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Neurotrophic factor structures reveal clues to evolution, binding, specificity, and receptor activation

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Abstract. The neutrophin family, the glial-derived neurotrophic factor family, and the ciliary neurotrophic factor are the best described growth factors specific for developing neurons and neutral crest cells. As might be expected for regulatory molecules of the complex central and peripheral nervous system, these factors show considerable receptor specificity and cross-talk. Thanks to a decade of intense research by numerous laboratories, the structures of many of these factors are now available. This review discusses the structural bases of receptor binding, specificity, and activation in each of these systems. Using structure-based sequence alignments, the evolutionary implications of these molecules and their receptors are discussed, followed by suggestions for further directions for research on the structure and function of these neurotrophic factors.

Key words. Neurotrophin; glial-derived neurotrophic factor; ciliary neurotrophic factor; structure; alignment.

Introduction

Many growth factors play a critical role in the development, maturation, maintenance, and programmed cell death of the nervous system. While dozens of proteins are implicated in these roles, there are currently three families of well-understood growth factors that are specific to the nervous system: the neurotrophin (NT) family, the glial cell-derived neurotrophic factor (GDNF) family, and the ciliary neurotrophic factor (CNTF). The members of these families signal the developing and mature organism to grow, form differentiated cells, and survive disease or injury.

Nerve growth factor (NGF) is the prototype member of the neurotrophin family, which has at least five other members. NGF was discovered in the 1940s by Stanley Cohen and Rita Levi-Montalcini in moccasin snake venom [1]. NGF and its siblings, brain-derived neurotrophic factor (BDNF), NT-3, NT-4/5, NT-6, and NT-7, provide the signal for growth, differentiation, and programmed cell death for developing and mature neurons and other neural crest-derived cells. Neurotrophin signaling underpins the development of numerous biological systems, including the brain and peripheral nervous system. Beyond these, neurotrophins have been found to play a role in retinal [2], cochlear [3], and heart development [4, 5]. Moreover, neutrotrophins are essential for maintaining the function of neurons in the adult. In addition, derangements of the neurotrophin signaling system are implicated in Alzheimer's disease [6], peripheral neuropathy [7, 8], neuroblastoma [9–11], and many neurodegenerative and neuronal storage diseases. Indeed, BDNF and NT-3 have been in clinical trials for peripheral neuropathy and adrenoleukodystrophy. The neurotrophins signal through two receptor types (fig. 1 A). First are the Trk receptors, a family of three nanomolar-affinity receptors (called TrkA, TrkB, and TrkC) each specific for a neurotrophin (NGF, BDNF or NT-4, and NT-3, respectively) with an intracellular tyrosine kinase domain that stimulates the mitogen-activated protein (MAP) kinase pathway when activated. The second receptor is p75, a nanomolar-affinity tumor necrosis factor receptor (TNFR) family member that signals through an intracellular death domain to activate the cellular apoptosis machinery. Excess p75 receptors may also act in consort with Trk when neurotrophin concentrations are low, producing a sub-nano-

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molar binding site [12], though details of this interaction are not well under-stood.

The GDNF was found in a search for secreted factors that promoted neuronal survival in primary culture by Lin in 1993 [13]. GDNF was cloned from a glial cell line culture, and quickly established prominence as a potent stimulant of dopaminergic neurons. Three other members of the GDNF family have been identified: persephin [14], neurturin [15], and artemin (also called enovin and neublastin) [16]. GDNF plays a critical role in kidney [17–19] and enteric nervous system [20] development. The GDNF system has an important medical role, as mutations have been linked with medullary thyroid tumors [21], the multiple endocrine neoplasia type 2 (MEN2A and MEN2B) syndrome [22], and Hirschprung's disease [23, 24]. Like the neurotrophins, the GDNF family signals through two receptor families (fig. 1B): the GDNF family receptor alpha (GFRα) family of glycosylphosphoinositol (GPI)-linked receptors and Ret, a tyrosine kinase receptor. GDNF family members predominantly signal in a two-step mechanism, first uniting two GFRα receptors, which then join with two Ret receptors, forming a heterohexameric complex. This then activates numerous intracellular signaling mechanisms including the MAP kinase and phosphatidylinositol 3-kinase pathways [25]. Each GDNF family member has a preferred GFRα receptor and vice versa, through there is considerable cross-talk between the receptors (fig. 1B) [26–28].

CNTF was discovered in 1979 by Varon's group in a search for growth factors in the ciliary ganglion [29, 30]. The primary role of CNTF remains elusive, though it contributes to the development and differentiation of retinal rod and other neurons. It may also contribute to weight regulation [31] by its influence on the hypothalamic arcuate nucleus, and is currently in clinical trials for this purpose. CNTF is structurally similar to the interleukin (IL)-6 family of hematopoietic cytokines, and it signals using common cytokine receptor components. CNTF first associates with CNTFRα, a specific GPI-linked receptor, then binds to the cytokine receptors gp130 and leukemia inhibitory factor receptor (LIFR), which activate the Janus kinase/signal transducer and activator of trancription (JAK/STAT) signaling pathway [32].

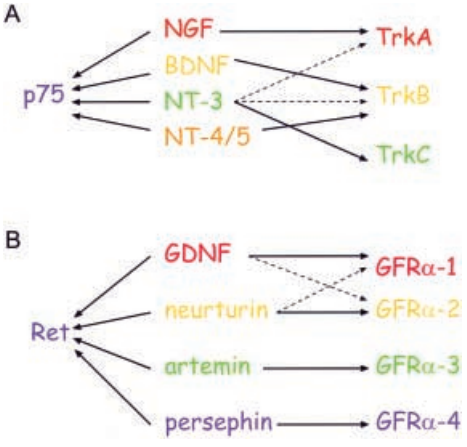

Figure 1. (*A*) The neurotrophins signal through two separate receptor systems, the Trk family of tyrosine kinase receptors, and p75, a death domain-containing tumor necrosis factor receptor-like receptor. While significant specificity is seen among the Trk receptors (solid lines), there is some cross-talk that may be evolutionarily reconciled (dashed lines). p75 binds all the neurotrophins equally. (*B*) The GDNF family of neurotrophic factors also binds two separate receptor systems: Ret, a glucosylphosphoinositol-linked surface receptor, and the GDNF family receptor alpha (GFRα). Like the neurotrophins, considerable specificity and cross-talk are seen.

After a decade of intense research, the crystal structures of many neurotrophic factors are now available. In addition, the recent explosion in genomics research has given us dozens of amino acid sequences of neurotrophic factors from dozens of species. Evidence from these sources in additon to the results from biochemistry experiments reveal clues to the evolution, binding, and specificity of these molecules. This review attempts to summarize these results and suggest further directions for research on the structure and function of the neurotrophic factors.

Sequence alignments and molecular evolution

Comparing the amino acid sequences of the neurotrophic factors can give insight into the evolutionary history of these families and can help reveal functionally important regions of the molecules. A structure-based sequence alignment was prepared using all currently known neurotrophins (fig. 2), representing over 30 species. As the figure shows, there are six major sub-families of neurotrophins, including NGF, BDNF, NT-3, NT-4/5, the human NT-6s, and the fish NT-6 and NT-7. Two NGF-like molecules from the fowlpox virus genome are included, although it is not known if these proteins are even transcribe; however a virus that can secrete NGF would clearly be at a competitive advantage including promoting survival of infected cells [33].

Previous works have divided neurotrophin residues into two categories: conserved and variable, based on early sequence alignments [34]. Most of the residues that participate in secondary structure are strongly conserved both within and between sub-families, though there are numerous exceptions. The N and C termini and loops 2 and 3 show modest diversity between families. Loop 3 has a large insert in the NT-4 and NT-6 sub-families. In fish NT-6, this region is thought to confer binding to heparin as a regulatory control mechanism [35]. Interestingly, two cysteines that make up the knot are missing from the human NT-6 molecules. Those residues implicated in binding that are conserved across families are thought to represent a common interface to the Trk re-

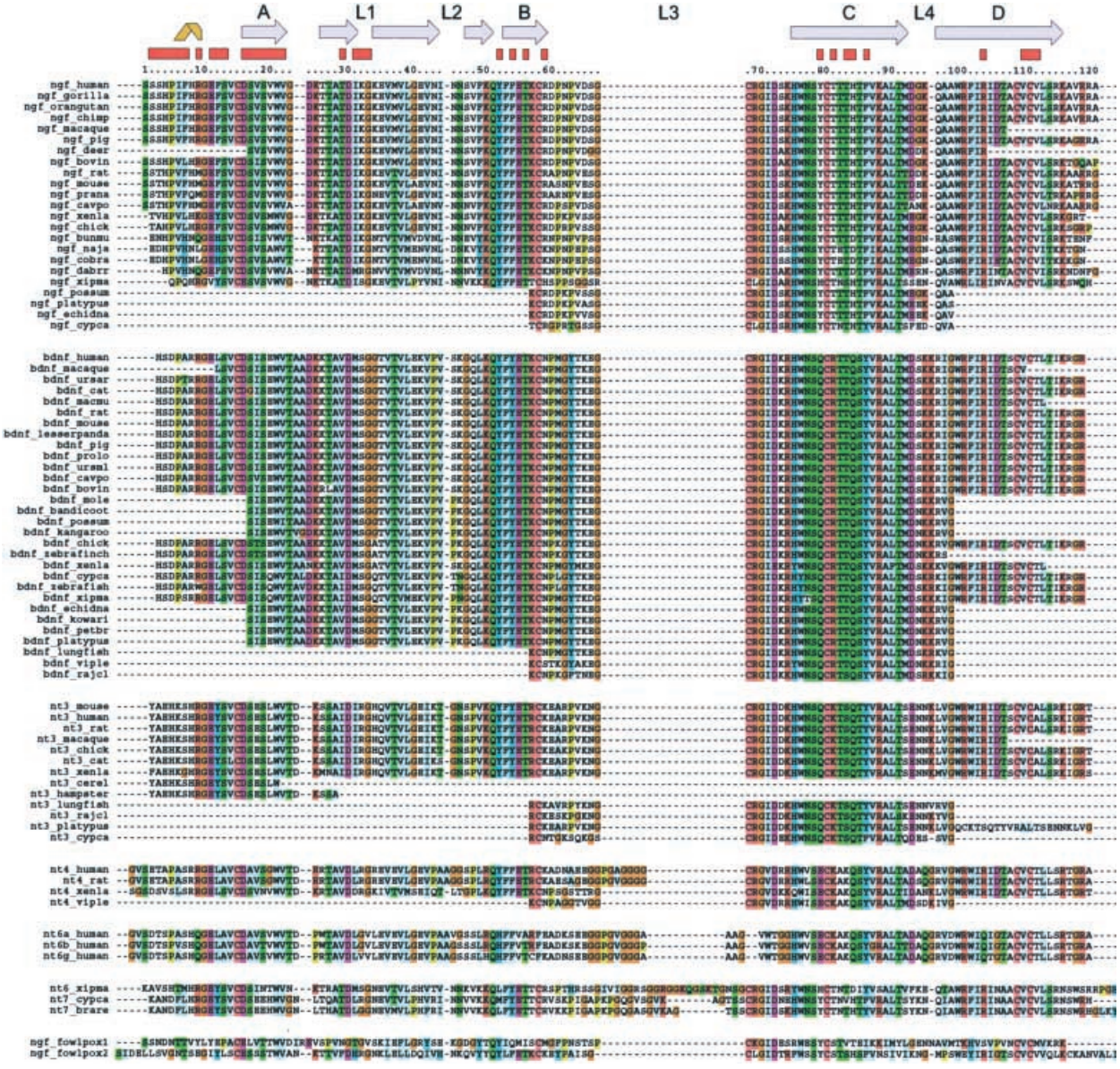

Figure 2. Structure-based sequence alignment of the known neurotrophins, grouped by sub-family. The secondary-structure assignments from PDB structure 1www are at the top (orange; helix; purple arrow; beta strand). The red bars along the top indicate residues seen in the interface with TrkA. The four core beta strands are designated by letter, and the four loops are indicated as well. Sequence numbering is based on NGF at the top. Many of the sequences are only fragments, and they are grouped at the bottom of each subfamily. The families clearly share similarities, especially along regions of secondary structure. Two NGF-like neurotrophins from the fowlpox virus are included at the bottom. The species is indicated next to each neurotrophin. Certain abbreviations include: prana, *Praomys natalensis* (African soft-furred rat); cavpo, *Cavia porcellus* (domestic guinea pig); xenla, *Xenopus laevis* (African clawed frog); bunmu, *Bungarus multicinctus* (many-banded krait); dabr; *Daboia russelli russelli* (Russell's viper); xipma, *Xiphophorus maculates* (southern platypus); cypca, *Cyprinus carpio* (common carp); macmu, *Macaca mulatta* (rhesus monkey); prolo, *Procyon lotor* (raccoon); petbr, *Petaurus breviceps* (sugar glider); is *Raja clavata* (thornback ray); viple, *Macrovipera lebetina* (Levantine viper).

ceptors, while the unique ones may represent elements of specificity [36].

Neurotrophin evolution has been studied using phylogenetic trees that organize the relationships between their amino acid sequences [37]. Early work used limited subsets of the currently known neurotrophins [37–43], though the general conclusions have been consistent: the ancestral neurotrophin underwent a gene duplication to form the branches that would lead to NGF and NT-3 on one side and BDNF and NT-4 on another. Another round of gene duplications resulted in the current set of four principal neurotrophins in the tetrapods. No ortholog of the NT-4 gene has been found in teleost fishes, and no ortholog of the NT-6 or NT-7 genes has been found in tetrapods (although the names are similar, the human NT-6 α, β, and γ human genes are closer to NGF). These findings

place constraints on the timeline of gene duplications to before 400 million years ago [43]. If a similar tree is prepared for the Trk receptors, the general pattern of interrelationships is upheld: TrkA and TrkC share a common branch of a tree, while TrkB lies on a separate branch [37]. That the receptor and ligand trees are 'parallel' supports the notion of co-evolution of these two families, as seen in other protein families [44].

A similar sequence alignment was prepared for the known GDNF family members (fig. 3). This reveals strong conservation, especially within secondary-structure elements. Within each sub-family, most changes are conservative. GDNF shows a significantly larger N-terminal tail than other members of the family. This tail is not important for GFRα1 binding [26], and its role is currently unknown. The helical region in persephin is likely one turn shorter than in the other members, and the succeeding loop region is more glycine rich, perhaps to accommodate more dramatic structural turns need to return to the next beta strand.

Until very recently, CNTF was in a sub-family by itself. The dramatic contrast between the phenotypes of the CNTF and CNTRFα knockout mice suggested the existence of another ligand for CNTFRα, which was recently discovered [45]. Figure 4 shows the structure-based sequence alignment of the known CNTFs as well as the newly identified molecule, cardiotrophin-like cytokine (CLC). The alignment shows strong conservation among the CNTFs, with the exception of the chicken homolog, which may reflect a different role for CNTF in that species [46]. The alignment also shows that CLC is only weakly related to the CNTF family, with 23–27% identity between CLC and the various CNTFs and with a significantly longer N-terminal region. A handful of charged and bulky hydrophobic residues are preserved, especially in helices B – D. More importantly, the residues identified by mutagenesis to be responsible for binding CNTFRα (site I, discussed below) appear to be well-conserved between CNTF and CLC, suggesting a common mechanism of binding.

Neurotrophin structures

The neurotrophins and GDNF family members are members of a large superfamily of growth factors that contain a cysteine 'knot.' This family includes transforming-growth factor (TGF)-β, human chorionic gonadotropin, platelet-derived growth factor, vascular endothelial growth factor, and many others. The cysteine knot consists of three

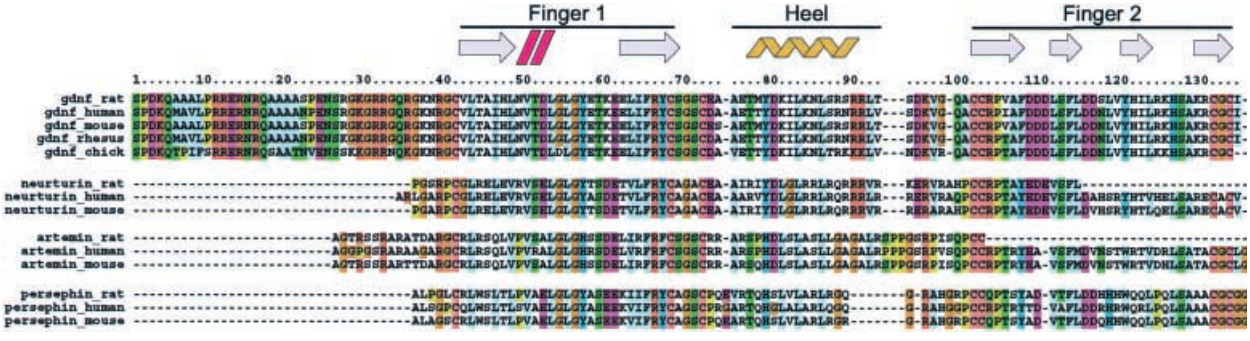

Figure 3. Structure-based sequence alignment of the known GDNF family members. The secondary-structure assignments are at the top (red, turn; orange, helix; purple arrow, beta strand). Residue numbering is based on GDNF at the top. The regions called the fingers and the heel are designated as well.

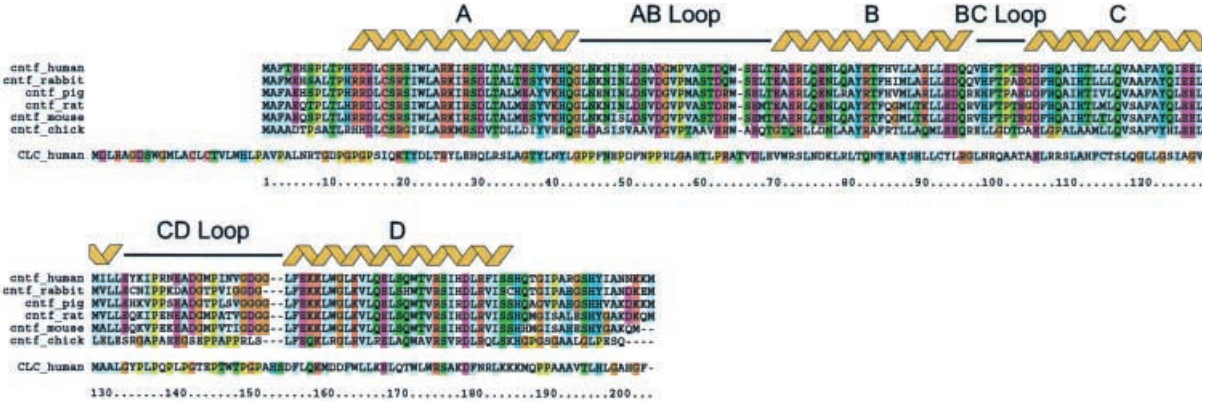

Figure 4. Structure-based sequence alignment of the known CNTFs, including the newly identified cardiotrophin-1-like cytokine (CLC).

Table 1. List of all the neurotrophic factor structures determined to date. Protein data bank (PDB) entries can be found at http://www.pdb.org.

Molecule	PDB ID code	Highest resolution (Å)	Reference
NGF homodimer	1bet	2.3	97
NGF homodimer	1btg	2.5	98
BDNF/NT-3 heterodimer	1bnd	2.3	99
NT-3 homodimer	1nt3	2.4	49
NT-4 homodimer	1b98	2.75	100
NT-3 homodimer	1b8k	2.15	100
BDNF/NT-4 heterodimer	1b8m	2.75	100
NGF + TrkA domain 5	1www	2.2	36
GDNF	1agq	1.9	101
CNTF	1cnt	2.4	80

disulfide bonds that form a true knot of the polypeptide chain. All the members of this family exist exclusively as dimers, though a variety of protomer arrangements are seen, including heterodimers and homodimers, covalent and non-covalent association of the promoters, and different spatial configurations of the promoters (head-to-toe, head-to-head, and skew). Oddly, there are other non-growth factor proteins that have three disulfide bonds in a knotted arrangement, but with a different configuration of disulfide bonds. One example is the invertebrate toxin omega-conotoxin, which curiously acts as a calcium channel antagonist.

The three-dimensional structures of most of the human neutrotrophins have been determined (table 1). These structures can be aligned to reveal that the regions of similarity are much larger than was suggested from sequence alignments [34]. The core structure consists of two pairs of intertwined two-strand beta sheets, joined by three disulfide bonds (fig. 5). There are also three shorter beta strands leading to beta turns and loops. The four core beta strands are virtually superimposable across all the structures with less than 1 Å deviation. Loop 3 is the most different among the four neurotrophin structures. NT-3 has a single loose helical turn in this region. NT-4 has a large insertion in this portion, including a small eight-residue beta turn, but the structure of much of this region has not been clearly determined (PDB entry 1b8m chain B or 1b98). This region also appears unstructured in the TrkA-NGF co-crystal structure, and does not appear to lie on the interface with TrkA domain 5. It may play a minor role in the interface with the p75 receptor [47–49] in NT-3 and NGF. The N- and C-termini are highly variable in both sequence and structure among the neurotrophins, and the temperature factors of these loosely structured residues are comparatively high. The N-terminus assumes a helical structure upon binding the Trk receptor, as will be discussed below. The role of the C-terminal residues is not known, though they may participate in p75 binding in NT-3 [48] and NGF [50].

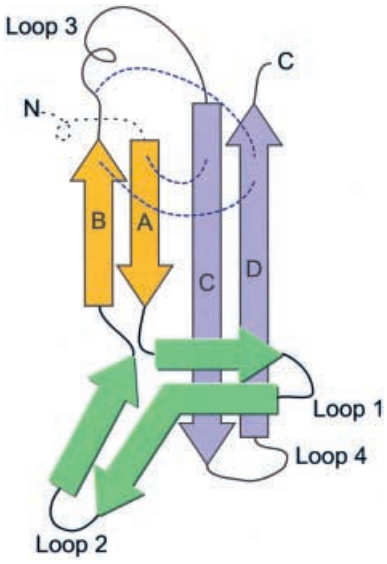

Figure 5. Schematic of the neurotrophin molecule. Dashed blue lines represent the three disulfide bonds of the cysteine knot. The N terminus is disordered in the unbound structures and is shown by a dashed line.

Structural details can also be used in conjunction with alanine-scanning mutagenesis, chimeras, and deletion mutations to synthesize a coherent model of receptor binding. However, at least three major factors relevant to the neurotrophin literature confound simply mapping the results of loss-of-function mutation experiments to reveal functionally important regions. Numerous early experimenters produced but failed to purify mutant neurotrophins [see for example refs 50, 51]. In a heterologous expression and assay system, such as transiently transfected PC12 cells, wild-type neurotrophins additionally produced by the cells could easily confound experiments [52]. Unpurified proteins might be mixed with other cellular products like kinase inhibitors, which could explain away otherwise significant kinetics results or biological assays. Second, some mutations, especially ones that cause significant structural rearrangement or instability, for example by damaging the dimer interface, can result in limited expression of the mutant protein. Critical residues that lie on the binding interface might thus be unintentionally excluded from analysis merely because of low production yield. Finally, mutations in one part of a molecule may show loss-of-function, and thus be incorrectly included in the putative interface, by inducing structural changes in a receptor contact surface in another part of the molecule. For these reasons, the actual interface has always been found to be a subset of those residues identified by mutagenesis [53].

With these limitations in mind, site-directed and alanine-scanning mutagenesis can still be useful tools for inferring the binding interface in the absence of a co-crystal structure [53]. The results of several mutagenesis experi-

ments over the past 10 years have revealed a consistent pattern of spatially distinct portions of the neurotrophin dimer molecule that are critical for Trk and p75 receptor binding. The structure of the NGF dimer in complex with domain 5 of the TrkA receptor validates many of these experimental predictions (fig. 6A). By coparing the residues involved in the interface between TrkA-d5 and NGF with the corresponding sequence alignments for the neurotrophins and the Trk receptors, they can be seen to form two groups of conserved and non-conserved residues.

One set of residues making up a large patch and centered on Arg 103 form a common set across all the neurotrophins and receptors (fig. 6B). As these residues are highly homologous and consist mainly of hydrophobic and aromatic side chains, they probably mediate the bulk of common binding affinity between neurotrophin and receptor. Another example is the hydrophobic region centered on Trp 21, a universally conserved residue that is principally buried by Phe 86 (which is either Phe or Tyr in all neurotrophins) and Phe 101 (which is either Phe or Trp in all neurotrophins) on the neurotrophin side, and on the receptor side, by His 353 (universally conserved), the side chain of Met 379 (either Met or Leu in TrkA, B, and C), and Pro 382 (universally conserved).

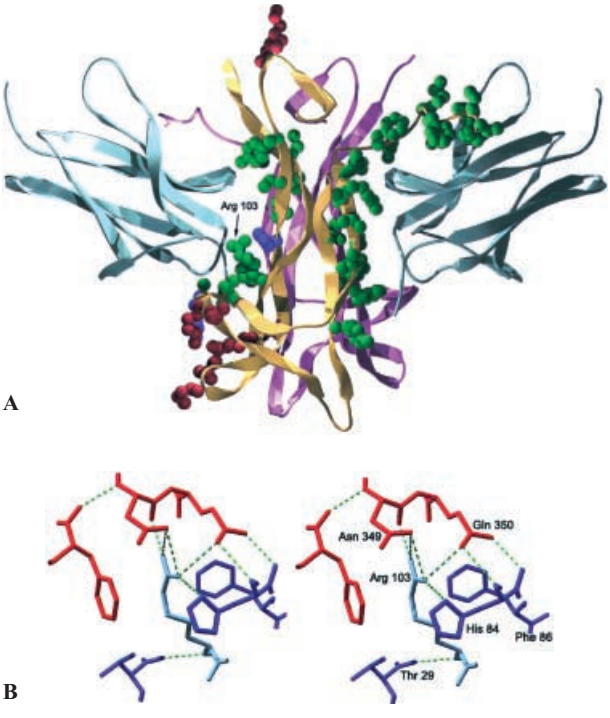

Figure 6. (*A*) Structure of the NGF homodimer with residues of the gold protomer highlighted in red or green if they affected binding to p75 or TrkA, respectively, upon mutagenesis (those that affected both are highlighted in blue). TrkA domain 5 is shown in silver. (*B*) Stereo view of NGF Arg 103, the centerpiece of the common binding region, and its intimate spatial relationship with key residues in TrkA domain 5 (in red) and NGF (in blue). TrkA residue Phe 327 is conserved as Phe, Tyr, or Ile in TrkB and TrkC; Asn 349 is either Asn or Gln; and Gln 350 is either Asn or Lys. TrkA residue His 84 is conserved as Gln in BDNF, NT-3, and NT-4; Phe 86 is either Phe or Tyr; and Thr 29, which forms a main chain hydrogen bond, is either Val or Ile with similar-sized side chains.

The other portion of the TrkA interface centers on the N-terminal resiues of the neurotrophin. As mentioned before, these residues show little conservation, either on the neurotrophin or the receptor side. The N-terminus, which was disordered in all the unbound neurotrophin structures, forms a one-and-a-half-turn helical arrangement in the complex between TrkA and NGF. When NT-3 and BDNF chimeras containing the NGF N-terminus were constructed, they showed enhanced ability to bind TrkA [54, 55], supporting the importance of this region for NGF specificity. NGF binding buries two helical hydrophobic residues and creates a salt bridge across the interface. BNDF, NT-3, and NT-4 do not share the same pattern of residue types at the N-terminus, and TrkB and TrkC also differ in their corresponding interacting residues [36]. Together, these results suggest that the N-terminal residues help determine receptor binding specificity and that each neurotrophin probably uses a different specific interface with its cognate receptor in this region. Other specificity-determining residues appear to lie scattered within the common binding site. For example, NGF point mutants introduced from the NT-3 sequence that conferred TrkC binding were centered around Gly 23 in one study [56]. Another study suggested TrkC specificity is determined by broadly separated patches across the length of the NT-3 molecule [57].

The putative neurotrophin-binding site for the p75 receptor has received similar attention as the Trk rerceptors. Unlike the Trk receptors, p75 binds all the neurotrophins equally, with high affinity [58] and much faster on- and off-rates [12, 59]. While the neurotrophins can clearly signal in the presence of either the Trk or p75 receptor alone, evidence suggests that excess p75 can improve Trk signaling by 75-fold when neurotrophin concentrations are low [12, 60]. This may imply either simultaneous binding of p75 and Trk receptor to a neurotrophin (the hand-off model), which is not strucutrally excluded [36, 49] (fig. 6A), or in serial steps that improve Trk kinetics by increasing the local concentration of neurotrophin. Supporting these models is recent evidence suggesting that p75 and Trk receptors may lie in preformed heteroreceptor complexes on the cell surface [61–63].

One unique feature of the p75-neurotrophin interface is the contribution of charged residues rather than buried surface area to stabilization of the complex [64]. Primarily positively charged residues on the neurotrophin side of the interface corrrespond to negative charges on p75, creating a network of paired charges. Mutating these charged residues significantly affects the local net charge and disrupts binding [65–67]. NGF, NT-3, and NT-4/5 display positively charged Arg, His, and Lys residues on loop 1, while BDNF displays these residues on the spa-

tially close loop 4 (fig. 5 and 6A). Besides these charged clusters, residues in loop 3 [47, 68] and the C terminus [48, 50] play a less significant role in p75 binding.

While neurotrophin structural research is the most advanced among the three families discussed here, numerous questions remain open. Though the structure of TrkA domain 5 in complex with NGF significantly advanced understanding of their interaction, evidence supporting the role of other TrkA domains still requires explanation. In additon, the determinants of specificity for BDNF and NT-3 could be elucidated by co-crystal structures of these neurotrophins with their receptors. The structural basis of the facilitation of Trk activation by p75 also remains unclear. The structure of the p75-neurotrophin complex would clarify the structural bases of binding and promiscuity of this receptor. Finally, the increasing interest in peptides [69–71] and small molecules [72] that mimic the neurotrophin-receptor interaction should drive structural examinations into these alternatives to define the minimal interface needed for receptor activation.

GNDF structure

Like the neurotrophins, members of the GDNF family can interact with two different receptors, probably simultaneously. Within the GDNF family, only the structure of GDNF itself has been determined. It shows the usual cysteine knot fold, with two pairs of anti-parallel twisted beta strands tightly joined by three disulfide bonds. The dimer arrangement, however, is anti-parallel, has an interchain disulfide bond, and due to a large lack of overlap has the appearance of two 'fingers' on each side of the molecule (fig. 7). This places GDNF in the TGF-β subfamily of cysteine knot growth factors. Because the protomers are arranged anti-parallel, an explicit left-right symmetry is created, suggesting symmetric binding sites for a dimerized receptor. Between strands three and four, where the neurotrophins have a large variable region, GDNF has a three-turn alpha helix. The large N terminus was not seen in the crystal structure, suggesting that this region is flexible in solution.

Figure 7. The structure of GDNF, a cysteine knot growth factor dimer arranged in head-to-toe manner. Residues from each protomer are highlighted in red and greeen if they affected binding to the GFRα1 receptor upon mutagenesis.

Limited mutagenesis has been performed on the GDNF molecule to determine its interaction site with the Ret and GFRα receptors. Eketjäll and colleagues [73] extensively changed surface-exposed GDNF residues to alanine and determined binding to Ret and GFRα1 using a steady-state competitive-binding assay. These results improve on other studies [74] and show that there are two principal modes of GDNF signaling. The first involves GDNF activating a preformed Ret-GFRα1 complex. This model is supported by evidence that GDNF mutant Tyr 120 $\rightarrow$ Ala is deficient in binding to GFRα1 but can still activate Ret [73], and that ligand-independent Ret signaling is enhanced by the presence of GFRα1 [75]. Acidic residues along finger 1 were shown to be critical for binding to GFRα1 (fig. 7) [73]. Baloh et al. [26] showed that two patches along finger 2 transplanted from GDNF into a persephin backbone are sufficient to induce activation of GFRα1-Ret, suggesting overlapping sites for each receptor. Surprisingly, the positively charged band of residues at the center of the dimer appeared to play no role in GFRα1 binding. Overlapping sites do not necessarily preclude simultaneous binding of both receptor types to GDNF, as the site on each end of the molecule could be used to associate with a different receptor. GFRα2 and GFRα3 binding and activation appear to require both the finger 2 regions needed in GFRα1 activity as well as part of the loop and helix in the heel region [26].

A second structural model of GDNF signaling, launched via Ret-independent GFRα1 activation, has been discussed [27, 76]. Alanine-scanning mutagenesis revealed that acidic and hydrophobic residues along GDNF fingers 1 and 2 affected binding to GFRα1 but not Ret activation. Increasing evidence supports the notion that GPI-linked receptors, like GFRα1, which have no transmembrane or intracellular region, can signal via activation of Src family tyrosine kinases by virtue of sharing a special, detergent-insoluble patch of membrane [77–79]. Clustering of the extracellular receptors is thought to induce activation of the intracellular kinases by proximity. Whether this functionality of GFRα1 has a biological role remains to be demonstrated.

These results suggest that acidic and hydrophobic residues along the exposed fingers play a critical role in mediating GDNF activation of both the Ret-GFRα 1 complex and GFRα1 alone. Further work is needed to clarify the biological roles of each of these signaling mechanisms. Structures of GDNF in complex with each of the receptor types, taking advantage of GDNF mutants that confer specific binding, would help reconcile apparently overlapping binding sites and inconsistencies in the biochemical evidence. Furthermore, structures of the other GDNF family members are needed to detail the structural basis of receptor specificity and cross-talk.

CNTF structure

CNTF is not a cysteine knot growth factor, but rather a four-helix bundle belonging to the IL-6 family of hematopoietic cytokines. The crystal structure revealed a dimer, thought to be an artifact of the high concentrations used in crystallization [80]. The basic structure shows four helices, named A–D, with two long cross-over loops (AB and CD) and one short loop (BC) (fig. 8). Much of the AB and the C-terminal part of the CD loops were not seen well in the electron density map.

CNTF is thought to bind three separate receptor partners: CNTFRα, gp130, and LIFR. Both immunoprecipitation [81, 82] and gel filtration with analytical ultracentrifugation experiments performed on IL-6 and CNTF support the notion that the active CNTF receptor ceomplex is hexameric with stoichiometric ratios of 2:2:1:1 CNTF:CNTFRα:gp130:LIFR [83]. However, these experiments were performed at concentrations well above the relatively weak dimerization constant of CNTF (40 µm), which could explain why large complexes were seen. There is also evidence from molecular modeling that the hexameric complex is inconsistent with the known size of CNTF [84]. In contrast, two additional models of the CNTF receptor complex have been proposed that are consistent with published mutagenesis data (fig. 9) [85]. The simplest of these suggests a tetrameric complex and still preserves the known interactions.

The CNTF molecule is thought to contain three binding sites, for each of its three receptors. These sites are necessarily distinct, as these receptors bind simultaneously, and are numbered sequentially in analogy to the growth hormone receptor model. Careful regional mutagenesis and chimeras of CNTF and IL-6 have shown that site I, which is responsible for binding the respective alpha receptor (CNTFRα or IL-6α), comprises the C-terminal AB loop and the C-terminal D helix [80, 84, 86–91]. Site II is the gp130-binding site (the primary of two such sites

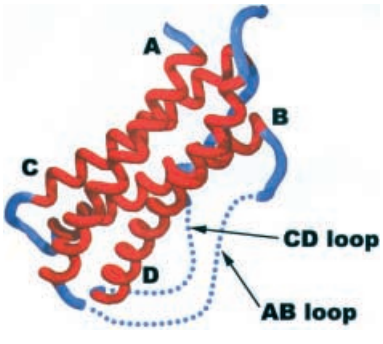

Figure 8. The structure of CNTF, a four-helix bundle protein similar to the IL-6-family of hematopoietic cytokines. Letters are positioned near the N terminus of each helix. Two long loops AB and CD were not resolved in the structure and are respresented by dashed lines.

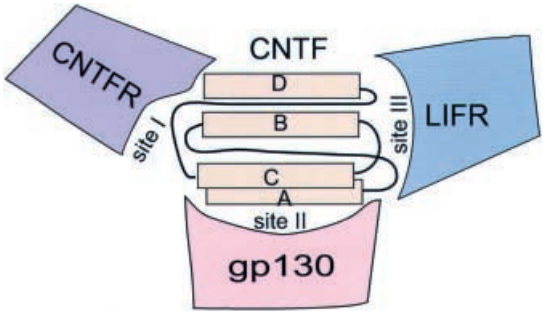

Figure 9. Scheme showing one possible binding model of CNTF with its receptors CNTFRα, gp 130, and LIFR in a 1:1:1:1 stoichiometry.

in IL-6, and the only one in CNTF), which has been localized to residues on the A and C helices [80, 92–94]. While site III in IL-6 plays the role of an additonal gp130-binding region, in CNTF it is responsible for binding LIFR. This surface is distributed across three regions that are spatially located at one end of the helical bundle, comprising the C-terminal A helix and N-terminal AB loop, the BC loop, and the C-terminal CD loop and N-terminal D helix [85]. The minimal surface needed to bind LIFR may be smaller, as is seen in the LIF interaction surface with LIFR [95, 96].

Structural and biochemical research on CNTF is the least complete of the three families discussed here. Open questions remain concerning the specific meachanism of receptor recruitment and activation. While IL-6-related results can be extrapolated to the CNTF system, obvious differences in gp130 and LIFR interaction highlight the need for specific investigations into CNTF binding. The structure of CNTF in complex with each of the receptors would refine understanding of the binding sites and lend support to particular models of receptor assembly. Furthermore, the discovery of the new CNTFRα ligand, CLC, demands a structural investigation. Common site I residues between CNTF and CLC should be validated by structural investigation, as these would circumscribe the surface needed for CNTFRα interaction.

Conclusion

This review has considered the three major families of neurotrophic growth factors: the neurotrophins, the GDNF family, and CNTF. The crystal structures of the neurotrophins show the classic cysteine knot growth factor structure with head-to-head sub-units forming a noncovalently linked dimer. The only significant differences between neurotrophins are found in the loops and turns between beta strands, especially loop 3. Mutagenesis and other biochemical techniques, as well as the co-crystal structure of TrkA with NGF, suggest that neurotrophin-Trk binding revolves around a patch of common interac-

tions centered around Arg103 and a patch of specific interactions primarily centered on the N terminus. GDNF and its family members are cysteine knot growth factor dimers arranged head-to-toe, covalently linked by a disulfide bond. Structural and sequence analysis of the GDNF family suggests that acidic and hydrophobic residues along fingers 1 and 2 are necessary for binding and activation of the Ret-GFRα1 preformed complex, the principal form of GDNF activity. A distinct site may be required for Ret-independent GFRα1 activation, although how this signal is transduced is not well understood. CNTF is a four-helix bundle in the IL-6 family of hematopoietic cytokines. It binds to three different receptors simultaneously to activate a signal for neuronal survival. Drawing from mutagenesis and chimera experiments with IL-6, the three binding sites have been localized to distinct surfaces on the CNTF molecule. Open questions remain about the true stoichiometry of the receptor complex and the structural basis of co-ordinated receptor binding.

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