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Perspective

Molecular Basis of Neurotrophin−Receptor Interactions

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1. Neurotrophins and Their Receptors

Neurotrophins are dimeric growth factor hormones that regulate development and maintenance of central and peripheral nervous systems.[1−3] Members of this protein family include nerve growth factor (NGF), neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF), and neurotrophin-4/5 (NT-4/5).[1−4] They regulate growth, survival, differentiation of neurons, and many other neuroectoderm tissues.[5]

Most "positive" signaling from neurotrophins is mediated by docking to tyrosine kinase (Trk) receptors, to which they bind selectively with high affinity ($K_d \approx 10^{-11}$ M).[6] While the neurotrophins have selectivities (NGF for TrkA, BDNF for TrkB, and NT-3 for TrkC), they are not specific (Figure 1). For instance, NT-3 binds TrkA but with lower affinity than for TrkC.[7,8] The rates of dissociation are slow for interactions of the Trk receptors with neurotrophins ($t_{1/2} > 10$ min) and follow the order of NGF > NT-3 > BDNF.[9]

All the neurotrophins also bind with similar affinities ($K_d \approx 10^{-9}$ M)[10] to the p75 receptor. The on and off rates for interacting with neurotrophins are much faster for the p75 receptor ($t_{1/2} \approx 3$ s).[11] Current understanding of the literature indicates that the roles of the p75 receptor include promotion of apoptosis, survival, and regulation of other Trk activities.[12−18] Expression of, and presumably binding to, p75 can also determine whether NT-3 binds and activates TrkA.[19,20]

Abnormal neurotrophin action is evident in neurodegenerative diseases such as Alzheimer's or stroke,[2,21−26]

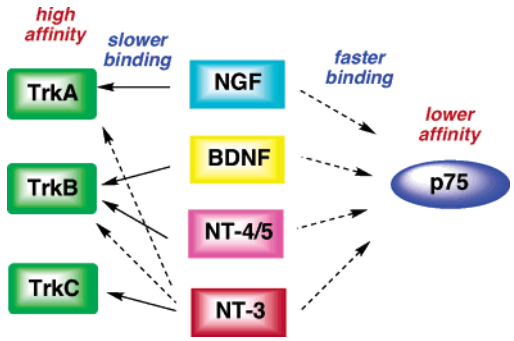

Figure 1. Relationships of neurotrophins and their receptors.

pain/neuropathy,[27−32] and cancer.[33−39] The rationale for using Trk and/or p75 receptor agonists or antagonists for certain disorders is strong,[40] but the neurotrophins themselves have short lifetimes in vivo, induce unfavorable side effects, and are expensive to produce.[25,26,41,42] Some of the undesirable side effects can be attributed to neurotrophins binding multiple receptors (NT-3: p75, TrkA, and TrkC) and activating multiple signaling pathways.[40,43] Small, proteolytically stable molecules that specifically bind certain neurotrophin receptors are therefore highly desirable. They have potential applications in pain management, control of neuropathy, and acute (e.g., stroke) or chronic (e.g., Alzheimer's disease) neurodegeneration.[44,45] Concentration of TrkA receptors in regions of the brain means that agonistic NGF mimics have particular potential for treatment of Alzheimer's disease. In fact, recombinant NGF has been used in clinical trials for the treatment of Alzheimer's disease[46] and for HIV-associated sensory neuropathy.[47] The TrkC receptor, however, is selectively expressed on motor neurons, and therefore, TrkC activating agents such as

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NT-3 are highly selective for disease states such as amyotrophic lateral sclerosis (ALS, or Lou Gehrig's disease).[48,49] While TrkC is a validated pharmacological target to prevent or delay motor neuron degeneration,[50,51] the use of NT-3 and related growth factors as therapeutics failed in clinical trials for ALS.[40,52,53]

Antagonists acting at the Trk receptors would be useful in treatment of cancer types that involve upregulation of these.[54] Increased levels of TrkA expression have been specifically associated with abnormal growth in prostate endothelium,[55] while NGF and NT 4/5 have been linked with tumor metastasis involving the prostate.[56] Breast cancer cells overexpress TrkA,[57−59] though cross-talk between NGF and the HER-2 receptor may be involved in activating the growth of these cells.[60] Even molecules that bind the receptors without causing a functional response may be useful for diagnostics, e.g., radio- or chemotherapeutic targeting of tumors.[61] It is less clear whether p75 agonists or antagonists are required for anticancer applications because the role of this receptor is less understood.[14] It has recently been shown that amyloid β binds to p75 receptors (but not TrkA) and activates that receptor initiating apoptotic cell death. These observations strongly imply that the amyloid β/p75 interaction is related to neuronal loss in Alzhiemer's disease.[62] Antagonists that block this interaction therefore have some therapeutic potential, though in general, p75 antagonists that nonselectively block apoptotic cell death could also have undesirable side effects.

TrkA signaling occurs via receptor reorganization and conformational changes that cause phosphorylation of intracellular tyrosines[63] located in a loop centered around residues 670−675.[64] The previously accepted model in which receptor dimerization plays the dominant role[65,66] is probably overly simplistic. Other Trk receptors are probably activated via similar events, since the receptors are highly homologous. Consequently, there is an opportunity to devise therapeutic interventions based on small molecules that mimic or disrupt interactions of the neurotrophins with the extracellular domains of the Trk (or p75) receptors. Such approaches are significantly different to more widely applied strategy of inhibiting the kinase function of the receptors. This perspective focuses on molecular interactions that would be relevant to this approach. It does not deal with inhibitors of kinase function[67] or small molecules that stimulate the production of neurotrophins.[40,68]

Rational design of small molecules that mimic the neurotrophins requires an understanding of the structures of the neurotrophins, Trk and p75 receptors, and of the "hot-spots" for their interactions on a molecular level. A decade ago, there were relatively little crystallographic data available in this field; most of what was known about neurotrophin−receptor interactions was inferred from in vitro tests on mutated or chimeric proteins. More recently, however, crystallographic studies of the neurotrophins, fragments of their receptors, and neurotrophins cocrystallized with receptor fragments have been reported (Table 1). It is now possible to consider the earlier biological studies in the context of molecular contacts. This area has been reviewed previously but before much of these data emerged.[69] The purpose of this Perspective is to evaluate the area to facilitate design of small-molecule neurotrophin mimics.

Table 1. Crystal Structures of Neurotrophins and Their Receptors

structure	pdb filename	ref
murine NGF dimer[a]	1bet	McDonald 1991[70]
murine NGF dimer[a]	1btg	Holland 1994[71]
NGF/TrkA-d5	1www	Wiesmann 1999[72]
human NT-3 dimer	1nt3	Butte 1998[73]
human NT-3 dimer	1b8k	Robinson 1999[74]
BDNF/NT-3	1bnd	Robinson 1999[75]
BDNF/NT-4/5	1b8m	Robinson 1999[74]
NT-4/5 dimer	1b98	Robinson 1999[74]
NT-4/5/TrkB-d5	1hcf	Banfield 2001[76]
human TrkA-d5	1he7	Robertson 2001[77]
human TrkA-d5	1wwa	Ultsch 1999[78]
human TrkB-d5	1wwb	Ultsch 1999[78]
human TrkC-d5	1wwc	Ultsch 1999[78]

[a] A crystal structure of mouse NGF complexed with four binding proteins (two sets of two protease molecules) has also been solved.[79]

2. Structure of Neurotrophins: General Considerations

Neurotrophins are ∼25 kDa proteins that belong to the cysteine knot superfamily; these share a common structural feature consisting of three intertwined disulfide bridges. Structures and sequence alignments of neurotrophin monomers are shown in Figure 2. Each monomer consists of three antiparallel pairs of β-strands connected to four β-hairpin loops. The core structure of the neurotrophins is formed by three disulfide bonds that constitute the cysteine knot.

The neurotrophins exhibit high sequence homology (ca. 50%) and exist exclusively as dimers.[1,2] In these dimers, the two subunits pack against each other by several hydrophobic residues along the β-strands that stabilize the structure. The residues along the β-strands are highly conserved among neurotrophins and are believed to be important in maintaining the tertiary structure. On the other hand, residues in the N-termini, C-termini, and turn regions are highly variable and seem to play a functional role in receptor binding and activation.[1,8,80]

3. Neurotrophin Receptors

The Trk receptors are highly homologous in sequence.[81] The precise overall three-dimensional structure of any of the Trk receptors is unknown, but their extracellular domains have been classified into five subdomains based on sequence similarities to other known receptors (Figure 3).[82] These domains consist of a leucine-rich region (LRR, domain 2) flanked by two cysteine rich (CR) clusters (domain 1 and 3), followed by two immunoglobulin (Ig)-like domains (4 and 5) located closer to the membrane.[5,81,83] The intracellular tyrosine kinase domain is thought to be linked to the extracellular parts via a membrane-spanning region that may be helical. The intracellular domain encodes a tyrosine kinase active site. Activation of Trk results in phosphorylation of the intracellular tyrosine residues that trigger downstream signaling pathways that mediate neurite outgrowth, neuronal differentiation, or survival.[84−86] Factors that govern these specific cellular responses are not yet well understood. Nevertheless, it has been shown that small-molecule ligands can selectively inhibit or promote one of these pathways.[87−90]

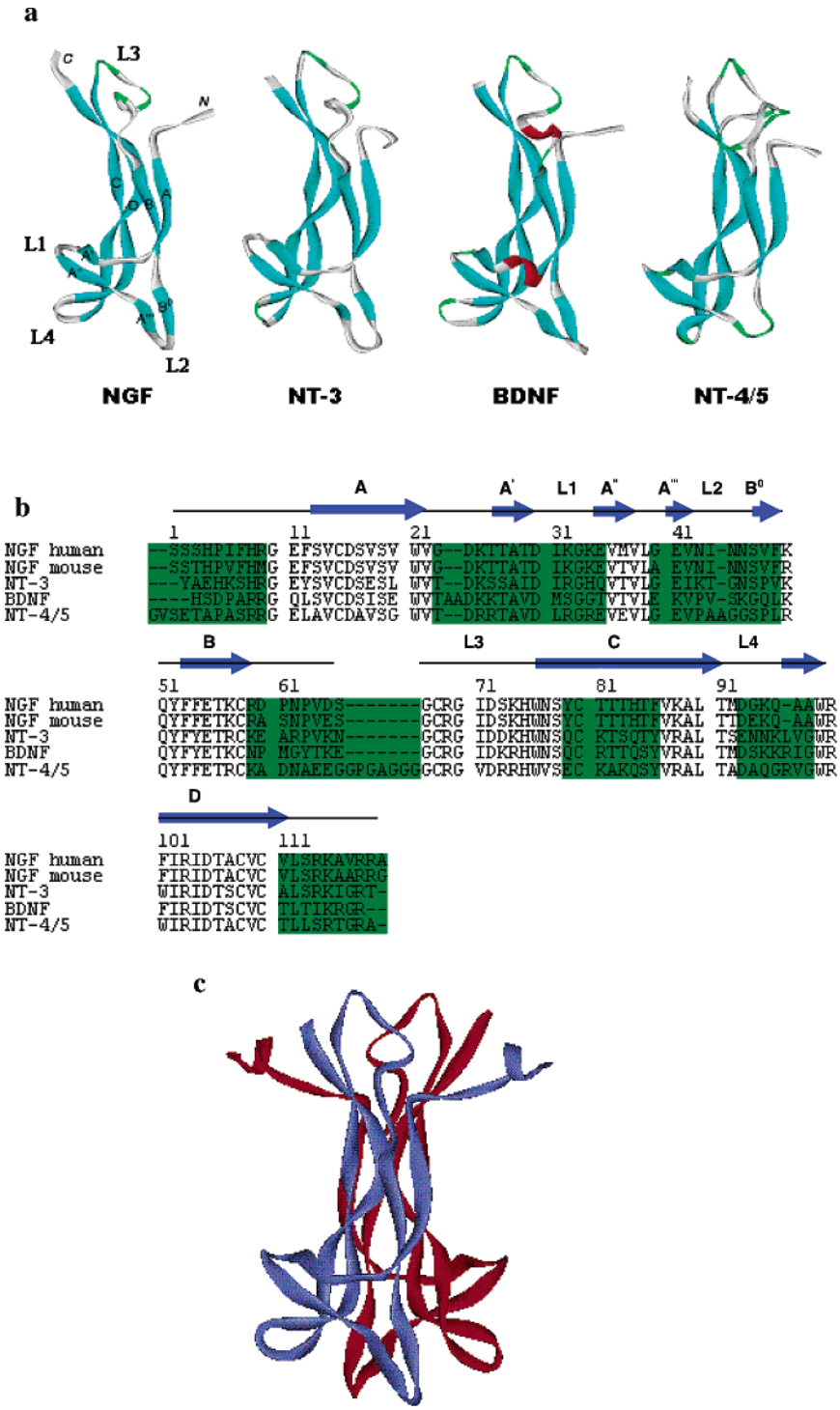

Figure 2. (a) Structure of the neurotrophins NGF, NT-3, BDNF, and NT-4/5 monomers with loops 1−4 (i.e., L1−L4) and the sheet regions A−D indicated on NGF; (b) sequence alignments of the neurotrophins where the numbers correspond to positions in the NGF sequence, dashes represent gaps introduced for the sake of alignment, and regions with low sequence homology are shaded; (c) structure of the NGF dimer.

The following conclusions about the binding of NGF to TrkA may be relevant to all the neurotrophin−Trk interactions, since the hormones and their receptors are so similar. Domain 5 (d5 or Ig2 in Figure 3) of TrkA is involved in high-affinity NGF binding,[91−94] and the leucine repeat regions (d2) may also be involved.[95−97] It is domain 5 of the Trk receptors that has been shown to be the most important for induction of biological responses,[92,94,98] as deduced by the following studies. Exchanging this domain between Trk receptors caused ligand specificity interchange,[98] while deletion of this domain in TrkA and TrkC resulted in diminished NGF and NT-3 affinities.[94] In addition, TrkA-d5 by itself has been shown to bind to NGF with an affinity similar to the binding of the intact receptor with NGF.[94,98]

The crystal structures of the fifth domain (d5) in TrkA, TrkB, and TrkC have recently been determined. Unfortunately, they crystallized as dimers with the N-terminal strand of each molecule unfolded and bound to the other molecule in the dimer (Figure 4).[78] The true

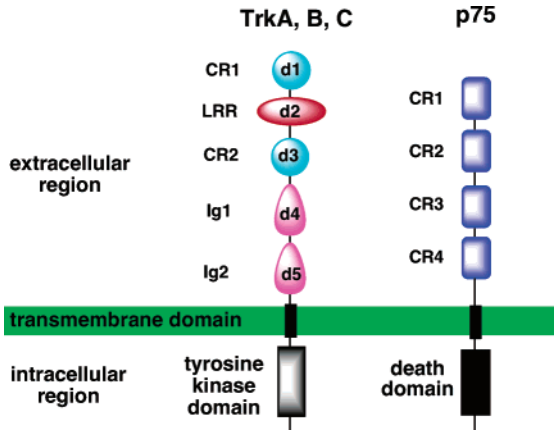

Figure 3. Schematic representation of the neurotrophin receptors Trk and p75.

structure of the d5 monomer is thought to have this N-terminus folded back against the rest of the molecule. However, the N-terminal sequence is not thought to be critical for binding. Figure 4b shows the monomeric fragment with the N-terminus deleted and the receptor regions that are important for binding (for TrkA and TrkB) highlighted in red. We composed this diagram primarily from crystallographic data of the Trk-d5 regions cocrystallized with the corresponding neurotrophins. The key binding regions for TrkC are not known because data for TrkC-d5 cocrystallized with NT-3 have not yet been reported. Binding of the neurotrophins to their Trk receptors will be discussed in more detail later in this review.

While nearly all the crystallographic work involving the Trk receptors focuses on domain 5 (hence, much of this review discusses that work), it is important to remember that other parts of the receptor may also be significant. The role of domain 4, for instance, is unclear, but mutation, deletion or chimeric exchange of this region between Trk receptors can cause constitutive, ligand-independent activation of the kinase activity;[99,100] consequently, it may also be a target for small-molecule interventions. Artificial ligands that bind hot-spots anywhere in the Trk receptors may regulate signal transduction by activating or deactivating the tyrosine kinase activity.

The p75 receptor is a member of the tumor necrosis factor (TNF) receptor superfamily, which in contrast to Trk receptors does not possess kinase activities.[101] Its overall three-dimensional structure is also unknown; there are no crystallographic data reported for any part of the receptor alone or as a cocrystal. It has been proposed that the extracellular domain of p75 contains four cysteine repeat motifs (Figure 3),[101] while the intracellular portion comprises death domains that regulate apoptosis and cell death.[102] The exact role of the p75 receptor is not known, but it induces various responses depending on the cellular context in which it is expressed. Binding of neurotrophin to p75 can cause apoptosis in cells that exclusively expressed high levels of the receptor,[14,102,103] but it can also cooperate with Trk to mediate Trk activities at low neurotrophin concentration in cells that coexpress both receptors.[6,10,84,104,105] The p75 receptor binds neurotrophins through CR2 and CR3 domains. One problem in defining functional p75 receptor domain activity relationships is that there are no established, robust, reliable biological assays for binding at this receptor. However, it is clear the p75 receptor modulates the effects of docking at the Trk receptors in subtle ways. For instance, it appears that binding to the TrkA-d5 tends to activate the Trk receptor, but docking to TrkA-d2 only gives activation when the p75 receptor is coexpressed.[104] This hints at an area of investigation that could potentially be very fruitful: the signaling that would be observed using ligands that selectively bind *only certain regions* of the neurotrophin receptors. Such ligands could cause all, some, or none of the key intracellular tyrosines to be phosphorylated, and this could result in selected signaling events and specific biological responses (e.g., trophic or neuritogenic activities). Successful induction of selected responses could be pivotal in the pharmaceutical development of neurotrophin analogues.

4. Approaches to Determining the Molecular Basis of Neurotrophin Action

Basic research has the potential to shed light on the following issues that are pivotal to rational design of small molecule interventions based on neurotrophin−receptor interactions: (1) key hot spots that govern the binding affinities of the neurotrophins to their receptors; (2) key hot spots that determine the specificities of neurotrophin−Trk receptor interactions; (3) correlations, if any, between induction of specific biological responses and docking to specific regions, or combinations of regions, of the neurotrophin receptors.

Studies in these areas are at relatively early stages of development. One of the best ways to approach these issues is to consider data from crystallographic analyses of neurotrophins and their receptors (Table 1),[71,72,74−76,78,106−108] with mutagenesis studies to access loss-of-function effects (Table 2)[109−122] and gain-of-function approaches based on chimeric recombination of neurotrophins (Table 3).[93,112,117,123−125]

In loss-of-function approaches, individual residues or small fragments of neurotrophins are deleted, replaced with alanine (alanine scanning mutagenesis), or substituted with homologous sequences from another family member (i.e., “homologue scanning mutagenesis”, in which case there is also a significant possibility of gain-of-function). Loss-of-function in these mutants is generally evaluated by measuring receptor binding affinities, Trk autophosphorylation, neurite outgrowth, and neuronal survival. In gain-of-function approaches, chimeric molecules having residues or small domains exchanged between different members of the neurotrophin family are prepared and the gain-of-function effects are examined.

5. Binding of NGF to TrkA

NGF is the most studied ligand in the neurotrophin family. There have been extensive site-directed mutagenesis experiments on this protein,[109−114,116,119,121−125] and crystal structures have been obtained for NGF,[71,107] its receptor (TrkA-d5),[78] and NGF/TrkA-d5 complex.[72]

Deletion,[109,113,116] point mutation,[116] and chimeric recombination experiments[112] (Tables 2 and 3) indicate that the NGF N-terminus is the most important region governing its specificity to TrkA. Indeed, some mutated

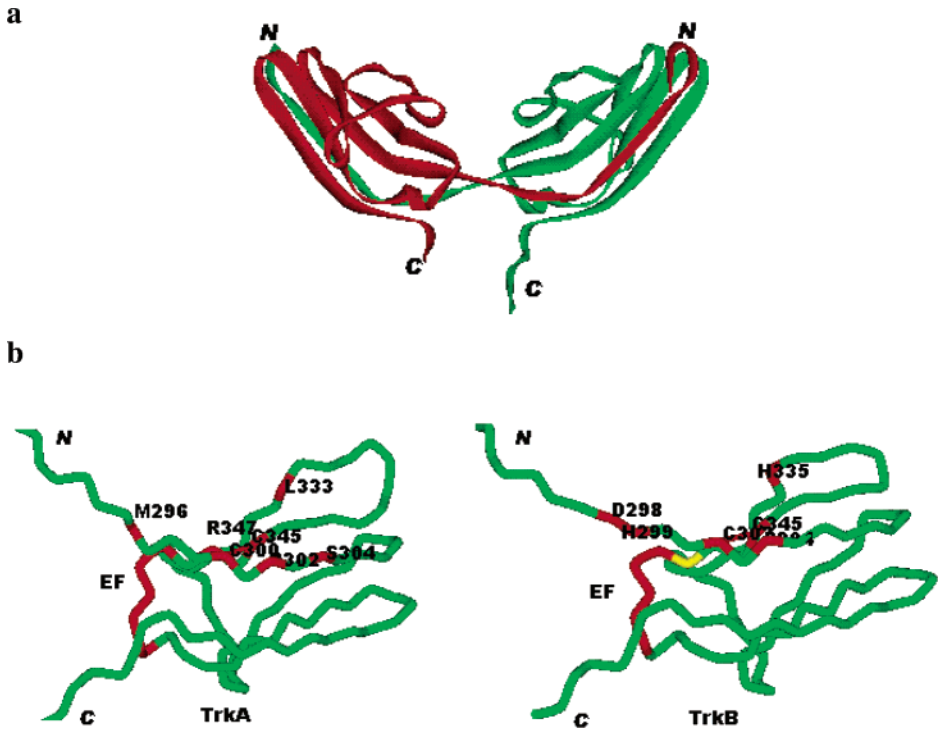

Figure 4. (a) Trk A dimer showing "strand swapping" at the N-terminus; (b) TrkA and TrkB molecules showing the key residues for binding to NGF and NT-4/5, respectively.

forms of NGF that lack the N-terminus bind the TrkA receptor 500× less strongly than the parent protein.[138,139] These mutants have even been used as probes for binding to p75 but not TrkA.[140]

The NGF C-terminal residues 112−118 are implicated in TrkA receptor activation.[109,119] Mutations of the β-hairpin loops also affect binding of NGF to TrkA. Replacing residues in loops 2 and 4 of NGF with their BDNF counterparts caused deleterious effects on association with TrkA.[112] Additional studies by point mutations reveal that residues 48−49 in loop 2 and 96−98 in loop 4 also induce NGF-like activity when substituted into NT-3 skeleton.[124,125] Fewer biological studies have focused on changes to the conserved regions, and scattered points in the β-strand bundles have also been shown to be critical for binding to TrkA.[111,122]

Crystallographic analysis of the NGF/TrkA-d5 complex supports the inference from mutagenesis studies that residues of the NGF N-terminus and along its β-sheet regions contribute significantly to TrkA-binding.[72] The crystal structure reveals two ligand−receptor binding interfaces (Figure 5): the "specific patch" and the "conserved patch". The specific patch involves the N-terminus (residues 2−13) of NGF. This adopts a single helical structure upon complexation with the hydrophobic pocket on the ABED sheet of TrkA. This name "specific patch" is intended to imply that the N-terminus of NGF governs the specificity for binding to TrkA, and the corresponding helical motif may be modified or absent in other neurotrophins. The conserved patch involves NGF residues along the β-strand bundle and in loop 1 (30−35), packing against the loops at the C-terminal end of TrkA-d5. This interface was called the "conserved patch" because residues in this region are highly conserved in all the neurotrophins.

On a molecular level, the role of the specific patch in NGF in Trk interactions is as follows. NGF residues H4 and I6 fill a hydrophobic pocket formed by the ABED sheet of TrkA (Figure 5b), while P5 and F7 also interact with the lypophilic residues in TrkA, further shielding the hydrophobic pocket. Simultaneously, the H4 and E11 amino acids of NGF form hydrogen bonds with S304 and R347 in TrkA. From this, we conclude that small molecules to mimic the NGF specificity patch should present hydrophobic groups in the same disposition as H4 and I6, possibly with other contacts corresponding to P5 and F7. Some form of helical mimic might be appropriate for this, though the N-terminal strand of NGF only forms one turn of a helix, and the key residues 4−7 are not situated in the middle of that motif.

In the conserved patch (Figure 5c), the NGF R103 side chain stacks against the F327 phenyl group of TrkA and forms a hydrogen bond with the carbonyl functionality of N349. Simultaneously, H84 of NGF forms a hydrogen bond with Q350 of TrkA. Residues I31 and F56 identified by mutagenesis studies[111] also appear to be in close contact with TrkA in this conserved patch.

The crystallographic data presented above provide some interesting insights into the NGF/TrkA interaction, but since only part of the TrkA receptor is involved, it does not reveal anything about some other interactions that may be important. For instance, it is not clear on how residues in loops 2 and 4 interact with TrkA, though it was proposed that these may dock with the linker region connected by the C-terminus of TrkA.[72]

6. Binding of NGF to p75

The key structural feature for binding to the p75 receptor appears to involve charged residues located on two discontinuous regions of the ligand.[110,115,117,119,121] Alanine mutation of three lysine residues (K32, K34 in loop 1 and K95 in loop 4) resulted in a complete loss of p75 binding,[110] while in similar studies, mutation of

Table 2. Binding Determinants Identified by Loss-of-Function Experiments

neurotrophin	ref	identified residues[a]	methods	effects
NGF	Ibánez 1990[126]	β-sheet: V21, R99+R102	point mutation (V21A, R99/102G)	significantly reduce TrkA binding and biological activities[c]
	Ibánez 1992[127]	N-terminus: H4, L1:I31	alanine scan	decrease receptor binding and activities[c]
		L1: K32+K34+L4:K95	alanine scan	loss of p75 binding affinity but maintain TrkA binding and activities[c]
	Ibánez 1993[129]	N-terminus: 1−9	chimeric NGF/BDNF[b]	3- to 5-fold reduce affinity to TrkA
		N-terminus: 1−9+L1: 23−25 +L2:45−49+L4:94−98 +β-sheet:79−88	chimeric NGF/BDNF[b]	reduce binding and activities[c] to <1% of wide type NGF
	Drinkwater 1993[130]	C-terminus: 111−118	deletion	no measurable NGF activities[c]
	Shih 1994[116]	N-terminus: 1−9	deletion	300-fold less potency[d]
		N-terminus: H4	alanine scan	1000-fold lower affinity to TrkA and 10- to 30-fold less potent[c,d]
	Guo 1996[122]	β-sheet: F53, F54	alanine scan	decrease affinity to TrkA and decrease potency[c−e]
	Kruttgen 1997[119]	C-terminus: 111−115, L112	alanine scan	decrease activity[d] (TrkA) and impair binding to p75
	Rydén 1997[132]	L3:D72, K74, H75	alanine scan	4- to 10-fold reduce affinity to p75 but not to TrkA
NT-3	Urfer 1994[133]	β-sheet: R103	alanine scan	2 order of magnitude decrease affinity to TrkC and loss activities[c,d]
		β-sheet: K80+Q83, R56, E54, T22, Y51, E10	alanine scan	some loss in affinity and activity[c,d] for TrkC
		β-sheet: R68, R114+K115, L1:R31, H33, L3:K73	alanine scan	significant decrease binding to p75
	Rydén 1995[134]	L1:R31+H33	alanine scan	50-fold reduce affinity to p75, retain ability to activate TrkC[d,e]
	Rydén 1996[135]	L1:R31+ H33	alanine scan	loss of affinities to TrkA and TrkB and unable to activate TrkA and TrkB[d,e]
BDNF	Rydén 1995[134]	L4:K95+ K96+R97	alanine scan	decrease affinity to p75, retain ability to activate TrkB[d,e]
NT-4/5	Rydén 1995[134]	L1:R34+R36	alanine scan	loss binding to p75, no effect on TrkB activation[d,e]

[a] Residues with plus signs are simultaneously identified. [b] BDNF residues were placed into the NGF skeleton. [c] Activity as assayed by neurite outgrowth stimulation. [d] Activity as assayed by induction of tyrosine phosphorylation. [e] Activity as accessed in cell-survival screens.

Table 3. Binding Determinants Identified by Gain-of-Function Experiments

neurotrophin	ref	identified residues[a]	chimeric proteins[b]	effects
NGF	Ibánez 1993[129]	N-terminus: 1−8	NT-3/NGF	gain TrkA activity,[c] retain full NT-3 capability[c]
	Ilag 1994[136]	L2:40−49	NT-3/NGF	activate both TrkA and TrkC[c]
	Urfer 1994[133]	N-terminus: 1−8, L4:91−98	NT-3/NGF	gain NGF-like activity[c,d]
	Kullander 1994[125]	L2:N43, I44, N45, V48, F49	(NT-3/NGF)	gain NGF-like activity[c,d]
	Kullander 1997[120]	L2:V48, P49, L4:Q96	NT-3/NGF	gain NGF-like activity[c,d]
NT-3	Ilag 1994[136]	L2:39−48	NGF/NT-3	gain TrkC activity,[c,d] retain full NGF capability[c,d]
	Urfer 1997[93]	β-sheet: E18, L20, T23, T29, Q84, Y86	NGF/NT-3	gain TrkC activity[c,d]
	Kullander 1997[114]	β-sheet:D72+Y85+R87+W101+S107+A111 +L4:91−98	NGF/NT-3	gain NT-3-like activity[c,d]
BDNF	Ibánez 1991[137]	L1:26−35+L2:45−49	NGF/BDNF	gain ability to activate TrkB[c]
	Ibánez 1993[129]	L4:K96+R97	NGF/BDNF	low affinity to TrkB but induce TrkB activity[c]
		β-sheet: 79−88+L4:91−97	NGF/BDNF	gain BDNF-like activity[c]
NT-4/5	Ilag 1994[136]	L2:43−53	NT-3/NT-4	gain BDNF-like activity[c,d]

[a] Residues with plus signs were simultaneously identified. [b] Neurotrophins prior to the slash (/) denote the main skeleton of the chimeras that were replaced at the corresponding positions given for identified residues. [c] Activity as assayed by neurite outgrowth stimulation. [d] Activity as assayed by induction of tyrosine phosphorylation.

residues in loop 3 (D72, K74, H75) and the C-terminus (111−115) reduced p75 affinity of these mutants.[119,121] Both these loops are predominantly positively charged, while hydrophobic residues in loops 1 and 4 are evident.

At present, there are no crystallographic data available for binding of NGF, or any of the other neurotrophin receptors, with p75. At least two groups have attempted to model the interactions,[141] but their results differ. One of these[141] models places the NGF in an orientation opposite (upside-down) relative to that observed in the NGF-d5 complex via crystallographic studies. Thus, there is a significant degree of uncertainty in the conclusions that can be drawn from such studies.

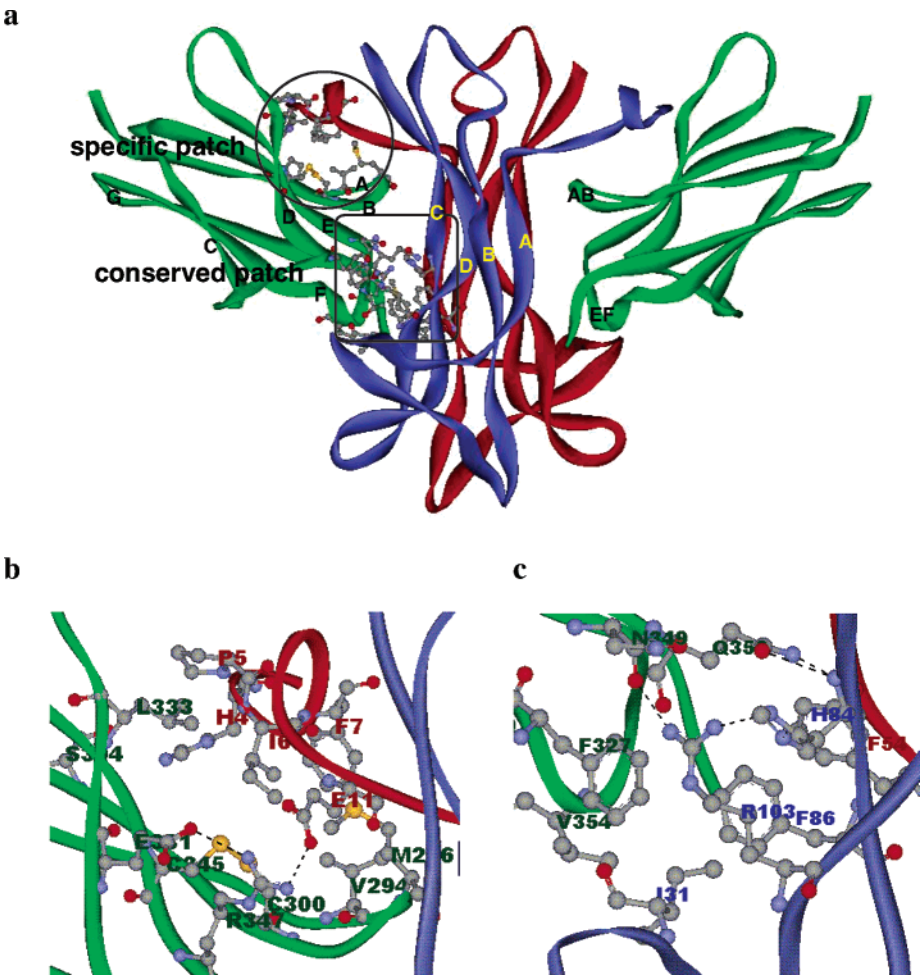

Figure 5. NGF/TrkA-d5 complex: (a) binding epitopes of NGF to TrkA-d5; (b) the specific patch; (c) the conserved patch. NGF monomers are in blue and red; TrkA-d5 is in green.

7. Interactions between NT-3 and TrkC

A crystal structure of NT-3 complexed with the fifth domain of TrkC has not yet been determined, but several studies with mutants and chimeric proteins have revealed key structural information concerning the hot spots that seem to determine NT-3 affinity to TrkC.[93,114,117,124]

The most striking difference between the interaction of NT-3 with TrkC and NGF/TrkA binding is at the N-terminus. It was found that the N-terminus of NT-3 is not essential for binding of NT-3 to TrkC; replacing the N-terminus of NT-3 with that of NGF did not affect the ability of the chimera to bind and activate TrkC.[112] Instead, the residues that cause maximum effect on TrkC binding after mutation were found to be located in the central β-strand bundles.[93,117] Mutation of R103 of NT-3 to alanine causes a total loss of NT-3 activity, suggesting that arginine is the most important binding determinant for association with TrkC.[117] Hydrophilic residues along the β-strands (T22, Y51, E54, R56, K80, and Q83) were also identified (alanine scanning mutagenesis) as critical for TrkC binding affinity and activity.[117] Replacing residues 18, 20, 23, 29, 84, and 86 of the NGF β-sheet regions with corresponding residues in NT-3 was shown to be sufficient to induce NT-3-like activities.[93] However, the loop regions must also play a role because it has also been shown that a significant level of TrkC activity can be induced by a chimera of NGF in which the loop 2 residues (40−49) are replaced with those of NT-3.[124] Key binding determinants for interaction of NT-3 with TrkC are shown in Figure 6 (red).

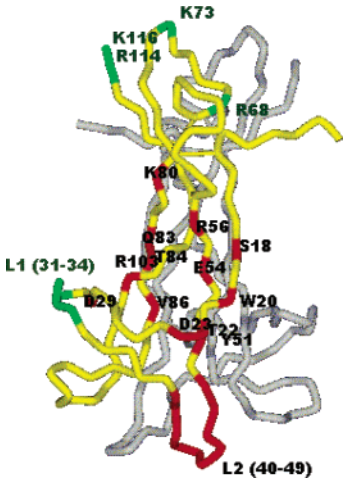

Figure 6. Binding epitopes of NT-3 to TrkC (red) and p75 (green) indicated on the NT-3 portion (yellow) of the NT-3/BDNF dimer structure.

8. Interactions between NT-3 and p75

The key recognition elements of NT-3 for binding to the p75 receptor have also been investigated via mu-

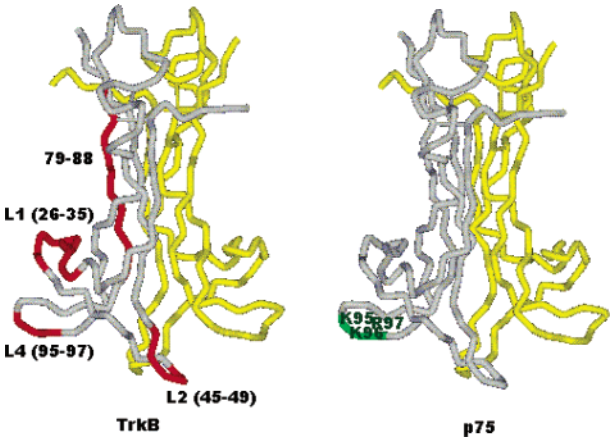

Figure 7. Binding epitopes of BDNF to TrkB (red) and p75 (green) indicated on the BDNF portion (gray) of the NT-3/BDNF dimer structure.

tagenesis.[106,115,117] Positively charged residues, as previously observed in NGF, seem to contribute mostly to the receptor binding. These residues included R31, H33, Q34 in loop 1 and K73 in loop 4.[115,117] Figure 6 shows the binding determinants of NT-3 for TrkC and p75.

9. Binding Determinants of BDNF to TrkB and p75

BDNF has not been studied as much as NGF and NT-3. There are no crystallographic data reported for the BDNF-TrkB complexes, and only crystal structures obtained for this hormone are heterodimers with NT-3 and with NT-4/5 (Table 1). Studies of chimeric proteins[112,123] and point mutants[112] indicated that the association of BDNF with TrkB involves similar regions to those found in NGF interacting with TrkA. Critical residues were identified on the β-strands and in loops 2 (45−49) and 4 (95−97) (Figure 7). The N-terminus of BDNF is not essential for TrkB binding, since chimeric NGF with the N-terminus from BDNF exhibited no additional specificity with TrkB.[112]

The BDNF regions that interact with the p75 receptor appear to overlap with the TrkB binding site, since alanine replacements in loop 4 (95−97) not only affected TrkB binding but also resulted in a significant decrease in p75 binding and activation (Figure 7).[115]

10. Docking of NT-4/5 to TrkB and to p75

The crystal structure of NT-4/5 complexed with the fifth domain of TrkB (TrkB-d5) has recently been determined.[74] It showed that interaction of NT-4/5 with TrkB is very similar to NGF/TrkA binding.[76] Two ligand−receptor interfaces were observed as in the NGF/TrkA complex, i.e., specific and conserved patches (Figure 8).[76] In the specific patch, a hydrophilic pocket on the ABED sheet of TrkB-d5 is occupied by the R11 side chain from the N-terminus of NT-4/5, which also forms a salt bridge with D298 on TrkB. The side chain of R10 (NT-4/5) stacks against the ring of H343 on TrkB-d5, while a main chain hydrogen bond is also observed between E13 (NT-4/5) and H299 (TrkB-d5). The conserved patch involves interaction between the EF loop of TrkB with residues along the β-stand bundles of NT-4/5. NT-4/5 residues Q94, Y96, and R114 form hydrogen bonds with D349, N350 on TrkB.

Few biological data have been collected on the TrkB/NT-4/5 interaction. The mutation experiments that have been done revealed that residues in loop 2 (42−53) are important for NT-4/5 binding to TrkB while the positively charged residue in loop 1 (R34, R36) is critical for interacting with p75. [115,124]

11. Neurotrophin−Receptor Interactions: Conclusions

The data summarized above indicate that the exposed disulfide-bridge pocket on the surface of the Trk-d5 appears to be critical for the receptor specificity.[78] In TrkA-d5 (Figure 9), the hydrophobic pocket formed by V294, M296, and L333 is able to accommodate the nonpolar N-terminal residues of NGF. Conversely, in TrkB-d5, this pocket is formed by hydrophilic residues (T296, D298, and H335) and, as a result, can bind preferentially to the polar residues of the NT-4/5 N-terminus. In TrkC-d5, the pocket is shielded by E322, which forms a salt bridge with R316 on top of the disulfide bond. This observation is inconsistent with mutagenesis studies that indicate no significant contribution of the NT-3 N-terminus toward TrkC binding.[112]

In summary, although there are similarities in sequences and structures within neurotrophin family, each neurotrophin−receptor interaction is distinct and seems to involve a large number of discontinuous contacts. Data from the above studies suggest both the conserved and variable regions are important for receptor binding affinity and specificity. The binding site for the Trk receptors seems to cover an elongated surface parallel to the 2-fold axis of the neurotrophin molecules. While the N-termini appear to be critical for specificity of NGF and NT-4/5 binding to their Trk receptors, the same region is less likely to be important in NT-3 and BDNF. The other binding epitope seems to scatter from the middle region of β-strand bundles up to the loop regions. Small local differences within this binding site allow discrimination between different members of the Trk family.

Critical hot spots of neurotrophins interacting with p75 appear to be located in the loop regions of neurotrophins.[115] These include positively charged residues in loop 1 (K32, K34) and loop 4 (K95) of NGF, loop 1(R31 and H33) in NT-3, loop 4 (K95, K96 and R97) in BDNF, and loop 1 (R34, R36) in NT-4/5.

Much of the data presented in this Perspective probably gives a useful guide to neurotrophin−receptor interactions, but all of it should be evaluated with skepticism. Throughout, it is widely appreciated that the act of forming mutants or protein chimeras may change conformational as well as the primary sequence. Most, if not all, of the experiments with mutants or chimeras cited here cannot exclude the possibility that changes in biological function are due to conformational effects more than switching residues or protein regions. Even the crystallographic data, which looks so compelling when presented in color with detailed explanations of molecular interactions, involve some significant degree of uncertainty and notable omissions. All the receptor fragments expressed and cocrystallized are unglycosylated, and this can lead to destabilization or aggregation effects. Consequently, the structures of the fragments observed may be significantly perturbed from

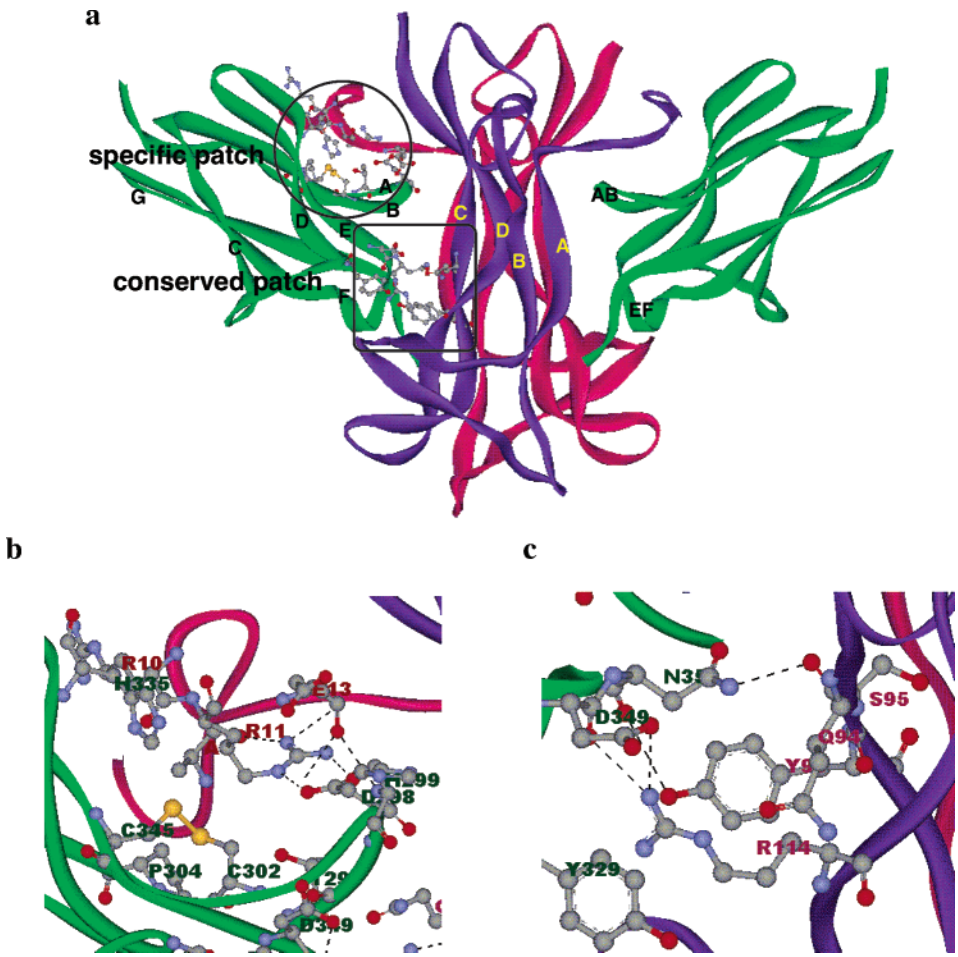

Figure 8. Structure of the TrkB-d5/NT-4/5 complex showing (a) the binding epitopes of NT-4/5 to TrkB-d5, (b) the specific patch, and (c) the conserved patch. The NT-4/5 monomers are in purple and red; TrkB-d5 is in green.

their true states. The d5 fragment featured in many of the crystallographic studies does not contain all the receptor regions necessary for neurotrophin binding. For instance, crystals of the d5-NGF complex do not include the receptor parts that connect the d5 region to the putative transmembrane helix. If the orientation of the neurotrophins in the d5 complex is correct, then these linker residues pack against the NGF loops 2 and 4 (Figure 2a). The last TrkA residue in the d5-NGF complex packs against the bottom of the NGF β-sheet with the C-terminus projecting toward loops 2 and 4 (Figure 5a). Experiments in which part of the hinge region between TrkA-d5 and the transmembrane region was deleted show this is important for binding to NGF,[142] and mutations in this region affect the selectivity of the receptor for NGF.[92] Other mutagenesis studies concluded that TrkA residues 402, 405, 406, and 408−419 were important to binding, but residues in this region of the Trk receptors were not observable in the d5/neurotrophin crystallographic studies. Finally and perhaps most disconcerting of all, the whole notion that neurotrophins bind to TrkA *or* p75 in isolated events may be incorrect. Indeed, there is a considerable set of data in the literature indicating this is so in some cases. For instance, patching experiments featuring fluorescently labeled monoclonal antibodies for TrkA and p75 indicate these receptors interact to form oligomers that have higher affinities for NGF than the isolated receptors.[143] Further, the cell signaling pathways that are induced by stimulating the receptor oligomers are different from those associated with the receptor oligomers.[144] A complete understanding of neurotrophin−receptor interactions on a molecular level must involve a degree of sophistication we cannot even begin to comprehend with the data available today. Nevertheless, there is enough data to formulate working hypotheses, and the potential applications of small-molecule neurotrophin mimics are sufficiently interesting that a close consideration of this area is timely.

12. Toward Small Molecules That Bind Neurotrophin Receptors

Neurotrophins are large polypeptides that have poor proteolytic stabilities and blood−brain barrier permeabilities.[145] They are also expensive to produce and cause effects that arise from activation of multiple receptors and several signaling pathways. Therefore, small proteolytically stable molecules that can selectively mimic or disrupt neurotrophin function are highly desirable.

High-throughput screens have led to identification of a few small-molecule ligands that can mediate responses that are associated with NGF (Figure 10), but they seem to function via mechanisms other than mimicking or disrupting interactions with the TrkA receptor.[40] Small molecules related to cyclosporins such as GPI-1018 **A** mimic NGF-like responses, but these do not act via p75 or TrkA nor do they require expression of neurotrophin

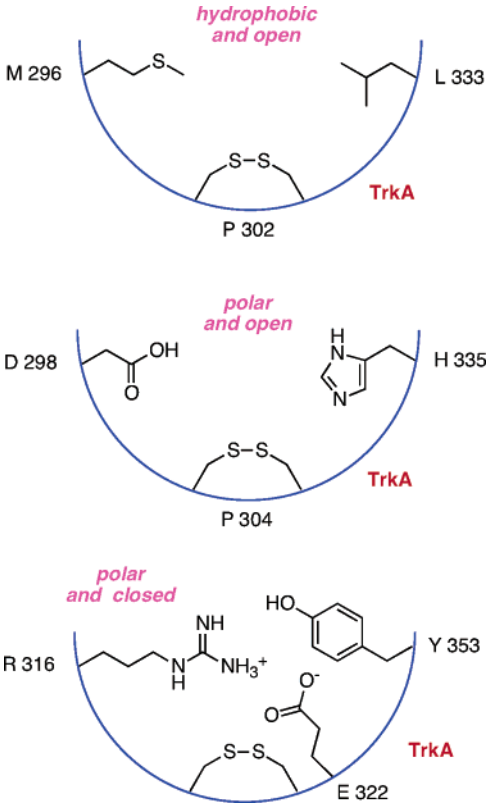

Figure 9. Representation of the Trk receptor pockets in the d5 region that can bind with the neurotrophin N-termini.

receptors; instead, they bind and affect immunophilins.[146,147] SR57748A **B** induces NGF activities by increasing production and release of NGF.[148] NG-061 (**C**) was shown to enhance and mimic trophic effects of NGF, but there is no evidence that it binds to TrkA.[149] Of those small molecules that exhibit antagonistic activities, kyanurenic acid derivatives such as **D** have been shown to inhibit the NGF/p75 interaction by binding to NGF.[150] Similarly, the K-252a **E** does not bind directly to TrkA but binds to an unknown target downstream in the signal transduction pathway.[151] ALE-0540 **F** also was found to act as a competitive inhibitor of NGF for binding to p75 and TrkA.[152] It was claimed that this compound does not bind to NGF, but no direct binding to TrkA is evident. In general, uncertainties regarding the mode of action of these compounds and the lack of confirmed leads mean that relating the pharmacophores in these molecules with the NGF structure is meaningless at this stage.

An alternative approach to identify small molecules that bind neurotrophin receptors is via structure-based design. The sequence diversity observed on the loop regions of the neurotrophins suggests that these turns are potentially important in defining the specificities of the hormone to the Trk receptors. Many cyclic peptides derived from these loop regions have been shown to function via the neurotrophin receptors. Disulfide-linked cyclic hexapeptides based on NGF loops 2 (43−48) and 4 (92−97) were shown to antagonize NGF by associating with TrkA and possibly p75, e.g., **G**.[43,61,153−155] Similarly, related cyclic peptides derived from NGF have been reported to bind to p75 while cyclic peptide analogues of the loop regions on BDNF were found to be BDNF antagonists, e.g., **H**.[131,156−159] The biological activities of

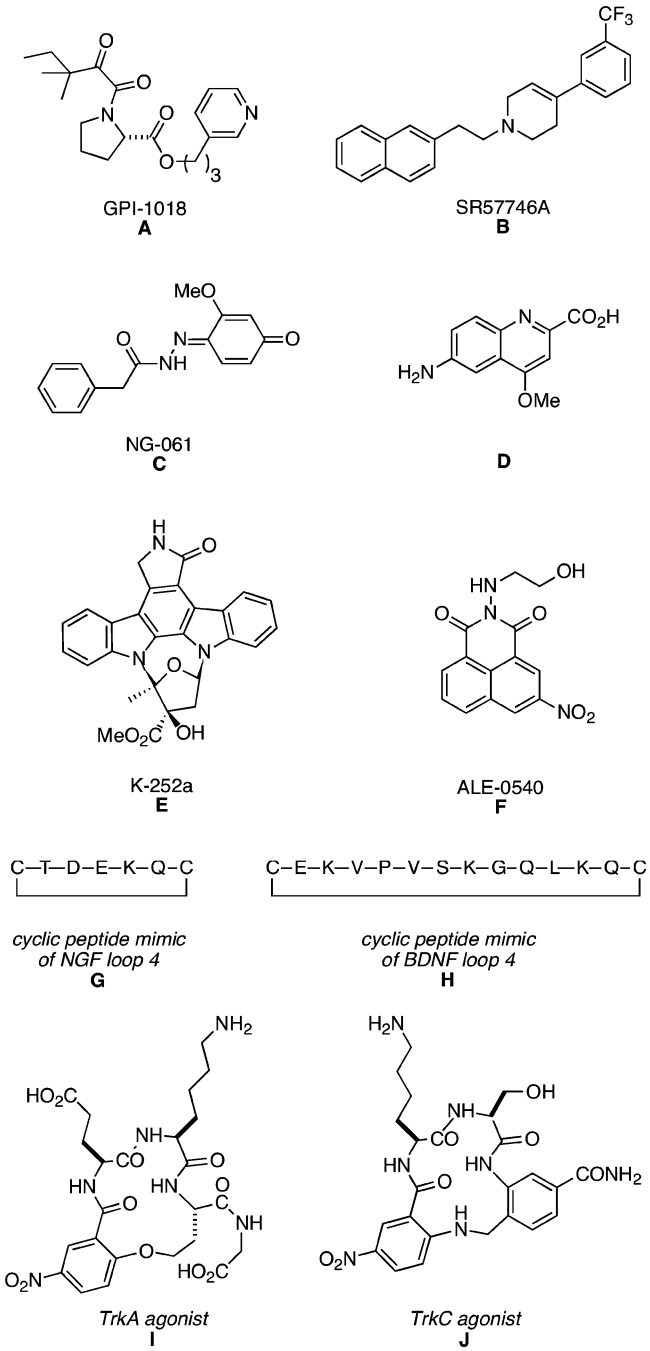

Figure 10. Small molecules that induce neurotrophin-like effects.

these compounds seem to strongly correlate with β-turn conformations because the respective linear analogues exhibited no response.[160,161]

Compounds **I** and **J** are representative of a large number of compounds prepared in our laboratories. These are designed to mimic the neurotrophin β-turns by presenting the $i + 1$ and $i + 2$ amino acids residues corresponding to a loop region in appropriate conformations. These molecules are smaller, more proteolytically stable, less hydrophilic, and generally more like small-molecule pharmaceuticals than the disulfide linked cyclic peptides and the parent neurotrophins. It is remarkable that **I** and **J** have agonist-like properties; they actually synergize with suboptimal concentrations of the neurotrophins and enhance the response obtained. In unpublished work, similar compounds that

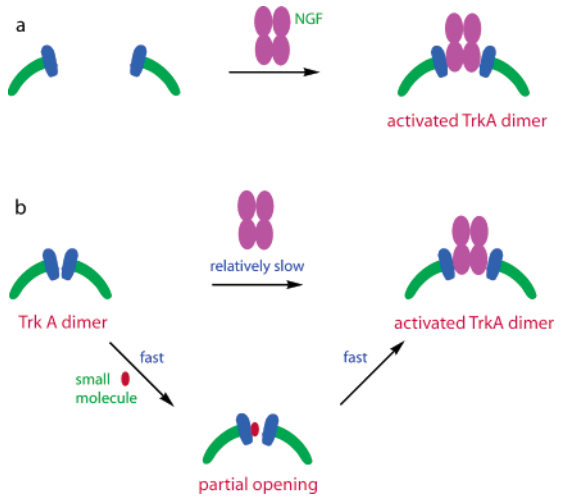

Figure 11. Perspective on the role of some small molecules that synergize with the neurotrophins to enhance their activities: (a) the established concept that NGF (and related molecules) bring receptor fragments together; (b) an alternative hypothesis in which NGF separates preformed NGF dimers (or oligomers) in a process that can be facilitated by small molecules.

have antagonistic character have been identified, but more of the hits have these types of agonistic properties. Mimic **I** has the EK dipeptide sequence corresponding to the $i + 1$ and $i + 2$ residues in NGF loop 4. Compound **J** was prepared to mimic a target that is not a neurotrophin; its dipeptide fragment does not exactly correspond to any $i + 1$ and $i + 2$ of a β-turn in NT-3, and its activity is a welcome but unexpected surprise.

Why would small molecules such as **I** and **J** synergize with suboptimal amounts of NGF to produce agonistic responses? Extensive experimental evidence has emerged to challenge the dogma that cell surface receptors tend to exist as isolated monomers that dimerize or oligomerize in the presence of an appropriate protein ligand (Figure 11a). For instance, there is data that indicate that the following receptors exist as preformed dimers or oligomers that are disrupted by their appropriate natural ligands (techniques used in parentheses): EPO−receptor (from crystallographic studies of part of the receptor and from immunofluorescence patching),[162] Fas (from fluorescence resonance energy transfer {FRET} studies),[163] TNF−receptors (chemical cross-linking and FRET),[164] β2-AR (from bioluminescence energy transfer {BRET} and chemical cross-linking),[165] CCR5 (BRET),[166] TGF-βR (immunofluorescence copatching),[167] p75 (Western blotting assays),[62] and notably, TrkA (freeze−fracture microscopy).[63] We speculate that small molecules such as **I** and **J** may induce conformational changes in Trk dimers or oligomers that kinetically facilitate binding of the neurotrophins, hence they appear to have agonist-like properties (Figure 11b).

Research on the loop analogues emerged from early biological studies that indicated these regions could be important and the observation that, in the neurotrophin series, the loops have a variable sequence indicative that they perhaps play a part in determining binding and specificity. Recent insights now available from crystallographic studies involving the Trk-d5 regions, and the parts of the neurotrophins that dock there, indicate that rational design of peptidomimetics for those particular neurotrophin hot spots should be possible. Generally, the only neurotrophin turn that is involved with docking to the Trk-d5 region is part of loop 1, so the structural insights could lead to peptidomimetics that have a different mode of action to the β-turn analogues described above.

If the idea presented in Figure 11b is valid, then small-molecule interventions might be designed around the concept that small molecules that bind to one of the Trk (or p75) receptor hot spots could synergize with endogenous neurotrophins. Alternatively, true agonists could be pursued by designing "bivalent molecules" capable of binding two Trk hot spots simultaneously. A strategy based on this logic was communicated for our laboratories recently.[168] Acceptance of such approaches will require a more liberal interpretation of the term "small molecule" than most medicinal chemists currently use. Common sense indicates that few molecules of less than 500 MW could simultaneously provide strong intermolecular contacts at two hot spots separated by 10 Å or more. Fortunately, quite apart from current interest in protein−protein interactions, the community appears to be moving away from the rigidly held Lipinski rule-of-five ideals toward more sophisticated interpretations that recognize molecular weight is not a fundamental parameter for pharmaceutical-like properties.[169] This broadens the horizons for those of us striving to design synthetic compounds that exploit the types of molecular interactions described here.

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Biographies

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Kevin Burgess is a Professor at Texas A & M University, where he has been since September 1992. His research interest focuses on projects in peptidomimetics of protein−protein interactions, fluorescent dyes for DNA sequencing, and catalysis, all with an emphasis on high-throughput and combinatorial chemistry.

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