emulsion autoradiography of the binding of iodinated neurotrophins to sections of rat medial septum. Note that pan-neurotrophin-1 (PNT-1) **(A)**, but not neurotrophin-3 (NT-3) **(B)**, has the ability to label neurones in the rat medial septum that normally respond to nerve growth factor (NGF).

central neurones depend on multiple neurotrophins has led to the suggestion that the combined influence of several of these molecules may be more physiologically representative of the *in vivo* situation than the individual effect of each trophic factor[50]. Neurotrophin molecules with multiple specificities, acting on several different neuronal populations, may be of great advantage, because they would greatly simplify the problems inherent in the use of heterogeneous mixtures of molecules (i.e. neurotrophin cocktails), such as differences in the diffusion and stability of the components that, for example, might affect their efficacy as therapeutic drugs. An NGF/BDNF chimeric molecule, previously shown to have the combined specificities of these two neurotrophins on peripheral neurones[23,31], has recently been shown to exhibit synergistic actions, and promote the survival of the basal forebrain cholinergic neurones[50] that degenerate in Alzheimer's disease. The chimera was 100-fold more potent than native BDNF, and this effect could be reproduced by the simultaneous addition of NGF and BDNF. Therefore, the chimeric factor revealed a synergy that may normally occur in the brain. This constitutes a potentially novel therapeutic agent with a greater potency than the naturally occurring individual neurotrophins.

Despite the fact that the different sub-populations of sensory neurones in the DRG, and the spinal cord motorneurones, respond to different trophic factors, their axons extend through the same peripheral nerves. Therefore, functional regeneration of damaged peripheral nerves is likely to require the concomitant action of different neurotrophins. Another multifunctional chimeric neurotrophin, engineered using an NT-3 skeleton in which residues 94–98 and 1–9 were replaced by corresponding amino acid residues from BDNF and NGF, respectively, has been shown to be capable of efficiently activating and initiating biological responses from all three Trk receptors[23,51]. An analysis of receptor-specific neurotrophic activities elicited by this molecule, called pan-neurotrophin-1 (PNT-1), demonstrated that it efficiently promoted the survival of multiple sub-populations of embryonic peripheral neurones expressing different Trk receptors. More importantly, PNT-1 showed robust retrograde transport to DRG neurones *in vivo* after injection into the sciatic nerve[51] (Fig. 4). Radiolabelled PNT-1 accumulated in small-sized neurones (presumably the nociceptive neurones stimulated by pain and heat, and that respond to NGF), medium-sized neurones (responding to BDNF) and large-sized neurones (presumably proprioceptive neurones conveying muscle-positional information, and that respond to NT-3). Co-injection with different unlabelled neurotrophins inhibited PNT-1 transport in distinct sub-populations of neurones of different sizes, suggesting that this molecule affects sensory neurones of different modalities, which are normally responsive to different neurotrophins.

These results indicate that PNT-1 is a multispecific neurotrophic factor that may be useful in the treatment of peripheral neuropathies and nerve damage. The multispecific activities of this factor have also been demonstrated in the brain. Iodinated PNT-1 (but not the parental molecule NT-3) was able to label neurones specifically in the medial septum, which is affected in Alzheimer's disease (Fig. 5). The labelling could be competed with excess of unlabelled NGF, indicating that the engineered factor was targeting cells that normally do not respond to NT-3, but to NGF (C. A. Altar, unpublished). Other multispecific neurotrophins have more recently been generated[24,34], although their specificities towards different neuronal populations and biological activities *in vivo* remain to be characterized.

Selectively active neurotrophins

In certain circumstances, molecules with a very selective receptor specificity may be appropriate for therapeutic purposes. Although most neurotrophins display a high degree of specificity in their interaction with Trk receptors, NT-3 is able to interact, albeit to a lesser extent, with TrkA and TrkB, in addition to

TrkC, its preferred receptor[12]. The efficiency of the interaction of NT-3 with non-cognate receptors appears to depend on the cellular context[7], and on the presence of accessory receptor molecules[52,53]. NT-3 is the only neurotrophin that is able to influence the survival of neurones from the locus coeruleus[42], a population of central noradrenegic neurones affected in patients with Parkinson's and Alzheimer's diseases. Although the specificity of this action makes NT-3 an attractive therapeutic candidate for the prevention of degeneration of these neurones, its promiscuous interactions with other Trk receptors may result in undesirable side-effects. A mutant NT-3 has recently been created that exhibits normal activities towards TrkC, although it is unable to bind or activate TrkA and TrkB (C. F. Ibáñez, unpublished). This molecule may be more effective than native NT-3 for the treatment of disorders in which neurones in the locus coeruleus are compromised.

Conclusions

The study of structure–function relationships in the neurotrophins has given an insight into the mechanisms of binding and activation of neurotrophin receptors. It has also provided a structural explanation for the different biological specificities displayed by different family members. This information can now be used to manipulate the pharmacological properties of these factors, and to generate agonists and antagonists with exciting scientific and clinical applications.

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