Protein Science (1999), *8*:2589–2597. Cambridge University Press. Printed in the USA.

The structures of the neurotrophin 4 homodimer and the brain-derived neurotrophic factor/neurotrophin 4 heterodimer reveal a common Trk-binding site

ROBERT C. ROBINSON,[1] CZESLAW RADZIEJEWSKI,[2] GLEN SPRAGGON,[3] JASON GREENWALD,[1,4] MIKEY R. KOSTURA,[1,4] LESLIE D. BURTNICK,[5] DAVID I. STUART,[6] SENYON CHOE,[1] AND E. YVONNE JONES[6]

[1]Structural Biology Laboratory, The Salk Institute, La Jolla, California 92037
[2]Regeneron Pharmaceuticals Inc., 777 Old Saw Mill River Road, Tarrytown, New York 10591
[3]Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0634
[4]Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92093-0634
[5]Department of Chemistry, University of British Columbia, Vancouver, British Columbia V6T 1Z1, Canada
[6]Laboratory of Molecular Biophysics & Oxford Centre for Molecular Sciences, Department of Biochemistry, Rex Richards Building, South Parks Road, Oxford University, Oxford OX1 3QU, United Kingdom

(RECEIVED June 15, 1999; ACCEPTED September 9, 1999)

Abstract

The neurotrophins are growth factors that are involved in the development and survival of neurons. Neurotrophin release by a target tissue results in neuron growth along the neurotrophin concentration gradient, culminating in the eventual innervation of the target tissue. These activities are mediated through trk cell surface receptors. We have determined the structures of the heterodimer formed between brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NT4), as well as the structure of homodimer of NT4. We also present the structure of the Neurotrophin 3 homodimer, which is refined to higher resolution than previously published. These structures provide the first views of the architecture of the NT4 protomer. Comparison of the surface of a model of the BDNF homodimer with the structures of the neurotrophin homodimers reveals common features that may be important in the binding between the neurotrophins and their receptors. In particular, there exists an analogous region on the surface of each neurotrophin that is likely to be involved in trk receptor binding. Variations in sequence on the periphery of this common region serve to confer trk receptor specificity.

Keywords: crystallography; nerve growth factor; receptor

Brain-derived neurotrophic factor (BDNF; Barde et al., 1982), originally purified from pig brain, neurotrophin 3 (NT3; Maisonpierre et al., 1990), and neurotrophin 4 (NT4; Ip et al., 1992), discovered through homology cloning, together with nerve growth factor (NGF; Levi-Montalcini, 1987), constitute a family of structurally and functionally related proteins known as the neurotrophins. The neurotrophins are responsible for the maintenance, proliferation, and differentiation of subsets of neurons bearing specific tyrosine kinase receptors, the trks (reviewed in Barbacid, 1995). Trk activation by neurotrophins, possibly mediated by dimerization of the trk receptors, promotes neuron survival through the negation of programmed cell death (Davies, 1994). Ligand specificity is such that both BDNF and NT4 activate trkB, NGF interacts with trkA, and NT3 is the preferred partner for trkC, although NT3 can also interact with both trkA and trkC, albeit with lower affinities than with trkC. During development, neurotrophins act as target-derived factors, attracting neurons to appropriate tissues. By limiting the concentrations of neurotrophin, the target tissue regulates the number of innervating neurons (reviewed in Johnson & Oppenheim, 1994). Autocrine mechanisms, at least in the case of BDNF (Davies & Wright, 1995), are also known to contribute to the stability of some neurons.

The trk receptors are modular, single-pass membrane proteins consisting of an N-terminal signal peptide followed by a cysteine-rich domain, three leucine-rich motifs, a second cysteine-rich domain, two immunoglobulin-like domains, a transmembrane domain, a juxtamembrane domain, a kinase domain, and a C-terminal tail (reviewed in Barbacid, 1995). The C-terminal immunoglobulin-like domain and the second leucine-rich motif have been implicated in direct interaction with neurotrophins (Urfer et al., 1995; Windisch et al., 1995). There is, however, some evidence of different modes of BDNF and NT4 binding to trkB, as a mutant trkB

Reprint requests to: Robert Robinson, Structural Biology Laboratory, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, California 92037; e-mail: bob@sbl.salk.edu.

(bearing the single mutation C345S in the extracellular C-terminal immunoglobulin-like domain) can be activated only by BDNF (Ip et al., 1993). TrkB and trkC may exist in several isoforms, as nonkinase containing cDNA clones have been identified. It has been suggested that these variant molecules may play a role in recruiting neurotrophins either for subsequent activation of full length trk receptors or for clearance from body fluids (Barbacid, 1995).

A second receptor, p75, which shows no kinase activity, is also able to interact with the neurotrophins. p75 has been implicated in retrograde transport of NT4 in dorsal root ganglia (Curtis et al., 1995). Loss of p75 binding affects receptor activation for cells expressing both p75 and trkB in response to NT4, but not to BDNF (Rydén et al., 1995). Contrary to this, p75 knockout mice appear to have a normal responsiveness to NT4, with effects limited to NGF-sensitive neurons (Lee et al., 1994).

Besides existing as homodimers, the neurotrophins, at least in vitro, are capable of existing as heterodimers (Radziejewski & Robinson, 1993). The existence of neurotrophin heterodimers in vivo has yet to be established; however, preliminary experiments have shown that co-expression of neurotrophins in several different cell lines has resulted in neurotrophin heterodimer production (Jungbluth et al., 1994; Heymach & Shooter, 1995), fueling the speculation for a role for neurotrophin heterodimers in vivo. NGF, BDNF, NT3, and NT4 have all been shown to form heterodimers, although those that involve NGF tend to be relatively less stable, reverting back to their respective homodimers (Radziejewski & Robinson, 1993).

The biological activity of neurotrophin heterodimers has been probed in the case of BDNF/NT3, which was reported to be 10-fold less active in neuronal survival assays than a 50/50 mixture of BDNF and NT3 homodimers (Jungbluth et al., 1994). In an apparently conflicting report, the BDNF/NT3 heterodimer was found to be as active as single parent homodimers in similar neuronal survival assays (Arakawa et al., 1994). The reconciliation of these data may lie in that distinct, but overlapping, subpopulations of neurons are responsive to BDNF and NT3, and, indeed, combinations of neurotrophins may have additive survival effects, rendering the control experiments incompatible in each study. The NGF/NT4 heterodimer has been shown to produce neuronal differentiation in PC12 cells as effectively as NGF (Treanor et al., 1995). To date, no biological activity studies for the BDNF/NT4 heterodimer have been reported. Such studies may be particularly illuminating, as the functions of BDNF and NT4 are both mediated through trkB.

The biological role of neurotrophin heterodimers in vivo is uncertain. If the neurotrophin heterodimers are significantly less active than their parent homodimers, as reported by Jungbluth et al. (1994), then neurotrophin heterodimers may act in a neurotrophin dominance switching mechanism (Robinson et al., 1995). In the intermediate stages, when a cell changes production from one neurotrophin to another, the major neurotrophin would sequester the minor one as the less active heterodimer, sharpening the switch between neurotrophins. Alternatively, if neurotrophin heterodimers prove to be of similar activity to their parent homodimers, the existence of heterodimers may simply reflect promiscuity among the neurotrophins.

The three-dimensional crystal structure of NGF (McDonald et al., 1991; Holland et al., 1994) revealed a pair of eight β-stranded protomers each held together, in part, by a distinctive cystine knot. The extended dimer interface consists mainly of main-chain–main-chain hydrogen bonds and of hydrophobic interactions. The amino acids that participate in the hydrophobic contacts tend to be conserved throughout the neurotrophin family. The crystal structure of the BDNF/NT3 heterodimer (Robinson et al., 1995) demonstrated the NGF protomer fold is typical of the neurotrophin family members and established that the neurotrophin protomers are structurally compatible as stable heterodimers. Here, we present the structure of a second stable neurotrophin heterodimer, BDNF/NT4, and the homodimeric structure of NT4. We also report the structure of the NT3 homodimer at a resolution of 2.15 Å, which is at a slightly higher resolution to that previously published 2.3 Å structure (Butte et al., 1998). These new structures detail the first view of the NT4 protomer structure and invite comparison of the BDNF, NT3, and NT4 protomer structures from different dimer molecular environments. From the analysis of the surface features of the neurotrophins, we provide new structural insight into the receptor binding of these ligands, each of which is responsible for binding to both a common and a specific receptor.

Results

Structure determination

Details of the crystals, data collection statistics, and refinement statistics are recorded in Table 1. In each case, molecular replacement resulted in an unambiguous solution. The present BDNF/NT4 model consists of residues 8–116 in BDNF and residues 12–63 and 69–130 in NT4. It was not possible to interpret electron density corresponding to residues 64–68 in NT4 or for the terminal regions of either protomer. Similarly, the NT3 model is comprised of residues 11–41, 48–59, and 67–113, the NT4 homodimer molecule 1 of residues 13–44, 52–102, and 108–124 and molecule 2 of residues 12–64, 69–103, and 106–126. Interestingly, lack of electron density in loop regions correlates extremely well with the absence of crystal contacts in these regions (Fig. 3; Table 2), indicating that the conformations of these loops are intrinsically flexible and only stabilized in the presence of crystal contacts. In the NT4 electron density maps, no extra electron density was found to accommodate the receptor mimetic peptide; hence, it was assumed to not have bound. Where electron density is observable in the models, the electron density is good throughout the length of the main chain of all the models, although poor in the region 52–55 of NT4 from the BDNF/NT4 heterodimer with a break in the main-chain density (1σ level) at Thr53. In this region (52–55) and in several regions from each model in which the main-chain temperature factors rise above 60 $Å^2$ (Table 1), the general direction of the main chain is unambiguous; however, the peptide plane orientations may be ill determined. No residues lie in disallowed regions of the Ramachandran plots of these structures.

The common fold of the neurotrophins

The common fold of the BDNF, NT3, NT4, and NGF protomers (McDonald et al., 1991; Holland et al., 1994; Butte et al., 1998) comprises eight β-strands that contribute to four antiparallel pairs of twisted β-strands (Fig. 1). This extended structure is locked by a cystine knot (McDonald & Hendrickson, 1993) formed by three disulfide bonds. Molecular superposition of BDNF, NT3, and NT4 determined from different molecular complexes (Table 3; Fig. 1) reveals the common structural scaffold with the structural variations being displayed in loop regions of low sequence similarity

Table 1. *Summary of crystal, data collection, and refinement statistics for BDNF/NT4, NT3, and NT4 models*

	BDNF/NT4	NT3	NT4
Space group	$P2_12_12$	$P2_12_12$	$P2_1$
Cell	$a = 50.7$, $b = 105.9$, $c = 51.8$ Å $\alpha = \beta = \gamma = 90°$	$a = 38.3$, $b = 52.3$, $c = 67.7$ Å $\alpha = \beta = \gamma = 90°$	$a = 43.8$, $b = 50.8$, $c = 53.0$ Å $\alpha = \gamma = 90°$, $\beta = 109.4°$
Asymmetric unit	1 Heterodimer	1 Protomer	1 Homodimer
Bragg spacing	20.00–2.75 Å	20.00–2.10 Å	20.00-2.35 Å
Reflections[a]	7,858	7,414	8,334
% Data[b]	98	82	93
R_{merge}[c]	0.060	0.047	0.031
R_c[d]	19.8	23.8	23.5
R-free[e]	27.9	30.2	33.6
Nonhydrogen atoms	1724	719	1621
Ordered waters	52	53	49
RMSD bonds	0.014 Å	0.015 Å	0.015 Å
RMSD angles	2.00°	2.15°	0.048 Å[f]
Mean temperature factor	36.9 Å^2	42.3 Å^2	46.3 Å^2
Residue with main chain *B*-factors > 60 Å^2	NT4 12–14, 45–51, 63, 69–76, 128–130 BDNF 116	NT3 11–12, 58–59, 67–77, 93–97	NT3 11–12, 58–59, 67–77, 93–97 Mol 1 26–28, 36–44, 83–86, 108–110 Mol 2 44–53, 69–72

[a] Number of unique reflections.
[b] Percentage of theoretically possible data measured.
[c] R_{merge} $(\sum|I - \langle I\rangle|/\sum\langle I\rangle)$.
[d] R_c $(\sum||F_o| - |F_c||/\sum|F_o|)$.
[e] Based on 5% of the data.
[f] RMSD on 1,3 distance.

between the neurotrophins (Loops II, III, and V). The two BDNF protomer structures, from the BDNF/NT3 and BDNF/NT4 heterodimers, are particularly closely related. The BDNF protomer appears to be ignorant of its partner and, hence, it could be expected that the structure will be essentially the same in the BDNF homodimer. This assumption is enforced by the high structural conservation, at the dimer interface between the homodimeric and heterodimeric structures of NT3 and NT4 (Table 3; Fig. 1).

Table 2. *A comparison of crystal contacts vs. the presence of loop structure in the BDNF/NT4, NT3, and NT4 models*

	Loop I	Loop II	Loop III	Loop V
BDNF[a]	Yes ✓	Yes ✓	Yes ✓	Yes ✓
NT3[b]	Yes ✓	No ×	No ×	Yes ✓
NT4[a]	Yes ✓	Yes ✓	Partial ✓	Yes ✓
NT4[c]	Yes ✓	No ×	Yes ✓	No ×
NT4[d]	Yes ✓	Yes ✓	No ×	No ×

[a] From the BDNF/NT4 heterodimer crystals.
[b] From the NT3 homodimer crystals.
[c] Molecule 1 from the NT4 homodimer crystals.
[d] Molecule 2 from the NT4 homodimer crystals. The loops are labeled on BDNF in Figure 1. Yes and no refer to the involvement and noninvolvement of a loop in a crystal contact. Ticks and crosses specify whether the particular loop is present or absent in the final models. This table shows these two parameters are highly correlated.

Molecular superposition between the different protomers of the neurotrophin family reveals strong structural homology in the β-sheet regions that form the dimer interface, with slightly more variation exhibited by the loop regions (Fig. 2; Table 3). These loop regions are typified by relatively higher *B*-factors than the average *B*-factors for each of the neurotrophin structures determined to date, suggesting that the loops may be mobile. Indeed, structural determinations of individual neurotrophins from different crystal environments show loop deviations of a similar amplitude to those observed between different members of the neurotrophin family (Figs. 1, 2; Holland et al., 1994) even though these regions display strong sequence variation, suggesting that these regions are innately flexible. Structural alignment analysis shows NT3 to be closest to the average structure with both protomers from BDNF/NT4 being distant. This pattern of structural relatedness mirrors to some extent the activities of the neurotrophins for the trk receptors. NT3 is able to interact with all three trk receptors (A, B, and C), and, for this reason, may exhibit a collection of structural features found only individually on each of the more specific neurotrophins (NGF being specific for trkA, while BDNF and NT4 exhibit preference for trkB). The NT3 structure reported here agrees well with that determined by Butte et al. characterized by a Cα RMS distance of 0.55 Å between the two structures (Butte et al., 1998).

As in the structures of the NGF homodimer (McDonald et al., 1991) and the BDNF/NT3 heterodimer (Robinson et al., 1995), the BDNF/NT4, NT3, and NT4 dimer interfaces are formed by main-chain–main-chain hydrogen bonding interactions and hydrophobic side-chain interactions that occur primarily between residues in the β-strands (Fig. 3). Superposition of the NGF, BDNF,

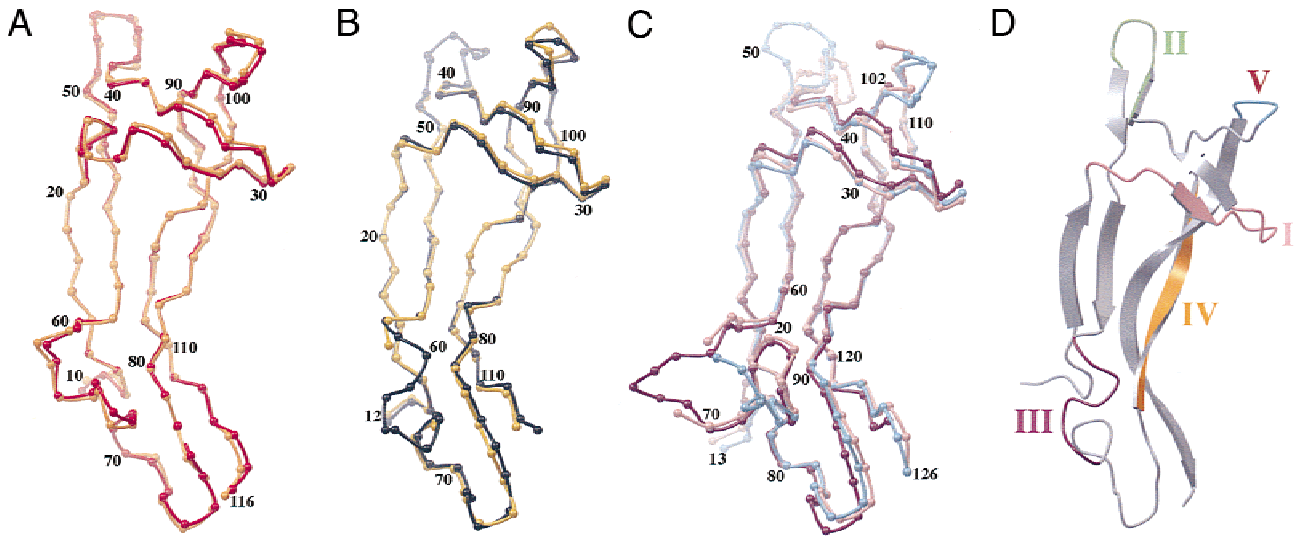

Fig. 1. Cα superpositions of the neurotrophin protomers with every tenth residue labeled. **A:** BDNF from the BDNF/NT3 heterodimer (orange) compared with BDNF from the BDNF/NT4 heterodimer (red). **B:** NT3 from the homodimer (yellow) overlaid on the structure of NT3 from the BDNF/NT3 heterodimer (gray). **C:** The three structures of NT4: molecule 1 (purple) and molecule 2 (pink) from the NT4 homodimer and NT4 from the BDNF/NT4 heterodimer (sky blue). **D:** A schematic representation of the secondary structural elements of the NGF protomer as a reference. There are five highly sequence variable regions of the neurotrophins, four of which form loops, which are colored and labeled: variable regions I (pink), II (light green), III (purple), IV (orange), and V (cyan).

NT3, and NT4 protomer structures onto each other reveals that these hydrophobic residues are structurally conserved, consistent with the propensity of the neurotrophins to form heterodimers. The BDNF/NT4, NT3, and NT4 dimer interfaces each form a cavity filled with ordered waters, which are also conserved in the NGF and BDNF/NT3 dimers.

Structural variations between the neurotrophins

Subtle variations on the general topology are observed: BDNF has a short α-helix between strands 1 and 2, which accommodates a two-residue insertion; BDNF also has a break in the C-terminal–most strand; NT3 lacks a strand pair (4 and 5) due to the absence of residues 42–47 in the model, as does NT4 molecule 1, which is missing residues 45–51; NT4 from NT4/BDNF and from NT4 molecule 2 has an additional N-terminal strand, and NT4 molecule 2 has strands 5 and 6 fused into a single strand. NT4 contains a seven amino acid insertion (residues 64–70) when compared to the other neurotrophins. This insertion is accommodated by a loop structure extending away from the bulk of the molecule in the BDNF/NT4 crystal (Fig. 3). The loop is involved in a crystal contact with a neighboring molecule in the BDNF/NT4 crystals and, therefore, may be stabilized by this contact. Even so, residues 64–68 are absent from the electron density maps. The position of Gly70 requires these missing residues to form extended loop structure out into the solvent. This loop in NT4 molecule 1 is completely visible, but takes an alternative path due to a different and more extensive crystal contact. This loop structure from NT4 molecule 2 lacks residues 65–68 and is not stabilized by crystal contacts; however, the C-terminal portion of the loop is more similar to NT4 from molecule 1 than to NT4 from BDNF/NT4. The NT4 protomer in solution, without the stabilization of crystal contacts, may be expected to be even more mobile in this region.

Table 3. *The structural relatedness of the neurotrophin protomers as indicated by molecular superpositions**

	NGF	BDNF[a]	BDNF[b]	NT3[a]	NT3	NT4[b]	NT4[c]	NT4[d]
Consensus	0.59	0.44	0.81	0.34	0.46	0.93	0.48	0.86
NT4[d]	1.03	1.20	1.59	0.83	1.24	0.48	1.10	
NT4[c]	0.85	0.62	0.76	0.68	0.65	1.20		
NT4[b]	1.09	1.22	1.64	0.92	1.28			
NT3	0.83	0.54	0.70	0.55				
NT3[a]	0.74	0.65	1.00					
BDNF[b]	1.13	0.59						
BDNF[a]	0.81							

*The values specify Cα RMS distances (Å) between each pair of protomers based on 80 equivalencies. "Consensus" refers to the average structure calculated from the eight models. Protomers labeled "a" are from the BDNF/NT3 complex (Robinson et al., 1995); "b" from BDNF/NT4; "c" refers to NT4 molecule 1; "d" to NT4 molecule 2. The NGF structure is that of murine NGF (McDonald et al., 1991).

Discussion

Consequences for neurotrophin structure

The structures of the BDNF/NT4 heterodimer, NT3 homodimer, and NT4 homodimer presented here, when compared to the previously determined neurotrophin structures, shows the strong structural similarity of the neurotrophin protomers to each other, particularly at the dimer interfaces. Within the context of these structures, the BDNF protomer structure can be used to form a model for the BDNF homodimer that exhibits no stereochemical

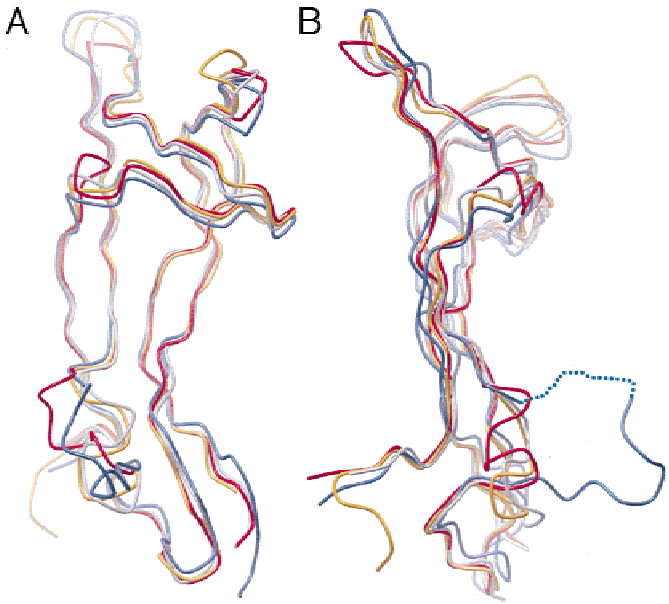

Fig. 2. Superpositions of the four neurotrophins. **A:** mouse NGF (green), human BDNF from the BDNF/NT3 heterodimer (red), NT3 from the BDNF/NT3 heterodimer (yellow), and NT4 from the BDNF/NT4 heterodimer (blue) in the same orientation as Figure 1. **B:** Figure 2a rotated 90° around the vertical. The dotted cyan line represents the unknown path of the main chain that is absent from this NT4 structure, which is included to aid chain tracing rather than to infer three-dimensional structure.

clashes. Indeed, protomer structures of BDNF from the two heterodimer structures (BDNF/NT4; BDNF/NT3; Robinson et al., 1995; Fig. 1; Table 3) are highly similar, showing no more variation than the structures of NGF (Holland et al., 1994), NT3, and NT4 from different crystal forms. Hence, we would expect that comparison of the protomer structure for BDNF in these hetero-

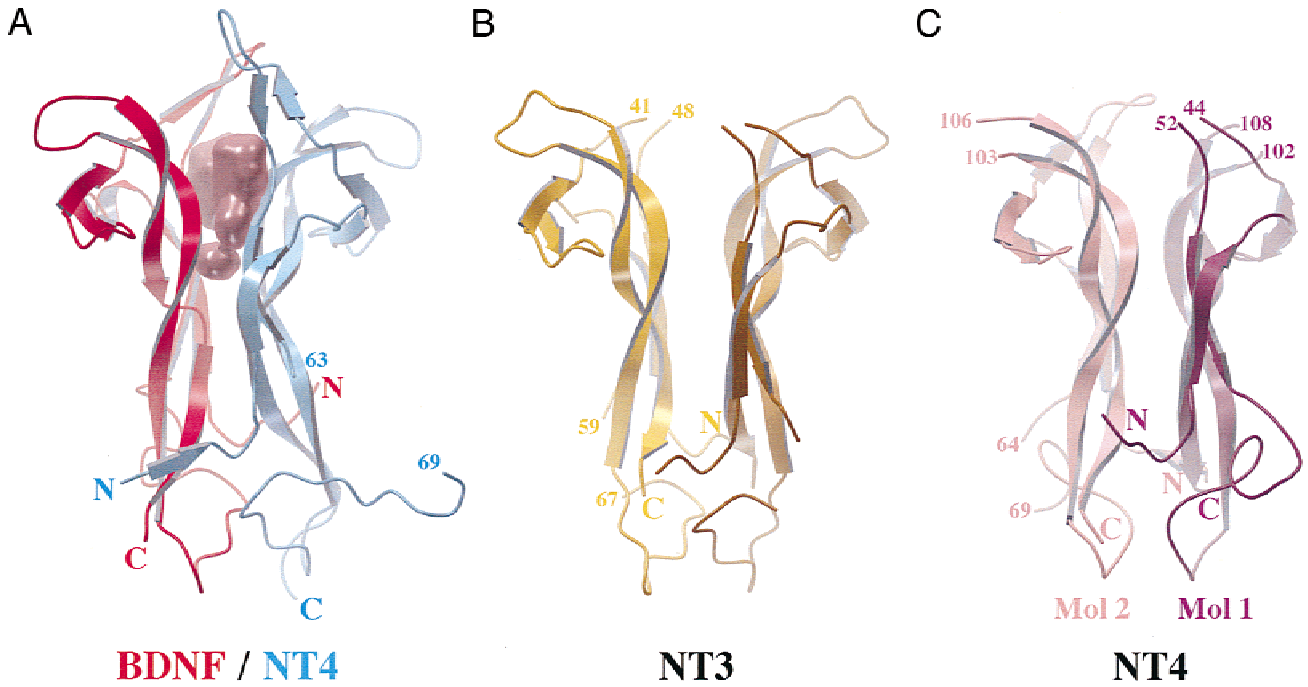

Fig. 3. Schematic representations of the BDNF/NT4 heterodimer and the NT3 and NT4 homodimers. In each case the N- and C-termini are labeled as are residues preceding and following chain breaks. **A:** BDNF(red)/NT4(sky blue). The representation highlights the cavity at the dimer interface (purple), which is not shown for NT3 and NT4 as the absent loop structure renders the cavity open. **B:** NT3 (yellow and darker yellow). This dimer results from crystallographic symmetry as such only one of the protomers is labeled. **C:** NT4. Molecule 1 is shown in purple and molecule 2 sketched in pink.

dimers with its structure in the homodimer, as yet not revealed, would display only minor adjustments to residues at the dimer interface, with the general positions of the solvent-exposed residues being unaltered by dimer composition. This exercise produces a model for the BDNF homodimer (Figs. 4, 5) that can be compared with the determined structures of NGF, NT3, and NT4 to analyze surface features that may be involved in receptor binding.

As there are no gross structural deviations within the neurotrophin family, variations in specificity for receptors among the neurotrophins may be due largely to sequence differences. The loops in which the majority of the reported p75 and trk binding specificity has been located (reviewed in Ibáñez, 1995) are mobile, suggesting that they may be molded by the receptors. This is implied by these loops showing variable conformations in different crystal environments and are only visible when they are stabilized by direct crystal packing (Table 2; Figs. 1, 3). The one distinctive structural variation that is found in the neurotrophin family is in the flexible loop III of NT4 (which includes the insertion of residues 64–70). This loop has yet to be shown to be important in receptor binding, for any neurotrophin (Ibáñez, 1995), and in the case of NT4, its extra bulk, flexibility, and protrusion into the solvent may preclude receptor binding in its vicinity.

Consequences for p75 binding

Mutagenesis studies on the neurotrophins have identified two clusters of residues that are involved in p75 receptor binding. The first cluster of residues includes basic residues in the variable regions I and V on NGF, BDNF, and NT3 (Ibáñez et al., 1991, 1992; Urfer et al., 1994; Fig. 4, and shown in light green in Fig. 5a) are implicated in binding this acidic receptor (estimated pI 4.4; Radeke et al., 1987). NT4 may be expected to interact with p75 in a similar manner because these residues are conserved. When such residues are viewed on the structures of NGF, NT3, and NT4 and the model of BDNF homodimer, they group in a distinctive area, which is duplicated due to twofold symmetry within the dimer.

A more detailed inspection of this first p75 binding site reveals that, while the putative interactive residues are located in close proximity to each other, they are in slightly different positions and conformations in the different neurotrophins (Fig. 4). Furthermore, analysis of the surface charge of the region implicated in binding to the p75 receptor shows it to be highly positively charged for all neurotrophins, but the exact arrangement of positive charges still exhibits variation among the neurotrophins. Loop flexibility may therefore be a factor in neurotrophin binding to a p75 receptor. p75 may be able to mold the flexible loops of the neurotrophins in an analogous fashion to which they are modified by crystal environ-

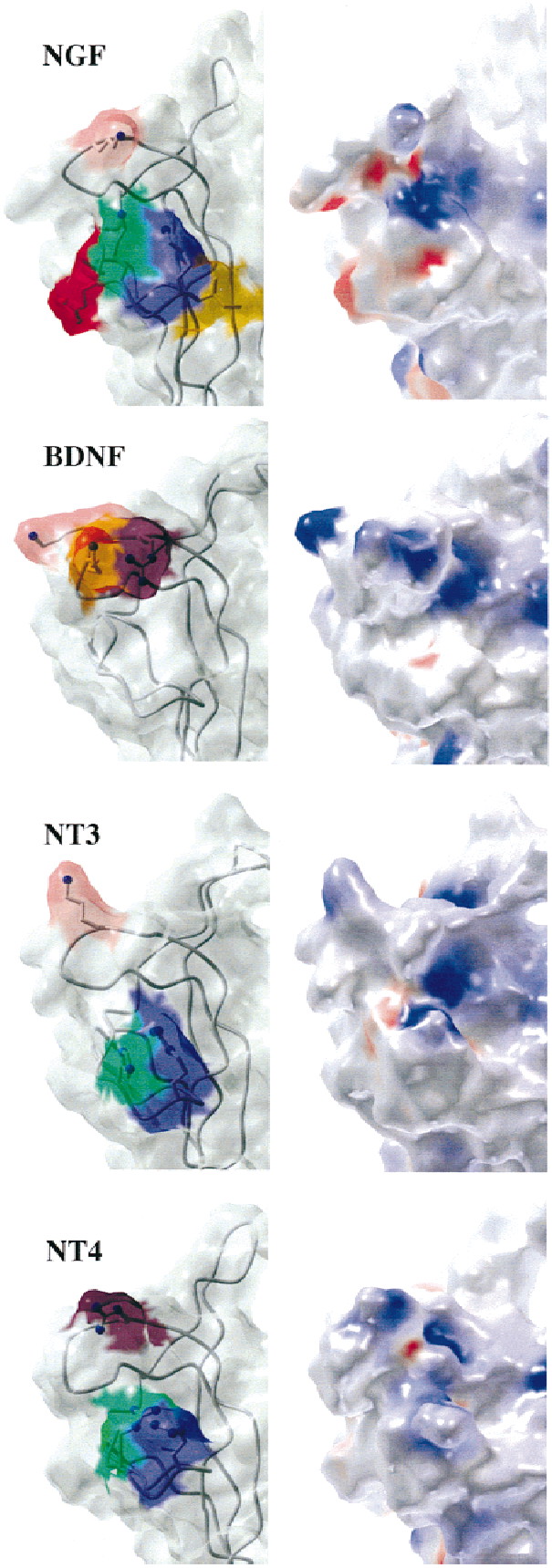

Fig. 4. Surface representations of a region of the neurotrophins homodimers demonstrated to be important in p75 binding. The left panel shows a transparent surface on top of a wire frame (gray) of each neurotrophin. Residues that are known to bind to p75 are displayed and their surface contribution colored: Ile31 (NGF), gold; Lys32 (NGF), Arg31 (NT3), Arg34 (NT4), blue; Lys34 (NGF), His33 (NT3), Arg36 (NT4), green; Lys95 (BDNF), orange; Lys95 (NGF), Lys96 (BDNF), Lys97 (NT3), pink; Arg97 (BDNF), Arg107 (NT4), purple, and Glu35 (NGF), red. The right panel shows an opaque surface charge representation of each neurotrophin dimer in the same orientation as the left panel. Blue regions represent positive and red denotes negative charged regions. This figure is rotated by 70° around the vertical in relation to the orientation of Figure 5.

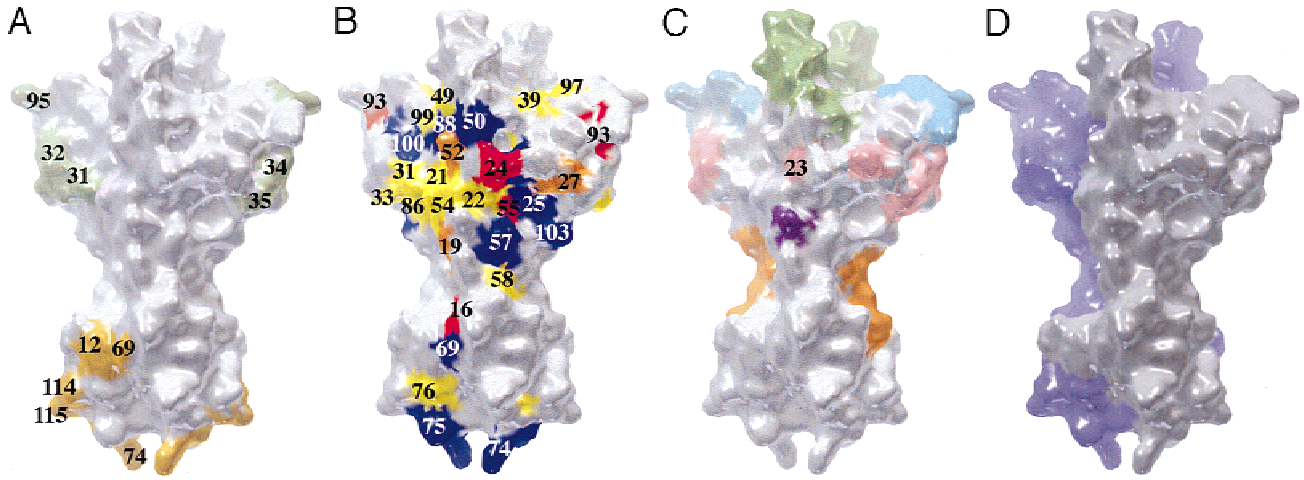

Fig. 5. Surface representations of NGF colored to highlight the common and receptor-binding features of the neurotrophin family. **A:** Residues that are known to interact with the p75 receptor. These residues cluster into two regions. Residues shown in light green form one region, which are highlighted in Figure 4. The second region shown in gold includes residues 11, 69, 74, 114, and 115. **B:** Conserved residues with surface area larger than 20 Å^2 in all of the neurotrophin structures are highlighted. Basic residues are shown in blue, acidic residues in red, polar residues in orange, and hydrophobic residues in yellow. **C:** Nonconserved resides within variable regions I (pink), II (light green), IV (orange), V (cyan), and residue 20 (purple), which are known, in at least one neurotrophin, to interact with the trk receptors. Residue 23 is labeled for comparison with **B**. **D:** The surface of NGF showing the contribution from each protomer. One protomer is colored lilac the other gray.

ment. This would explain the variation in circular dichroism spectra observed among the different neurotrophins binding to this receptor (Timm et al., 1994). There are two additional charged, conserved surface residues (D-93 and R-100, NGF numbering) that lie just proximal to the residues already implicated in binding p75 receptors (Figs. 4, 5A,B). These residues also must be considered as a candidate for participation in the binding process. Furthermore, this region is flanked by a number of conserved hydrophobic residues (Fig. 5A,B), including Trp21, which is known to bind p75 (Ibáñez et al., 1990; Drinkwater et al., 1991). These hydrophobic residues may in turn contribute to the p75 binding interface.

The second cluster of p75-binding residues (Urfer et al., 1994) is shown in gold in Figure 5A. These residues are proximal to three surface conserved residues (D-16, W-76, and H-75; Fig. 5B), which must be considered as a potential part of this common p75-binding region. Again this second cluster of residues will be duplicated due to twofold symmetry within the dimer. The overall duplication of p75 binding sites might allow the symmetrical interaction of one neurotrophin dimer with two molecules of p75. The two clusters of interactive residues are contributed to mainly by only one protomer from each neurotrophin dimer, with the exception of residues 12 (conservative substitution, NGF numbering, Fig. 5), 69 (conserved), and potential interactive residue 16 (conserved). Therefore, a heterodimer might be expected to interact with p75 in a manner characteristic of both of the relevant parent homodimers.

Consequences for trk binding

The residues implicated in trk binding fall into several spatially diverse regions of neurotrophin structure. Residues within variable region I (NGF, BDNF, NT3; Fig. 5C, pink), variable region II (all neurotrophins, light green), variable region IV (NGF, BDNF, NT3; orange), variable region V (NGF, BDNF; cyan), and residue 20 (purple, NGF numbering; Urfer et al., 1994), as well as some N- and C-terminal residues, have been shown to be involved in trk receptor binding (reviewed in Ibáñez, 1995). In surface representations, these regions are seen to be scattered mainly in the top half of the molecule and do not form a single, cohesive site (Fig. 5C). The residues implicated in trk receptor binding were generally identified by the mutation of residues that are not conserved in the primary structure among the neurotrophins. Hence, these represent residues that are involved in receptor specificity. These residues fall into either flexible loop regions or structurally conserved regions. Therefore, the choice of amino acid, in these regions, is important for receptor specificity rather than for structural variation between the ligands. A proportion of residues that are conserved among the neurotrophins may be expected to contribute to shared trk-binding features. To determine whether there may be a common region for trk receptor binding, we pasted all residues that are conserved among all four neurotrophins and that have an exposed surface area of at least 20 Å^2 onto the surface of NGF (Fig. 5B). The vast majority of these residues cluster around regions that have been demonstrated to be important for trk receptor specificity (Fig. 5C). Furthermore, approximately half of these residues cluster together in an area that connects all four of the regions involved in trk binding specificity. There is existing evidence that this surface region is involved in trk binding. Conserved residues Val22, Arg100, and Arg103 on NGF (Ibáñez et al., 1993) and residues Tyr51, Glu54, Arg56, and Arg103 from NT3 (Urfer et al., 1994) have been shown to bind their respective receptors. We propose that this conserved region, consisting of residues 19, 21, 22, 24, 25, 31, 33, 49, 50, 52, 54, 55, 57, 58, 86, 88, 93, 99, 100, and 103 (NGF numbering), presents shared elements of a neurotrophin-trk binding site that is duplicated due to dimer symmetry. The surrounding, highly variable regions confer specificity to individual neurotrophin-trk receptor pairings. In particular, the cluster of conserved hydrophobic residues (residues 21, 31, 33, 54, and 86) could constitute a typical protein–protein interaction interface (Janin & Chothia, 1990; Clackson & Wells, 1995), which

is surrounded by charged and specificity-conferring residues. Clearly, there is some overlap between neurotrophin residues known to bind p75 and those that bind to the trk receptors (Fig. 5). This suggests that receptor binding is either mutually exclusive or that the two classes of receptor may tightly abut each other to allow simultaneous binding to a single neurotrophin.

The proposed common features of the trk binding site on the neurotrophins include residues from both protomers of the neurotrophin dimer (Fig. 5B,D). The specificity-conferring elements (Fig. 5C) also are contributed by both protomers. In our trk-binding model, variable regions II and V from different protomers will form part of each of the trk-binding sites in the dimer, whereas variable regions I and IV from the same protomer may be expected to contribute to both trk-binding sites. The implication for neurotrophin heterodimers is that each trk-binding site will be a hybrid and, therefore, may be expected to display reduced but more promiscuous activity than observed with the parent homodimers.

Materials and methods

Crystal growth and data collection

All recombinant human proteins, purified from *Escherichia coli*, were supplied by Regeneron Pharmaceuticals Inc. (Tarrytown, New York). Details of crystallization and data collection protocols for NT3 and the BDNF/NT4 heterodimer have been reported previously (Robinson et al., 1995). NT4 homodimer crystals were grown at 15 °C from a 10 mg/mL solution of NT4 in 10 mM Tris pH 8.0 using a reservoir solution of 22% PEG 8000, 100 mM PIPES pH 6.5. The crystallization drop also contained a 1:2 ratio (NT4 protomer:peptide) of a 24 amino acid peptide reported to mimic trkB binding (Windisch et al., 1995). Data to 2.3 Å from a single frozen NT4 crystal, stabilized by 20% glycerol as a cryoprotectant, were collected at a wavelength of 0.97 Å on beamline 1-5, SSRL, Stanford (ADSC, Quantum 4 CCD).

Molecular replacement and refinement

The molecular replacement solutions were determined using AMORE (Navaza, 1994). In the case of BDNF/NT4, the search model consisted of a model of the BDNF dimer generated from the crystal structure of the BDNF/NT3 heterodimer (Robinson et al., 1995) by superimposing the structure of the BDNF protomer onto the structure of the NT3 protomer. From the refined structure of BDNF/NT4, a NT4 dimer model was similarly constructed and used in the case of the NT4 homodimer, whereas the structure of the NT3 protomer from the BDNF/NT3 structure provided the search model for NT3, as this crystal form has a single protomer in the asymmetric unit. Subsequent positional, simulated annealing and *B*-factor refinements (grouped *B*-factors in the case of BDNF/NT4) were applied using either X-PLOR version 3.1 (Brünger, 1992) or REFMAC (CCP4, 1994).

The $2|F_o| - |F_c|f_{calc}$ and $|F_o| - |F_c|f_{calc}$ electron density maps were viewed using the interactive computer graphics programs FRODO (Jones, 1985) or O (Jones et al., 1991). The NT4 protomer was readily identifiable in the BDNF/NT4 map by two insertions (one of seven amino acids and the other of a single amino acid), as well as by a deletion (two amino acids) in comparison to BDNF. The correct amino acids were inserted, the models were rebuilt manually, and further rounds of positional refinement and simulated annealing were applied. Molecular restraints between the two protomers of NT4 in the NT4 homodimer were discarded once clear differences between the structures were observed.

The program PROCHECK (Laskowski et al., 1993) was utilized to assess the quality of final structures. Secondary structural assignments and solvent accessibilities were calculated in the program DSSP (Kabsch & Sander, 1983). Cavities were located using a probe accessible radius of 1.2 Å, and molecular surfaces were calculated using GRASP (Nicholls & Honig, 1991). Final structures were displayed using a variety of programs: FRODO (Jones, 1985), MOLSCRIPT (Kraulis, 1991), and O (Jones et al., 1991). Molecular superpositions were implemented using MNYFIT (Sutcliffe et al., 1987). The coordinates have been deposited in the Brookhaven Protein Data Bank with accession codes 1B8K (NT3 homodimer), 1B8M (BDNF/NT4 heterodimer), and 1B98 (NT4 homodimer).

Acknowledgments

We thank the staff at the SRS, Daresbury, and Henry Bellamy at the SSRL, Stanford, for help with X-ray data collection, and R. Bryan, R. Esnouf, and W. Kwiatkowski for computing facilities. We are very grateful to W. Kwiatkowski for help with the preparation of the figures. The Oxford Centre for Molecular Sciences is supported by BBSRC and the MRC. E.Y.J. is supported by a Royal Society Research Fellowship, D.I.S. by the MRC, L.D.B. by the Natural Sciences and Engineering Council of Canada and by the Royal Society Canada-UK Exchange Program, R.C.R. was supported by a MRC Research Studentship, by the Oxford Centre for Molecular Sciences, by a Hoffman Fellowship and by a Klingenstein Foundation Fellowship.

References

Arakawa T, Haniu M, Narhi LO, Miller JA, Talvenheimo J, Philo JS, Chute HT, Matheson C, Carnahan J, Louis J-C, et al. 1994. Formation of heterodimers from three neurotrophins, nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor. *J Biol Chem 269*:27833–27839.

Barbacid M. 1995. Neurotrophic factors and their receptors. *Curr Opin Cell Biol 7*:148–155.

Barde Y-A, Edgar D, Thoenen H. 1982. Purification of a new neurotrophic factor from mammalian brain. *EMBO J 1*:549–553.

Brünger AT. 1992. *X-PLOR version 3.1. A system for X-ray crystallography and NMR.* New Haven, Connecticut: Yale University Press.

Butte MJ, Hwang PK, Mobley WC, Fletterick RJ. 1998. Crystal structure of neurotrophin-3 homodimer shows distinct regions are used to bind its receptors. *Biochemistry 37*:16846–16852.

CCP4 (Collaborative Computing Project Number 4). 1994. The CCP4 suite: Programs for crystallography. *Acta Crystallogr D50*:760–763.

Clackson T, Wells JA. 1995. A hot spot of binding energy in a hormone–receptor interface. *Science 26*:383–386.

Curtis R, Adryan KM, Stark JL, Park JS, Compton DL, Weskamp G, Huber LJ, Chao MV, Jaenisch R, Lee KF, et al. 1995. Differential role of the low affinity neurotrophin receptor (p75) in retrograde axonal transport of the neurotrophins. *Neuron 14*:1201–1211.

Davies AM. 1994. Switching neurotrophin dependence. *Curr Biol 4*:273–276.

Davies AM, Wright EM. 1995. Neurotrophic factors. Neurotrophin autocrine loops. *Curr Biol 5*:723–726.

Drinkwater CC, Suter U, Angst C, Shooter EM. 1991. Mutation of tryptophan-21 in mouse nerve growth factor (NGF) affects binding to the fast NGF receptor but not induction of neurites on PC12 cells. *Proc R Soc Lond B Biol Sci 246*:307–313.

Heymach JV, Shooter EM. 1995. The biosynthesis of neurotrophin heterodimers by transfected mammalian cells. *J Biol Chem 270*:12297–12304.

Holland DR, Cousens LS, Meng W, Matthews BW. 1994. Nerve growth factor in different crystal forms displays flexibility and reveals zinc binding sites. *J Mol Biol 239*:385–400.

Ibáñez CF. 1995. Neurotrophic factors: From structure–function relationships to designing effective therapeutics. *Trends Biotechnol 13*:217–227.

Ibáñez CF, Ebendal T, Barnaby G, Murray-Rust J, Persson H. 1992. Disruption of the low affinity receptor binding site in NGF allows neuronal survival and differentiation by binding to the trk gene products. *Cell 69*:329–341.

Ibáñez CF, Ebendal T, Persson H. 1991. Chimeric molecules with multiple neurotrophic activities reveals structural elements determining the specificities of NGF and BDNF. *EMBO J 10*:2105–2110.

Ibáñez CF, Hallbook F, Ebendal, Persson H. 1990. Structural studies of nerve growth factor: Functional importance of highly conserved amino acid residues. *EMBO J 9*:1477–1483.

Ibáñez CF, Ilag LL, Murray-Rust J, Persson H. 1993. An extended surface of binding to trk tyrosine kinase receptors in NGF and BDNF allows the engineering of a multifunctional panneurotrophin. *EMBO J 12*:2281–2293.

Ip NY, Ibáñez CF, Nye SH, McClain J, Jones PF, Gies D, Belluscio L, Le Beau MM, Espinosa R, Squinto SP, et al. 1992. Mammalian neurotrophin-4: Structure, chromosomal localization, tissue distribution and receptor specificity. *Proc Natl Acad Sci USA 89*:3060–3064.

Ip NY, Stitt TN, Tapley P, Klein R, Glass DJ, Fandle J, Greene LA, Barbacid M, Yancopoulos GD. 1993. Similarities and differences in the way neurotrophins interact with the Trk receptors in neuronal and nonneuronal cells. *Neuron 10*:137–149.

Janin J, Chothia C. 1990. The structure of protein–protein recognition sites. *J Biol Chem 265*:16027–16030.

Johnson J, Oppenheim R. 1994. Neurotrophins. Keeping track of changing neurotrophic theory. *Curr Biol 4*:662–665.

Jones TA. 1985. Interactive computer graphics: FRODO. *Methods Enzymol 115*:157–171.

Jones TA, Zou J-Y, Cowan SW, Kjeldgaard M. 1991. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr A47*:110–119.

Jungbluth S, Bailey K, Barde Y-A. 1994. Purification and characterization of brain-derived neurotrophic factor/neurotrophin 3 (BDNF/NT3) heterodimer. *Eur J Biochem 221*:677–685.

Kabsch W, Sander C. 1983. Dictionary of protein secondary structure: Pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers 22*:2577–2637.

Kraulis P. 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J Appl Crystallogr 24*:946–950.

Laskowski RJ, Macarthur MW, Moss DS, Thornton JM. 1993. PROCHECK: A program to check the stereochemical quality of protein structures. *J Appl Crystallogr 26*:283–290.

Lee KF, Davies AM, Jaenisch R. 1994. p75-Deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. *Development 120*:1027–1033.

Levi-Montalcini R. 1987. The nerve growth factor 35 years later. *Science 237*:1154–1162.

Maisonpierre PC, Belluscio L, Squinto SP, Ip NY, Furth ME, Lindsay RM, Yancopoulos GD. 1990. Neurotrophin-3—A neurotrophic factor related to NGF and BDNF. *Science 247*:1446–1451.

McDonald NQ, Hendrickson WA. 1993. A structural superfamily of growth factors containing a cystine knot motif. *Cell 73*:421–424.

McDonald NQ, Lapatto R, Murray-Rust J, Gunning J, Wlodawer A, Blundell TL. 1991. New protein fold revealed by a 2.3 Å resolution crystal structure of nerve growth factor. *Nature 354*:411–414.

Navaza J. 1994. AMORE: An automated package for molecular replacement. *Acta Crystallogr A40*:157–163.

Nicholls A, Honig B. 1991. A rapid finite difference algorithm, utilizing successive over relaxation to solve the Poisson–Boltzmann equation. *J Comput Chem 12*:435–445.

Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM. 1987. Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Nature 325*:593–597.

Radziejewski C, Robinson RC. 1993. Heterodimers of the neurotrophic factors: Formation, isolation and differential stability. *Biochemistry 32*:13350–13356.

Robinson RC, Radziejewski C, Stuart DI, Jones EY. 1995. Structure of the brain-derived neurotrophic factor/neurotrophin 3 heterodimer. *Biochemistry 34*:4139–4146.

Rydén M, Murray-Rust J, Glass D, Ilag LL, Trupp M, Yancopoulous GD, McDonald NQ, Ibáñez CF. 1995. Functional analysis of mutant neurotrophins deficient in low-affinity binding reveals a role for p75LNGFR in NT-4 signalling. *EMBO J 9*:1979–1990.

Sutcliffe MJ, Haneef I, Carney D, Blundell TL. 1987. Knowledge based modelling of homologous proteins, Part II: Rules for the conformations of substituted sidechains. *Protein Eng 1*:377–384.

Timm DE, Ross AH, Neet KE. 1994. Circular dichroism and crosslinking studies of the interaction between four neurotrophins and the extracellular domain of the low affinity neurotrophin receptor. *Protein Sci 3*:451–458.

Treanor JJS, Schmelzer C, Knusel B, Winslow JW, Shelton DL, Hefti F, Nikolics K, Burton LE. 1995. Heterodimeric neurotrophins induce phosphorylation of Trk receptors and promote neuronal differentiation in PC12 cells. *J Biol Chem 270*:23104–23110.

Urfer R, Tsoulfas P, O'Connell L, Shelton DL, Parada LF, Presta LG. 1995. An immunoglobulin-like domain determines the specificity of neurotrophin receptors. *EMBO J 14*:2795–2805.

Urfer R, Tsoulfas P, Soppet D, Escandon E, Parada LF, Presta LG. 1994. The binding epitopes of neurotrophin-3 to its receptors trkC and gp75 and the design of a multifunctional human neurotrophin. *EMBO J 13*:5896–5909.

Windisch JM, Marksteiner R, Lang ME, Auer B, Schneider R. 1995. Brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 bind to a single leucine-rich motif of TrkB. *Biochemistry 34*:11256–11263.