ACS Chemical Neuroscience

Research Article

pubs.acs.org/chemneuro

A Small Linear Peptide Encompassing the NGF N-Terminus Partly Mimics the Biological Activities of the Entire Neurotrophin in PC12 Cells

Alessio Travaglia,[†] Adriana Pietropaolo,[‡] Rossana Di Martino,[§] Vincenzo G. Nicoletti,[∥,⊥] Diego La Mendola,[#] Pietro Calissano,*[,∇] and Enrico Rizzarelli*[,○]

[†]Center for Neural Science, New York University, 4 Washington Place, New York, New York 10003, United States

[‡]Dipartimento di Scienze della Salute, Università di Catanzaro, Viale Europa, 88100 Catanzaro, Italy

[§]Istituto di Bioimmagini e Fisiologia Molecolare (IBFM)-CNR, C.da Pietrapollastra-Pisciotto, Cefalù, Palermo 90015, Italy

[∥]Dipartimento di Scienze Biomediche e Biotecnologiche - Sezione di Biochimica Medica, Università degli Studi di Catania, Viale Andrea Doria 6, 95125 Catania, Italy

[⊥]Istituto Nazionale Biostrutture e Biosistemi (INBB) − Sezione Biomolecole, Consorzio Interuniversitario, Viale Medaglie d'Oro 305, 00136 Roma, Italy

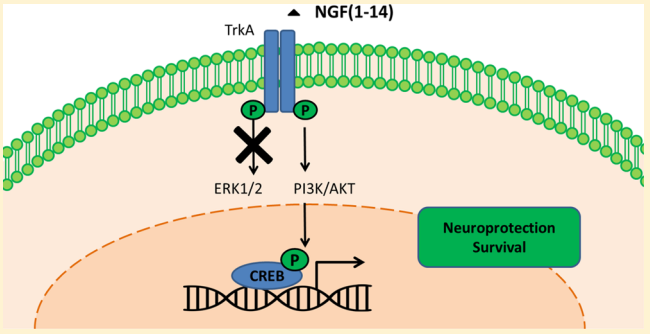
[#]Department of Pharmacy, University of Pisa, Via Bonanno Pisano 6, 56126 Pisa, Italy

[∇]European Brain Research Institute (EBRI), Via del Fosso di Fiorano, 64-65, 00143 Rome, Italy

[○]IBB-CNR, UOS Catania, via Paolo Gaifami 18, 95126 Catania, Italy

Supporting Information

ABSTRACT: Ever since the discovery of its neurite growth promoting activity in sympathetic and sensory ganglia, nerve growth factor (NGF) became the prototype of the large family of neurotrophins. The use of primary cultures and clonal cell lines has revealed several distinct actions of NGF and other neurotrophins. Among several models of NGF activity, the clonal cell line PC12 is the most widely employed. Thus, in the presence of NGF, through the activation of the transmembrane protein TrkA, these cells undergo a progressive mitotic arrest and start to grow electrically excitable neuritis. A vast number of studies opened intriguing aspects of NGF mechanisms of action, its biological properties, and potential use as therapeutic agents. In this context, identifying and utilizing small portions of NGF is of great interest and involves several human diseases including Alzheimer's disease. Here we report the specific action of the peptide encompassing the 1−14 sequence of the human NGF (NGF(1−14)), identified on the basis of scattered indications present in literature. The biological activity of NGF(1−14) was tested on PC12 cells, and its binding with TrkA was predicted by means of a computational approach. NGF(1−14) does not elicit the neurite outgrowth promoting activity, typical of the whole protein, and it only has a moderate action on PC12 proliferation. However, this peptide exerts, in a dose and time dependent fashion, an effective and specific NGF-like action on some highly conserved and biologically crucial intermediates of its intracellular targets such as Akt and CREB. These findings indicate that not all TrkA pathways must be at all times operative, and open the possibility of testing each of them in relation with specific NGF needs, biological actions, and potential therapeutic use.



KEYWORDS: *Peptidomimetic, neuroprotection, nerve growth factor, ERK, CREB, PC12*

Neurotrophins are small proteins that induce cell growth, differentiation, and survival of neurons,[1,2] as well as of a growing number of non-neuronal cell types.[3,4] They include nerve growth factor (NGF), brain-derived growth factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5).[1,2,5] Each neurotrophin selectively binds to its tyrosine kinase membrane receptor (Trk) and not selectively to a 75 kDa neurotrophin receptor (p75NTR). Trk signaling occurs through different pathways, including the mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide 3-kinase (PI3) pathway. These pathways tune the fate of neurotrophin signaling in terms of cell survival and cell differentiation,[6] while neurotrophin binding to p75NTR can trigger neuron apoptosis (see Figure1).[7]

Received: February 17, 2015
Revised: April 13, 2015
Published: May 4, 2015

DOI: 10.1021/acschemneuro.5b00069
ACS Chem. Neurosci. 2015, 6, 1379−1392

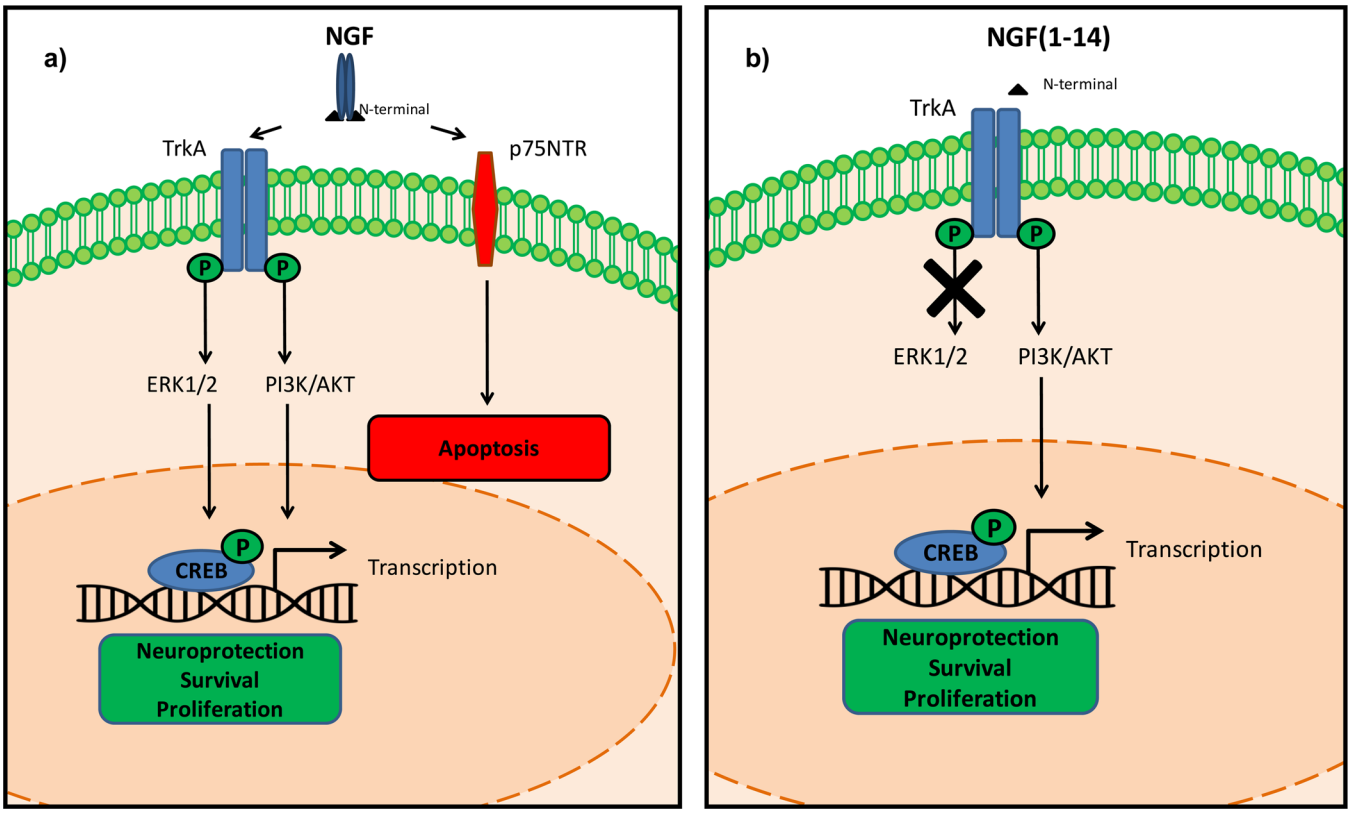


Figure 1. Schematic representation of biochemical signaling activated by NGF (a) and NGF(1−14) (b).

NGF has been the first member of the neurotrophin family to be discovered. It is able to cause outgrowth of fibers from sensory or sympathetic nerve cells.[8−10] It is a secreted growth factor synthesized as a precursor, proNGF, which is cleaved to give the mature form, a homodimer of approximately 26 kDa.

Elevated concentrations of NGF mRNA have been found in the spinal cord and dorsal root ganglia (DRG),[11] and, in the brain, it is produced in cortex and hippocampus. Since the discovery of NGF more than 50 years ago,[8,9] neurotrophins have been indicated as therapeutic agents against a number of brain disorders due to their ability to prevent or reverse neuronal degeneration, to promote neurite regeneration and to enhance synaptic plasticity.[12,13]

Despite their overall promise, the use of neurotrophins as therapeutic tools has encountered significant contraindications, because of their suboptimal pharmacological properties.[14] These include low serum stability, poor oral bioavailability, and undetectable penetration of the blood-brain barrier.[15,16]

The pleiotropic actions of neurotrophins, due to the activation of their multireceptor signaling networks (see Figure 1), is also responsible of adverse effects, such as the promotion of neurodegeneration[13,17,18] or pain,[19,20] with further issues for their clinical applications.

To overcome these limitations that hamper its use as drug, different routes of NGF and neurotrophin protein administration have been investigated. Such studies include neurotrophin receptor-specific antibodies to modulate signaling processes that are relevant to pathological states[21−25] and expression of neurotrophins via viral and cell-based delivery systems.[26−30] A more recent development of a painless mutant still endowed with full biological activity has been recently produced,[31] in order to avoid the painful adverse action of NGF when administered via systemic or local administration.

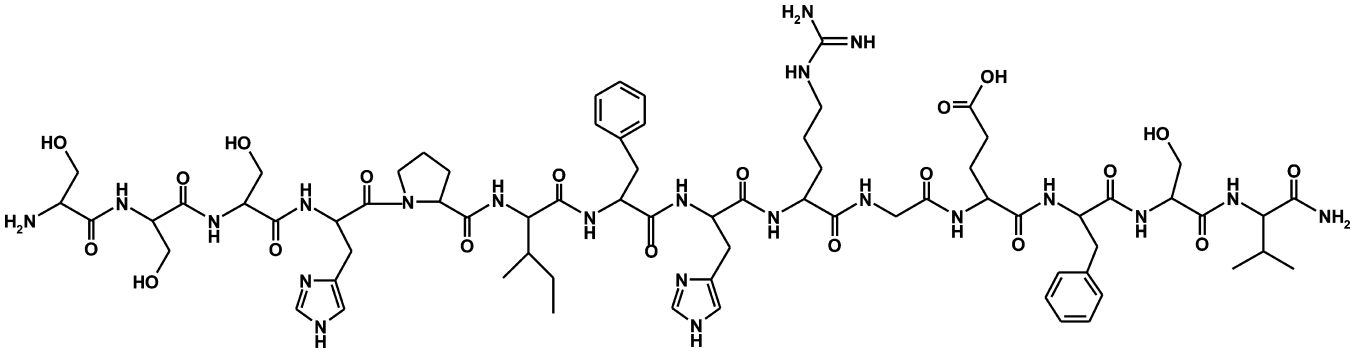
An alternative strategy is centered on the ability of small molecules to differentially interact with neurotrophin receptors. Such small molecules, either as agonists or as antagonists, aim to achieve distinct signaling profiles, thus resulting in more selective effects than those activated by neurotrophins,[32] representing the potential solution for the above pharmacokinetic drawbacks.[33]

Mature neurotrophins contain a central "saddle" region[34−36] that represents their main binding site with their cognate receptors. Neurotrophins also have three hairpin β-turn loops (loops 1, 2, and 4), which display a relatively low degree of amino acid similarity across the neurotrophin family and represent three of the five neurotrophin variable domains.[37] These variable regions contribute to multidomain receptor interfaces and receptor specificity, playing a key role in the structure-based design of receptor-specific small-molecules. The binding of NGF to TrkA induces the dimerization of the receptor[38] (although preformed TrkA multimers may occur[39,40]) and autophosphorylation of TrkA receptors at multiple tyrosine residues. This event, in turn, leads to the recruitment of different intracellular signaling components and the activation of downstream pathways (Figure 1).[2,3]

Considerable efforts have turned to develop drugs able to interact selectively with the TrkA receptor mimicking or antagonizing the NGF activities. These drugs include NGF peptides,[41−45] small natural compounds,[46] and synthetic peptidomimetics.[47−51] Among the NGF different domains, the relevance of the N-terminus tail has been brought into evidence.[52,53] Kahle et al. reported that a truncated form of NGF, which lacks the first nine residues, displayed more than 300-fold lower affinity for TrkA than the whole NGF.[53] Such truncated NGF has also been reported to have 50-fold lower capacity in eliciting TrkA phosphorylation.[53] Of note, such

DOI: 10.1021/acschemneuro.5b00069
ACS Chem. Neurosci. 2015, 6, 1379−1392

Scheme 1. Schematic View of the N-Terminal Domain of NGF



NGF(1-14) = SSSHPIFHRGEFSV-NH_2

biological effects are not due to a different folding, stability, or conformation of the NGF molecule.[54] Along the residues of the N-terminal domain, the His-4 has been reported to be critical.[55] Diethyl pyrocarbonate modification of His-4 and His-8 in a NGF double mutant abolished neuritogenesis, binding to both receptors, and phosphorylation of TrkA in PC12 cells. NGF(H4D) mutant showed a lower binding affinity for TrkA (compared with wild type NGF), less efficacy in the TrkA autophosphorylation, and less capacity in PC12 cell differentiation.[55] These findings have been confirmed by computational simulations,[56] which identified the residues lying at the ligand−receptor interface. Among them, the His 4 and Glu 11 residues of the N-terminal domain provide highly persistent H-bond interactions with the receptor, as well as Ile 6 and Phe 7 residues stabilize the binding of the N-term of NGF to TrkA through hydrophobic interactions. Taken together, these studies converge to suggest that the first residues of the NGF N-terminus could play a crucial role for the trophic activities of the neurotrophin. However, no experimental studies have been reported on the ability of a linear peptide encompassing the N-terminus sequence of the neurotrophin to directly activate the NGF pathways and to mimic the whole protein activities.

In this context, the small peptide encompassing the 1−14 sequence of the human NGF (NGF(1−14)) (Scheme 1) has been synthesized to ascertain its ability to (1) activate the TrkA pathway; (2) act as biomimetic agent of NGF; and (3) induce the phosphorylation of CREB as a prerequisite of its activity. The binding mode between the peptide fragment of the NGF N-terminus and the TrkA receptor was also determined by means of a computational study.

RESULTS

Activity of NGF(1−14) on the Proliferation and Morphology of PC12 Cells. We have previously reported the ability of NGF(1−14) to increase the proliferation rate of neuroblastoma cells SHSY5Y.[57] PC12 pheochromocytoma cells have been employed to study the trophic effects of NGF and to validate the efficiency of NGF-like molecules.[42,58] In order to ascertain the NGF(1−14) ability to mimic NGF and to stimulate PC12 differentiation, cells were cultured in complete medium and treated with NGF(1−14) (1, 10, and 100 μM) or NGF (100 ng/mL, corresponding to 3.84 nM). Cell morphology and confluence was analyzed 24 h (data not shown) and 7 days after the treatment (Figure 2a−d). As largely reported in previous studies, NGF is sufficient to trigger PC12 differentiation and leads to the formation of a complex neuronal network within 7 days (Figure 2d). At the same time point, untreated PC12 continued to proliferate, still showing the undifferentiated phenotype (Figure 2b). The treatment with NGF(1−14), in the concentration range explored (1−100 μM), was not able to induce any detectable sign of differentiation on PC12, within 7 days (Figure 2c).

Although we have not been able to obtain reliable data indicating that NGF(1−14) can induce differentiation, we observed a reduced cell confluence 7 days after the treatment with the NGF(1−14) peptide (Figure 2c), compared with the untreated PC12 (Figure 2b). Indeed, the treatment with NGF(1−14) 10 μM had a significant effect on the reduction of cell proliferation and the consequent decrease in cell number (79.6% ± 3.7, $p < 0.05$) (Figure 2e). However, we also observed a significant difference between NGF and NGF(1−14) treated cells ($p < 0.01$).

Finally, we verified the ability of NGF(1−14) to maintain the differentiated phenotype. In agreement with previous data, morphological analysis reveal that differentiated PC12 underwent degeneration of the neuronal processes 24h after deprivation (Supporting Information Figure S1). In parallel, we observed a significant decrease in cell viability, compared with NGF treated cells, both at 24h ($p < 0.01$) and 48h ($p < 0.01$) after the deprivation (Figure 2f).

A striking effect was observed when differentiated PC12 cells were deprived from serum and NGF, but treated with NGF(1−14). The treatment with NGF(1−14) was unable to maintain the morphology of the differentiated phenotype. However, in growth factor withdrawal conditions, differentiated PC12 cells were kept alive by treatment with NGF(1−14) 10 μM, and we did not find any significant difference with NGF treated cells ($p > 0.05$) (Figure 2f).

Interaction with TrkA Receptor. The induction of biochemical pathways in NGF-sensitive cells, after NGF binding to TrkA and its dimerization, is first related to the autocatalytic intracellular domain receptor phosphorylation.[2,3] To ascertain whether such autophosphorylation could be induced by NGF(1−14) peptide, we performed Western blot (WB) analyses of protein extracts from PC12 cell cultures exposed for 5, 15, and 30 min to different concentrations (1, 10, and 100 μM) of NGF(1−14). Negative controls consisted in

DOI: 10.1021/acschemneuro.5b00069
ACS Chem. Neurosci. 2015, 6, 1379−1392

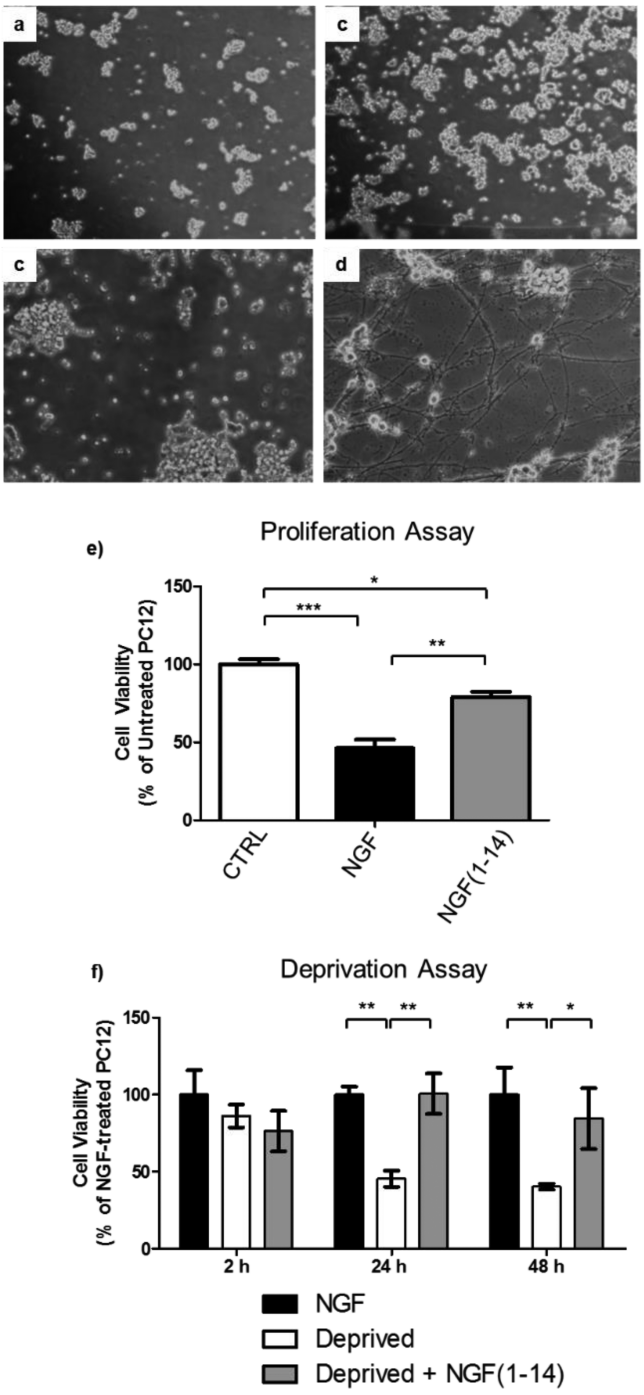


Figure 2. NGF-like mimetic activity of NGF(1−14) on PC12 cells. Representative cell morphology and confluence of untreated PC12 cells, at the time of plating (a) and 7 days after (b). Representative cell morphology and confluence of PC12 cells 7 days after the treatment with NGF(1−14) (10 μM) (c) or NGF (100 ng/mL, corresponding to 3.84 nM) (d). (e) Proliferation assay of PC12 cells treated with NGF (100 ng/mL) or NGF(1−14) 10 μM. Data are reported as percent of untreated control. One-way ANOVA followed by Bonferroni post hoc test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (f) Differentiated PC12, after deprivation from serum and also from NGF, treated with NGF(1−14) 10 μM or NGF (100 ng/mL, corresponding to 3.84 nM). In growth factor withdrawal conditions, differentiated PC12 cells are kept alive by treatment with NGF(1−14) (10 μM). Two-way ANOVA followed by Bonferroni post hoc test, *$p < 0.05$, **$p < 0.01$.

untreated PC12 cells, whereas NGF (100 ng/mL) was used as positive control.

Quantitative WB analysis indicated that NGF(1−14) is able to activate TrkA, inducing its autophosphorylation (Figure 3a,b). A dose-dependent response is evident 5 min after the treatment with NGF(1−14) 1 μM (165% of control, $p > 0.05$), 10 μM (185% of control), and 100 μM (215% of control) (Figure 3c). At the same time point, NGF is able to elicit its strongest effect (312% of control).

However, the pattern of activation induced by NGF and NGF(1−14) showed some interesting differences over time. NGF TrkA phosphorylation peaks at 5 min after the treatment and slowly declines. Conversely, 15 and 30 min after the treatment, the levels of phosphorylation induced by NGF(1−14) are higher or unchanged with respect to those at 5 min.

Furthermore, 15 and 30 min after the treatment with both NGF and NGF(1−14), at all the concentrations, a significant increase in the phospho-TrkA level, compared with control ($p < 0.001$) was observed (Figure 3d, e).

Molecular Models and Binding Hypothesis. The domain 5 of TrkA (TrkA-D5) encompasses several residues involved in the binding with the N-terminal section of NGF.[34,56] We therefore assessed at molecular level if the N-terminal portion of NGF can still preserve the typical NGF binding features.

In order to investigate the binding between the 14-residue long NGF-mimicking peptide and the domain 5 of TrkA, we initially run parallel tempering simulations (PT) on the NGF(1−14) peptide. Those simulations have the feature to enhance the sampling of flexible molecular structures, as those of peptide sections are.

The former simulation was validated through the calculations of the backbone ^{1}H chemical shift and compared with those experimentally recorded[57] (Supporting Information Tables S1 and S2).

The conformations of NGF(1−14) obtained during the course of PT simulations (Figure 4a) show a tendency to preserve a loop state in the central peptide section. The former loop can be further stabilized via backbone hydrogen bonds to form a short α helix involving ^{5}PIF^{7} and a turn states in the ^{8}HR^{9} section. These conformational motifs are present in the structure of NGF in complex with TrkA-D5, obtained by X-ray diffraction (PDB code 1WWW).[34]

Upon docking NGF(1−14) with TrkA-D5, specific differences in their binding poses have been observed (Figure 4, bottom). In particular, three main binding poses were detected for the peptide sections. The peptide adopts similar binding motifs to those found in the solved X-ray structure of the cocrystallized NGF/TrkA complex,[34] only when the short α helix involving ^{5}PIF^{7} is preserved.

In particular, the first binding pose indicates that S1 belonging to NGF(1−14) is close to H343 of TrkA-D5 and H4 of NGF(1−14) faces H291 belonging to TrkA-D5. Moreover, V14 of NGF(1−14) approaches H297 of TrkA-D5 (Figure 4, bottom). The second binding pose suggests a weak intramolecular interaction between the amine group of S1 belonging to NGF(1−14) and E295 belonging to TrkA-D5. H297 and H298 also face the N-terminal section of NGF(1−14). The C-terminal tail of NGF(1−14) faces through E11 and V14 the residues H291, H343 of TrkA-D5 (Figure 4, bottom). The third binding pose shows again a salt-bridge between the amine group of S1 belonging to NGF(1−14) and E295

DOI: 10.1021/acschemneuro.5b00069
ACS Chem. Neurosci. 2015, 6, 1379−1392

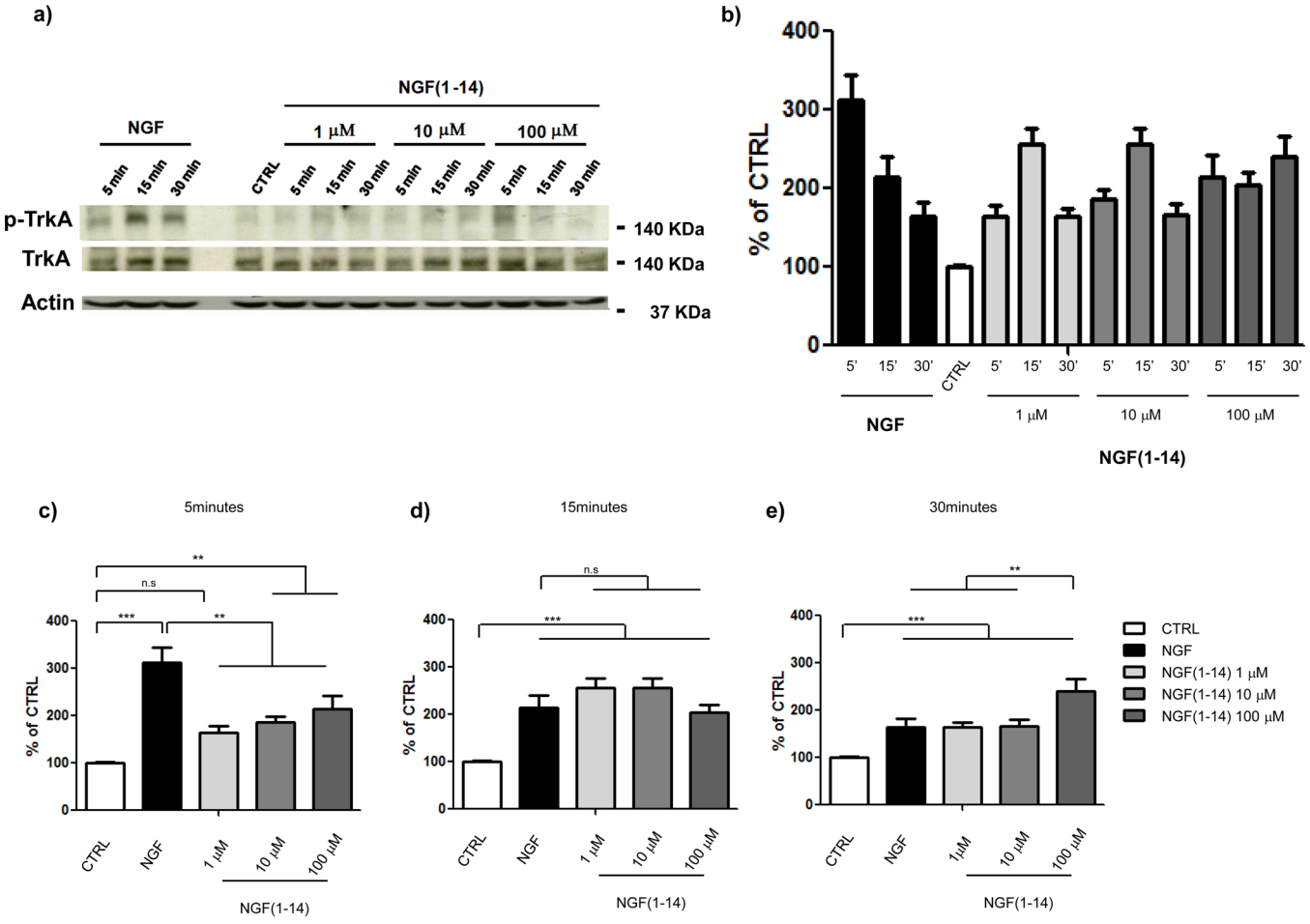


Fig. 3

Figure 3. NGF(1−14) triggers the phosphorylation of TrkA receptor. Representative Western blot (a) and densitometric quantitative analyses (b−e) of PC12 protein extracts from untreated (CTRL) and cell incubated with NGF (100 ng/mL, corresponding to 3.84 nM), NGF(1−14) (in the concentration range 1−100 μM), for 5, 15, or 30 min. All values are expressed as percentage of control and normalized on the basis of actin values. Data represent the mean ± SEM (bars) of at least four independent experiments. One-way ANOVA followed by Bonferroni post hoc test, *p = 0.05, **p < 0.01, ***p < 0.001.

belonging to TrkA-D5, but at variance with the second binding pose, V14 of NGF(1−14) faces H343 of TrkA-D5.

On the whole, only the first binding pose preserves the typical interaction between the 1−14 segment of NGF with domain 5 of TrkA. This behavior can be ascribed to the much more pronounced α helix conformation adopted in the ^{5}PIF^{7} region of the first NGF(1−14) cluster with respect to the other two clusters (Figure 4, top).

NGF(1−14) Activates the Signaling Cascade Downstream of TrkA Receptor. The NGF binding to the TrkA receptor can activate a series of downstream signaling cascades regulating neuronal survival, differentiation, and/or trophism. PI3−K/Akt and ERK1/2, are the two major NGF-mediated signaling pathways, involved in neuronal survival and differentiation, respectively.[59,60]

In order to determine which molecular pathways are coupled with TrkA activation, we examined whether NGF(1−14) is able to trigger the typical phosphorylation cascade induced by NGF.

Specifically, we performed WB analyses of protein extracts from PC12 cell cultures (treated as above). We tested the effect of NGF(1−14) on the phosphorylation and/or expression levels of different classes of proteins that are known to be crucial steps of NGF signaling including the phosphorylation of PI3-K, Akt, GSK-3, and ERK1/2 (Figure 5)

PI3-Ks, and their downstream target Akt, belong to a conserved family of signal transduction enzymes. The PI3-K/Akt axis phosphorylation is one of the earliest step of the pathway, with relevant insights in neuronal survival.[61] WB analyses revealed an induction of PI3-K phosphorylation versus control after 5, 15, and 30 min of treatment with NGF(1−14) (range +150% to +200%) (Figure 5a). However, it can be also observed that although the levels of PI3-K phosphorylation showed a time dependent decrease of phosphorylation after NGF treatment, the levels of p-PI3-K were maintained practically unchanged by NGF(1−14) along the time points. The time related differential effect between NGF and NGF(1−14) observed for TrkA (Figure 3) activation was therefore reproduced downstream also for PI3-K phosphorylation (Figure 5a).

Next in the downstream of phosphorylation cascade, Akt represents a central link in the network of signaling, and has been associated with pro-survival and neuroprotective effects of neurotrophins.[62−64] In line with TrkA activation and p-PI3-K upregulation, an increase in phosphorylation was observed also

DOI: 10.1021/acschemneuro.5b00069
ACS Chem. Neurosci. 2015, 6, 1379−1392

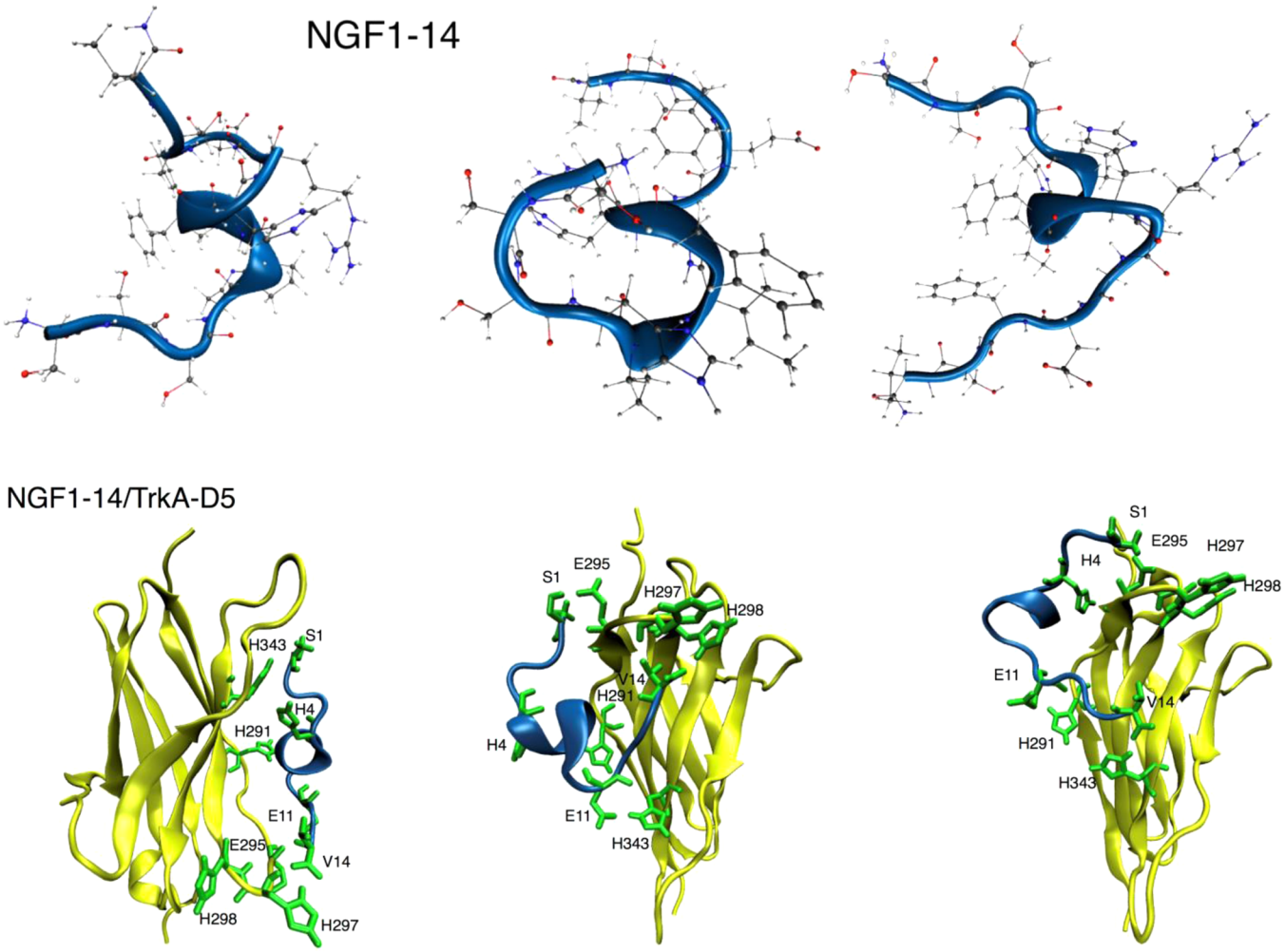


Figure 4. Main clusters of NGF(1−14) and lowest energy binding modes for the NGF(1−14)/TrkA. Three main clusters of NGF(1−14) (top). The central α helix conformation involving ^{5}PIF^{7} occurs among the three clusters differently. Carbon atoms are shown in gray, nitrogen in blue, and oxygen in red spheres. NGF is shown in blue and TrkA-D5 is shown in yellow. N- and C-termini are shown from up to bottom, respectively. (Bottom) The three lowest energy binding modes for the NGF(1−14)/TrkA. NGF(1−14) sections are shown by blue ribbons, TrkA-D5 sections are shown by yellow ribbons. NGF(1−14) and TrkA-D5 backbone are shown by sticks. N- and C-termini of NGF(1−14) are shown from up to bottom, respectively.

for Akt (Figure 5b). In the NGF and NGF(1−14) group, compared to the control, the treatment with both high and low doses of peptides significantly increased the phosphorylation of Akt.

GSK-3 is a critical regulator of diverse signaling pathways. It must be inhibited, through its phosphorylation, in order to let the pathway to function properly. GSK-3 participates to cell survival decisions in the brain regulating growth factor signaling through Akt.[65]

The neurotrophin-induced PI3-K/Akt activation phosphorylates GSK-3 at an N-terminal serine residue (Ser21 on the GSK-3α isoform, Ser9 on GSK-3β),[66] allowing activation of downstream effectors such as glycogen synthase and the mammalian target of rapamycin (mTOR)[67,68] or antiapoptotic stimuli.[69,70]

GSK-3β phosphorylation (Ser9) (Figure 5c) showed to be also positively affected by NGF(1−14) treatment, with levels of p-GSK-3β (Ser9 ranging from +100% to +250% vs control), similar to the effect induced by NGF. The values were stably maintained until 30 min of treatment.

ERK1/2 is a central knot in the canonical signaling cascade mediated by neurotrophins. Growth factor stimuli can induce different cell fates by activating ERK. For example, PC12 cells can proliferate after transient ERK activation by epidermal growth factor,[71] but they can terminally differentiate after more sustained ERK activation by NGF.[72,73] In our model, strikingly, ERK1/2 phosphorylation was absolutely not triggered by treatment of PC12 cell (Figure 5d) with NGF(1−14), as a further evidence of differential features of the NGF(1−14) in comparison with the NGF protein. In summary, the Western blot analysis of kinase phosphorylation cascade (Figure 5) shows that the NGF(1−14) effects are largely comparable with those induced by NGF, with the exception of ERK1/2, whose phosphorylation was indeed unaffected by NGF(1−14) treatment.

NGF(1−14) Triggers the Phosphorylation of CREB. The transcription factor cAMP response element-binding protein (CREB) has long been known to be a major transcriptional mediator of neuronal responses to neurotrophins[74] and has been shown to be a key regulator in developmental and adaptive responses that require stimulus-dependent transcription including neuronal plasticity, axonal regeneration,[75] memory,[76−78] as well as metabolism.[79]

DOI: 10.1021/acschemneuro.5b00069
ACS Chem. Neurosci. 2015, 6, 1379−1392

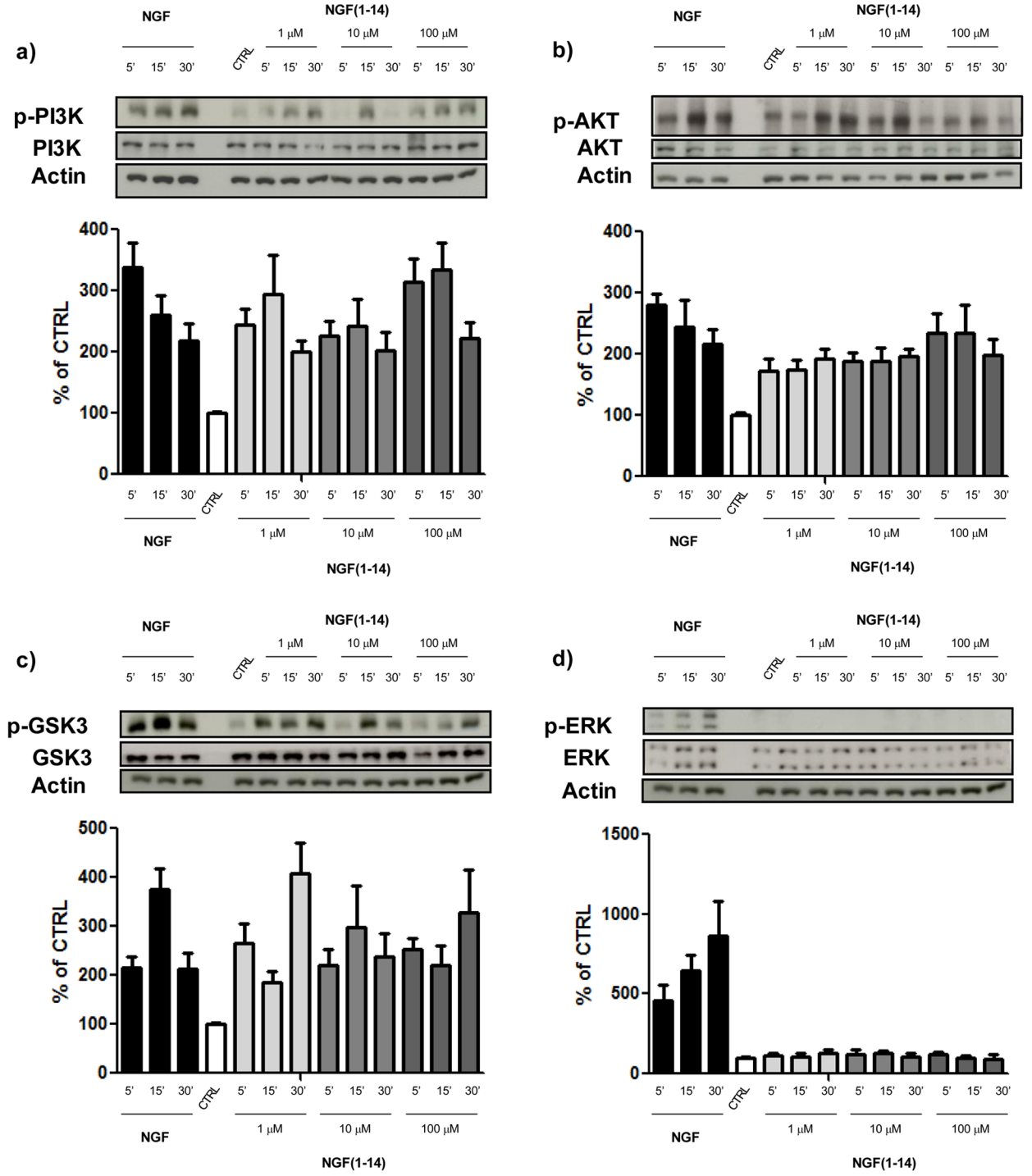


Figure 5. NGF(1−14) selectively activates the PI3K/AKT/GSK3 pathway, without inducing the phosphorylation of ERK1/2. Densitometric analyses of PC12 protein extracts from untreated (CTRL) and cell incubated with NGF (100 ng/mL, corresponding to 3.84 nM), NGF(1−14) (in the concentration range 1−100 μM), for 5, 15, or 30 min. All values are expressed as percentage of control and normalized on the basis of actin values. Data represent the mean ± SEM (bars) of at least four independent experiments.

Indeed, neurotrophins trigger the phosphorylation and activation of CREB (pCREB) at Ser-133. In turn, pCREB binds specific DNA sequences, thus enhancing gene expression that results in long lasting functional and structural changes.[77]

It was therefore particularly important to check the ability of the NGF(1−14) peptide to activate the full pathway to this crucial knot of neurotrophins signaling. Our results demonstrate that NGF(1−14) was indeed able to activate CREB through Ser133 phosphorylation in a dose- and time-dependent manner (Figure 6a, b).

Considering the different times of cell exposure to NGF(1−14) (Figure 6c−e), NGF(1−14) triggered a dose-dependent response in the phosphorylation of CREB. NGF(1−14) 1 μM does not increase the phosphorylation of CREB, compared to control, 5 min after the treatment (Figure 6c). Both NGF (152% of control, $p > 0.05$) and NGF(1−14) 10 μM are responsible for a nonsignificant trend toward induction,

DOI: 10.1021/acschemneuro.5b00069
ACS Chem. Neurosci. 2015, 6, 1379−1392

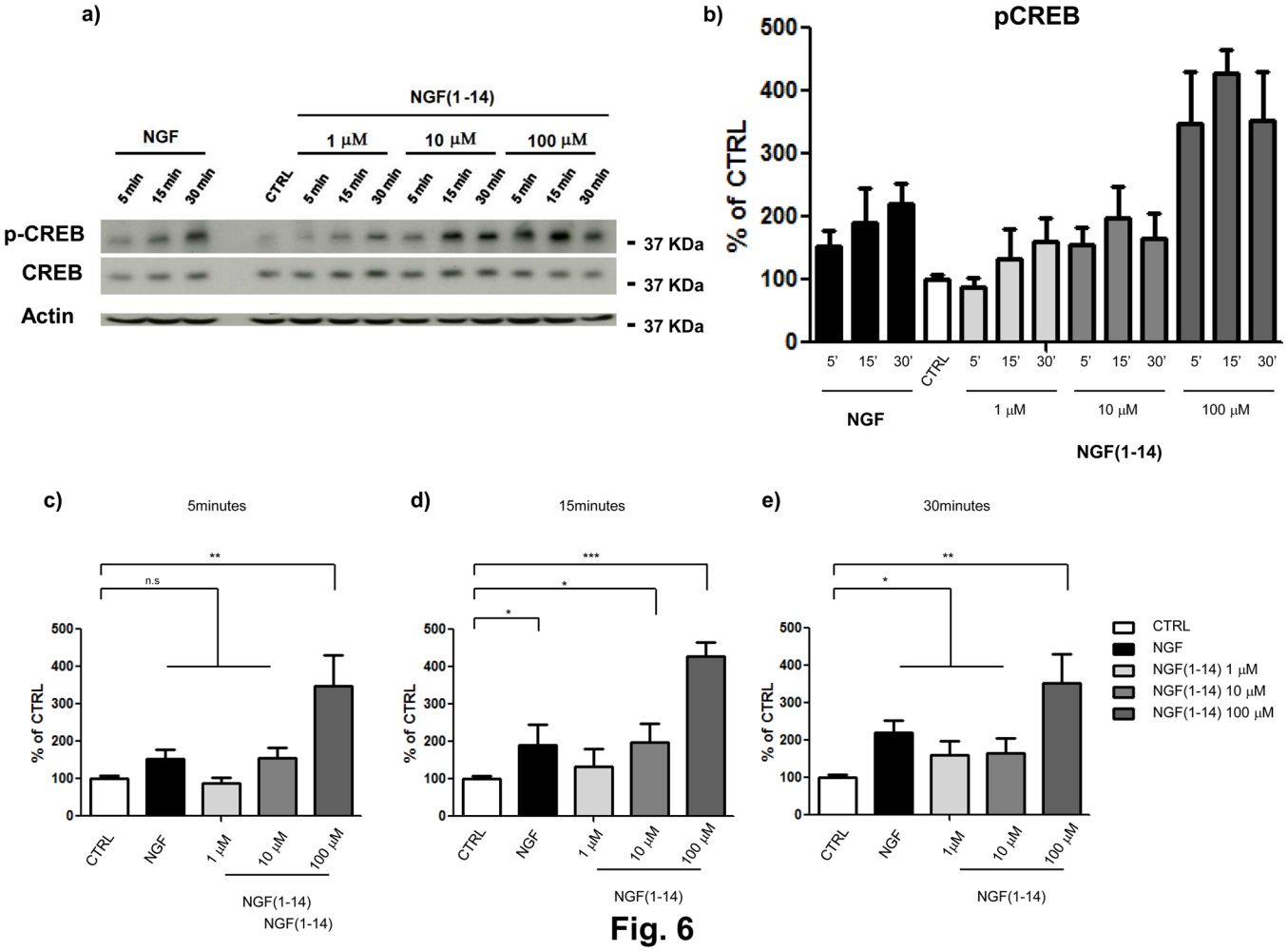


Fig. 6

Figure 6. NGF(1−14) triggers the phosphorylation of CREB. Representative Western blot (a) and densitometric quantitative analyses (b−e) of PC12 protein extracts from untreated (CTRL) and cell incubated with NGF (100 ng/mL, corresponding to 3.84 nM), NGF(1−14) (in the concentration range 1−100 μM), for 5, 15, or 30 min. All values are expressed as percentage of control and normalized on the basis of actin values. Data represent the mean ± SEM (bars) of at least four independent experiments. One-way ANOVA followed by Bonferroni post hoc test, *p = 0.05, **p < 0.01, ***p < 0.001.

whereas NGF(1−14) 100 μM significantly increases the phosphorylation of CREB already at this early time point (346% of control, p < 0.01). Then, 30 min after the treatment, NGF(1−14) triggered a significant increase in the phosphorylation of CREB (Figure 6c−e), in all the range of concentration explored (1−100 μM) (Figure 6e).

DISCUSSION

A promising strategy to the neurotrophin-based therapies involves the use of peptide mimics. These molecules can act either as agonist or as competitive antagonist and retain some neurotrophic activities, thus being potentially useful for the treatment of any disease involving their use as therapeutic agents, including Alzheimer's disease.[80−84] As for NGF, such peptides have been shown to exhibit neurotrophic effects by binding to TrkA.[42−44,47,58,85]

A NGF mimicking molecule would be expected to encompass structural domains of one or more NGF active sites that interact with NGF receptors. Many efforts have been devoted to design NGF peptide fragments to identify which regions of the protein interact with its receptors.[86]

Experimental and modeling studies[34,41,44,87−93] revealed that TrkA binding sites consist of three hydrophilic β-hairpin loop residues in NGF: loop 1 (residues 29−35), loop 2 (residues 40−49), loop 4 (residues 91−97). Furthermore, the N terminus (residues 1−8) and the C terminus (residues 111−115) have been found to be involved in the binding of the Trk/NGF complex.

We report on a small linear N-terminus peptide fragment of NGF that activates the TrkA neurotrophin receptor, induces its autophophorylation in PC12 cells, and largely activates the intracellular signal transduction pathway. These findings demonstrate not only that a small peptide can activate a tyrosine kinase neurotrophin receptor, normally involved in the binding to a relatively large protein ligand, but also that a linear peptide encompassing the first 14 amino acid of the NGF N-terminus domain demonstrates a NGF mimicking activity. This finding is in contrast with previous reports concerning linear peptides:

(i) The N-terminal octapeptide of NGF (SSTHPVFH) did not show trophic activity and did not inhibit in vitro biological effects of NGF (NGF-induced neurite outgrowth).[94]

(ii) Differently from monomeric cyclized peptides encompassing the amino acid sequence of loop 2 or loop 4, their

DOI: 10.1021/acschemneuro.5b00069
ACS Chem. Neurosci. 2015, 6, 1379−1392

linear analogues did not demonstrate antagonist activity and did not inhibit NGF-mediated PC12 cell neurite projections.[42]

(iii) Linear peptide mimics of NGF loop 4 not only did not promote cell survival in serum-free medium and induce TrkA tyrosine phosphorylation conversely showed by their cyclic monomeric analogues[56] but also did not activate ERK and Akt and promote NGF-like neurotrophic effects as reported for dimeric peptidomimetics.

(iv) Bicyclic peptides mimicking the NGF two loops (1 and 4) linked together with an appropriate spacer, without the N-terminal region, have been found to induce differentiation of DRGs and PC12 cells and display neurotrophic activities, at variance both with the analogous peptide containing also the N-terminus domain and its linear form.[45]

In an attempt to identify a short peptide as a mimic of the interaction occurring between the TrkA-D5 domain with the NGF N-terminus (see next section, on ligand binding site), we analyzed through molecular simulations and docking models NGF residues exhibiting significant contact surface with the receptor. Among those, the residues forming significant electrostatic interaction and/or binding-energy hot spots[95,96] were localized. The experimental results of the N-terminus encompassing the complete sequence of the first 14 residues of NGF indicate that also linear peptide can activate TrkA and promote neurotrophic effects.

Ligand Binding Sites. The Trk extracellular domain consists of five subdomains: the N-terminal leucine-rich motif (LRM), two cysteine-rich clusters, and two imunoglobulin (Ig)-like subdomains, IgC1 and IgC2 (also respectively known as D4 and D5).[82] The D5 subdomain is the main site for NGF/TrkA, BDNF/TrkB, or NT-3/TrkC binding and functionality.[97]

The D4 subdomain regulates the levels of receptor autophosphorylation, and its mutagenesis can result in oncogenic autoactivation.[38,97−99] A second activation site lies on the LRM subdomain and mediates NGF/TrkA functionality.[97] These regulatory or activation sites named functional "hot spots" of the receptors have been considered main targets for the development of ligands[81,100,101] that bind a defined activation or regulatory hot spot (e.g., only the D5 or only the LRM subdomains).

We reasoned that ligands binding to these hot spots could cause partial receptor activation or regulation, as opposed to full receptor activation. In order to assess the molecular recognition between the NGF N-terminal segment and the domain 5 of TrkA, we performed a combined parallel tempering/docking studies achieved through the structural data provided by the NGF/TrkA cocrystallization structure.[34] The former molecular simulations predicted that the short central α helix in the ^{5}PIF^{7} section is crucial in preserving the binding features observed in the cocrystallized structure of NGF/TrkA-D5. This observation is in line with what has been previously reported on the importance of TrkA in retaining the typical contacts within NGF to enhance the peptidomimetic activity.[48]

Moreover, different allosteric models were postulated in the years for the TrkA activation.[35,97,102] In particular, a dynamic model[97,102] and a rigid one[35] have been proposed. In the dynamic model, Zaccaro et al. indicated a subtle interaction between TrkA and NGF that is regulated through the p75 receptor.[97] The former interaction induces specific conformational changes leading to an optimal docking of the NGF and TrkA proteins. The conformational changes were postulated to occur primarily on the NGF binding to IgG-C2 subdomain, while the surface-exposed IgG-C1 subdomain can induce larger conformational changes on the NGF/TrkA complex architecture. Moreover, TrkA was suggested to include allosteric binding sites regulated through interdomain rotations.

The rigid model instead is deemed since Wehrman et al. observed that NGF only contacts TrkA at the D5 domain.[35] As for the allosteric domains, the authors reported that a high energetic cost is required to disrupt the interdomain interactions leading to the large conformational changes.[35]

The strength of the interdomain interactions has been assessed in the years through molecular simulations. In particular, MD simulations of human NGF/TrkA-D5 complex indicated that H-bond interactions at the protein−protein interface have a crucial role for the NGF/TrkA-D5 binding.[103] Furthermore, the specificity of NGF N-terminal segment was also observed during MD simulations on the NGF/TrkA-D5 complex with and without the N-terminal portion of NGF.[56] The complete complex is stable during the dynamics, but when the N-terminal domain of NGF is missing, the two moieties detach. A strong plasticity of the NGF(1−16) and NT-4(1−16) N-terminal peptides was also recently addressed through molecular simulations.[104] All these observations strengthen the subtle role of the exposed residues of TrkA for tailoring the typical TrkA/NGF interactions.

Mechanism of Action of NGF(1−14). The native NGF is a symmetrical dimer known to activate TrkA via its homodimerization.[2,3] Conversely, NGF(1−14) is not a dimer and it has no detectable propensity to dimerize even at high millimolar concentrations in solution. However, NGF(1−14) triggers a slower, but longer lasting, activation and phosphorylation of TrkA in comparison with NGF.

A crucial question is how NGF(1−14) activates TrkA. Different hypotheses have been suggested to explain the ability of small peptides to activate TrkA receptor: (i) specific and peculiar conformational changes that favor direct dimerization; (ii) increased receptor mobility with a consequent increase in spontaneous dimerization of receptors.

The extent of TrkA mobility in the cell membrane is strictly ligand-dependent. It has been reported that each ligand promotes a distinct pattern of membrane-associated TrkA receptor lateral trajectories, inducing distinct membrane mobility patterns.[105] The fate-determination of neurotrophin signaling, and specific biological effects, is strongly affected by ligand binding and receptor activation. A differential combination of receptor-binding affinities, which subsequently affects the docking of intracellular cofactors, can basically produce discrete signaling routes. The mechanisms that regulate the ligand binding to Trk receptors still require deeper understanding. In this context, the experimental data here presented do not allow to distinguish among the different hypotheses, although the NGF-like activation pathway appears well established. NGF(1−14) is able to induce the PI3-k-Akt-GSK-3β kinase cascade and CREB phosphorylation while it is not able to trigger ERK1/2 phosphorylation.

Together, these data raise the question whether canonical full-blown pattern of receptor activation is actually triggered by the NGF(1−14), with particular attention to the signaling which discriminate differentiating from survival effects.[13,106]

Studies now in progress clearly show that this peptide can exert a neurite growth promoting activity (similar to that elicited by the full NGF molecule) in chick embryo sensory ganglia, the prototypical target of NGF.[8,10] Further studies on this experimental paradigm, as compared to those reported in

DOI: 10.1021/acschemneuro.5b00069
ACS Chem. Neurosci. 2015, 6, 1379−1392

this paper, should provide a specific answer to the crucial question dealing with the possible distinct and/or alternative pathways operating in the trophic, neurite outgrowth and differentiative action exerted by NGF.

CREB is a transcription factor activated through its phosphorylation at Ser133 by multiple signaling cascades induced by different extracellular stimuli and different kinases.

It represents a key mediator of intracellular signaling events that regulate a wide range of biological functions in developmental and adaptive responses that require stimulus-dependent transcription, such as memory formation.[77,107,108]

The pro-survival effect of NGF treatment is known to be due to its signaling, cell internalization, and CREB phosphorylation, followed by CREB induced gene expression. This is a very critical step and target common in many neurodegenerative diseases. Strikingly, many genes downstream of CREB are required in both long-term memory formation and neuronal regeneration. Thus, the identification of specific events and factors involved in discrete mechanisms of signal transduction and transcription is a strong challenge to find new and more specific therapeutic applications.

In summary, NGF (1−14) is the first monomer and linear N-terminal NGF peptide sequence able to activate the NGF signaling cascade. It induces CREB phosphorylation and represents a good candidate for tests in NFG-like activities. The ability to activate CREB is extremely important and open new perspectives for further studies to evaluate its potentials as drug for neurodegenerative diseases, as well as related disorders of mood, memory strengthening and neuroregeneration.

METHODS

Peptide Synthesis. The peptide fragment NGF(1−14), encompassing the 1−14 sequence of the human NGF amino-terminal domain, SSSHPIFHRGEFSV-NH2, was synthesized with the solid-phase peptide synthesis strategy, by using a Pioneer Peptide Synthesizer. The peptide was synthesized with the C-termini amidated to mimic the peptide within the whole protein. All amino acid residues were added according to the TBTU/HOBT/DIEA activation method in Fmoc chemistry on Fmoc-PAL-PEG resin. Other experimental details have already been reported.[109]

Parallel Tempering Simulations. The initial NGF1−14 coordinates were taken from the solved X-ray structure between NGF and domain 5 of TrkA (TrkA-D5) (PDB code 1WWW).[34] Those underwent 20 ns of parallel tempering (PT) simulations in explicit solvent with a total volume of 40 × 40 × 40 Å^3. Both systems were first equilibrated through 2 ns of MD in explicit solvent. GROMACS 4.5.6 package was used.[110] The overall charge of the system was neutralized by adding 1 chloride ion for NGF(1−14). Periodic boundary conditions were applied. The AMBER99SB force field was used for the biomolecules and counterions,[111] and the TIP3P force field was used for water molecules.[112] Electrostatic interactions were calculated using the particle mesh Ewald method.[113] A cutoff (0.9 nm) was used for the Lennard−Jones interactions. The time step was set to 2 fs. All bond lengths were constrained to their equilibrium values using the SHAKE algorithm for water[114] and the LINCS algorithm for the peptide.[115] We simulated 64 replicas distributed in the temperature range 300−400 K following a geometric progression. All the replicas were simulated in the NVT ensemble using a stochastic thermostat with a coupling time of 0.1 ps.[116] A thermostat that yields the correct energy fluctuations of the canonical ensemble is crucial in parallel tempering simulations.[117] Exchanges were attempted every 0.1 ps. The method of Daura and Van Gunsteren was used in postprocessing phase to cluster the resulting trajectories,[118] with a cutoff of 3 Å calculated on the backbone atoms as implemented in the clustering utility provided in the GROMACS package.[110] The conformational ensemble was validated through the calculation of the 1H backbone chemical shift using the SHIFTX2 software[119]

Docking Simulations. The starting coordinates of TrkA-D5 were considered from the X-ray structure of NGF/TrkA-D5 complex (PDB code 1WWW).[34] Docking simulations have been performed using HADDOCK interface.[120] All residues of NGF(1−14) were included as active residues for the Haddock docking. For TrkA-D5, we select the binding surface of the solved X-ray structure (PDB code 1WWW).[34] The former includes the protein segment H291 to I301. All the structures underwent rigid body energy minimization, semirigid simulated annealing in torsion angle space, with a final clusterization of the results.

Cell Lines and Cell Cultures. Rat pheochromocytoma PC12 cells were cultured in RPMI-1640 (GIBCO), supplemented with 10% horse serum (GIBCO), 5% fetal bovine serum (FBS; GIBCO). All cell lines were obtained from ATCC. Culture media were also supplemented with 2 mM L-glutamine and 50 IU/mL penicillin, and 50 μg/mL streptomycin. Cells were plated at 1 × 10^6 cells on 3.5 cm dishes precoated with collagen.

For deprivation experiments, PC12 cells were first cultured for 6−7 days in RPMI medium plus 10% HS and 5% FBS, in the presence of NGF (100 ng/mL, corresponding to 3.84 nM) to induce their differentiation. Then, differentiated PC12 cells were cultured for another 6−7 days in RPMI, without serum, but in the presence of NGF (100 ng/mL). To ensure maximum bioavailability, NGF was replaced every 2 days. Finally, the cultures were deprived also from the NGF, and treated with NGF(1−14). The percentage of viable cells was measured, in independent experiments, 2, 24, and 48 h after the serum and NGF deprivation. Viable PC12 cells were quantified by counting the number of intact nuclei as previously reported.[121]

Western Blot Analysis. PC12 were stimulated for 5−30 min with 1, 10, and 100 μM NGF(1−14) or 10 nM NGF as positive control. After stimulations PC12, cells were washed with ice cold PBS and lysed with cold RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 2 mM EDTA; 1 mM NaF; 1 mM sodium orthovanadate, 1% NP-40) in the presence of phosphatase and protease inhibitor cocktails (Sigma-Aldrich), incubated for 45 min at 4 °C, and then centrifuged at 12 000 rpm for 15 min at 4 °C. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories). Equal amounts of total protein (20−60 μg per lane) were separated via denaturing SDS-PAGE and transferred to PVDF membranes (Millipore) by electroblotting. Blotted membranes were dried, reactivated in methanol for 5 min, and then washed with three changes of water. The membrane was then blocked in 5% non fat dry milk in TBS for 1 h at room temperature, then incubated with the appropriate primary antibody overnight at 4 °C.

Antibodies to P-TrkA (Y490) (1:1000, Cat #9141), P-PI3k (1:100, Cat #4228), PI3K kinase (1:1000, Cat #4257), P-AKT (1:1000, Cat #4051), AKT (pan) (1:1000, Cat # 4685), P-GSK3β (1:2000, Cat # 9336), GSK3β (1:2000, Cat # 9315), P-ERK1/2 (1:2000, Cat # 9106), ERK1/2 (1:2000, Cat # 9107, P-CREB (Ser133) (1:1000, Cat # 9191), and CREB ((1:1000, Cat # 9197) were purchased from Cell Signaling Technology; antibodies to TRK (B-3, sc-7268) and actin−horseradish peroxidase (HRP) (1:4000) were from Santa Cruz Biotechnology. The membranes were washed and treated with secondary HRP-labeled donkey antibody to rabbit (1:4000) or sheep antibody to mouse (1:4000) for 1 h.

After staining with HRP-conjugated antibody, the reactions were visualized via the ECL detection system. Quantitative densitometric analysis was performed using ImageJ (U.S. National Institutes of Health). Actin was used as loading control for all markers.

Statistical Analysis. Values were expressed as mean ± SEM. Statistical analysis was performed with one- or two-way ANOVA, followed by Bonferroni post hoc tests. Statistical significance was accepted at the 95% confidence level ($P < 0.05$).

DOI: 10.1021/acschemneuro.5b00069
ACS Chem. Neurosci. 2015, 6, 1379−1392

■ ASSOCIATED CONTENT

Supporting Information

Predicted and experimental ^{1}HN backbone chemical shifts (ppm) of the peptide NGF(1-14) calculated from the parallel tempering simulations; predicted and experimental $\alpha C^{1}H$ backbone chemical shifts (ppm) of the peptide NGF(1-14) calculated from the parallel tempering simulations; representative cell morphology of differentiated PC12 in a deprivation experiment. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00069.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: p.calissano@ebri.it.

*E-mail: erizzarelli@unict.it.

Author Contributions

Alessio Travaglia, Nicoletti Giuseppe Vincenzo, Pietro Calissano and Enrico Rizzarelli designed and developed the study. Alessio Travaglia and Rossana Di Martino carried out the biochemical studies and analyses. Adriana Pietropaolo carried out the computational analyses. Diego La Mendola and Alessio Travaglia synthesize and purified the peptide. Alessio Travaglia, Nicoletti Giuseppe Vincenzo, Pietro Calissano and Enrico Rizzarelli wrote the manuscript.

Funding

MIUR is also acknowledged for financial support under the Project PRIN2010 M2JARJ and FIRB MERIT (RBNE08HWLZ_001).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Cineca is acknowledged for computational time provided under the Caspur Project std12-149.

■ REFERENCES

(1) Huang, E. J., and Reichardt, L. F. (2001) Neurotrophins: roles in neuronal development and function. *Annu. Rev. Neurosci. 24*, 677−736.

(2) Chao, M. V. (2003) Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat. Rev. Neurosci 4*, 299−309.

(3) Reichardt, L. F. (2006) Neurotrophin-regulated signalling pathways. *Philos. Trans. R Soc. London, Ser. B. 361*, 1545−1564.

(4) Caporali, A., and Emanueli, C. (2009) Cardiovascular actions of neurotrophins. *Physiol Rev. 89*, 279−308.

(5) Sofroniew, M. V., Howe, C. L., and Mobley, W. C. (2001) Nerve growth factor signaling, neuroprotection, and neural repair. *Annu. Rev. Neurosci. 24*, 1217−1281.

(6) Huang, E. J., and Reichardt, L. F. (2003) Trk receptors: roles in neuronal signal transduction. *Annu. Rev. Biochem. 72*, 609−642.

(7) Dechant, G., and Barde, Y. A. (2002) The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. *Nat. Neurosci. 5*, 1131−1136.

(8) Levi-Montalcini, R., and Hamburger, V. (1951) Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *J. Exp. Zool. 116*, 321−361.

(9) Cohen, S., Levi-Montalcini, R., and Hamburger, V. (1954) A Nerve Growth-Stimulating Factor Isolated from Sarcom as 37 and 180. *Proc. Natl. Acad. Sci. U. S. A. 40*, 1014−1018.

(10) Levi-Montalcini, R., and Cohen, S. (1960) Effects of the extract of the mouse submaxillary salivary glands on the sympathetic system of mammals. *Ann. N.Y. Acad. Sci. 85*, 324−341.

(11) Sobue, G., Yamamoto, M., Doyu, M., Li, M., Yasuda, T., and Mitsuma, T. (1998) Expression of mRNAs for neurotrophins (NGF, BDNF, and NT-3) and their receptors (p75NGFR, trk, trkB, and trkC) in human peripheral neuropathies. *Neurochem. Res. 23*, 821−829.

(12) Allen, S. J., and Dawbarn, D. (2006) Clinical relevance of the neurotrophins and their receptors. *Clin Sci. 110*, 175−191.

(13) Chao, M. V., Rajagopal, R., and Lee, F. S. (2006) Neurotrophin signalling in health and disease. *Clin Sci. 110*, 167−173.

(14) Weissmiller, A. M., and Wu, C. (2012) Current advances in using neurotrophic factors to treat neurodegenerative disorders. *Transl. Neurodegener. 1*, 14.

(15) Longo, F. M., Yang, T., Knowles, J. K., Xie, Y., Moore, L. A., and Massa, S. M. (2007) Small molecule neurotrophin receptor ligands: novel strategies for targeting Alzheimer's disease mechanisms. *Curr. Alzheimer Res. 4*, 503−506.

(16) Wilson, R. M., and Danishefsky, S. J. (2006) Applications of total synthesis to problems in neurodegeneration: Fascinating chemistry along the way. *Acc. Chem. Res. 39*, 539−549.

(17) Fahnestock, M., Michalski, B., Xu, B., and Coughlin, M. D. (2001) The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease. *Mol. Cell. Neurosci. 18*, 210−220.

(18) Mufson, E. J., He, B., Nadeem, M., Perez, S. E., Counts, S. E., Leurgans, S., Fritz, J., Lah, J., Ginsberg, S. D., Wuu, J., and Scheff, S. W. (2012) Hippocampal proNGF signaling pathways and beta-amyloid levels in mild cognitive impairment and Alzheimer disease. *J. Neuropathol. Exp. Neurol. 71*, 1018−1029.

(19) Dyck, P. J., Peroutka, S., Rask, C., Burton, E., Baker, M. K., Lehman, K. A., Gillen, D. A., Hokanson, J. L., and O'Brien, P. C. (1997) Intradermal recombinant human nerve growth factor induces pressure allodynia and lowered heat-pain threshold in humans. *Neurology 48*, 501−505.

(20) Bergmann, I., Reiter, R., Toyka, K. V., and Koltzenburg, M. (1998) Nerve growth factor evokes hyperalgesia in mice lacking the low-affinity neurotrophin receptor p75. *Neurosci. Lett. 255*, 87−90.

(21) Aboulkassim, T., Tong, X. K., Tse, Y. C., Wong, T. P., Woo, S. B., Neet, K. E., Brahimi, F., Hamel, E., and Saragovi, H. U. (2011) Ligand-dependent TrkA activity in brain differentially affects spatial learning and long-term memory. *Mol. Pharmacol. 80*, 498−508.

(22) Bai, Y., Xu, J., Brahimi, F., Zhuo, Y., Sarunic, M. V., and Saragovi, H. U. (2010) An agonistic TrkB mAb causes sustained TrkB activation, delays RGC death, and protects the retinal structure in optic nerve axotomy and in glaucoma. *Invest. Ophthalmol. Visual Sci. 51*, 4722−4731.

(23) Guillemard, V., Ivanisevic, L., Garcia, A. G., Scholten, V., Lazo, O. M., Bronfman, F. C., and Saragovi, H. U. (2010) An agonistic mAb directed to the TrkC receptor juxtamembrane region defines a trophic hot spot and interactions with p75 coreceptors. *Dev. Neurobiol. 70*, 150−164.

(24) Ugolini, G., Marinelli, S., Covaceuszach, S., Cattaneo, A., and Pavone, F. (2007) The function neutralizing anti-TrkA antibody MNAC13 reduces inflammatory and neuropathic pain. *Proc. Natl. Acad. Sci. U. S. A. 104*, 2985−2990.

(25) Sahenk, Z., Galloway, G., Edwards, C., Malik, V., Kaspar, B. K., Eagle, A., Yetter, B., Forgie, A., Tsao, D., and Lin, J. C. (2010) TrkB and TrkC agonist antibodies improve function, electrophysiologic and pathologic features in Trembler J mice. *Exp. Neurol. 224*, 495−506.

(26) Blesch, A., Uy, H. S., Diergardt, N., and Tuszynski, M. H. (2000) Neurite outgrowth can be modulated in vitro using a tetracycline-repressible gene therapy vector expressing human nerve growth factor. *J. Neurosci. Res. 59*, 402−409.

(27) Taylor, L., Jones, L., Tuszynski, M. H., and Blesch, A. (2006) Neurotrophin-3 gradients established by lentiviral gene delivery promote short-distance axonal bridging beyond cellular grafts in the injured spinal cord. *J. Neurosci. 26*, 9713−9721.

(28) Nagahara, A. H., Merrill, D. A., Coppola, G., Tsukada, S., Schroeder, B. E., Shaked, G. M., Wang, L., Blesch, A., Kim, A., Conner, J. M., Rockenstein, E., Chao, M. V., Koo, E. H., Geschwind, D.,

DOI: 10.1021/acschemneuro.5b00069
ACS Chem. Neurosci. 2015, 6, 1379−1392

Masliah, E., Chiba, A. A., and Tuszynski, M. H. (2009) Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. *Nat. Med. 15*, 331−337.
(29) Chattopadhyay, M., Wolfe, D., Mata, M., Huang, S., Glorioso, J. C., and Fink, D. J. (2005) Long-term neuroprotection achieved with latency-associated promoter-driven herpes simplex virus gene transfer to the peripheral nervous system. *Mol. Ther. 12*, 307−313.
(30) Travaglia, A., Satriano, C., Giuffrida, M. L., La Mendola, D., Rampazzo, E., Prodi, L., and Rizzarelli, E. (2013) Electrostatically driven interaction of silica-supported lipid bilayer nanoplatforms and a nerve growth factor-mimicking peptide. *Soft Matter 9*, 4648−4654.
(31) Capsoni, S., Covaceuszach, S., Marinelli, S., Ceci, M., Bernardo, A., Minghetti, L., Ugolini, G., Pavone, F., and Cattaneo, A. (2011) Taking pain out of NGF: A "painless" NGF mutant, linked to hereditary sensory autonomic neuropathy type V, with full neurotrophic activity. *PLoS One 6*, e17321.
(32) Longo, F. M., and Massa, S. M. (2013) Small-molecule modulation of neurotrophin receptors: a strategy for the treatment of neurological disease. *Nat. Rev. Drug Discovery 12*, 507−525.
(33) Liskamp, R. M., Rijkers, D. T., Kruijtzer, J. A., and Kemmink, J. (2011) Peptides and proteins as a continuing exciting source of inspiration for peptidomimetics. *ChemBioChem 12*, 1626−1653.
(34) Wiesmann, C., Ultsch, M. H., Bass, S. H., and de Vos, A. M. (1999) Crystal structure of nerve growth factor in complex with the ligand-binding domain of the TrkA receptor. *Nature 401*, 184−188.
(35) Wehrman, T., He, X., Raab, B., Dukipatti, A., Blau, H., and Garcia, K. C. (2007) Structural and mechanistic insights into nerve growth factor interactions with the TrkA and p75 receptors. *Neuron 53*, 25−38.
(36) Banfield, M. J., Naylor, R. L., Robertson, A. G., Allen, S. J., Dawbarn, D., and Brady, R. L. (2001) Specificity in Trk receptor:neurotrophin interactions: the crystal structure of TrkB-d5 in complex with neurotrophin-4/5. *Structure 9*, 1191−1199.
(37) Ibanez, C. F. (1998) Emerging themes in structural biology of neurotrophic factors. *Trends Neurosci. 21*, 438−444.
(38) Arevalo, J. C., Conde, B., Hempstead, B. L., Chao, M. V., Martin-Zanca, D., and Perez, P. (2000) TrkA immunoglobulin-like ligand binding domains inhibit spontaneous activation of the receptor. *Mol. Cell. Biol. 20*, 5908−5916.
(39) Mischel, P. S., Umbach, J. A., Eskandari, S., Smith, S. G., Gundersen, C. B., and Zampighi, G. A. (2002) Nerve growth factor signals via preexisting TrkA receptor oligomers. *Biophys. J. 83*, 968−976.
(40) Shen, J., and Maruyama, I. N. (2011) Nerve growth factor receptor TrkA exists as a preformed, yet inactive, dimer in living cells. *FEBS Lett. 585*, 295−299.
(41) Longo, F. M., Vu, T. K., and Mobley, W. C. (1990) The in vitro biological effect of nerve growth factor is inhibited by synthetic peptides. *Cell Regul. 1*, 189−195.
(42) LeSauteur, L., Wei, L., Gibbs, B. F., and Saragovi, H. U. (1995) Small peptide mimics of nerve growth factor bind TrkA receptors and affect biological responses. *J. Biol. Chem. 270*, 6564−6569.
(43) Maliartchouk, S., Debeir, T., Beglova, N., Cuello, A. C., Gehring, K., and Saragovi, H. U. (2000) Genuine monovalent ligands of TrkA nerve growth factor receptors reveal a novel pharmacological mechanism of action. *J. Biol. Chem. 275*, 9946−9956.
(44) Xie, Y., Tisi, M. A., Yeo, T. T., and Longo, F. M. (2000) Nerve growth factor (NGF) loop 4 dimeric mimetics activate ERK and AKT and promote NGF-like neurotrophic effects. *J. Biol. Chem. 275*, 29868−29874.
(45) Colangelo, A. M., Bianco, M. R., Vitagliano, L., Cavaliere, C., Cirillo, G., De Gioia, L., Diana, D., Colombo, D., Redaelli, C., Zaccaro, L., Morelli, G., Papa, M., Sarmientos, P., Alberghina, L., and Martegani, E. (2008) A new nerve growth factor-mimetic peptide active on neuropathic pain in rats. *J. Neurosci. 28*, 2698−2709.
(46) Xu, J., Lacoske, M. H., and Theodorakis, E. A. (2014) Neurotrophic natural products: chemistry and biology. *Angew. Chem., Int. Ed. Engl. 53*, 956−987.
(47) Maliartchouk, S., Feng, Y., Ivanisevic, L., Debeir, T., Cuello, A. C., Burgess, K., and Saragovi, H. U. (2000) A designed peptidomimetic agonistic ligand of TrkA nerve growth factor receptors. *Mol. Pharmacol. 57*, 385−391.
(48) Scarpi, D., Cirelli, D., Matrone, C., Castronovo, G., Rosini, P., Occhiato, E. G., Romano, F., Bartali, L., Clemente, A. M., Bottegoni, G., Cavalli, A., De Chiara, G., Bonini, P., Calissano, P., Palamara, A. T., Garaci, E., Torcia, M. G., Guarna, A., and Cozzolino, F. (2012) Low molecular weight, non-peptidic agonists of TrkA receptor with NGF-mimetic activity. *Cell Death Dis. 3*, e389.
(49) Lin, B., Pirrung, M. C., Deng, L., Li, Z., Liu, Y., and Webster, N. J. (2007) Neuroprotection by small molecule activators of the nerve growth factor receptor. *J. Pharmacol. Exp. Ther. 322*, 59−69.
(50) Jang, S. W., Okada, M., Sayeed, I., Xiao, G., Stein, D., Jin, P., and Ye, K. (2007) Gambogic amide, a selective agonist for TrkA receptor that possesses robust neurotrophic activity, prevents neuronal cell death. *Proc. Natl. Acad. Sci. U. S. A. 104*, 16329−16334.
(51) Jang, S. W., Liu, X., Chan, C. B., Weinshenker, D., Hall, R. A., Xiao, G., and Ye, K. (2009) Amitriptyline is a TrkA and TrkB receptor agonist that promotes TrkA/TrkB heterodimerization and has potent neurotrophic activity. *Chem. Biol. 16*, 644−656.
(52) Shih, A., Laramee, G. R., Schmelzer, C. H., Burton, L. E., and Winslow, J. W. (1994) Mutagenesis identifies amino-terminal residues of nerve growth factor necessary for Trk receptor binding and biological activity. *J. Biol. Chem. 269*, 27679−27686.
(53) Kahle, P., Burton, L. E., Schmelzer, C. H., and Hertel, C. (1992) The amino terminus of nerve growth factor is involved in the interaction with the receptor tyrosine kinase p140trkA. *J. Biol. Chem. 267*, 22707−22710.
(54) Woo, S. B., Timm, D. E., and Neet, K. E. (1995) Alteration of NH2-terminal residues of nerve growth factor affects activity and Trk binding without affecting stability or conformation. *J. Biol. Chem. 270*, 6278−6285.
(55) Woo, S. B., and Neet, K. E. (1996) Characterization of histidine residues essential for receptor binding and activity of nerve growth factor. *J. Biol. Chem. 271*, 24433−24441.
(56) Berrera, M., Cattaneo, A., and Carloni, P. (2006) Molecular simulation of the binding of nerve growth factor peptide mimics to the receptor tyrosine kinase A. *Biophys. J. 91*, 2063−2071.
(57) Travaglia, A., Arena, G., Fattorusso, R., Isernia, C., La Mendola, D., Malgieri, G., Nicoletti, V. G., and Rizzarelli, E. (2011) The inorganic perspective of nerve growth factor: interactions of Cu^{2+} and Zn^{2+} with the N-terminus fragment of nerve growth factor encompassing the recognition domain of the TrkA receptor. *Chemistry 17*, 3726−3738.
(58) Beglova, N., Maliartchouk, S., Ekiel, I., Zaccaro, M. C., Saragovi, H. U., and Gehring, K. (2000) Design and solution structure of functional peptide mimetics of nerve growth factor. *J. Med. Chem. 43*, 3530−3540.
(59) Yao, R., and Cooper, G. M. (1995) Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science 267*, 2003−2006.
(60) Zhang, Y., Moheban, D. B., Conway, B. R., Bhattacharyya, A., and Segal, R. A. (2000) Cell surface Trk receptors mediate NGF-induced survival while internalized receptors regulate NGF-induced differentiation. *J. Neurosci. 20*, 5671−5678.
(61) Cantley, L. C. (2002) The phosphoinositide 3-kinase pathway. *Science 296*, 1655−1657.
(62) Crowder, R. J., and Freeman, R. S. (1998) Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. *J. Neurosci. 18*, 2933−2943.
(63) Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science 275*, 661−665.
(64) Bartlett, S. E., Reynolds, A. J., Weible, M., Heydon, K., and Hendry, I. A. (1997) In sympathetic but not sensory neurones, phosphoinositide-3 kinase is important for NGF-dependent survival

DOI: 10.1021/acschemneuro.5b00069
ACS Chem. Neurosci. 2015, 6, 1379−1392

and the retrograde transport of 125I-betaNGF. *Brain Res. 761*, 257−262.
(65) Kaidanovich-Beilin, O., and Woodgett, J. R. (2011) GSK-3: Functional Insights from Cell Biology and Animal Models. *Front. Mol. Neurosci. 4*, 40.
(66) Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature 378*, 785−789.
(67) Dajani, R., Fraser, E., Roe, S. M., Young, N., Good, V., Dale, T. C., and Pearl, L. H. (2001) Crystal structure of glycogen synthase kinase 3 beta: structural basis for phosphate-primed substrate specificity and autoinhibition. *Cell 105*, 721−732.
(68) Wang, X., and Proud, C. G. (2006) The mTOR pathway in the control of protein synthesis. *Physiology 21*, 362−369.
(69) Pap, M., and Cooper, G. M. (1998) Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. *J. Biol. Chem. 273*, 19929−19932.
(70) Hetman, M., Cavanaugh, J. E., Kimelman, D., and Xia, Z. (2000) Role of glycogen synthase kinase-3beta in neuronal apoptosis induced by trophic withdrawal. *J. Neurosci. 20*, 2567−2574.
(71) Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell 77*, 841−852.
(72) Qui, M. S., and Green, S. H. (1992) PC12 cell neuronal differentiation is associated with prolonged p21ras activity and consequent prolonged ERK activity. *Neuron 9*, 705−717.
(73) Santos, S. D., Verveer, P. J., and Bastiaens, P. I. (2007) Growth factor-induced MAPK network topology shapes Erk response determining PC-12 cell fate. *Nat. Cell Biol. 9*, 324−330.
(74) Finkbeiner, S., Tavazoie, S. F., Maloratsky, A., Jacobs, K. M., Harris, K. M., and Greenberg, M. E. (1997) CREB: a major mediator of neuronal neurotrophin responses. *Neuron 19*, 1031−1047.
(75) Teng, F. Y., and Tang, B. L. (2006) Axonal regeneration in adult CNS neurons—Signaling molecules and pathways. *J. Neurochem. 96*, 1501−1508.
(76) Kim, J., Kwon, J. T., Kim, H. S., and Han, J. H. (2013) CREB and neuronal selection for memory trace. *Front. Neural Circuits 7*, 44.
(77) Alberini, C. M. (2009) Transcription factors in long-term memory and synaptic plasticity. *Physiol. Rev. 89*, 121−145.
(78) Bisaz, R., Travaglia, A., and Alberini, C. M. (2014) The neurobiological bases of memory formation: from physiological conditions to psychopathology. *Psychopathology 47*, 347−356.
(79) Leone, V., D'Angelo, D., Ferraro, A., Pallante, P., Rubio, I., Santoro, M., Croce, C. M., and Fusco, A. (2011) A TSH-CREB1-microRNA loop is required for thyroid cell growth. *Mol. Endocrinol. 25*, 1819−1830.
(80) Saragovi, H. U., and Gehring, K. (2000) Development of pharmacological agents for targeting neurotrophins and their receptors. *Trends Pharmacol. Sci. 21*, 93−98.
(81) Saragovi, H. U., Greene, M. I., Chrusciel, R. A., and Kahn, M. (1992) Loops and secondary structure mimetics: development and applications in basic science and rational drug design. *Biotechnology 10*, 773−778.
(82) Saragovi, H. U., and Zaccaro, M. C. (2002) Small molecule peptidomimetic ligands of neurotrophin receptors, identifying binding sites, activation sites and regulatory sites. *Curr. Pharm. Des. 8*, 2201−2216.
(83) Pollack, S. J., and Harper, S. J. (2002) Small molecule Trk receptor agonists and other neurotrophic factor mimetics. *Curr. Drug Targets: CNS Neurol. Disord. 1*, 59−80.
(84) Massa, S. M., Xie, Y., and Longo, F. M. (2003) Alzheimer's therapeutics: neurotrophin domain small molecule mimetics. *J. Mol. Neurosci 20*, 323−326.
(85) Zaccaro, M. C., Lee, H. B., Pattarawarapan, M., Xia, Z., Caron, A., L'Heureux, P. J., Bengio, Y., Burgess, K., and Saragovi, H. U. (2005) Selective small molecule peptidomimetic ligands of TrkC and TrkA receptors afford discrete or complete neurotrophic activities. *Chem. Biol. 12*, 1015−1028.
(86) Bradshaw, R. A., Murray-Rust, J., Ibanez, C. F., McDonald, N. Q., Lapatto, R., and Blundell, T. L. (1994) Nerve growth factor: structure/function relationships. *Protein Sci. 3*, 1901−1913.
(87) McDonald, N. Q., and Chao, M. V. (1995) Structural determinants of neurotrophin action. *J. Biol. Chem. 270*, 19669−19672.
(88) McDonald, N. Q., Lapatto, R., Murray-Rust, J., Gunning, J., Wlodawer, A., and Blundell, T. L. (1991) New protein fold revealed by a 2.3-A resolution crystal structure of nerve growth factor. *Nature 354*, 411−414.
(89) Holland, D. R., Cousens, L. S., Meng, W., and Matthews, B. W. (1994) Nerve growth factor in different crystal forms displays structural flexibility and reveals zinc binding sites. *J. Mol. Biol. 239*, 385−400.
(90) Ibanez, C. F., Ebendal, T., Barbany, G., Murray-Rust, J., Blundell, T. L., and Persson, H. (1992) Disruption of the low affinity receptor-binding site in NGF allows neuronal survival and differentiation by binding to the trk gene product. *Cell 69*, 329−341.
(91) Kruttgen, A., Heymach, J. V., Jr., Kahle, P. J., and Shooter, E. M. (1997) The role of the nerve growth factor carboxyl terminus in receptor binding and conformational stability. *J. Biol. Chem. 272*, 29222−29228.
(92) Kullander, K., Kaplan, D., and Ebendal, T. (1997) Two restricted sites on the surface of the nerve growth factor molecule independently determine specific TrkA receptor binding and activation. *J. Biol. Chem. 272*, 9300−9307.
(93) Drinkwater, C. C., Barker, P. A., Suter, U., and Shooter, E. M. (1993) The carboxyl terminus of nerve growth factor is required for biological activity. *J. Biol. Chem. 268*, 23202−23207.
(94) Mobley, W. C., Schenker, A., and Shooter, E. M. (1976) Characterization and isolation of proteolytically modified nerve growth factor. *Biochemistry 15*, 5543−5552.
(95) Kortemme, T., and Baker, D. (2002) A simple physical model for binding energy hot spots in protein-protein complexes. *Proc. Natl. Acad. Sci. U. S. A. 99*, 14116−14121.
(96) Kortemme, T., Kim, D. E., and Baker, D. (2004) Computational alanine scanning of protein-protein interfaces. *Sci. STKE 2004*, pl2.
(97) Zaccaro, M. C., Ivanisevic, L., Perez, P., Meakin, S. O., and Saragovi, H. U. (2001) p75 Co-receptors regulate ligand-dependent and ligand-independent Trk receptor activation, in part by altering Trk docking subdomains. *J. Biol. Chem. 276*, 31023−31029.
(98) Arevalo, J. C., Conde, B., Hempstead, B. I., Chao, M. V., Martin-Zanca, D., and Perez, P. (2001) A novel mutation within the extracellular domain of TrkA causes constitutive receptor activation. *Oncogene 20*, 1229−1234.
(99) Coulier, F., Kumar, R., Ernst, M., Klein, R., Martin-Zanca, D., and Barbacid, M. (1990) Human trk oncogenes activated by point mutation, in-frame deletion, and duplication of the tyrosine kinase domain. *Mol. Cell. Biol. 10*, 4202−4210.
(100) Jones, S., and Thornton, J. M. (1996) Principles of protein-protein interactions. *Proc. Natl. Acad. Sci. U. S. A. 93*, 13−20.
(101) Bogan, A. A., and Thorn, K. S. (1998) Anatomy of hot spots in protein interfaces. *J. Mol. Biol. 280*, 1−9.
(102) Heldin, C. H. (1995) Dimerization of cell surface receptors in signal transduction. *Cell 80*, 213−223.
(103) Settanni, G., Cattaneo, A., and Carloni, P. (2003) Molecular dynamics simulations of the NGF-TrkA domain 5 complex and comparison with biological data. *Biophys. J. 84*, 2282−2292.
(104) Stanzione, F., Esposito, L., Paladino, A., Pedone, C., Morelli, G., and Vitagliano, L. (2010) Role of the conformational versatility of the neurotrophin N-terminal regions in their recognition by Trk receptors. *Biophys. J. 99*, 2273−2278.
(105) Marchetti, L., Callegari, A., Luin, S., Signore, G., Viegi, A., Beltram, F., and Cattaneo, A. (2013) Ligand signature in the membrane dynamics of single TrkA receptor molecules. *J. Cell Sci. 126*, 4445−4456.
(106) Matusica, D., and Coulson, E. J. (2014) Local versus long-range neurotrophin receptor signalling: endosomes are not just carriers for axonal transport. *Semin. Cell Dev. Biol. 31*, 57−63.

DOI: 10.1021/acschemneuro.5b00069
ACS Chem. Neurosci. 2015, 6, 1379−1392

(107) Xing, J., Kornhauser, J. M., Xia, Z., Thiele, E. A., and Greenberg, M. E. (1998) Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. *Mol. Cell. Biol. 18*, 1946−1955.
(108) Lonze, B. E., Riccio, A., Cohen, S., and Ginty, D. D. (2002) Apoptosis, axonal growth defects, and degeneration of peripheral neurons in mice lacking CREB. *Neuron 34*, 371−385.
(109) Forte, G., Travaglia, A., Magri, A., Satriano, C., and La Mendola, D. (2014) Adsorption of NGF and BDNF derived peptides on gold surfaces. *Phys. Chem. Chem. Phys. 16*, 1536−1544.
(110) Hess, B., Kutzner, C., van der Spoel, D., and Lindahl, E. (2008) GROMACS 4: Algorithms for highly efficient, load-balanced, and scalable molecular simulation. *J. Chem. Theory Comput. 4*, 435−447.
(111) Hornak, V., Abel, R., Okur, A., Strockbine, B., Roitberg, A., and Simmerling, C. (2006) Comparison of multiple amber force fields and development of improved protein backbone parameters. *Proteins 65*, 712−725.
(112) Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W., and Klein, M. L. (1983) Comparison of Simple Potential Functions for Simulating Liquid Water. *J. Chem. Phys. 79*, 926−935.
(113) Essmann, U., Perera, L., Berkowitz, M. L., Darden, T., Lee, H., and Pedersen, L. G. (1995) A Smooth Particle Mesh Ewald Method. *J. Chem. Phys. 103*, 8577−8593.
(114) Miyamoto, S., and Kollman, P. A. (1992) Settle - an Analytical Version of the Shake and Rattle Algorithm for Rigid Water Models. *J. Comput. Chem. 13*, 952−962.
(115) Hess, B. (2008) P-LINCS: A parallel linear constraint solver for molecular simulation. *J. Chem. Theory Comput. 4*, 116−122.
(116) Bussi, G., Donadio, D., and Parrinello, M. (2007) Canonical sampling through velocity rescaling. *J. Chem. Phys. 126*, 014101.
(117) Rosta, E., Buchete, N. V., and Hummer, G. (2009) Thermostat Artifacts in Replica Exchange Molecular Dynamics Simulations. *J. Chem. Theory Comput. 5*, 1393−1399.
(118) Daura, X., Gademann, K., Jaun, B., Seebach, D., van Gunsteren, W. F., and Mark, A. E. (1999) Peptide folding: When simulation meets experiment. *Angew. Chem., Int. Ed. 38*, 236−240.
(119) Han, B., Liu, Y., Ginzinger, S., and Wishart, D. (2011) SHIFTX2: Significantly improved protein chemical shift prediction. *J. Biomol. NMR 50*, 43−57.
(120) De Vries, S. J., van Dijk, M., and Bonvin, A. M. J. J. (2010) The HADDOCK web server for data-driven biomolecular docking. *Nat. Protoc. 5*, 883−897.
(121) Volonte, C., Ciotti, M. T., and Battistini, L. (1994) Development of a method for measuring cell number: application to CNS primary neuronal cultures. *Cytometry 17*, 274−276.

DOI: 10.1021/acschemneuro.5b00069
ACS Chem. Neurosci. 2015, 6, 1379−1392