Emerging themes in structural biology of neurotrophic factors

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Neurotrophic factors control the survival, differentiation and maintenance of neurons in the peripheral and central nervous systems. Their discovery and characterization have been instrumental to our understanding of a wide range of phenomena in the development, plasticity and repair of the nervous system. Their potential importance in the development of therapeutic agents against neurodegenerative disorders and nerve injury has led to a flurry of activity towards understanding their structure, function and signaling mechanisms. This knowledge has increased dramatically in recent years, in particular due to the elucidation of three-dimensional structures, the discovery of families of structurally related neurotrophic factors and the characterization of receptors and downstream signaling components. Common themes are emerging from these recent studies that allow us to make new insights and predictions as to the function and possible clinical utility of these molecules.

Neurotrophic factors, like all polypeptide hormones, deliver their message to the cell interior via interaction with cell surface receptors. More often than not, neurotrophic factors interact with multicomponent receptors consisting of several distinct protein subunits. In some cases, such as the receptor complexes for ciliary neurotrophic factor (CNTF) and glial cell line-derived neurotrophic factor (GDNF), the tasks of ligand binding and downstream signaling are segregated among the different subunits (Fig. 1). In a simplified model of receptor activation, a ligand-binding component, the α subunit, first associates with the hormone. This complex is subsequently recognized by the signaling receptor, the β subunit, which thereby becomes activated. A number of variations of this basic model are known to exist. The α subunits of the GDNF and CNTF receptor complexes are shed to some extent after cleavage of their glycosylphosphatidylinositol (GPI) anchors. Soluble forms of α-receptor components are believed to capture ligand from the extracellular environment for subsequent assembly into a functional receptor complex on the membrane of cells carrying signaling subunits. In the case of GDNF, receptor binding and crosslinking studies indicate that GDNF contacts both the α subunit, GFRα-1, and the signaling subunit, the receptor tyrosine kinase RET (Refs 3, 4, 7). In addition, GFRα-1 has some intrinsic affinity for RET in the absence of ligand (Ref. 8), suggesting that pre-formed complexes of α and β subunits are also present at the cell surface. GFRα-1 has also been shown to modulate negatively the constitutive levels of RET tyrosine phosphorylation in the absence of ligand. Thus, at least in this system, GFRα receptors could also provide a gain control mechanism to increase the signal-to-noise ratio of the response to ligand.

For other neurotrophic factors, however, ligand binding and signaling are not segregated into different receptor polypeptide chains. The neurotrophin nerve growth factor (NGF) binds to two different receptors, the p75 neurotrophin receptor (p75NT) and the receptor tyrosine kinase TrkA, each with distinct signaling capabilities (Ref. 9) (Fig. 1). Although multimeric receptor complexes and functional interactions between both receptors have been observed, it is clear that NGF can bind to and elicit biological actions through each of these two receptors independently.

Most neurotrophic factors belong to families of structurally and functionally related molecules. This raises the issue of the specificity of the interaction of structurally similar but distinct ligands with their corresponding receptors. This specificity problem has been solved in different ways during the diversification of the different families of neurotrophic factors (Fig. 2). Subunits of the α-receptor can help in the discrimination of related ligands. Thus, for example, although CNTF and another structurally related member of the neurokine family, leukemia inhibitory factor (LIF), utilize the same signaling receptor subunits (that is, LIFR-β and gp130), CNTF, but not LIF, can only activate these receptors via prior binding to CNTFRα (Ref. 23). Similarly, both GDNF and the related factor neurturin (NTN) utilize RET as a signaling receptor subunit. However, while GDNF binds preferentially to GFRα-1 (previously called GDNFR-α), NTN shows binding specificity for GFRα-2 (Refs 14, 15; but see also Refs 8, 16), the second member of an expanding family of structurally related GPI-linked receptors. The ligand specificities of two recently discovered members of this family, GFRα-3 (Refs 9, 17–20) and GFRα-4 (Ref. 21) are unknown. Interestingly, although GFRα-3 does not bind GDNF directly, it is able to interact with GDNF in the presence of RET (Ref. 9).

In contrast to the neurokine and GDNF families, specificity in the neurotrophin family is achieved through interaction of the different ligands with distinct members of the Trk family of receptor tyrosine kinases.
ligand binding p75NTR may be of functional importance is underlined. Glial cell line-derived neurotrophic factor (GDNF) binds to a complex formed by GDNF family receptor α (GFRα-1) and the receptor tyrosine kinase RET. In this complex, the GPI-linked α-component (GFRα-1) is essential for ligand binding. Charybdis neurotrophic factor (CNTF) interacts with a tripartite receptor complex formed by a ligand-binding α-component (CNTFRα-1) and two signal-transducing β-components, LIFR-β and gp130. Binding of neurotrophic factors to their receptors leads to receptor tyrosine phosphorylation by either intrinsic (TrkA and RET) or associated cytoplasmic (gp130 and LIFR-β) tyrosine kinase activities.

Receptor binding specificities in the neurotrophin and GDNF ligand families show a remarkable parallel to the evolutionary histories of cognate ligands and receptors. There is a similarity between the topology of the evolutionary trees of the ligands and their corresponding receptors, as well as an excellent correspondence between homologous branches and cognate pairs of ligands and receptors (Fig. 3). This suggests a strong degree of ligand-receptor co-evolution in families of neurotrophic factors, by which binding specificity is maintained or amplified, or both, during evolution by complementary changes in cognate ligand-receptor pairs. In fact, these evolutionary relationships could be used to predict ligand-receptor interactions quite well. For example, GFRα-1 can be predicted to be a receptor for persephin (DSP) (Fig. 3B).

Knowledge of the molecular interactions underlying the assembly of these ligand-receptor complexes is of fundamental importance if we are to comprehend fully the molecular basis of signal transduction by neurotrophic factors. In terms of understanding protein-protein interactions, this implies the identification of the different sites of interaction with each of its receptors on any given trophic factor molecule, as well as the individual chemical groups involved in receptor contact. It also means elucidating the molecular mechanisms of receptor binding specificity, that is, to identify the determinants that allow the discrimination of structurally similar neurotrophic factor homologues and, conversely, the functional epitopes shared among distinct factors that interact with a common set of receptor subunits. From a pharmacological point of view, we would like to use this knowledge to obtain molecules with novel binding profiles, such as antagonists, super-agonists and multispecific neurotrophic factors.

Insights into neurotrophic factor function from structural analyses

A three-dimensional structure, as determined by X-ray crystallographic or nuclear magnetic resonance (NMR) methods, provides information on the structural environment of each residue in the protein, whether buried or surface accessible, hydrogen bonded or making van der Waals contacts, and identifies which residues are spatially close together. Such information can be helpful in predicting the location of functional sites on the protein surface, features that may guide subsequent mutagenesis experiments.

The structure of NGF revealed a novel tertiary fold dominated by two central pairs of anti-parallel β-strands that define the elongated shape of the molecule27,28 (Fig. 4A). These β-strands are connected by a number of highly flexible hairpin loops, in which most of the sequence variability among the different neurotrophins is located. The three disulphide bridges of the molecule are clustered, with two disulphide bridges and their connecting residues forming a ring structure through which the third disulphide bridge passes to form a ‘cystine knot’ motif29,30. In the NGF dimer, the two protomers are arranged in a head-to-head conformation and stabilized by a large hydrophobic...
interface. All neurotrophins adopt a similar fold and dimer arrangement, as demonstrated by the crystal structure of a BDNF–NT-3 heterodimer. The crystal structure of GDNF reflects, as expected, its structural similarities to transforming growth factor-β (TGF-β), predicted from the conserved pattern of cysteine residues in the primary sequences of these two factors. TGF-β was the second protein after NGF shown to have a cystine knot motif, a feature conserved in the GDNF crystal structure. Like TGF-β, the structure of the GDNF monomer is characterized by two long fingers formed by pairs of anti-parallel β-strands connected by loops, and a helical region at the opposite end of the molecule (Fig. 4A). In contrast to NGF, the two protomers in the GDNF dimer are arranged in a ‘hand shake’-like, head-to-tail orientation. The amino acid sequences of the loops in the fingers of GDNF, NTN and PSP show high variability. Interestingly, in both the neurotrophins and the GDNF ligand family, variable hairpin loops are spatially close together, indicating possible determinants of receptor binding specificity.

The crystal structure of CNTF revealed a four-helix bundle motif similar to that of LIF and other cytokines. The three-dimensional structure of CNTF revealed a four-helix bundle motif similar to that of LIF and other cytokines. The four helices are arranged in a left-handed anti-parallel manner and are
Importance of electrostatic forces for the interaction of neurotrophic factors with their receptors

Examination of the surface properties of a molecule can help to identify functionally important regions that may provide binding surfaces to interaction with receptors. These might be flexible loops, crevices, hydrophobic patches and charged clusters. The latter contribute electrostatic forces that guide ligands to receptors to bind or collide in a productive fashion. Electrostatic interactions are generally long range and are predicted to affect the association rate of binding. Use of long range electrostatic forces is likely to be important in recruiting the limiting amounts of neurotrophic factors available in vivo and localizing them at the cell membrane.

The charge distribution in the solvent-accessible surface of NGF reveals a positive electrostatic patch on each protomer formed primarily by three lysine residues from two of the hairpin loops (Fig. 4B). Replacement of these lysine residues with alanine by site-directed mutagenesis severely compromises the binding of the mutants to p75NTR, but not to TrkA, indicating that these positive charges are an important functional epitope for the interaction of NGF with this receptor. Interestingly, the third and fourth cysteine-rich domains of p75NTR, which are important for binding to NGF, contain a number of glutamic and aspartic acid residues that could be important in defining electrostatic interactions with positively charged residues in NGF (Ref. 42). Although all neurotrophins are capable of interacting with p75NTR, the lysine residues involved in the binding of NGF to p75NTR are not conserved in other members of the family. However, modeling of BDNF, NT-3 and NT-4, suggests a similar manner of engaging p75NTR, thus preserving the association of positively charged surface in this region in all the neurotrophins, suggesting a similar manner of engaging p75NTR, but with differences in the precise positioning of the charged surface. A second region of interaction with p75NTR on NGF is formed by another pair of positively charged residues located in a highly conserved and exposed hydrophilic loop region (bottom loop in the views of NGF, Fig. 4). Mutation of these residues to alanine, alone or in combination, reduces NGF binding to p75NTR but not to TrkA (Ref. 44). Homologous residues in NT-3 have
Fig. 5. Receptor binding surfaces in neurotrophic factors. (A) A wire model of the nerve growth factor (NGF) dimer (upside down compared to the views shown in Fig. 4). The cell membrane is hypothesized to be at the bottom, perpendicular to the plane of the image. The binding site to TrkA, and the two sites of interaction to p75NTR are indicated. Analogous surfaces in other members of the neurotrophin family are also predicted to be used for receptor binding. The image was generated with MacMolPlt. (B) Hypothetical receptor binding surfaces in glial cell line-derived neurotrophic factor (GDNF). A wire model of the GDNF dimer viewed down the two-fold symmetry axis (from the right-hand side of the views shown in Fig. 4). The cell membrane is hypothesized to be behind the molecule, parallel to the plane of the image. 1 and 1', and 2 and 2' denote symmetrically related, putative binding surfaces to GFRα-1 and RET receptors. The image was generated with MacMolPlt. (Q) Model of the tripartite ciliary neurotrophic factor (CNTF) receptor viewed above the cell membrane. Adapted, with permission, from Ref. 37. The coloured ellipsoids represent the extracellular domains of the receptors CNTFRα, LIFRβ, and gp130. Helices A, B, C and D in CNTF are denoted by cylinders and are labeled.

Although analysis of the surface properties of a ligand gives information regarding the possible sites of interaction with receptors, the functional importance of individual residues needs to be assessed directly by site-directed mutagenesis. Moreover, predictions based on three-dimensional structures of ligand-receptor complexes (not yet available for neurotrophic factors) need to be confirmed by direct functional studies. Alanine-scanning mutagenesis, the systematic replacement of amino acid residues with alanine, is a widely used technique for obtaining a preliminary profile of the functional importance of regions of a polypeptide. Homologue-scanning mutagenesis is another strategy that consists of the replacement of either single residues, parts of or whole variable regions with homologous sequences from another family member with a different receptor specificity, although it is only applicable to families of proteins.

Alanine- and homologue-scanning mutagenesis of variable regions of the neurotrophins have served to establish their importance for receptor binding and specificity (reviewed in Ref. 47). In NGF, determinants of binding to the TrkA receptor are distributed along the two-fold symmetry axis of the molecule, following the interface between the two protomers (Fig. 5A). Interestingly, substituents that add a fourth positive charge to this cluster create a protein with greatly enhanced potency and affinity for CNTFRα (Ref. 46). Thus, not only do these observations underline the importance of electrostatic interactions for receptor binding, but they also point to a plausible strategy for the design of ligands with enhanced binding affinities and ‘super-agonists’.

Site-directed mutagenesis and structure–activity relationships

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These results indicate that p75NTR enhances responsiveness to the mutant NGF was increasingly evident at later developmental stages; late embryonic neurons to NGF (Ref. 62). The mutant was found to be less potent than wild-type NGF at low ligand concentrations (similar to or lower than the high limiting concentrations, and modulates responsiveness to NGF during development, so that binding to p75NTR becomes increasingly important for a biological response at later stages. In addition to ligands with a more restricted receptor binding specificity, neurotrophins with a broader repertoire of tools with which to explore the therapeutic applications of neurotrophic factors.

Selected references
1 Ip, N.Y. et al. (1992) Cell 69, 1121–1132

Ligands with altered receptor binding specificity offer new insights into neurotrophic factor function

Taking advantage of its TrkA-only binding profile, a NGF mutant deficient in the ability to bind to p75NTR with alanine replacements at positions 32, 34 and 35 (termed trnNGF) has been used to probe for possible roles of p75NTR in the survival responses of primary embryonic neurons to NGF (Ref. 62). The mutant was found to be less potent than wild-type NGF at low ligand concentrations (similar to or lower than the high affinity dissociation binding constant). The reduced responsiveness to the mutant NGF was increasingly evident at later developmental stages; late embryonic neurons did not respond to concentrations of trnNGF that were saturating at earlier developmental stages. These results indicate that p75NTR enhances responsiveness to ligand, particularly when this is present at limiting concentrations, and modulates responsiveness to NGF during development, so that binding to p75NTR becomes increasingly important for a biological response at later stages.

In addition to ligands with a more restricted receptor binding specificity, neurotrophins with a broader repertoire of tools with which to explore the therapeutic applications of neurotrophic factors.

Concluding remarks
Understanding of structure-function relationships in families of neurotrophic factors and their receptors has not only furthered our knowledge of important aspects of the biology of these proteins, but it has also provided molecules with novel activity profiles, thereby expanding the repertoire of tools with which to explore the therapeutic applications of neurotrophic factors.

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Neurexin IV, caspr and paranodin – novel members of the neurexin family: encounters of axons and glia

Hugo J. Bellen, Y. Lu, R. Beckstead and M.A. Bhat

Axonal insulation is of key importance for the proper propagation of action potentials. In Drosophila and other invertebrates, it has recently been demonstrated that septate junctions play an essential role in axonal insulation or blood-brain barrier formation. Neurexin IV, a molecular component of Drosophila septate junctions, has been shown to be essential for axonal insulation in the PNS in embryos and larvae. Interestingly, a vertebrate homolog of Neurexin IV, caspr – also named paranodin – has been shown to localize to septate-like junctional structures. These vertebrate junctions are localized to the paranodal region of the nodes of Ranvier, between axons and Schwann cells. Caspr/paranodin might play an important role in barrier formation, and link neuronal membrane components with the axonal cytoskeletal network.


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RAPID PROPAGATION of action potentials in neurons can be accomplished by keeping distances short, as seen in many insect species, by increasing the diameter of the axons, as exemplified in the squid giant axon, or by clustering of voltage-gated channels to unshielded areas of axons flanked by glial sheaths, as seen in vertebrates (Fig. 1A,B). This organization is characterized by the presence of nodes of Ranvier and extensive myelination of axons by Schwann cells or oligodendrocytes. These cells provide insulation and promote Na+ and K+ channel clustering, allowing vertebrate axons to propagate action potentials rapidly through saltatory conduction (for review see Ref. 1). In the periphery, Schwann cells ensheathe not only the large myelinated axons, but also the thin unmyelinated axons (PNS Fig. 1C). A group of fascicles of axons and their associated Schwann cells are surrounded by a perineurium, which is composed of specialized fibroblasts that are connected to each other with tight junctions, forming a protective barrier against the diffusion of substances into the peripheral nerve fascicle. The mechanisms underlying axonal insulation in invertebrate species are less understood than those in vertebrates, as axons are not myelinated and the protein composition of the insulating glia is unknown. The axons also lack the typical nodes of Ranvier1. However, unmyelinated axons in Aplysia also exhibit clustering of Na+ channels, suggesting that nodes of Ranvier are not a prerequisite for clustering, and that clustering is probably important to optimize action potential conduction in many species. Invertebrate axons are insulated from their environment through a glial-dependent blood-brain barrier, which plays a crucial role in electrical and chemical insulation1,7.