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Rapid Communication

Structural Determinants of Trk Receptor Specificities Using BDNF-Based Neurotrophin Chimeras

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Neurotrophins play very important roles in the development and maintenance of the vertebrate nervous system. In mammals, there are four members of the family: NGF, BDNF, NT-3, and NT-4/5. Members of the neurotrophin family activate different receptors that belong to a class of receptor tyrosine kinases known as "Trks." For example, NGF is the specific ligand of TrkA, while BDNF activates TrkB. To elucidate which regions of the two neurotrophins determine the receptor specificities, chimeric neurotrophins were constructed using BDNF as the backbone, with various regions being substituted by the corresponding regions of NGF. The activity of the chimeras on the Trk receptors was assayed in transfected fibroblasts ectopically expressing the Trk receptors. Our findings revealed that, although BDNF is absolutely conserved in mammals, substitution of several small variable regions from NGF into the BDNF backbone did not lead to significant loss in TrkB activity or gain in TrkA activity. Moreover, important determinants of TrkB activation might be located in the carboxy-terminal half of BDNF. On the other hand, critical elements for TrkA activation might be located within the amino-terminal half of the mature NGF molecule. © 1996 Wiley-Liss, Inc.

Key words: chimeric neurotrophins, receptor tyrosine kinase, nerve growth factor, brain-derived neurotrophic factor

INTRODUCTION

The discovery of the prototypic nerve growth factor (NGF) and the purification and subsequent cloning of brain-derived neurotrophic factor (BDNF) led to the concept of the "neurotrophin" family (Levi-Montalcini and Hamburger, 1953; Cohen et al., 1954; Barde et al., 1982; Leibrock et al., 1989). The strategy of homology cloning revealed the presence of two additional mammalian members of this family, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Ernfors et al., 1990; Hohn et al., 1990; Jones and Reichardt, 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990; Hallböök et al., 1991; Berkemeier et al., 1991; Ip et al., 1992). The neurotrophins are basic proteins of about 120 amino acids after processing from larger precursors and share about 50% amino acid identity to each other. Moreover, there are six conserved cysteine residues that are present in the mature forms of all known neurotrophins and are thought to maintain the conformation of the molecule by disulfide-bridge formation. The regions around five of the six cysteine residues in the mature protein are also highly conserved among neurotrophins, whereas the amino acids between these conserved framework domains are less conserved and are referred to as variable regions.

It is well known that the neurotrophins play a very important role in the development and maintenance of the vertebrate nervous system (for review, see Davies, 1994). In vitro studies suggest that different neurotrophins can have both overlapping and specific activities on neurons. For example, both NGF and BDNF are able to support the survival and differentiation of dorsal root ganglion neurons (Lindsay, 1988). However, only NGF can support the survival and differentiation of sympathetic ganglion neurons, while nodose ganglion responds only to BDNF and NT-3 but not to NGF (Levi-Montalcini, 1987; Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990; Ruit et al., 1990). The specific actions of the neurotrophins are largely due to the differential expression of their cognate receptors in different neurons.

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There are two classes of neurotrophin receptor. The low-affinity NGF receptor, or p75, binds to all neurotrophins with low affinity; the dissociation constant of binding is $\sim 10^{-9}$ M (Chao et al., 1986; Radeke et al., 1987). On the other hand, a family of receptor tyrosine kinases known as "Trks" bind to the neurotrophins with higher affinity; the dissociation constant is $\sim 10^{-11}$ M (Martin-Zanca et al., 1990; Meakin and Shooter, 1992). It is generally accepted that the Trk receptors are responsible for mediating the responses to neurotrophins, while the role played by p75 remains controversial (Bothwell, 1995). Upon ligand binding, the Trk receptors dimerize and undergo autophosphorylation (Jing et al., 1992). Furthermore, ectopic expression of Trk receptors in fibroblasts leads to cell proliferation, rather than growth inhibition and neurite outgrowth, in response to neurotrophins, suggesting that the Trk receptors utilize signaling pathways similar to that employed by conventional growth factor receptors like fibroblast growth factor (FGF) receptors (Cordon-Cardo et al., 1991; Glass et al., 1991; Jing et al., 1992; Ip et al., 1993; Ip and Yancopoulos, 1994).

There are three members of the Trk receptor family; each of them interacts with specific neurotrophin(s). In vitro studies of both neuronal and nonneuronal cells suggest that NGF is the specific ligand of TrkA (Kaplan et al., 1991), BDNF and NT-4/5 interact specifically with TrkB (Klein et al., 1991, 1992; Ip et al., 1992), whereas NT-3 preferentially activates TrkC and, to a lesser extent, TrkA and TrkB in certain cellular contexts (Glass et al., 1991; Klein et al., 1991; Lambelle et al., 1991; Soppet et al., 1991; Squinto et al., 1991; Ip et al., 1993; Davies et al., 1995). The specific interactions with different Trk receptors enable each neurotrophin to act on distinct neuronal populations. This is supported by recent gene targeting studies in which expression of each of the Trk receptors was individually knocked out (reviewed by Snider, 1994; Klein et al., 1993, 1994; Smeyne et al., 1994; Ip and Yancopoulos, 1996). Distinct neuronal deficits can be observed in mice that lack different Trk receptors, suggesting that each neurotrophin plays distinct roles in the development of the nervous system through activation of different Trk receptors in vivo.

In the present study, we examined the structural elements of NGF and BDNF that determine their receptor specificities. Because of their structural homology, it is feasible to elucidate the molecular basis of the receptor specificity using chimeric molecules. A number of studies that involved construction of chimeras using NGF or NT-3 as the backbone have been reported (Ibáñez et al., 1991, 1993; Suter et al., 1992; Ilag et al., 1994; Kullander and Ebendal, 1994; Urfer et al., 1994). In one study, seven variable regions between NGF and BDNF were defined and exchanged individually or in combinations between the two neurotrophins (Ibáñez et al., 1993). The results of these studies suggested that cooperation of amino acids located at different variable regions is necessary to determine the receptor specificity of NGF and BDNF. In particular, the amino acids located at the amino terminus and region II (amino acids 40–49 which constitute a β-hairpin loop according to the three-dimensional conformation) of NGF were shown to be important in the interaction with TrkA (McDonald, 1991; Ibáñez et al., 1993). However, none of the previous studies utilized BDNF as the backbone for the construction of chimeras. Amino acid sequence comparison revealed that BDNF is much more conserved throughout evolution than NGF (Maisonpierre et al., 1991, 1992; Götz et al., 1992). Indeed, the amino acid sequence of BDNF of all mammalian species examined thus far is absolutely conserved (Maisonpierre et al., 1991). This suggests that the selective pressure on the structure of BDNF during evolution appears to be greater than that of NGF, possibly due to a more stringent requirement for its interaction with TrkB. It would be of interest to examine whether substitution of short regions from the BDNF backbone, which are variable between NGF and BDNF, leads to any loss in TrkB activation. Moreover, any gain in TrkA activity as a result of the introduction of NGF segments into the BDNF backbone might supplement previous reports on the structural elements in NGF that are responsible for TrkA activation. To assay the receptor specificities of the chimeric neurotrophins, survival of transfected fibroblasts ectopically expressing either TrkA or TrkB was utilized in the present study.

MATERIALS AND METHODS

Construction of Chimeras

The cDNA of human NGF and human BDNF subcloned into XhoI and NotI sites of the expression vector pCDM8 were used as templates in polymerase chain reaction (PCR) to generate the chimeric neurotrophins. For the construction of CHIM 1–5, which involved the substitution of a relatively long region, a patch PCR technique that utilized two templates (BDNF and NGF in pCDM8) and three primers was used. In the PCR, the amino-terminal part of the chimeric molecule was amplified from BDNF using a BDNF-specific sense primer upstream of the NarI site and an anti-sense primer that spanned the BDNF/NGF junction of the chimera. The 3′-end of the resulting fragment would anneal to the corresponding region of NGF due to sequence homology. The chimera was subsequently amplified by the BDNF sense primer and an NGF-specific anti-sense primer located at the NotI site of pCDM8 and then subcloned into the NarI and NotI sites of the BDNF template. For the construction of CHIM 6–9, in which seven

or eight amino acids were substituted, the amino- and carboxy-terminal fragments were amplified from BDNF in two separate PCR reactions. The short sequence of NGF that was to be introduced to the BDNF backbone was located at the ends of both fragments. After gel purification, the two fragments were pooled together in a second PCR in which the opposite ends of the two fragments would anneal because of the overlapping NGF sequence, and the chimera was amplified by the primers flanking the cloning sites (XhoI and NotI) of pCDM8. Similarly, to exchange the pre-pro regions of the chimeras with that of NGF, the pre-pro region of mouse NGF and the mature region of each chimera were amplified separately by PCR and pooled together in a second PCR that generated the chimera with the mouse NGF pre-pro region. All chimeric constructs as well as the cDNA of NGF and BDNF were subcloned into the expression vector pMT21 after digestion by XhoI and NotI. To confirm the identities of the chimeras, constructs were subjected to double-stranded DNA sequencing based on the dideoxy sequencing method of Sanger et al. (1977).

Production of Neurotrophins in COS-5 Cells

Chimeric constructs as well as BDNF and NGF subcloned in pMT21 were transiently transfected into COS-5 cells using the diethylaminoethanol (DEAE)-dextran-chloroquine method as previously described (Ip et al., 1992). Briefly, COS-5 cells (1×10^6) were plated onto 100 mm culture plates. Twenty-four hours later, 8 ml of the transfection cocktail that contained 0.4 mg/ml DEAE/dextran, 0.1 mM chloroquine, and 10 μg plasmid in RPMI was added to each plate and incubated at 37°C for 3.25 hr. The cells were shocked by 10% dimethylsulfoxide (DMSO) for 3 min at room temperature and recovered in Dulbecco's Modified Eagle Medium (DMEM) + 10% Fetal bovine serum (FBS) after washing once with the medium. Twenty-four hours later, cells were switched to serum-free defined medium after washing once with phosphate-buffered saline (PBS) and further incubated for 3 days before the COS cell supernatant was collected. The levels of expression of the chimeric neurotrophins were not determined by Western blot analysis, due to the lack of a specific antibody that can recognize all the chimeras.

Survival Assays of Fibroblasts

Survival assays of fibroblasts that expressed TrkA or TrkB were utilized to determine the receptor specificities of the various chimeras. In the cotransfection assay, NIH-3T3 fibroblasts (1×10^6) were plated onto 100 mm culture plate. Twenty-four hours later, cells were cotransfected with 5 μg of plasmid encoding the chimeric neurotrophins and 5 μg of either TrkA or TrkB subcloned in the expression vector pVCosNeo, using a calcium phosphate precipitation method in the presence of 20 μg of human placental DNA (Glass et al., 1991; Ip et al., 1993). After 16 hr, cells were washed twice in PBS before incubation with 10 ml of DMEM + 10% calf serum for 24 hr. Plates were then split 1:10 for those cotransfected with TrkA or 1:50 for those cotransfected with TrkB in DMEM + 10% calf serum containing 500 μg/ml G418 (Geneticin; GIBCO, Grand Island, NY). The G418 selection lasted for 7 days, during which the medium was changed every 3 days. Cells were switched to serum-free defined medium after washing with PBS, and the medium was changed every other day. After 8 days, surviving colonies were stained by hematoxylin. For the growth assay of stable transfectants that overexpressed either TrkA or TrkB, 10^4 cells were plated in serum-free defined medium into each well of the Primaria-coated 96-well plates, in which serially diluted COS cell supernatant containing the neurotrophin was added. After incubation with the chimeric neurotrophin for 4 days, survival of fibroblasts was monitored by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay as previously described (Ip et al., 1992).

Tyrosine Phosphorylation Assay

TrkA-expressing fibroblasts (4×10^6 cells) were plated onto a 100 mm culture dish. On the next day, cells were starved for 60 min in serum-free defined medium before being treated with COS cell supernatant containing the neurotrophin for 5 min at 37°C. They were then lysed by RIPA buffer containing 1% Nonidet-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, 1 mM penylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 0.14 U/ml aprotinin in PBS. Lysates were immunoprecipitated by the pan-Trk antibody RG145 at 4°C overnight and subsequently bound to protein A sepharose. Immunoprecipitated products were run on SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membrane, and immunoblotted with the antiphosphotyrosine antibody (Upstate Biotechnologies, Lake Placid NY).

RESULTS

Construction of Chimeric Neurotrophins

Using PCR, nine chimeras were constructed by substituting various regions of the BDNF backbone with the corresponding regions of NGF (Figs. 1, 2). For CHIM 1–4, relatively long regions of BDNF carboxy-terminal sequences were substituted by the corresponding regions of NGF (e.g., CHIM 1 represented NGF with a pre-pro region of BDNF). These chimeras might reveal which regions of the NGF and BDNF molecules contained structural elements important in Trk receptor activation. On the other hand, short stretches of amino

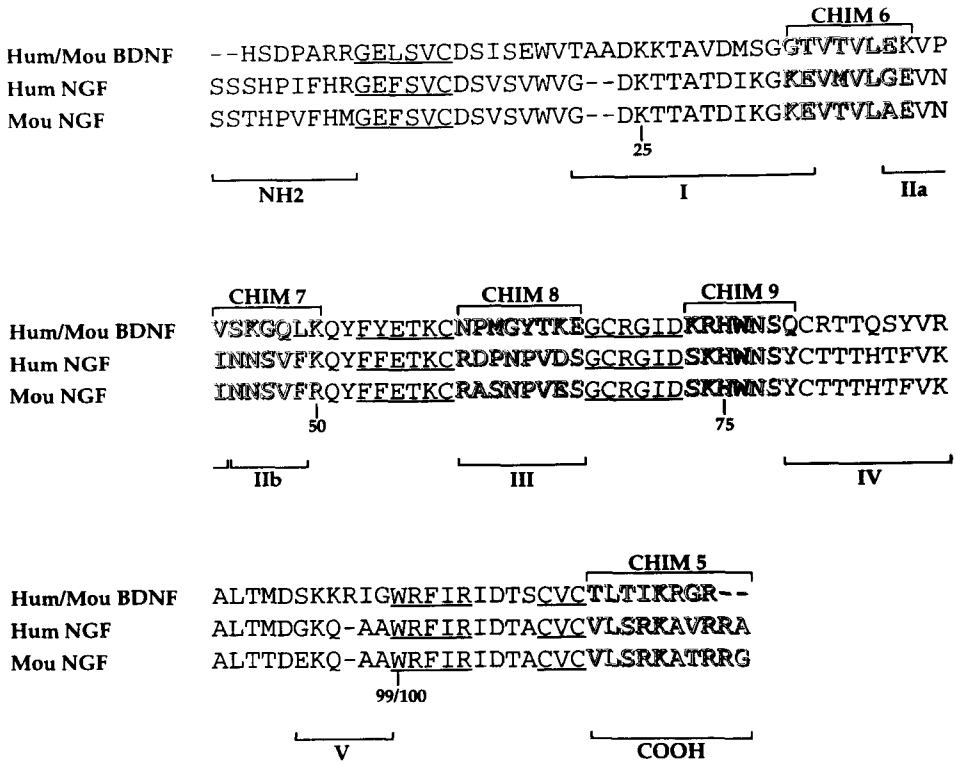

Fig. 1. Alignment of the amino acid sequence of the mature regions of human (HUM) and mouse (MOU) BDNF and NGF. The short stretches of amino acids that are highlighted represent those which were substituted in constructing CHIM 5–9. They either were highly variable or contained amino acids that carry different charges at the corresponding positions of NGF and BDNF. The regions that are more conserved among the two neurotrophins which are located near the cysteine residues, are underlined. The positions of some of the amino acids are numbered, while the variable regions, as defined by Ibáñez et al. (1993), are bracketed at the bottom.

acids at various locations of BDNF were replaced by the homologous regions of NGF in CHIM 5–9. These regions were either highly variable between NGF and BDNF or contained amino acids that carried different charges in the homologous positions of the two neurotrophins (for example, the regions replaced in CHIM 6 and CHIM 9). These amino acids might be important in determining the receptor specificities of NGF and BDNF. In addition, nine more chimeras were constructed which were the same as CHIM 1–9 except that the pre-pro region was derived from mouse NGF; they were denoted as NGF/CHIM 1–9. The exchange of pre-pro region between BDNF and NGF had been shown to increase the production of recombinant BDNF in mammalian as well as insect cells, possibly due to an increase in efficiency of processing from the larger precursor (Ip et al., 1992; Meyer et al., 1994). The pre-pro exchange might therefore increase the production of the chimeric neurotrophins.

Formation of Surviving Colonies After Cotransfection of Fibroblasts With TrkB and Chimeric Neurotrophins

Previous studies demonstrated that cotransfection of plasmids encoding TrkB and BDNF, but not NGF, into NIH-3T3 fibroblasts could lead to formation of surviving colonies in serum-free defined medium (Glass et al., 1991). This cotransfection assay can therefore indicate chimeric neurotrophin activities on TrkB. In our study, it was found that CHIM 1–4, like NGF, could not give rise to any surviving colony after cotransfection with TrkB (Fig. 3). In contrast, CHIM 5–9 produced surviving colonies after cotransfection with TrkB in a manner similar to BDNF (Fig. 3), suggesting that replacement of the short regions from BDNF did not lead to apparent loss in TrkB activation. Similar results were observed for the nine chimeras that were subjected to pre-pro exchange with NGF (NGF/CHIM1–9; data not shown).

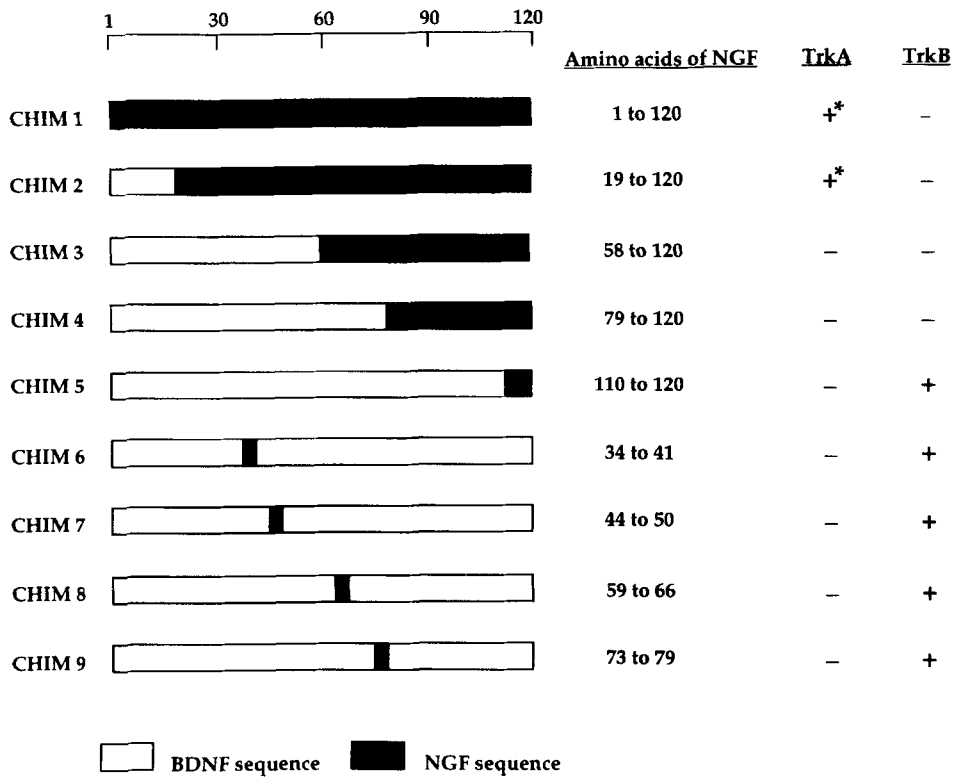

Fig. 2. Schematic diagram of the chimeras, constructed by substituting various regions from the BDNF backbone with the homologous regions of NGF. Construction of CHIM 1–4 involved the introduction of relatively long regions of NGF into the BDNF molecule, while short segments of NGF were substituted into CHIM 5–9. Numbering of NGF amino acids started from the amino-terminus at the mature region. In addition, there were nine more chimeras that were composed of the same mature region except that the pre-pro region was exchanged with that of NGF. The + and – signs represent the presence or absence, respectively, of functional activity on TrkA or TrkB according to the cotransfection and fibroblast survival assays in our study. *Positive TrkA activity was observed for CHIM 1 and 2 only after the pre-pro exchange with NGF.

Formation of Surviving Colonies After Cotransfection of Fibroblasts With TrkA and Chimeric Neurotrophins

To determine whether any of the chimeras could gain the ability to activate TrkA, fibroblasts were cotransfected with the chimeras together with plasmid-encoding TrkA. Surprisingly, all nine chimeras in which the pre-pro region was derived from BDNF produced very few surviving colonies (similar to that produced by BDNF) after co-transfection with TrkA (data not shown). Even CHIM 1, in which the entire mature region was composed of NGF, did not result in activity higher than background. However, after exchanging the pre-pro region with that of NGF, NGF/CHIM 1 and NGF/CHIM 2 could give rise to significant numbers of surviving colonies (Fig. 4), albeit lower than that of NGF. On the other hand, NGF/CHIM 3–9 remained inactive in promoting colony formation (Fig. 4).

Survival of Fibroblasts Overexpressing TrkB in Response to Chimeric Neurotrophins

The activities of the chimeras on TrkB were verified by survival of fibroblasts that ectopically expressed TrkB. The chimeric neurotrophins were produced in COS cells, and the fibroblast survival in serum-free defined medium in response to serially diluted COS cell supernatant was determined using the MTT assay. Consistent with the cotransfection assay, CHIM 1–4 were unable to promote the survival of fibroblasts that expressed TrkB (Fig. 5A), while the activities of CHIM 5–9 regarding fibroblast survival were comparable to that of BDNF (Fig. 5B). Although the activity of CHIM 6 was considerably higher than that of the other chimeras and BDNF in the MTT assay (Fig. 5B), this difference was not observed in the cotransfection assay. Moreover, the higher activity on TrkB-expressing fibroblasts was not observed for CHIM 6 with the pre-pro region of NGF

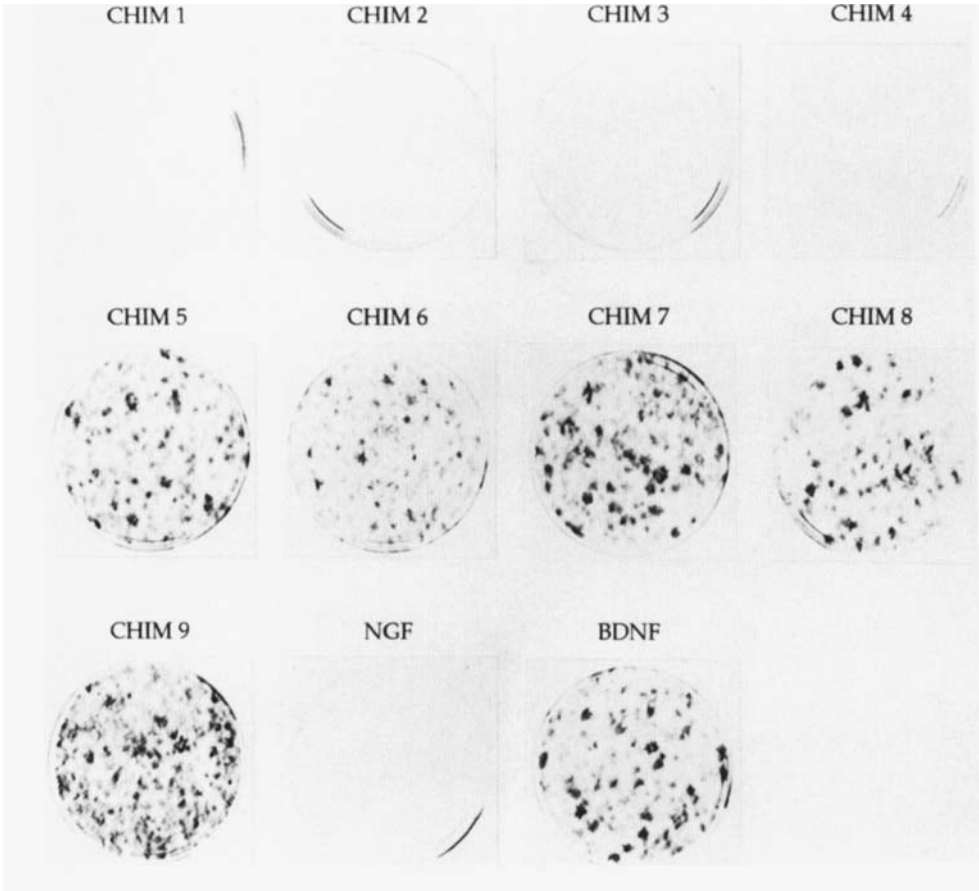

Fig. 3. Photographs showing the surviving colonies (stained by hematoxylin) of NIH-3T3 fibroblasts in serum-free defined medium after cotransfection of the respective chimera with plasmid-encoding TrkB. CHIM 5–9 represent BDNF, with individual short segments of amino acids being substituted by the corresponding ones from NGF; these chimeras produced comparable numbers of surviving colonies as BDNF. In contrast, CHIM 1–4 could not produce any surviving colony after cotransfection. Similar results were obtained for the nine chimeras in which the pre-pro region was exchanged with that of NGF.

(NGF/CHIM6; data not shown). A similar trend in TrkB activation was observed for the rest of the chimeras that were subjected to pre-pro exchange (data not shown).

Survival of Fibroblasts Overexpressing TrkA in Response to Chimeric Neurotrophins

The survival of TrkA-expressing fibroblasts in response to the chimeras was also examined. Similar to the results of the cotransfection assay, none of the nine chimeras which contained the pre-pro region of BDNF showed any TrkA activity (data not shown). Only NGF/CHIM 1 and NGF/CHIM 2, which were subjected to pre-pro exchange, could support the survival of TrkA-expressing fibroblasts in serum-free defined medium (Fig. 6A). Consistent with the cotransfection assay, the TrkA activity of either chimera was lower than that of wild-type NGF. For the other seven chimeras, pre-pro exchange could not enable them to promote the survival of fibroblasts via TrkA activation (Fig. 6).

DISCUSSION

Advantages of Fibroblast Survival Assays

The fibroblast survival assay utilized in our study (Glass et al., 1991; Ip et al., 1993) offers a simpler system to investigate the interactions between Trk receptors and neurotrophins compared to neuronal systems (such as survival of dissociated primary neurons or neurite extension of the pheochromocytoma PC12 cells). Interpretations of neurotrophin-Trk interactions in neurons might be complicated by the presence of accessory molecules such as p75, which has been reported to affect the binding and activation of Trk receptors by the neurotrophins (Barker and Shooter, 1994; Hantzopoulos et al., 1994; Verdi et al., 1994). Some previous studies on the structure-function relationships of neurotrophins had utilized tyrosine phosphorylation of Trk receptors expressed in fibroblasts to assay the activities of chimeric neurotrophins (Ibáñez et al., 1993; Ilag et al., 1994).

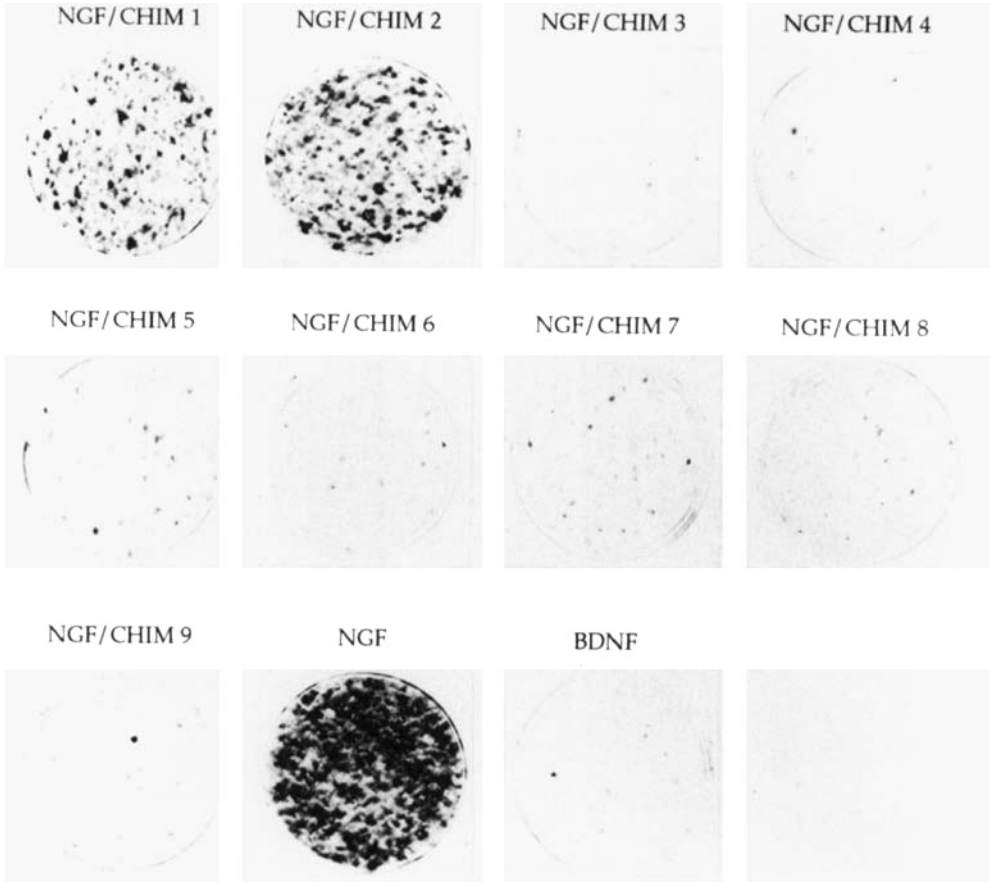

Fig. 4. Photographs showing the surviving colonies of NIH-3T3 fibroblasts in serum-free defined medium after cotransfection of cells with NGF/CHIM 1–9 (i.e., CHIM 1–9 with the pre-pro region derived from NGF) and plasmid-encoding TrkA. Only NGF/CHIM 1 and 2 produced comparable but fewer numbers of surviving colonies as NGF. All the other chimeras could not give rise to more colonies than the negative control (cotransfection of BDNF and TrkA). All nine chimeras that contained a pre-pro region derived from BDNF could not produce significantly more surviving colonies than the background.

However, tyrosine phosphorylation is the initial step in the signaling cascade activated by neurotrophin-Trk receptor interaction; survival response of fibroblasts can be achieved only when the pathways downstream of the Trk receptors are activated. Thus, survival of fibroblasts that express the Trk receptors is important in demonstrating the activities of chimeric neurotrophins in nonneuronal environments.

Structural Elements of BDNF That Determine TrkB Receptor Specificity

The short regions selected for replacement in CHIM 5–9 either are highly variable or contain amino acid residues that carry different charges between NGF and BDNF; these regions are therefore expected to play a role in determining the receptor specificity of BDNF. However, the individual replacement of short regions from the BDNF backbone by the homologous regions of NGF could not lead to any apparent loss in TrkB activation according to fibroblast survival assays. This finding is analogous to a previous report in which individual substitution of short segments of BDNF into the NGF backbone did not lead to any significant loss in NGF action (Suter et al., 1992). However, BDNF is much more conserved throughout evolution than NGF, suggesting less flexibility in the structure of the BDNF molecule. It is therefore a surprising finding that individual replacement of some of the short variable regions did not interfere with the ability of BDNF to activate TrkB.

Despite no apparent defect in the function of BDNF after individual substitutions, the short regions being substituted in the construction of CHIM 5–9 might still play a role in determining the TrkB activation of BDNF. The relative importance of each short segment might be revealed if it was substituted in combination since it is possible that the receptor binding surface is made up of

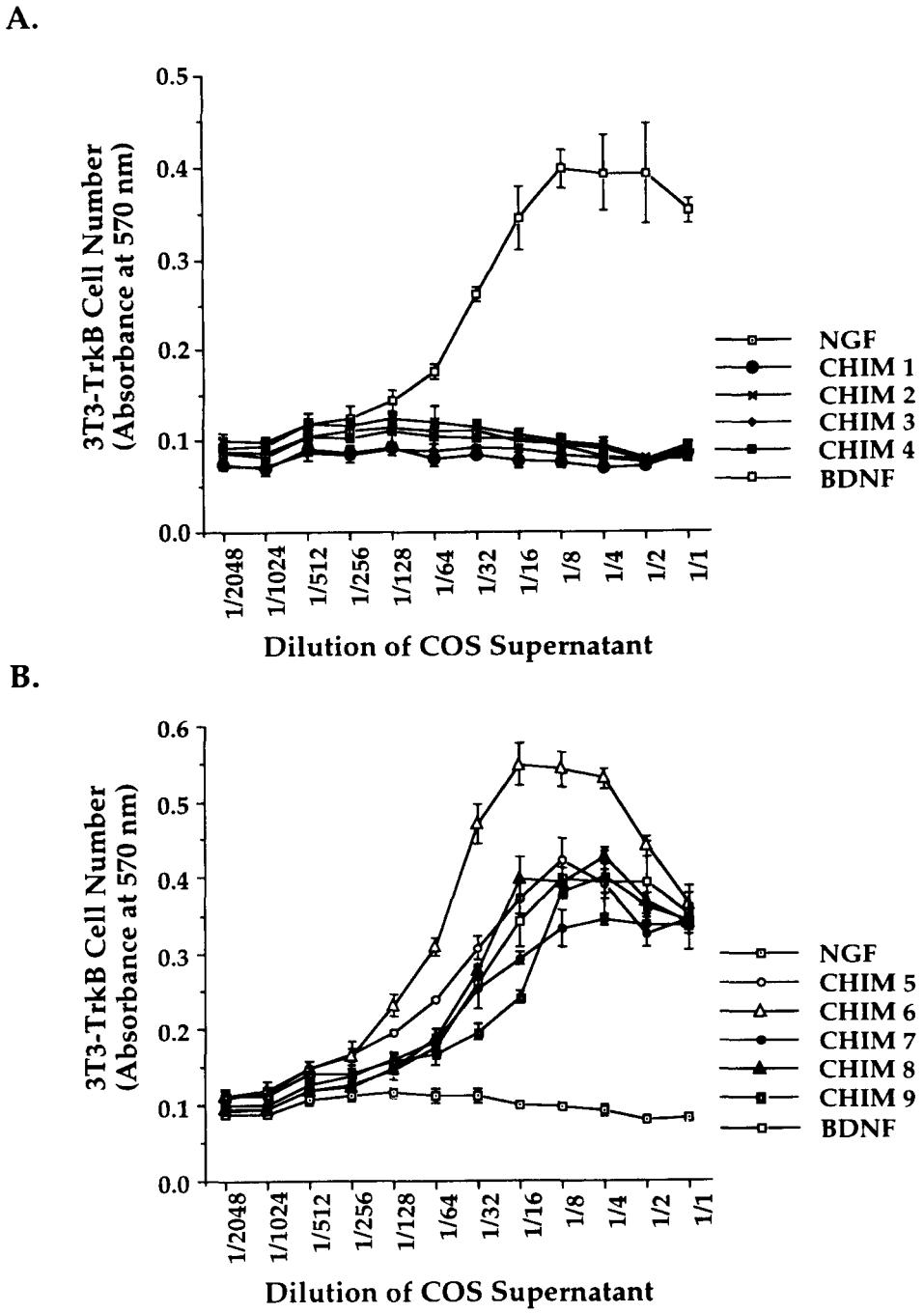

Fig. 5. Survival of TrkB-expressing fibroblasts in MTT assay in response to CHIM 1–4 (**A**) and CHIM 5–9 (**B**). NIH-3T3 cells stably expressing TrkB (denoted as 3T3-TrkB) were treated with supernatants from COS transfected with neurotrophin chimera at different dilutions as indicated. CHIM 1–4 failed to support fibroblast survival via TrkB interaction, while the activities on TrkB of CHIM 5–9 were comparable to that of BDNF. The higher activity of CHIM 6 compared with BDNF was observed in duplicate experiments. The corresponding greater activity was not observed for NGF/CHIM 6 (data not shown). Each data point represents mean ± SD, n = 4.

more than one segment of amino acids, which are brought together by protein folding. Furthermore, there are other variable regions between NGF and BDNF that were not individually substituted in our study. Interestingly, comparing the activities of CHIM 4 and 5 on TrkB revealed the importance of the 32 amino acids (79–110)

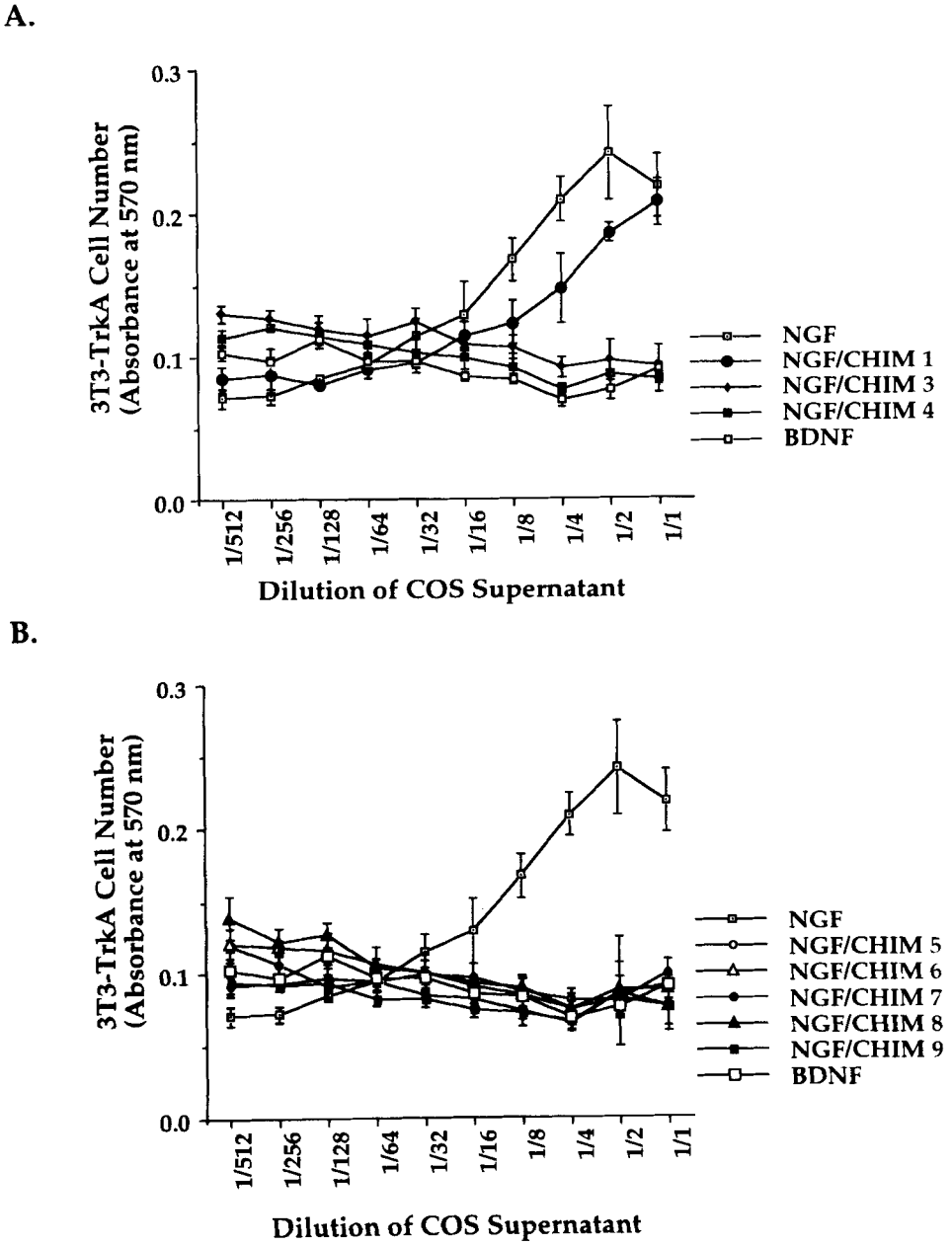

Fig. 6. Survival of TrkA-expressing fibroblasts in MTT assay in response to the nine chimeras that were subjected to pre-pro exchange, i.e., NGF/CHIM 1, 3, and 4 (**A**) and NGF/CHIM 5–9 (**B**). NIH-3T3 cells stably expressing TrkA (denoted as 3T3-TrkA) were treated with supernatants from COS transfected with neurotrophin chimera at different dilutions as indicated. Of those shown, only NGF/CHIM 1 could support the survival of TrkA-expressing fibroblasts; results for NGF/CHIM 2, which could also support the survival of fibroblasts, is not shown. The remaining chimeras did not show significant activity compared with wild-type NGF. Each data point represents the mean ± SD, n = 4.

in the carboxy-terminal half of BDNF. Much of CHIM 4 (about 70%) was derived from BDNF, yet it was unable to promote the survival of TrkB-expressing fibroblasts as a result of the substitution of the 32 amino acid residues. On the other hand, CHIM 5, which contained those amino acids, showed TrkB activity comparable to that of wild-type BDNF. It was noteworthy that the region substituted in CHIM 4 included two variable regions (regions IV and V) which had been shown to confer the ability to activate TrkB after their individual introduction into the NGF backbone (Ibáñez et al., 1993). Our present finding, therefore, provides additional evidence for the

requirement of these two variable regions for BDNF to activate TrkB.

CHIM 1–4 failed to show any TrkB activity in the fibroblast survival assay even after the pre-pro exchange with NGF, which might increase the production of the mature protein (see below). CHIM 1–4 might already lose the ability to interact with TrkB completely after the amino acid substitution and, therefore, could not support the survival of TrkB-expressing fibroblasts even after increasing production or processing of the chimeric neurotrophins.

Structural Elements of NGF That Determine TrkA Receptor Specificity

Of the nine chimeras, only CHIM 1 and 2 could support survival of TrkA-expressing fibroblasts after exchanging the pre-pro region with NGF. The requirement for the NGF pre-pro region can be explained by the increase in processing, stability, and/or secretion of the chimeras after the pre-pro exchange. However, even with the NGF pre-pro region, the activity of both CHIM 1 and 2 on TrkA was considerably lower than that of wild-type NGF. One possibility is that the pre-pro region of NGF/CHIM 1 and 2 was derived from mouse NGF, while the mature region was derived from human NGF; this species difference might result in lower stability of the chimeric molecules.

With the first 18 amino acids of NGF replaced by the corresponding ones from BDNF in the mature region, CHIM 2 showed activity on TrkA similar to NGF/CHIM 1, in which the entire mature region was derived from NGF. This finding appears to contradict results from previous studies demonstrating the importance of the amino-terminus of NGF in TrkA interaction (Ibáñez et al., 1993; Urfer et al., 1994). However, it is possible that NGF/CHIM 2 binds to TrkA with somewhat lower affinity but that the activity on the receptor is not substantially reduced because the chimera contains other structural elements essential for TrkA activation. Indeed, substitution of the first nine amino acid residues of the mature region of NGF has been shown to greatly reduce the binding, but not the biological activity, of NGF on TrkA, suggesting that the binding and activation of TrkA are determined by different structural elements in the NGF molecule (Ibáñez et al., 1993).

Apart from CHIM 1 and 2, none of the other chimeras could support the survival of TrkA-expressing fibroblasts after the pre-pro exchange. There is one common feature in these chimeras: the lack of the 17 amino acids (34–50 from the amino-terminus of the mature region) which had been shown to be critical in the production of NGF (Suter et al., 1992; Ibáñez et al., 1993). Accordingly, two more chimeras were constructed by introducing the 17 amino acids to NGF/CHIM 3 and NGF/CHIM 4, and tyrosine phosphorylation of TrkA in fibroblasts was utilized to examine whether TrkA activation could be restored. It was found that the introduction of those amino acids could only slightly enhance TrkA phosphorylation by NGF/CHIM 3 but not by NGF/CHIM 4 (data not shown). Moreover, the activity of either chimera was much lower than that of NGF/CHIM 1 (which contained the entire NGF mature region). The modest activity on TrkA by NGF/CHIM 3 was therefore attributed to the absence of other regions, i.e., the amino-terminus and region I (Ibáñez et al., 1993) as well as the two more conserved regions (10–22 and 51–57) which form an anti-parallel β-strand according to the three-dimensional conformation of NGF (McDonald et al., 1991). Previous structure-function studies on NGF mainly focused on the role of the highly variable regions such that the importance of the more conserved regions might be overlooked. Since NGF and BDNF differ in four amino acids in the two conserved regions, the lack of significant TrkA activation of NGF/CHIM 3 after introduction of the 17 amino acids might be attributed to the substitution in the two conserved regions together with the amino-terminus and variable region I.

The failure to achieve significant TrkA activity after introduction of the 17 amino acids (34–50) to the BDNF backbone also illustrates one interesting point. It has been shown that introducing amino acids of NGF variable region II into NT-3 could result in significant interaction with TrkA in both neuronal and nonneuronal cells (Kullander and Ebendal, 1994; Ilag et al., 1994). However, our present study demonstrates that introduction of the 17 amino acids (which included the variable region II) of NGF to a chimera in which the carboxy-terminal half was composed entirely of NGF could not give rise to significant TrkA phosphorylation. Thus, it seems easier to model NT-3 according to the behavior of NGF than to model BDNF according to the behavior of NGF; this is consistent with the fact that the structural and evolutionary relationships between NT-3 and NGF are closer than those between BDNF and NGF (Hallböök et al., 1991) and that NT-3 has the ability to weakly activate TrkA (Ip et al., 1993). Indeed, two chimeric neurotrophins that possess multiple Trk specificities were made from NT-3 with substitution of short segments by the corresponding sequence of NGF or BDNF (Ibáñez et al., 1993; Urfer et al., 1994).

The present study examined the structure-function relationships of NGF and BDNF by constructing chimeric neurotrophins using BDNF as the backbone. The survival of fibroblasts that expressed TrkA or TrkB provided a simplified model to study the activity between the chimeric neurotrophins and the Trk receptors. Our present findings provide the first demonstration that some of the variable regions of BDNF, despite being

absolutely conserved during the evolution of mammals, are dispensable for the activation of TrkB. Moreover, the exchange of the entire carboxy-terminal half with NGF does not allow BDNF to gain any activity on TrkA. It remains to be seen whether substitution of the entire amino-terminal half of NGF into the BDNF backbone would result in any enhancement of TrkA activation.

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