An extended surface of binding to Trk tyrosine kinase receptors in NGF and BDNF allows the engineering of a multifunctional pan-neurotrophin

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Neurotrophin-mediated cell survival and differentiation
of vertebrate neurons is caused by ligand-specific binding
to the Trk family of tyrosine kinase receptors. However,
sites in the neurotrophins responsible for the binding to
Trk receptors and the mechanisms whereby this inter-
action results in receptor activation and biological activity
are unknown. Here we show that in nerve growth factor
(NGF) and brain-derived neurotrophic factor (BDNF),
discontinuous stretches of amino acid residues group
together on one side of the neurotrophin dimer forming
a continuous surface responsible for binding to and
activation of TrkA and TrkB receptors. Two symmetrical
surfaces are formed along the two-fold axis of the
neurotrophin dimer providing a model for ligand-
mediated receptor dimerization. Mutated neurotrophins
inducing similar levels of receptor phosphorylation
showed different biological activities, suggesting that
structural differences in a ligand may result in dissimilar
responses in a given tyrosine kinase receptor. Our results
allowed us to combine structural elements from NGF,
BDNF and neurotrophin-3 to engineer a pan-neuro-
trophin that efficiently activates all Trk receptors and
displays multiple neurotrophic specificities.
Key words: receptor binding/signal transduction/site-directed
mutagenesis/tyrosine phosphorylation

Introduction

Many polypeptide growth factors mediate their biological
responses by binding to and activating cell surface receptors
with intrinsic tyrosine kinase activity. Upon ligand binding,
these receptors become autophosphorylated on multiple
tyrosine residues and subsequently associate with intracellular
molecules important for signal transduction (Ullrich and
Schlessinger, 1990). However, little is known about the
structural determinants of the specificity of ligand – receptor
binding and how this interaction results in receptor activation
and in the transduction of pleiotropic biological effects.

Recent work on the Trk family of tyrosine kinase recep-
tors has established that these molecules constitute signal-
transducing receptors for a family of structurally and
functionally related neurotrophic factors collectively known
as the neurotrophins. Most of our knowledge of neurotrophic
factors comes from work on the nerve growth factor (NGF),

a 118 amino acid polypeptide that controls the maturation
and survival of sympathetic neurons, as well as subpopula-
tions of sensory and central neurons (Levi-Montalcini and
Angeletti, 1968; Thoenen and Barde, 1980; Thoenen et al.,
1987). Other neurotrophins include brain-derived
neurotrophic factor (BDNF; Barde et al., 1982; Leibrock
et al., 1989), neurotrophin-3 (NT-3; Ernfors et al., 1990;
Hohn et al., 1990; Jones and Reichardt, 1990; Kaisho et al.,
1990; Maisonpierre et al., 1990; Rosenthal et al., 1990) and
neurotrophin-4 (NT-4; Hallböök et al., 1991; Ip et al.,
1992), also named neurotrophin-5 (Berkemeier et al., 1991).
The ability of the neurotrophins to promote survival of
peripheral and central neurons during development and after
neuronal damage has stimulated the interest in these
molecules as potential therapeutic agents for the treatment
of neurodegenerative diseases and nervous system injuries.

The neurotrophins show ~50% amino acid sequence
identities and display both overlapping and specific sets of
neurotrophic activities on peripheral and central neurons.
For example, all neurotrophins, in various proportions,
support the survival of embryonic neural crest-derived
sensory neurons (Thoenen and Barde, 1980; Lindsay et al.,
1985; Hohn et al., 1990; Ip et al., 1992; Ibáñez et al.,
1993). In contrast, survival of embryonic sympathetic
neurons is only supported by NGF, while placode-derived
sensory neurons are supported by BDNF and NT-3, but not
by NGF (Thoenen and Barde, 1980; Lindsay et al., 1985;
Hohn et al., 1990). This specificity is believed to be achieved
in part by the selective interaction between the different
neurotrophins and the members of the Trk family of tyrosine
kinase receptors expressed on the surface of distinct neuronal
populations. Thus, whereas NGF binds only to p140^{trk}
(herein called TrkA) (Kaplan et al., 1991a,b; Klein et al.,
1991a), BDNF and NT-4 interact exclusively with p145^{trkB}
(herein called TrkB) (Soppet et al., 1991; Squinto et al.,
1991; Klein et al., 1991b, 1992; Ip et al., 1992) while NT-3
interacts with p145^{trkC} (herein called TrkC) and, to a lesser
extent, also with TrkA and TrkB (Cordon-Cardo et al.,
1991; Lambelle et al., 1991; Klein et al., 1991b; Squinto
et al., 1991). Unlike Trk receptors, the low-affinity nerve
growth factor receptor p75^{NGFR} (herein called LNGFR)
(Johnson et al., 1986; Radeke et al., 1987) recognizes each
of the neurotrophins with a similar affinity (Ernfors et al.,
1990; Rodriguez-Tébar et al., 1990, 1992; Hallböök et al.,
1991). Although LNGFR was initially postulated as a
component of functional high-affinity NGF receptors
(Hempstead et al., 1989, 1991) involved in mediating
biological activity (Hempstead et al., 1989; Yan et al.,
1991), emerging evidence disputes the direct role of this
molecule in signal transduction (Glass et al., 1991;
Weskamp and Reichardt, 1991; Ibáñez et al., 1992; Jing
et al., 1992).

Most of the sequence variations among the neurotrophins
occur in distinct regions, and initial studies using chimeric
molecules between NGF and BDNF have shown that specific

combinations of some of these variable sequences allow a broader spectrum of neurotrophic activities than those of the two wild type (wt) proteins (Ibáñez *et al.*, 1991a; Suter *et al.*, 1992). However, these studies did not identify specific receptor-binding sites in the neurotrophins responsible for the observed differences in biological specificities. The recent elucidation of the crystal structure of NGF has localized three-quarters of the variable residues in three β-hairpin loops and a reverse turn (McDonald *et al.*, 1991). Other variable regions include part of a β-strand and the NH₂ and COOH-termini. The fact that most of the conserved amino acid residues in the neurotrophins play structural roles suggests that all four neurotrophins have very similar conformations, with individual differences restricted to the variable regions (McDonald *et al.*, 1991). Surface loop regions are in general thought to be important for receptor-binding of growth factors (Daopin *et al.*, 1992; Oefner *et al.*, 1992; Sclunegger and Grüter, 1992), although so far no such sequences have been identified in ligands of tyrosine kinase receptors. In the present study, we have used site-directed mutagenesis combined with biochemical and biological assays to define structural elements in the neurotrophins responsible for the specificity of binding and activation of Trk receptors. The results allowed us to construct a multifunctional neurotrophin agonist that efficiently activates all Trk receptors and displays neurotrophic specificities recruited from NGF, BDNF and NT-3.

Results

We have previously defined five variable regions in which the NGF sequence differs from those of BDNF and NT-3 (Figure 1A) (Ibáñez *et al.*, 1991a). Three of these regions (variable regions I, II and V) correspond to β-hairpin loops exposed on the surface of the NGF molecule, whereas region III contains a reverse turn and region IV is a β-strand (Figure 1B) (McDonald *et al.*, 1991). Using oligonucleotide site-directed mutagenesis, the variable regions in the NGF molecule were systematically replaced either alone or in different combinations by the corresponding sequences from BDNF. Chimeric molecules including sequences from the BDNF NH₂- and COOH-termini were also constructed. In addition, point mutations were generated in either the wt NGF molecule or in the context of a chimeric molecule. Structural elements involved in the interaction of NGF and BDNF with their respective TrkA and TrkB receptors were investigated simultaneously by evaluating the effect of the mutations as loss of NGF function and gain of BDNF function, respectively.

Structural elements in NGF responsible for binding to and activation of TrkA
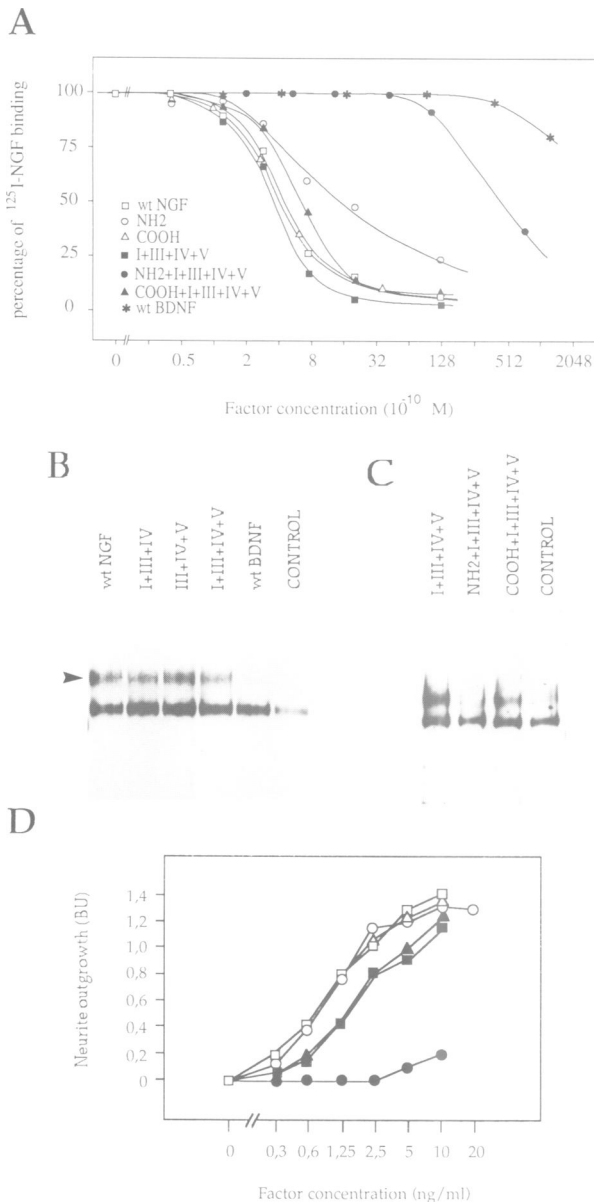
The individual replacement of variable regions I, II, III, IV or V generated chimeric molecules that were indistinguishable from wt NGF in TrkA receptor binding, activation or in their biological activity (not shown). Moreover, the chimeric molecule I+III+IV+V retained wt levels of TrkA binding and tyrosine phosphorylation although it showed reduced biological activity on sympathetic neurons [~50% of wt NGF activity (Figures 2A, B, D and Table I)]. These results suggested that the remaining NGF variable regions (i.e. region II and NH₂- and COOH-termini) could be responsible for the NGF activities of the I+III+IV+V molecule. To test this, the NH₂- and COOH-termini in wt
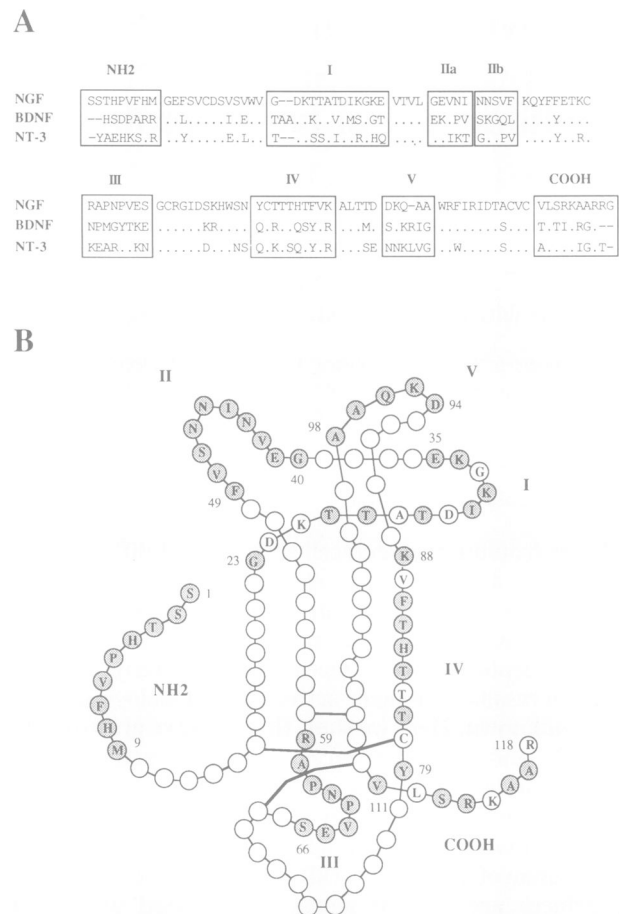
Fig. 1. Variable regions in the neurotrophins. (**A**) Alignment of the amino acid sequences (single-letter code) of rat NGF (Whittemore *et al.*, 1988), rat BDNF (Maisonpierre *et al.*, 1990) and rat NT-3 (Ernfors *et al.*, 1990). Variable regions are boxed and labelled. (**B**) Schematic representation of the three-dimensional structure of the NGF monomer (McDonald *et al.*, 1991). Residues in the different variable regions are indicated by their one-letter code. Residues changed in the NGF protein as a result of the replacement of variable regions from BDNF are shaded. Thick lines at the bottom indicate disulphide bridges.

NGF and in the I+III+IV+V chimera were replaced by those of BDNF. Replacement of the COOH-terminus had no effect on TrkA binding, TrkA activation or biological activity (Figure 2A, C, D and Table I). In contrast, replacement of the NH₂-terminus in wt NGF reduced the affinity of binding to TrkA 4- to 5-fold (Figure 2A), but TrkA phosphorylation and biological activity were not affected (Table I and Figure 2D). However, replacement of the NH₂-terminus in the I+III+IV+V chimera reduced binding, receptor phosphorylation and biological activity to <1% of wt NGF (Figure 2A, C and D). Identical results were obtained when only residues 3−9 in NGF (Figure 1A) were replaced by the NH₂-terminus of BDNF (Table I), indicating that residues in NGF important for TrkA binding are located between positions 3 and 9.

The differences observed when the NH₂-terminus was replaced in the context of the wt NGF or in chimeric molecule I+III+IV+V suggested that other variable regions could cooperate synergistically with NH₂-terminal sequences to promote binding, receptor activation and biological activity. To test this possibility, variable regions I, IV or V in the NH₂+I+III+IV+V chimera were

A

B

C

D

Table I. Relative TrkA receptor binding, receptor activation and specific biological activity of wild type, mutant and chimeric NGF proteins

Mutant protein[a]	Receptor binding[b] (% of wild type)	Receptor activation[c]	Biological activity[d] (% of wild type)
wt NGF	100	+ + +	100
wt BDNF	<0.2	−	<0.2
NH$_2$	23	+ + +	100
NH$_2$(3−9)	22	+ + +	100
COOH	90	+ + +	100
I31A	20	ND	25
I+III+IV	105	+ + +	100
III+IV+V	95	+ + +	50
I+III+IV+V	110	+ +	50
NH$_2$+I+III+IV+V	<1	−	<1
NH$_2$(3−9)+I+III+IV+V	<1	−	<1
NH$_2$+III+IV+V	12	+ +	7
NH$_2$+I+III+V	12	+ +	35
NH$_2$+I+III+IV	22	+ +	40
COOH+I+III+IV+V	75	+ +	50
I+IIa+III+IV+V	110	+ +	50
I+IIb+III+IV+V	96	±	10
I+II+III+IV+V	70	−	3
E41A+I+III+IV+V	90	+	12
N43A+I+III+IV+V	100	+ + +	50
I44A+I+III+IV+V	90	+ + +	50
N45A+I+III+IV+V	95	+ +	25
N46A+I+III+IV+V	100	+ + +	50
V48A+I+III+IV+V	100	+ + +	50

[a]Mutants are abbreviated by the wt residue (single amino acid designation), followed by its codon number and the mutant residue. Roman numerals denote variable regions in NGF that were replaced by homologous regions from BDNF.
[b]Data from at least three different dose−response experiments using rtrkA-3T3 cells varied by ±10%.
[c]Measured as receptor tyrosine phosphorylation stimulated by 100 ng/ml of ligand. Consistent results were obtained in three independent experiments.
[d]Measured as neurite outgrowth evoked from explants of embryonic sympathetic ganglia. Data from at least three different dose−response experiments varied by ±10%.
ND, not determined.

Fig. 2. TrkA receptor binding, receptor phosphorylation and biological activity of wt and chimeric molecules. (**A**) Serial dilutions of transfected COS cell conditioned medium containing equal amounts of wt NGF, wt BDNF, and chimeric molecules NH$_2$, COOH, I+III+IV+V, NH$_2$+I+III+IV+V and COOH+I+III+IV+V were assayed for their ability to displace [^{125}I]NGF from receptors on rtrkA-3T3 cells. Medium from mock-transfected cells failed to displace [^{125}I]NGF from these cells. Each point represents the mean of triplicate determinations. Standard deviation was ≤10%. (**B** and **C**) Tyrosine phosphorylation of TrkA receptors stimulated by wt NGF, wt BDNF and chimeric molecules. rtrkA-3T3 cells were treated with 100 ng/ml of the indicated recombinant proteins and assayed for tyrosine phosphorylation. Medium from mock-transfected COS cells was used as a negative control (CONTROL). Arrowhead indicates the migration of phosphorylated TrkA (p140^{trk}). The more rapidly migrating band corresponds to constitutively phosphorylated p140^{trk} precursors (Kaplan et al., 1991b). (**D**) Serial dilutions of transfected COS cell conditioned medium containing equal amounts of wt and chimeric factors were assayed for their ability to stimulate neurite outgrowth from E8 chick sympathetic ganglia. Data from three determinations varied by ±20% of the average values reported here. Key symbols as in (A).

changed back to those of NGF. Any of these three changes partially restored receptor binding (Figure 3A and Table I), supporting the argument that regions I, IV and V are also involved in the contact to TrkA. More receptor binding was rescued by region V than by regions I or IV, although none of these changes resulted in >20−25% of wt NGF binding (Table I). Receptor phosphorylation and biological activity in sympathetic neurons were restored to 40% of wt NGF (Figure 3B and Table I). In the biological assay, regions IV and V could rescue NGF responsiveness more efficiently than region I (Table I). Previous results from alanine-scanning mutagenesis of variable region I suggested that Ile31 is important for maximal NGF biological activity (Ibáñez et al., 1992). In agreement with this, replacement of Ile31 with Ala resulted in a 4-fold reduction in TrkA receptor binding (Figure 3A and Table I), indicating that this exposed hydrophobic residue is involved in the contact to TrkA. Within variable region IV, Tyr79, Thr81 and His84 are present in NGF from all species analysed to date but are replaced by non-conservative residues in other neurotrophins (Hallböök et al., 1991 and H.Persson et al., unpublished results), suggesting that they could be mediating the inter-action of NGF region IV with TrkA.

Replacement of regions IIa, IIb and IIa+IIb in the I+III+IV+V chimera resulted in molecules that still

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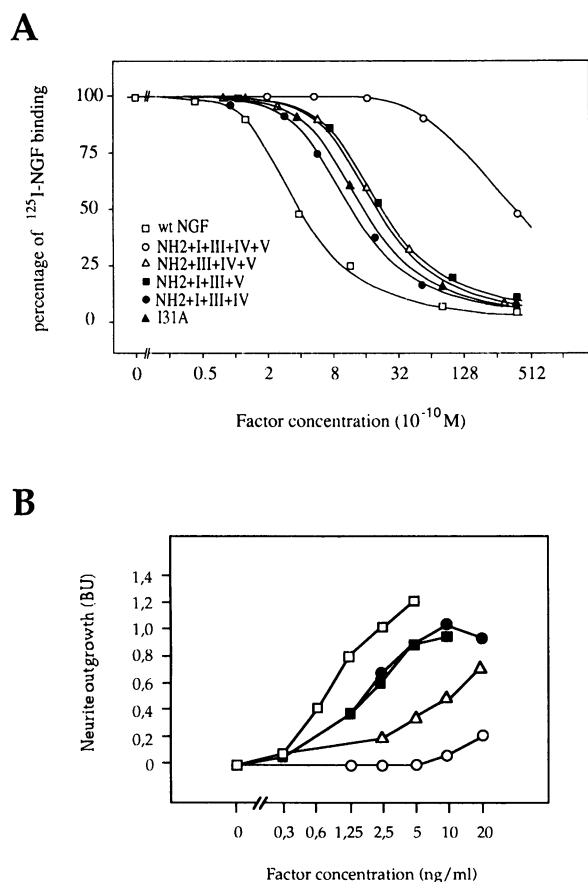
Fig. 3. NGF variable regions I, IV and V partially restore TrkA binding and biological activity of the $NH_2+I+III+IV+V$ chimeric molecule. (A) Serial dilutions of transfected COS cell conditioned medium containing equal amounts of wt NGF, chimeric molecules $NH_2+I+III+IV+V$, $NH_2+III+IV+V$, $NH_2+I+III+V$, $NH_2+I+III+IV$ and NGF mutant I31A were assayed for their ability to displace [^{125}I]NGF from receptors on rtrkA-3T3 cells. Medium from mock-transfected cells failed to displace [^{125}I]NGF from these cells. Each point represents the mean of triplicate determinations. Standard deviation was <10%. (B) Serial dilutions of transfected COS cell conditioned medium containing equal amounts of wt and chimeric factors were assayed for their ability to stimulate neurite outgrowth from E8 chick sympathetic ganglia. Data from three determinations varied by ±20% of the average values reported here. Key symbols are as in (A).

retained high levels of binding to TrkA (Figure 4A and Table I). Moreover, chimeric molecule I+IIa+III+IV+V was indistinguishable from I+III+IV+V in receptor phosphorylation and biological activity on sympathetic neurons (Figure 4B and D). In contrast, a drastic decrease in both receptor activation and biological activity was observed when region IIb (residues 45−49) was replaced (Figure 4B, D and Table I), indicating that the second half of this variable loop is important for activation of the TrkA receptor by NGF. Replacement of the complete region II resulted in even lower levels of receptor phosphorylation and bioactivity (Figure 4B, D and Table I). Interestingly, despite their low biological activity, these two molecules maintained high levels of TrkA binding, probably due to the fact that they retain the NH_2-terminus from NGF.

The functional importance of amino acid residues in variable region II was further investigated by alanine-scanning mutagenesis. Glu41, Asn43, Ile44, Asn45, Asn46 and Val48 were individually replaced with Ala in wt NGF.

A

B   **C**

D
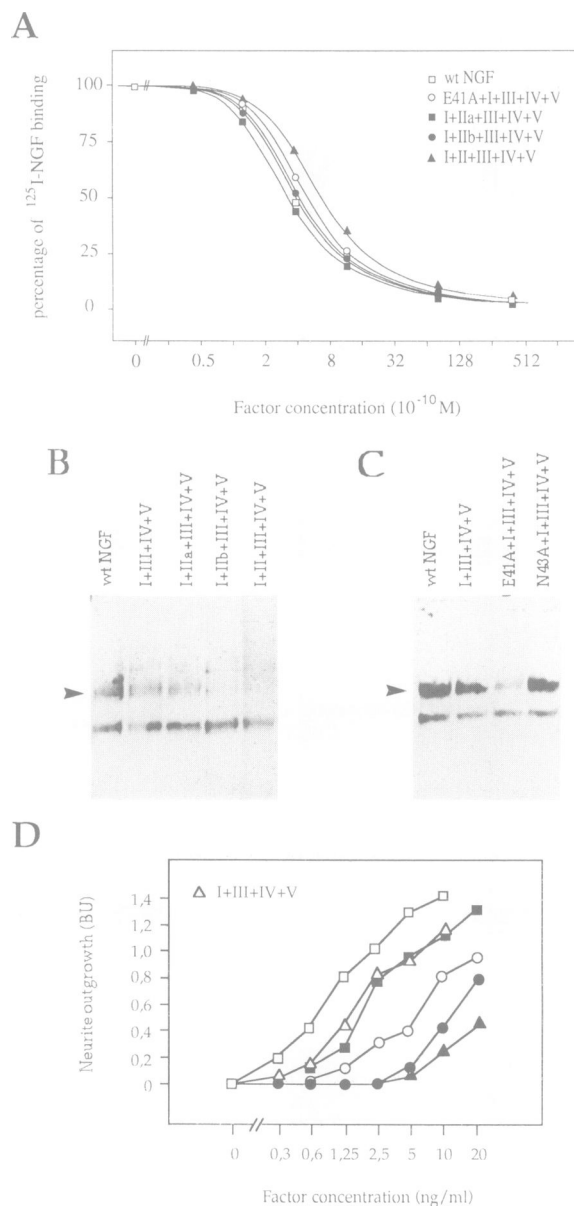
Fig. 4. Residues in variable region II of NGF are involved in TrkA activation and bioactivity but not in TrkA binding. (A) Serial dilutions of transfected COS cell conditioned medium containing equal amounts of wt NGF and chimeric molecules E41A+I+III+IV+V, I+IIa+III+IV+V, I+IIb+III+IV+V and I+II+III+IV+V were assayed for their ability to displace [^{125}I]NGF from receptors on rtrkA-3T3 cells. Medium from mock-transfected cells failed to displace [^{125}I]NGF from these cells. Each point represents the mean of triplicate determinations. Standard deviation was ≤10%. (B and C) Tyrosine phosphorylation of TrkA receptors stimulated by wt NGF and chimeric molecules. rtrkA-3T3 cells were treated with 100 ng/ml of the indicated recombinant proteins and assayed for tyrosine phosphorylation. Arrowhead indicates the migration of phosphorylated TrkA (p140^{trk}). The more quickly migrating band corresponds to constitutively phosphorylated p140^{trk} precursors (Kaplan *et al.*, 1991b). (D) Serial dilutions of transfected COS cell conditioned medium containing equal amounts of wt and chimeric factors were assayed for their ability to stimulate neurite outgrowth from E8 chick sympathetic ganglia. Data from three determinations varied by ±20% of the average values reported here. Key symbols are as in (A) except for chimeric molecule I+III+IV+V (△).

None of these mutations affected receptor binding, receptor phosphorylation or bioactivity of the NGF molecule (not shown). The same alanine-scanning mutagenesis was then

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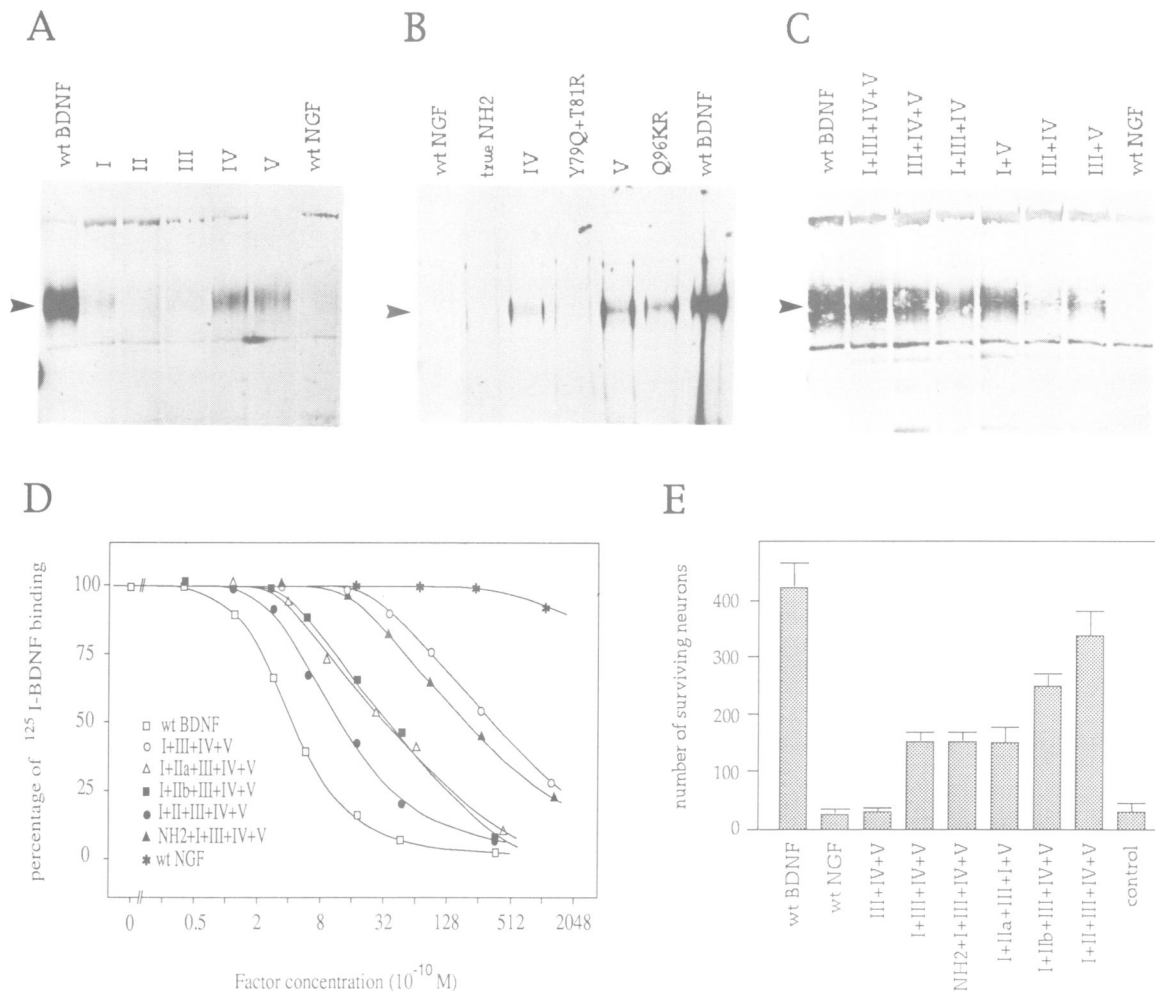
Fig. 5. TrkB receptor binding, receptor phosphorylation and biological activity of wt and chimeric molecules. (A, B and C) Tyrosine phosphorylation of TrkB receptors stimulated by wt NGF, wt BDNF and NGF mutant and chimeric molecules. rtrkB-3T3 cells were treated with 100 ng/ml of the indicated recombinant proteins and assayed for tyrosine phosphorylation. Arrowhead indicates the migration of phosphorylated TrkB (p145^{trkB}).
(D) Serial dilutions of transfected COS cell conditioned medium containing equal amounts of wt BDNF, wt NGF and chimeric molecules
I+III+IV+V, I+IIa+II+IV+V, I+IIb+III+IV+V, I+II+III+IV+V and NH$_2$+I+III+IV+V were assayed for their ability to displace
[^{125}I]BDNF from receptors on rtrkB-3T3 cells. Medium from mock-transfected cells failed to displace [^{125}I]BDNF from these cells. Each point
represents the mean of triplicate determinations. Standard deviation was ≤10%. (E) Neuronal survival of dissociated E8 chick neurons from the
nodose ganglion in the presence of saturating amounts (20−50 ng/ml) of wt BDNF, wt NGF and the indicated chimeric molecules. Medium from
mock-transfected COS cells was used as a negative control (control). Results are presented as the mean of triplicate determinations ± SD.

performed in the I+III+IV+V molecule. In this context, replacement of Glu41 with Ala substantially reduced TrkA phosphorylation and biological activity (Figure 4C, D and Table I). Mutation of Asn45 showed a less pronounced effect, while replacement of any of the other positions with Ala had no effect (Table I).

Structural elements in BDNF responsible for binding to and activation of TrkB

Replacements of variable regions IV and V in NGF by analogous regions from BDNF resulted in chimeric molecules that induced TrkB phosphorylation (Figure 5A). A weak but consistent signal was also detected with chimera I (Figure 5A). In contrast, no TrkB activation was seen when regions II, III or the NH$_2$- and COOH-termini were replaced (Figure 5A and Table II), although in the case of region II, this could be due to the low level of production of this chimeric molecule (Table II). Three main changes are introduced in NGF after replacement with BDNF variable region IV: Tyr79, Thr81 and His84 are replaced

with Gln, Arg and Gln, respectively (Figure 1A). Interestingly, these residues are also present in other neurotrophins reported to interact with TrkB, i.e. NT-3 and NT-4. Tyr79 and Thr81 (in region IV) were simultaneously replaced with Gln and Arg, respectively, but this mutant failed to stimulate TrkB phosphorylation at the level seen with region IV (Figure 5B). This suggests that Gln at position 84 is important, and/or that synergistic cooperation of these three residues is necessary for receptor activation. In variable region V, BDNF has three positively charged residues (95, 96 and 97), two of which are not present in the other neurotrophins. Mutation of Gln96 in NGF into the dipeptide Lys−Arg resulted in a molecule able to stimulate tyrosine phosphorylation of TrkB to the same level as chimera V (Figure 5B), suggesting that the positive charges in region V of BDNF are important for activation of TrkB. Additive effects on TrkB phosphorylation were seen in chimeric molecules containing different combinations of regions I, IV and V (Figure 5C).

Despite their ability to stimulate TrkB phosphorylation,

Table II. Relative TrkB receptor binding, receptor activation and specific biological activity of wild type, mutant and chimeric NGF proteins

Mutant protein[a]	Receptor binding[b] (% of wild type)	Receptor activation[c]	Biological activity[d] (% of wild type)
wt BDNF	100	+++	100
wt NGF	<0.2	−	<5
NH$_2$	<0.2	−	ND
I	ND	+	ND
II[e]	ND	−	ND
III	ND	−	ND
IV	ND	+	ND
Y79Q+T81R	ND	−	ND
V	ND	+	ND
Q96KR	ND	+	ND
COOH	ND	−	ND
I+V	ND	++	<5
III+IV	ND	+	ND
III+V	ND	+	ND
I+III+IV	0.3	++	<5
III+IV+V	0.6	++	<5
I+III+IV+V	1.2	+++	30
NH$_2$+I+III+IV+V	2.5	ND	30
COOH+I+III+IV+V	1	ND	30
I+IIa+III+IV+V	12	ND	30
I+IIb+III+IV+V	12	ND	60
I+II+III+IV+V	55	ND	80

[a]Mutants and chimeric molecules are abbreviated as in Table I.
[b]Data from at least three dose−response experiments using r*trkB*-3T3 cells varied by ±10%.
[c]Measured as receptor tyrosine phosphorylation stimulated by 100 ng/ml of ligand. Consistent results were obtained in three independent experiments.
[d]Measured as maximal cell survival in cultures of dissociated embryonic nodose neurons. Data from at least three experiments varied by ±10%.
[e]Yield of recombinant protein <0.05% of wt NGF. Assayed at 10 ng/ml.
ND, not determined.

these molecules showed very low levels of receptor binding in competitive binding assays. The TrkB binding affinity of chimera I+III+IV+V was 100-fold lower than of wt BDNF (Figure 5D and Table II), suggesting that variable region II and the NH$_2$- and COOH-termini of BDNF could be important for binding to the TrkB receptor. However, adding the NH$_2$-terminus of BDNF to the I+III+IV+V chimera produced only a minor improvement in binding to TrkB (Figure 5D and Table II) and adding the COOH-terminus had no effect (Table II). In contrast, adding regions IIa or IIb resulted in chimeric molecules with increased affinity to TrkB receptors, now reaching 12% of the level seen with wt BDNF (Figure 5D and Table II). These two sequences must cooperate synergistically since the I+II+III+IV+V molecule bound to TrkB with only 2-fold lower affinity than wt BDNF (Figure 5D and Table II).

From all the combinations of variable regions I, III, IV and V tested, only chimeric molecule I+III+IV+V promoted the survival of nodose neurons. However, only 30% of the neurons could be rescued with saturating amounts of this protein compared with wt BDNF (Figure 5E and Table II). Adding sequences from the NH$_2$- and COOH-termini of BDNF to the I+III+IV+V molecule did not improve the biological activity on nodose neurons (Figure 5E and Table II). However, adding region IIb, but not IIa,
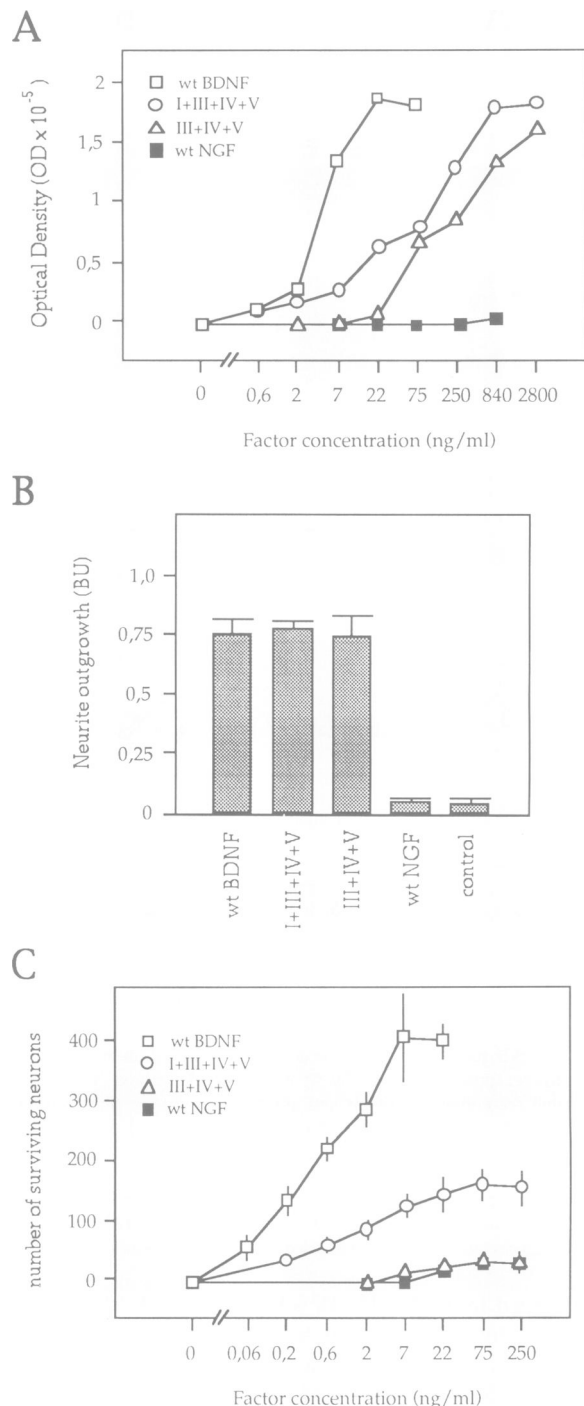
Fig. 6. Similar levels of TrkB receptor phosphorylation may result in different biological activities. (**A**) Serial dilutions of transfected COS cell conditioned medium containing equal amounts of wt BDNF, wt NGF and chimeric molecules I+III+IV+V and III+IV+V were assayed for their ability to stimulate tyrosine phosphorylation of TrkB expressed in r*trkB*-3T3 cells. Appropriately exposed autoradiograms of phosphotyrosine blots were used for densitometric scanning and the results are expressed in arbitrary units of optical density. Similar results were obtained in a duplicate experiment. (**B**) Stimulation of neurite outgrowth from explanted E8 chick nodose ganglia in the presence of 50 ng/ml of the wt BDNF, wt NGF and chimeric molecules I+III+IV+V and III+IV+V. Medium from mock-transfected COS cells was used as a negative control (control). Results are presented as the mean of triplicate determinations ±SD. (**C**) Serial dilutions of transfected COS cell conditioned medium containing equal amounts of the indicated wt and chimeric molecules were assayed for their ability to stimulate survival of dissociated E8 chick nodose neurons. Results are presented as the mean of triplicate determinations ±SD.
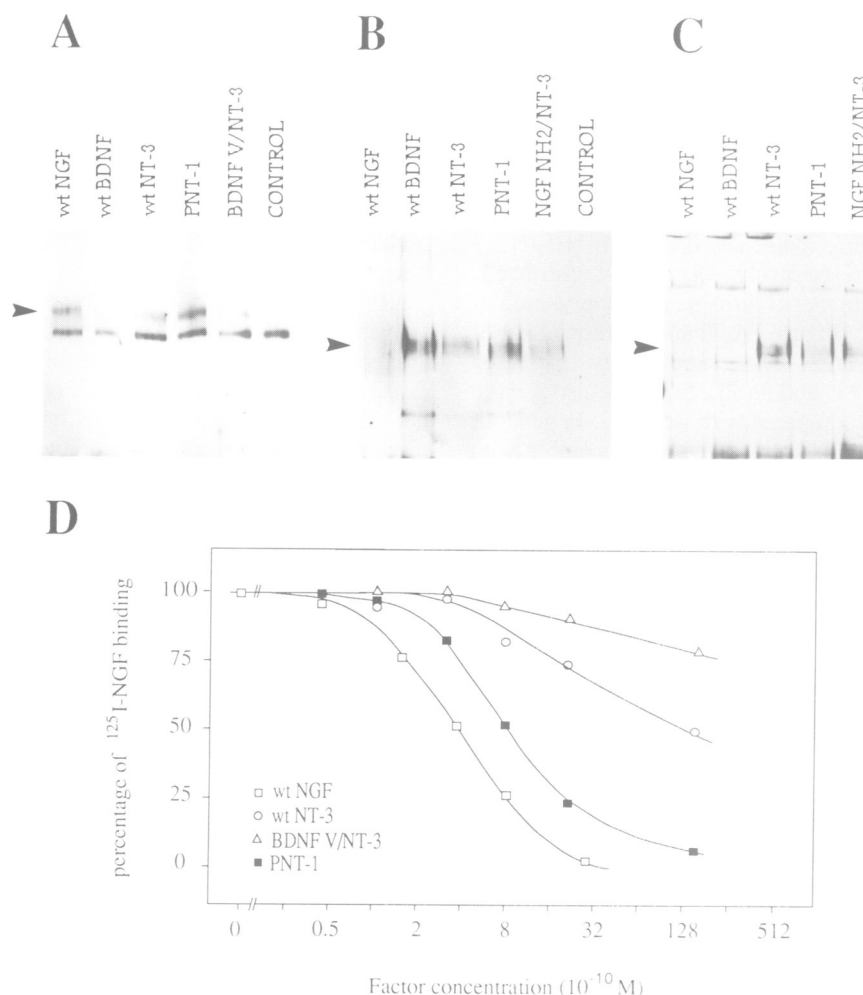
Fig. 7. PNT-1 interacts efficiently with TrkA, TrkB and TrkC receptors. (A, B and C) Tyrosine phosphorylation of TrkA (A), TrkB (B) and TrkC (C) receptors stimulated by wt NGF, wt BDNF, wt NT-3 and the NT-3-derived chimeric molecules PNT-1, BDNF V/NT-3 and NGF NH$_2$/NT-3. Trk-expressing cells were treated with 100 ng/ml of the indicated recombinant proteins and assayed for tyrosine phosphorylation. Arrowheads in A, B and C indicate the migration of phosphorylated TrkA (p140^{trk}), TrkB (p145^{trkB}) and TrkC (p145^{trkC}), respectively. (D) Serial dilutions of transfected COS cell conditioned medium containing equal amounts of wt NGF, wt NT-3, and NT-3-derived chimeric molecules BDNF V/NT-3 and PNT-1 were assayed for their ability to displace [^{125}I]NGF from receptors on rtrkA-3T3 cells. Medium from mock-transfected cells failed to displace [^{125}I]NGF from these cells. Each point represents the mean of triplicate determinations. Standard deviation was ≤10%.

resulted in a molecule able to rescue 60% of the nodose neurons compared with wt BDNF (Figure 4E and Table II). This indicates that residues 45−49 in BDNF are important for further activation of the TrkB receptor. Biological activity in nodose neurons reached 80% of wt BDNF levels when the complete region II was included (residues 40−49) (Figure 5E and Table II), suggesting that region IIa (residues 40−44) contributes indirectly by allowing a conformation of this loop that facilitates the contact of residues 45−49 with the TrkB receptor.

Similar levels of TrkB receptor phosphorylation may result in different biological activities

As shown in Figure 6A, similar dose−response curves of TrkB receptor phosphorylation were obtained with wt BDNF and chimeric molecules I+III+IV+V and III+IV+V, although the latter two were displaced towards higher concentrations. In this assay, the EC$_{50}$ for wt BDNF was 5 ng/ml, whereas the EC$_{50}$ for chimeric molecules I+III+IV+V and III+IV+V were 125 and 250 ng/ml, respectively (Figure 6A). Despite these differences, both of the chimeric molecules and wt BDNF stimulated similar levels of neurite outgrowth from explanted nodose ganglia

when assayed at concentrations ranging from 20 to 200 ng/ml (Figure 6B and unpublished data). However, clear differences were seen in their ability to promote neuronal survival of nodose neurons (Figure 6C). Chimeric molecule III+IV+V failed to promote neuronal survival even at concentrations that allowed substantial levels of receptor phosphorylation (compare Figure 6A and C). On the other hand, chimeric molecule I+III+IV+V was able to rescue some neurons but it reached a plateau which was 3-fold lower than that of wt BDNF, even though both molecules caused similar maximal levels of TrkB phosphorylation (compare Figure 6A and C). Comparison of Figure 6A, B and C shows that, when assayed at their respective EC$_{50}$ for receptor phosphorylation, all three molecules promote similar levels of neurite outgrowth, while they differ widely in their ability to promote survival of the same neurons, suggesting that they induce different modes of receptor activation.

Engineering of a multifunctional neurotrophin agonist, pan-neurotrophin-1

Next, we used the information from our structure−function studies on NGF and BDNF to design a multifunctional neurotrophin agonist which we named pan-neurotrophin-1

(PNT-1). Since NT-3 is the only neurotrophin that binds to and activates TrkC, NT-3 was used as a skeleton. The possibility was tested that NH_2-terminal sequences from NGF could elicit NT-3 binding to and activation of the TrkA receptor. Variable sequences from region V of BDNF were also introduced into NT-3, in an attempt to enhance inter-action with the TrkB receptor. As well as the double chimera (PNT-1), single chimeric molecules (i.e. NGF NH_2/NT-3 and BDNF V/NT-3, respectively) were also constructed.

PNT-1 efficiently stimulated tyrosine phosphorylation of TrkA, TrkB and TrkC (Figure 7A, B and C, respectively). Phosphorylation of TrkA was significantly increased compared with wt NT-3, and an increase was also seen in TrkB phosphorylation compared with wt NT-3. In general, PNT-1 activated Trk receptors at levels comparable with those obtained with each of their cognate wt ligands. Moreover, affinity of binding to TrkA receptors was improved in PNT-1 from ~3% to almost 50% of the wt NGF affinity (Figure 7D). Interestingly, the affinity of BDNF V/NT-3 was even lower than that of wt NT-3, in agreement with the notion that positive charges in region V interfere with binding to TrkA (Figure 7D). Binding to TrkB was not changed in PNT-1 or BDNF V/NT-3 as compared with wt NT-3 (not shown).

Biological activities of PNT-1 and related molecules were assayed in sympathetic neurons and in neurons from the nodose ganglion. Both PNT-1 and NGF NH_2/NT-3 efficiently stimulated neurite outgrowth from explanted sympathetic ganglia. In contrast, wt NT-3 displayed only a marginal effect when assayed at high concentrations (compare Figure 8A, B, C and D). Comparison of dose–response curves showed that both PNT-1 and NGF NH_2/NT-3 showed substantially higher NGF-like biological activity than wt NT-3 (Figure 8E). The potencies of NGF NH_2/NT-3 and PNT-1 were 8- and 15-fold lower, respec-tively than wt NGF. As suggested by the TrkA binding experiment (Figure 7D), the differences in bioactivity between NGF NH_2/NT-3 and PNT-1 are probably due to a structural interference from region V of BDNF on the interaction with TrkA. The ability of PNT-1 to stimulate neurite outgrowth from sympathetic ganglia correlated with survival of dissociated sympathetic neurons (Figure 8F). PNT-1 and NGF NH_2/NT-3 rescued about half the number rescued with wt NGF (Figure 8F), whereas no survival was seen with wt NT-3 (Figure 8F).

Previous studies have demonstrated additive effects of BDNF and NT-3 in the survival of nodose neurons, indicating that the two factors promote survival of different neuronal subpopulations in this ganglion (Hohn et al., 1990; Götz et al., 1992). The effects of PNT-1 on survival of
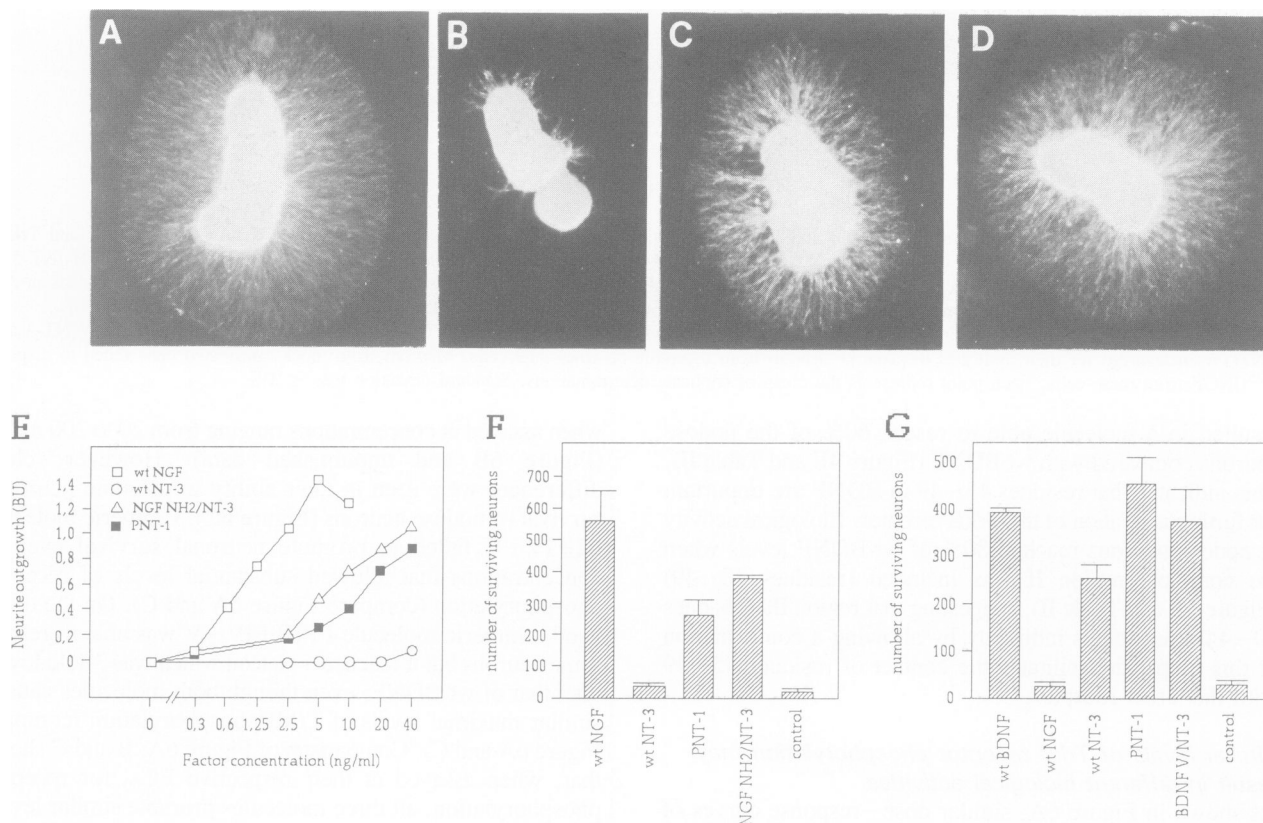
Fig. 8. PNT-1 displays multiple neurotrophic specificities. (A–D) Dark-field photomicrographs of E8 chick sympathetic ganglia cultured for 48 h in the presence of COS cell conditioned media containing 2.5 ng/ml wt NGF (A), 20 ng/ml wt NT-3 (B), 20 ng/ml PNT-1 (C) and 20 ng/ml NGF NH_2/NT-3 (D). (E) Dose–response curves of neurite outgrowth stimulation of E8 chick sympathetic ganglia by wt NGF, wt NT-3 and NT-3-derived chimeric molecules NGF NH_2/NT-3 and PNT-1. Data from three determinations varied by ±20% of the average values reported here. (F) Neuronal survival of dissociated E8 chick sympathetic neurons in the presence of 2 ng/ml of wt NGF, wt NT-3 and NT-3-derived chimeric molecules NGF NH_2/NT-3 and PNT-1. Medium from mock-transfected COS cells was used as a negative control (control). Results are presented as the mean of triplicate determinations ±SD. (G) Neuronal survival of dissociated E8 chick nodose neurons in the presence of saturating amounts (20–50 ng/ml) of wt BDNF, wt NGF, wt NT-3 and NT-3-derived chimeric molecules PNT-1 and BDNF V/NT-3. Medium from mock-transfected COS cells was used as a negative control (control). Results are presented as the mean of triplicate determinations ±SD.

nodose neurons was then assayed to evaluate the efficiency of this molecule as a BDNF and NT-3 agonist. As expected, wt NGF failed to rescue neurons from this ganglion, whereas saturating concentrations of wt BDNF and wt NT-3 rescued 40 and 25% of the neurons, respectively (Figure 8G). PNT-1 promoted the survival of 45% of the nodose neurons, almost a 2-fold increase compared with wt NT-3 (Figure 8G). Comparable survival effects were obtained with the BDNF V/NT-3 chimera, indicating that the increased activity was due to the replacement of region V in NT-3 with region V from BDNF. It should be noted, however, that the level of survival with PNT-1 did not reach the maximal level expected for additive effects of BDNF and NT-3 (~65%),

suggesting that PNT-1 is a partial BDNF agonist. In addition, preliminary experiments using chick dorsal root ganglia in culture and RNase protection analysis indicate that PNT-1 is able to support survival of neurons expressing either TrkA, TrkB or TrkC mRNAs (C.F.Ibáñez, unpublished).

Discussion

Receptor binding sites in neurotrophins to Trk receptors

The results of our study show that the interaction of neurotrophins with Trk receptors is mediated by multiple, although specific, contacts that cooperate synergistically to
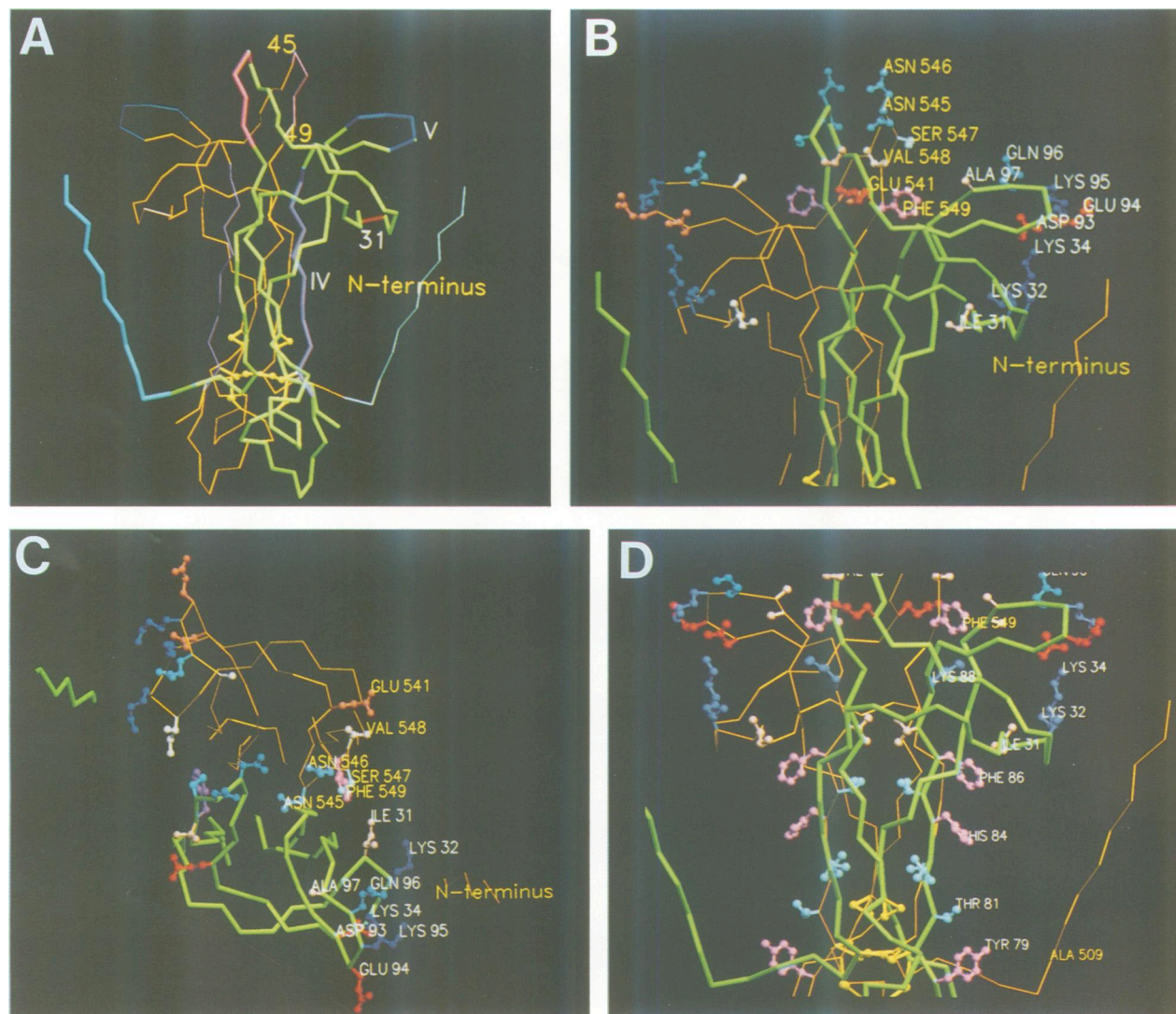
Fig. 9. Three-dimensional structure of the NGF receptor binding site to the TrkA receptor. The NGF dimer consists of two identical 118 residue peptides (protomers 1 and 2) related by a two-fold axis, but in the crystal structure the N-terminal 10 residues and the C-terminal six residues of each chain are not seen. The C_{α} backbone of the two chains are shown here with protomer 1 thicker and coloured green, and protomer 2 thinner and coloured orange. Disulfide bonds are shown in yellow. The residue labels of protomer 1 are white, and of protomer 2 are yellow; also the numbering of protomer 2 has 500 added: the equivalent residue number from protomer 1. (A) A backbone diagram of the NGF dimer viewed perpendicular to the two-fold axis, showing regions containing residues involved in TrkA binding. Each of the two symmetrically related sites includes Ile31 and residues in regions IV and V from the first protomer, and residues 45−49 from the second protomer; it also contains the N-terminal region, although residues 1−10 are not seen in the crystal structure, and are shown here in an arbitrary extended conformation. (B) A closer view of the top part of the NGF dimer in the same orientation as (A), with individual residues from one binding site labelled. (C) A view of the top part of the NGF dimer perpendicular to that in (B) i.e. along the two-fold axis. Residues contributing to each of the two binding sites come close together at the two-fold axis, and thus form a continuous plane stretching across the top of the dimer. The location of the N-termini is again speculative. Note that Lys32, Lys34 and Lys95, which mediate binding to LNGFR (Ibáñez et al., 1992), are also exposed in the binding surface to TrkA. (D) The 'middle' region of the NGF dimer in the same orientation as (A) showing the location of strand residues 79, 81 and 84 on the edge of protomer 1. The N-terminal chain of protomer 2 is in an arbitrary position, to indicate that it is potentially capable of contacting region IV from protomer 1.

receptor binding and activation. In particular, amino acid residues mediating the interaction of NGF with TrkA include residues 3−9 from the NH_2-terminus, Ile31 from region I, Glu41 and Asn45 from region II, Tyr79, Tyr81 and His84 from region IV and residues 94−98 from region V. This result indicates that the binding site is formed by discontinuous stretches of amino acid residues, suggesting that determinants of specificity and receptor activation may be distributed. However, when these residues are viewed in the three-dimensional structure of NGF (McDonald *et al.*, 1991), they appear grouped on one side of the NGF dimer, delineating a continuous surface extending approximately parallel to the two-fold axis of the molecule (Figure 9A, B, C and D). At the top of the dimer, amino acid residues from regions I and V from one protomer are in close proximity to residues 45−49 in region IIb from the other protomer, forming a patch of residues which contains most of the main contacts to the TrkA receptor (Figure 9B). Looking from the top of the molecule along the two-fold axis, residues 31−34 (region I) and residues 95−97 (region V) from the first protomer, and residues 41 and 45−49 (region IIb) from the second protomer cluster on a continuous plane stretching across the top of the NGF dimer (Figure 9C). Additional contacts may be provided in the middle region of the NGF dimer by residues 79, 81 and 84 (region IV) from the first protomer, thereby extending the binding surface down to about three-quarters of the molecule (Figure 9D). The NH_2-terminal residues (1−10) are not seen in the crystal structure (McDonald *et al.*, 1991) and, although they cannot be positioned with respect to the other regions of the molecule, it is tempting to speculate that they cooperate in the extension of the binding surface along the side of the dimer (Figure 9D and see discussion below). Overall, the binding site is a rather flat surface stretching across the top of the molecule up to loop region II which, due to its flexibility, could conceivably change conformation upon receptor binding.

Interestingly, structural elements mediating the contact of BDNF with the TrkB receptor were, in general, localized in variable regions analogous to those involved in the interaction of NGF with TrkA. Thus, residues in variable regions I, IV and V were shown to interact with TrkB and induce receptor phosphorylation. In region V, Lys96 and Arg97 are responsible for this effect, whereas in region IV, Gln84 appears to be important. Similarly, residues 45−49 in variable region IIb were implicated in TrkB binding and bioactivity in nodose neurons. A notable difference was however found for the NH_2-terminus, which seems to play a less important role for receptor binding in BDNF than in NGF. Further mutagenesis of these sequences in BDNF will be required to clarify their role in TrkB binding. Taken together, these results suggest that during evolution, neurotrophins have conserved a general mechanism of binding to their receptors, and that a parallel evolution of cognate ligands and Trks has developed specific contacts through different residues in the same variable regions of the neurotrophins.

Implications for the mechanisms of binding and signal transduction

The results obtained with chimeric molecules in the NH_2-terminus (residues 3−9) of NGF suggest that this region is more important for binding to TrkA than for

receptor activation or biological activity. Interestingly, replacement of the NH_2-terminus in wt NGF with that of BDNF reduced binding to TrkA but not biological activity. In addition, the fact that NGF-derived chimeric molecules with all variable regions but the NH_2-terminus replaced by sequences from BDNF still retain substantial binding to TrkA, indicates that this region is the main contributor to the energy of binding to this receptor. The role of the other variable regions in receptor binding seems to be secondary and probably synergistic with the NH_2-terminus, by stabilizing the ligand−receptor complex. On the other hand, variable residues in loop regions appear to be the main factor responsible for the contacts and conformational changes necessary for receptor activation and biological activity. This dissociation between receptor binding and biological activity is intriguing and may reflect the mechanism whereby NGF binds and activates the TrkA receptor. Due to the poor density of the NH_2-terminus on structural maps, it has been suggested that this region is both flexible and solvent accessible (McDonald *et al.*, 1991). It is possible that the NH_2-terminal chain of NGF functions as a docking domain for initial receptor contact in a way analogous to that proposed for the COOH-terminus of the insulin B-chain (Nakagawa and Tager, 1986). This initial binding would then lead to further conformational changes in the ligand and/or receptor to optimize binding interactions and allow receptor activation.

It is generally accepted that ligand-induced activation of tyrosine kinase receptors is mediated by receptor dimerization or oligomerization (Schlessinger, 1988). Cross-linking studies have revealed the formation of TrkA homodimers upon NGF binding to PC12 cells and fibroblasts ectopically expressing TrkA (Meakin and Shooter, 1991; Jing *et al.*, 1992). Recently, using kinase-deficient mutants of the TrkA receptor, Jing *et al.* (1992) demonstrated that formation of functional TrkA homodimers is necessary for NGF-mediated receptor autophosphorylation and signal transduction. Due to the two-fold symmetry of the NGF dimer, identical binding surfaces are formed on both sides of the molecule, each containing structural elements from both protomers (Figure 9C). It is therefore conceivable that a bivalent NGF dimer could mediate dimerization of neighbouring TrkA receptors. Binding of the NGF dimer to one TrkA molecule could then promote the association of the complex with a second molecule of TrkA through the remaining binding surface, resulting in receptor autophosphorylation and signal transduction. Although validation of this hypothesis will require determination of the stoichiometry of the NGF−TrkA complex, similar models of ligand-induced tyrosine kinase receptor dimerization have been proposed for other bivalent ligands like platelet-derived growth factor (PDGF) (Heldin *et al.*, 1989; Seifert *et al.*, 1989). In the case of PDGF, it was proposed that each PDGF monomer interacts with one distinct monomer of the receptor, whereas in the neurotrophins, both neurotrophin monomers seem to make contacts with each Trk receptor. The fact that native BDNF and NT-3 also appear to be dimeric molecules (Radziejewski *et al.*, 1992) suggests that this model could be applied to all the neurotrophins.

Amino acid residues mediating binding of NGF to LNGFR have recently been identified (Ibáñez *et al.*, 1992). Interestingly, these residues seem to overlap partially with the TrkA binding site identified in this study (Figure 9C).

Association between LNGFR and TrkA has been proposed as a necessary step for the formation of NGF high-affinity binding sites (Hempstead et al., 1991), although results from other laboratories have recently disputed that model (Klein et al., 1991a; Jing et al., 1992). Complexes between LNGFR and TrkA have so far not been detected in cross-linking experiments performed with either PC12 cells, fibroblasts or sensory neurons under conditions which allowed detection of LNGFR or TrkA homodimers (Hosang and Shooter, 1985; Meakin and Shooter, 1991; Jing et al., 1992). Our results imply that, provided that there are no steric impediments, binding of NGF to one molecule of TrkA or LNGFR still leaves one identical binding site free which could be used to form either a TrkA−LNGFR heterodimer or any of the two receptor homodimers (Figure 10). That receptor heterodimers have not yet been detected could be due to their very low number or because they represent a transient state in the dynamics of NGF receptor complexes on the membrane of responsive cells. It has been suggested that LNGFR contributes to the activation of Trk receptors by promoting ligand presentation or recruitment of circulating neurotrophins (Glass et al., 1991; Jing et al., 1992). Since the number of LNGFRs in the membrane of responsive cells is ~10-fold higher than the number of TrkA molecules and since NGF dissociates more quickly from LNGFR, it is possible that LNGFR−TrkA complexes function as intermediates in the transfer of ligand from LNGFR homodimers to TrkA homodimers (Figure 10). The proportion of LNGFR and TrkA homo- and heterodimers present in a cell could depend on the relative amount of each receptor species and on the membrane environment of a given cell type.

Upon ligand binding, activated TrkA homodimers auto-phosphorylate on specific tyrosine residues which are, in turn, supposedly recognized by intracellular proteins involved in signal transduction (Kaplan et al., 1991a). In this context, the observation that similar levels of TrkB receptor phosphorylation can lead to different biological effects is intriguing and may reflect activation of different components in the signal transduction pathway. Thus, qualitative rather than quantitative differences could exist between states of receptor activation achieved after stimulation by different, although structurally related ligands. These differences may be due to variations in the conformation of the receptor upon ligand binding or in the pattern of phosphotyrosines in the cytoplasmic domain of the receptor, or to both. The recent observation that distinct phosphotyrosines in the PDGF and fibroblast growth factor (FGF) receptors bind to specific molecules which mediate different signalling pathways (Fantl et al., 1992; Mohammadi et al., 1992; Peters et al., 1992) suggests that different phosphorylation patterns in Trk receptors could result in transduction of different biological responses such as neurite outgrowth or neuronal survival. Thus, chimeric molecules with different biological activities could be valuable tools for unravelling the signal transduction pathways leading to the pleiotropic biological effects of neurotrophins.

Engineering of neurotrophin agonists and antagonists

Based on the functional information obtained with chimeric and mutant molecules, a chimeric neurotrophin could be constructed that displayed biochemical and biological properties from three different neurotrophins. PNT-1 efficiently activated TrkA, B and C receptors and displayed biological specificities on neurons characteristic of NGF, BDNF and NT-3. This result demonstrates that the structural−functional information obtained can be used for
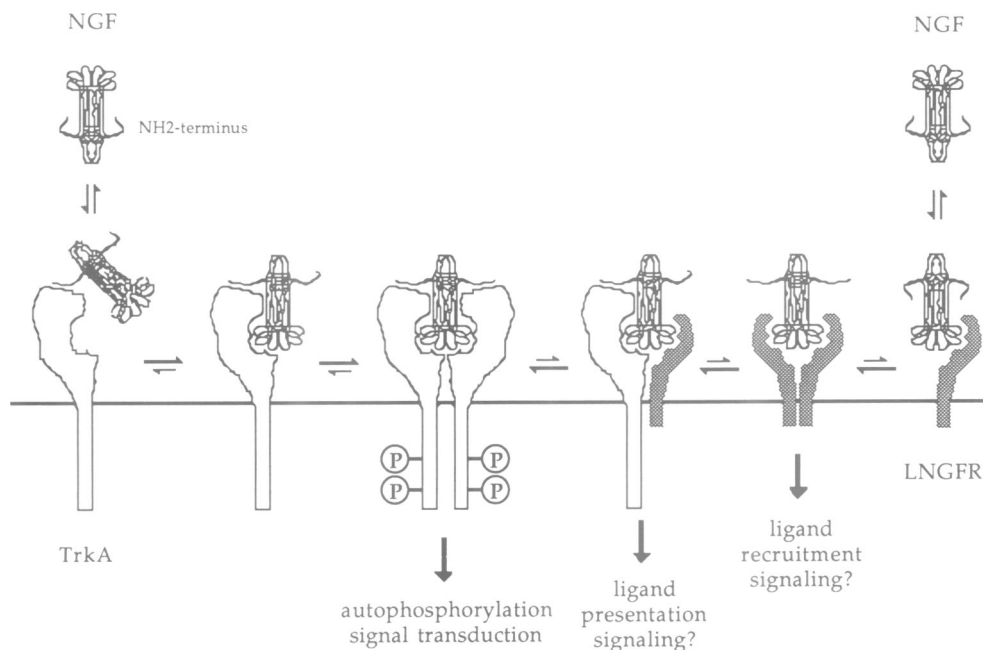
Fig. 10. Speculative model for the interaction of NGF with its receptors and the dynamics of receptor complexes on the membrane of responsive cells. The NH₂-terminus, shown in an arbitrary extended conformation, may act as a docking domain during the initial interaction of the NGF dimer with the TrkA receptor. Cross-linking experiments have so far only demonstrated the association of monomeric and homodimeric receptors with the ligand. The bivalent nature of the NGF dimer may allow the formation of heterodimeric receptor complexes which could be transient intermediates in the transfer of ligand from LNGFR to TrkA. The signalling capacity of homodimeric LNGFR and heterodimeric receptor complexes is controversial (Berg et al., 1991; Jing et al., 1992).

engineering of molecules with broader or novel biological specificities. In previous studies extensive replacements were necessary to combine biological activities from NGF and BDNF (Ibáñez *et al.*, 1991a), and most of the chimeric molecules generated only displayed some but not all of the activities of the wt molecules (Suter *et al.*, 1992). In the present study, information on neurotrophin binding sites to Trk receptors was applied to the rational design of a triple-functional molecule by exchanging only 10% of its original amino acid residues. The information presented here will make it possible to construct other pan-neurotrophins like PNT-1 by engineering with more precision, introducing single amino acid replacements and specific side chains which could modulate the role of different variable regions.

Previous studies have demonstrated the existence of distinct subpopulations of neurons within a given sensory ganglion (e.g. dorsal root, nodose and trigeminal ganglia) that are dependent on different neurotrophins for survival (Lindsay *et al.*, 1985; Hohn *et al.*, 1990; Ibáñez *et al.*, 1993). These results suggest that functional regeneration of lesioned sensory terminals is likely to require concomitant action of different neurotrophins. In this context, the use of a single molecule with multiple neurotrophic activities, like PNT-1, may be of great advantage. Recent studies have indicated that different neurotrophins have different rates of diffusion in nervous tissue (R.Lindsay, personal communication). Although the cause of these differences is not yet understood, they may affect the efficiency of neurotrophin 'cocktails' in the treatment of nervous injury and neuro-degeneration. The use of a pan-neurotrophin may therefore greatly simplify problems inherent to the heterogeneity of a mixture of molecules, such as differences in diffusion, stability, etc. among its components. In addition to the engineering of neurotrophin agonists, the fact that chimeric molecules such as I+II+III+IV+V retained high levels of receptor binding but showed low biological activity, open up a strategy to design neurotrophin antagonists, with potential uses for studies of neurotrophin function *in vivo*.

Materials and methods

DNA cloning and site-directed mutagenesis
Fragments containing the pre-pro-coding sequences from the rat NGF (Whittemore *et al.*, 1988), mouse BDNF (Hofer *et al.*, 1990) and rat NT-3 (Ernfors *et al.*, 1990) genes were cloned into pBluescipt KS$^+$ (Stratagene). Note that the amino acid sequence of mouse and rat BDNF are identical (Maisonpierre *et al.*, 1990). Single-stranded DNA from these plasmids was used as template for oligonucleotide based site-directed mutagenesis as described by Kunkel (1985) and detailed in Ibáñez *et al.* (1990). The replacements were confirmed by nucleotide sequence analysis by the chain termination method (Sanger *et al.*, 1977). For protein expression, DNA inserts containing the desired replacements were subcloned in pXM (Yang *et al.*, 1986).

Production and quantitation of recombinant proteins
COS cells grown to ~70% confluency were transfected with 25 μg plasmid DNA per 100 mm dish using the DEAE dextran—chloroquine protocol (Luthman and Magnusson, 1983). To correct for differences in the amounts of recombinant protein produced by the different constructs, 35 mm dishes transfected in parallel were maintained in the presence of 100 μCi/ml [^{32}S]cysteine (Amersham). Aliquots of conditioned media were then analysed by SDS—PAGE and the amounts of recombinant protein in the different samples were equalized after densitometer scanning of the corresponding autoradiograms as previously described (Ibáñez *et al.*, 1991b). The absolute amount of wt NGF protein was determined by quantitative immunoblotting of conditioned media and by measurement of biological activity in cultured sympathetic ganglia using standards of purified mouse NGF (Ibáñez *et al.*, 1990). The data obtained from these analyses were then used to determine the protein concentration in the samples containing wt BDNF, wt NT-3, chimeric and mutant proteins.

Binding assays
Purified mouse NGF and BDNF were labelled with ^{125}I by the lacto-peroxidase method to an average specific activity of 1×10^8 c.p.m./μg. NIH3T3 fibroblasts expressing TrkA, TrkB or TrkC were used at $2-10 \times 10^6$ cells/ml. Steady state binding was measured in competition assays performed at 4°C using 1.5×10^{-9} M [^{125}I]NGF or [^{125}I]BDNF and serial dilutions of conditioned media containing equivalent amounts of wt or mutated NGF protein. All components were added at the same time and the cells were collected by centrifugation after equilibrium was reached (90—120 min incubation). Cell pellets were then counted in a gamma counter. Control experiments using medium from mock transfected COS cells showed that other proteins present in the conditioned medium had no effect on the binding of [^{125}I]NGF or [^{125}I]BDNF to the cells. Non-specific binding was measured in a parallel incubation to which a 300- to 1000-fold molar excess of unlabelled purified factor was added. All results were corrected for this non-specific binding, which was <10% (for NGF) or 30% (for BDNF) of total binding. The concentration of each chimeric, mutant and wild type molecule that gave 50% binding (IC$_{50}$) was determined, and relative binding was calculated using the relationship: (mutant IC$_{50}$/wild type IC$_{50}$) $\times$ 100.

Phosphorylation assays
A confluent 10 cm plate containing $\sim 10^7$ *rtrk*-3T3 or *rtrkB*-3T3 cells was treated for 5 min at 37°C with wt, chimeric or mutant factors and subsequently lysed with 1 ml of ice-cold buffer containing 1% NP40, 20 mM Tris pH 8.0, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM PMSF, 0.15 U/ml aprotinin, 20 μM leupeptin and 1 mM sodium orthovanadate. Plates were incubated for 15 min at 4°C after which insoluble material was removed by centrifugation. Cell lysates were normalized for protein content before immunoprecipitation. Trk immunoprecipitation was performed by incubating lysates with 1 μl of anti-TrkB polyclonal antiserum 443 (Soppet *et al.*, 1991) which recognizes TrkA, TrkB and TrkC. After 2 h at 4°C, immunocomplexes were collected with protein A—Sepharose (Pharmacia, Sweden), washed in lysis buffer and boiled for 5 min before SDS—PAGE. After electrophoresis, gels were blotted to nitrocellulose membranes, reacted with anti-phosphotyrosine monoclonal antibody 4G10 (UBI, New York) and developed with the ECL Western Detection System (Amersham, UK). Appropriately exposed autoradiograms of phosphotyrosine blots were used for densitometric scanning.

Biological assays
Serial dilutions of conditioned media containing equivalent amounts of recombinant protein were assayed for stimulation of neurite outgrowth on explanted chick embryonic day 8 (E8) sympathetic (NGF-specific) and nodose (BDNF-specific) ganglia as previously described (Ebendal, 1989; Ibáñez *et al.*, 1991a). Fibre outgrowth was scored on a semiquantitative scale in biological units (BU) by comparison with standards obtained with purified mouse NGF, for which 1 BU is equivalent to ~5 ng/ml. The concentration of each protein that gave 0.6 BU in this scale was determined, and used to calculate the relative activity compared with that obtained with wt NGF.

Dissociated neurons from chick E8 sympathetic and nodose ganglia were preplated on plastic for 2 h and then cultured in 96-well plates coated with poly-L-ornithine and laminin at a density of 800—1000 cells/well. Serial dilutions of conditioned media containing equivalent amounts of recombinant proteins were added at the time of plating and neuronal survival was determined after 36—48 h by phase contrast microscopy, scoring the number of surviving neurons in the entire well.

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