An Intracellular Domain Fragment of the p75 Neurotrophin Receptor (p75NTR) Enhances Tropomyosin Receptor Kinase A (TrkA) Receptor Function

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Facilitation of nerve growth factor (NGF) signaling by the p75 neurotrophin receptor (p75NTR) is critical for neuronal survival and differentiation. However, the interaction between p75NTR and TrkA receptors for this activity is not understood. Here, we report that a specific 29-amino acid peptide derived from the intracellular domain fragment of p75NTR interacts with and potentiates binding of NGF to TrkA-expressing cells, leading to increased neurite outgrowth in sympathetic neurons as a result of enhanced Erk1/2 and Akt signaling. An endogenous intracellular domain fragment of p75NTR (p75ICD) containing these 29 amino acids is produced by regulated proteolysis of the full-length receptor. We demonstrate that generation of this fragment is a requirement for p75NTR to facilitate TrkA signaling in neurons and propose that the juxtamembrane region of p75ICD acts to cause a conformational change within the extracellular domain of TrkA. This finding provides new insight into the mechanism by which p75NTR and TrkA interact to enhance neurotrophic signaling.

Nerve growth factor (NGF) is crucial for neurite outgrowth, differentiation, and survival during development of both the central and peripheral nervous systems. NGF elicits its effects by binding two structurally unrelated receptors, the common p75 neurotrophin receptor (p75NTR) and the tropomyosin receptor kinase (Trk) family member TrkA (1). TrkA mediates the survival and neurite outgrowth-promoting effects of NGF during development (2–4), whereas p75NTR has been shown to promote apoptosis and neurite pruning following neurotrophin binding. Independently, p75NTR and TrkA have low-affinity binding rates for NGF (5, 6). However, p75NTR can increase the specificity and binding affinity of Trk receptors for their preferred neurotrophins, promoting neuronal survival during development (7–11).

Over the past two decades, a number of mechanistic models of the functional interactions between p75NTR and Trk receptors have been proposed (12), including (i) the formation of a classic 1:1 heterodimer complex with a 25-fold higher on-rate than that of the individual receptors (6, 13) and (ii) the ligand passing model, in which p75NTR first binds to NGF before releasing the ligand for TrkA to bind (12–14). However, no model is consistent with all the existing experimental data. The p75NTR- TrkA heterodimer model is not supported by structural data (15–17), and the ligand passing model, although consistent with the finding that a NGF mutant that cannot bind p75NTR has only low binding affinity (18), is inconsistent with the observation that the extracellular ligand-binding domain of p75NTR is not required to create high-affinity NGF binding sites (19, 20). Therefore, although a heteroreceptor complex may form, and ligand transfer from p75NTR to TrkA could occur, these mechanisms cannot be the sole basis for the formation of high-affinity binding sites.

p75NTR receptors lacking the ligand-binding domain are generated endogenously via proteolytic cleavage. An α-secretase removes the extracellular domain, leaving a membrane-bound carboxyterminal fragment (p75CTF), which is subsequently cleaved by γ-secretase, releasing the intracellular domain (p75ICD) into the cytoplasm (21, 22). It is now emerging that these p75NTR cleavage steps are important in mediating a number of the neurotrophin functions (23).

Trk-mediated signals have recently been reported to induce the generation of p75ICD, with this fragment being capable of promoting both TrkA- and TrkB-initiated trophic signaling (24–27). Furthermore, we have observed that a proximal-intracellular juxtamembrane 29-amino acid fragment of p75ICD, termed the “Chopper” domain, or a peptide comprising the Chopper fragment lacking a transmembrane linker (c29) inhibits neuronal death (28–30). Interestingly, removal of either the
whole p75NTR intracellular domain or just the Chopper domain results in diminished interactions with Trk receptors and a reduced number of neurotrophin high-affinity binding sites (8, 19, 20). In this study, we investigated the mechanism of action of the c29 peptide, investigating whether this juxtamembrane fragment of p75NTR has neurotrophic properties.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—The p75NTR, p75\(^{Δ\text{JUX}}\), and p75\(^{ICD}\) constructs have been described previously (29). YFP versions of p75NTR constructs were made as described in Ref. 27. The p75\(^{N-\text{Gly}}\) TrkA and TrkAK538R constructs have been described previously (31), as has Trk\(^{E203A}\) (32). The epidermal growth factor (EGF) receptor-GFP and control YFP constructs were generously provided by Rob Parton (The University of Queensland).

**Peptide Synthesis**—The 29-amino acid residue peptide of the juxtamembrane Chopper domain (29) (c29, KRWNSCK-QNKHGSNPRVQNTTPPGEK) and a randomly scrambled version (SC, SKGVQCNQPGQNKPEPANKSWKETPLRN) were synthesized as N-terminal fusions to a non-naturally occurring protein transduction domain peptide (YARAAARNARA) (33) using t-boc chemistry and then purified using reverse-phase HPLC by James I. Elliott (Yale University). No effects were seen in cells treated with the PTD alone or peptides without carrier. For pulldown experiments, the c29 peptide was labeled on the amino terminus with biotin via a six-carbon spacer. The biotinylated control peptide used for pulldowns mimics the p75NTR extracellular juxtamembrane domain LC1 (RGTTDNLIGGSC) and was manufactured by Auspep.

**SCG Assays**—For neurite outgrowth assays, mouse superior cervical ganglia (SCG) were dissected from c57BL6j and c57BL6j p75\(^{NTR(\text{exonIII})}\) knock-out (10) postnatal day 3–5 mouse pups, with approval from the institutional Animal Ethics Committee. SCG explants were cultured as described previously (34). Where indicated, explants were pre-incubated with 200 nM compound E (Calbiochem) and 1 μM c29 peptide or scrambled peptide before addition of 10 ng/ml NGF (Biosensis). Explants were fixed after 2 days in 4% paraformaldehyde containing PBS, stained with anti-β-III tubulin (Promega, G712A), and detected using an Odyssey Imaging System (LI-COR Biosciences). NIH ImageJ software was used for the quantification of Western blots.

**Cell Lysis and Immunoblotting**—Both PC12 and HEK293 cells were lysed for 20 min on ice (25). For immunoblotting, samples were separated by SDS-PAGE and transferred onto PVDF membranes and Western blotted using standard protocols. The following antibodies were used: rabbit anti-p75NTR (1:2000; Promega, catalog no. G323A, or 1:1000; Upstate, catalog no. 07-476), goat anti-p75NTR (1:1000; R&D Systems, catalog no. AF1157), rabbit anti-TrkA (1:500; Upstate, catalog no. 06-574), mouse anti-phospho-Erk1/2 (1:2000; Cell Signaling, catalog no. 9101S), rabbit anti-panErk (1:2000; Cell Signaling, catalog no. 9102), rabbit anti-phospho-Akt (1:2000; Cell Signaling; catalog no. 4060S), mouse anti-panAkt (1:2000; Cell Signaling; catalog no. 9272) and anti-β-III tubulin (Promega, catalog no. G712A). Immunoreactive bands were detected using Invitrogen anti-rabbit Alexa Fluor 680 (1:10,000) or anti-mouse Alexa Fluor 800 (1:50,000) secondary antibodies and imaged using an Odyssey Imaging System (LI-COR Biosciences). NIH ImageJ software was used for the quantification of Western blots.

**Cross-linking, Pulldown, and Immunoprecipitation Assays**—For peptide pulldown experiments, the biotinylated c29 or LC1 control peptides were incubated with Dynabeads MyOne streptavidin T1 (Invitrogen) overnight at 4 °C. Cell lysates were then added and incubated for 1 h at 4 °C, before being eluted by boiling in Laemmli sample buffer.

For p75NTR construct immunoprecipitations, transfected HEK293 cells were treated with NGF at 50 ng/ml for 10 min, harvested in ice-cold PBS, and lysed as described above. Lysates were precleared in 75 μl (1:2) Gammabind G-Sepharose beads (Amersham Biosciences) for 2 h at 4 °C and then incubated with 3–5 μg of mouse anti-GFP (Roche Applied Science; catalog no. 11814460001), rabbit anti-TrkA (Abcam, catalog no. ab8871), or rabbit anti-human p75NTR intracellular domain antibody (Promega, catalog no. G323A) for 2 h at 4 °C. Immunoprecipitation was performed by the addition of (1:2) Gammabind G-Sepharose beads incubated for 16 h at 4 °C. The immunoprecipitate was washed and eluted in 2X LDS sample buffer (Invitrogen).

For surface biotinylation and TrkA cross-linking experiments, PC12 cells were serum-starved prior to treatment with peptides and NGF for 1 h. Cellular proteins were then either biotinylated for 90 min with EZ-Link Sulfo-NHS-biotin...
Results

**SCG Neurons Exhibit an Enhanced Response to NGF in the Presence of c29**—We have previously demonstrated that the c29 cell-permeable peptide, encompassing 29 amino acids of the juxtamembrane intracellular domain of p75<sup>NTR</sup> fused to a synthetic protein transduction domain peptide (see “Experimental Procedures”), can inhibit p75<sup>NTR</sup>-mediated cell death (29). As p75<sup>NTR</sup> has a well characterized role in the survival and differentiation of sympathetic neurons, we tested whether c29 is neurotrophic by using SCG explants, which selectively express p75<sup>NTR</sup> and TrkA, and require NGF for neurite outgrowth and survival. SCG explants isolated from newborn mice and cultured in the presence of 10 ng/ml NGF and 1 μM c29 displayed significantly enhanced neurite outgrowth compared with explants exposed to NGF alone or NGF and scrambled peptide controls (Fig. 1, a and b). To determine the reason for this effect, we examined neurite outgrowth of dissociated SCG cultures. Time-lapse microscopy revealed that the speed of axonal growth and the number of axonal branches per dissociated SCG neuron were not significantly different between treatments (Fig. 1, c and d); however, the presence of c29 significantly reduced the time required for dissociated SCG neurons to initiate NGF-mediated neuritogenesis (Fig. 1e).

**PC12 Cells Exhibit an Enhanced Response to NGF in the Presence of c29**—To understand the biochemical events underlying this effect, we analyzed neurite outgrowth in PC12 cells, which differentiate into a neuron-like phenotype when treated with NGF, and are commonly used to model sympathetic neurons (40). PC12 cells treated with c29 and low concentrations of NGF (1 ng/ml or 10 ng/ml) extended neurites that were significantly longer than those observed in cultures treated with equivalent concentrations of NGF, either with or without scrambled peptide (Fig. 2, a and b). Indeed, the neurite length of cells treated with 1 ng/ml or 10 ng/ml NGF in the presence of c29 was comparable with that of cells exposed to 10-fold higher NGF concentrations alone (Fig. 2b); however, c29 had no effect on neurite outgrowth of PC12 cells in the presence of saturating concentrations of 100 ng/ml NGF (Fig. 2b). c29 also had no effect on PC12 cell neurite outgrowth in the absence of NGF (Fig. 2, a and b) or in the presence of EGF (Fig. 2e), which also stimulates neurite outgrowth via endogenous EGF receptors when applied at high concentrations. This suggested that c29 had no intrinsic trophic effect (41).

NGF-dependent differentiation and neurite outgrowth in PC12 cells depends on increased and sustained activation of Erk1/2 by TrkA (2, 42, 43) and serine/threonine-specific protein kinase Akt (44). We therefore analyzed phosphorylated Erk1/2 and Akt by immunoblotting lysates from cells treated for 24 h with NGF, either alone or in combination with c29 or
scrambled peptide. Addition of NGF to c29-treated cells produced a significant and dose-dependent increase in the levels of phosphorylated Erk1/2 (pErk1/2) (Fig. 2, d–f) and phosphorylated Akt (pAkt) (Fig. 2, d–f), indicating that c29 did not independently stimulate either Erk1/2 or Akt activity. These increases in Erk1/2 and Akt phosphorylation were observed between treatments (n = 18 neurons per condition). c29 increased the number of neurites sprouting from dissociated SCG neurons from the time of plating in NGF and c29 or scrambled peptide. No significant differences were observed between treatments (n = 18 neurons per condition). c29 enhanced the extent of neurite growth of dissociated SCG neurons during the first 6 h after plating in medium containing 10 ng/ml NGF and c29 or scrambled peptide. c29 treatment increased the level of neurite growth observed in PC12 cells treated with various concentrations of NGF and c29 or scrambled peptide. No significant differences were observed between experimental conditions (n = 18 neurons per condition). c29 treatment stimulated Erk1/2 and Akt signaling, indicating that c29 did not independently stimulate Erk1/2 or Akt activity. These increases in Erk1/2 and Akt phosphorylation were consistent with and accounted for the biological effects that we observed in the neurite outgrowth assays.

Endogenous p75ICD Enhances NGF-Stimulated Neurite Outgrowth-To determine whether c29 required endogenous p75ICD expression for its trophic actions, we treated SCG explants isolated from p75ICD−/− mice with c29 and NGF (Fig. 3a). NGF-treated p75ICD−/− deficient ganglia had less neurite outgrowth over 48 h than NGF-treated ganglia from wild-type mice (Fig. 3b). However, c29 treatment enhanced the extent of outgrowth in p75ICD−/− deficient explants, restoring it to the level of NGF-stimulated outgrowth of p75ICD−/− deficient ganglia (Fig. 3, a and c).

Similarly, when PC12 cells lacking p75ICD (p75KO) were treated with c29 and NGF, the cultures displayed enhanced neurite outgrowth (Fig. 3d). Addition of NGF to c29-treated p75KO PC12 cultures also resulted in an early and enhanced activation of downstream Erk1/2 signaling compared with that in cultures treated with NGF alone (Fig. 3e). These results demonstrated that endogenous p75ICD is not required for c29 to promote neurotrophic functions.

Endogenous p75ICD Enhances Trk Receptor Function-We next investigated whether the membrane-free p75ICD containing the c29 sequence (22, 26) had a similar neurite growth-enhancing activity in NGF-treated p75NTR-deficient ganglia (Fig. 3, a). NGF-treated p75NTR-deficient ganglia had less neurite outgrowth over 48 h than NGF-treated ganglia from wild-type mice (Fig. 3b). However, c29 treatment enhanced the extent of neurite growth of dissociated SCG neurons during the first 6 h after plating in medium containing 10 ng/ml NGF and c29 or scrambled peptide. c29 treatment increased the level of neurite growth observed in PC12 cells treated with various concentrations of NGF and c29 or scrambled peptide. No significant differences were observed between experimental conditions (n = 18 neurons per condition). c29 treatment stimulated Erk1/2 and Akt signaling, indicating that c29 did not independently stimulate Erk1/2 or Akt activity. These increases in Erk1/2 and Akt phosphorylation were consistent with and accounted for the biological effects that we observed in the neurite outgrowth assays.

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p75ICD Enhances Trk Receptor Function

Endogenous p75NTR is not required for c29 to enhance NGF-stimulated neurite outgrowth. a, representative photomicrographs of individual mouse p75NTR(exonIII) knock-out SCG explants cultured in 10 ng/ml NGF and 1 μM of c29 or scrambled control peptide and stained for βIII-tubulin (scale bar = 500 μm) show enhanced neurite outgrowth in the presence of c29 and NGF. b, quantification of axonal outgrowth of wild-type and p75NTR(exonIII) knock-out mouse SCG explants cultured in 10 ng/ml NGF for 48 h. c, quantification of axonal outgrowth from p75NTR(exonIII) knock-out mouse SCG explants cultured with NGF and c29 or scrambled control (SC) peptide (five explants quantified per condition; mean ± S.E.). d, quantification of neurite length of p75KO PC12 cells (n = 4 experiments; median ± S.E.; >50 cells per condition) 72 h after treatment with 10 ng/ml concentrations of NGF, c29, or scrambled peptide as indicated. e, representative Western blots of immunopositive bands of total (tERK) and phosphorylated forms of Erk1/2 and Akt in lysates of p75KO PC12 cells pretreated with or without c29 peptides and exposed to 10 ng/ml NGF for various times as indicated (n = 3 experiments; mean ± S.E.; *, p < 0.05; **, p < 0.01; ANOVA).

Because p75ICD has been reported to independently activate Erk1/2 signaling (45), we next determined whether the trophic effect of p75ICD expression required TrkA activity. Cells co-expressing p75ICD together with a dominant-negative TrkA construct containing a mutation in the kinase active site (TrkAK538R) had significantly shorter neurites than control YFP-expressing cells (Fig. 4, a–c) and displayed enhanced Erk1/2 phosphorylation (Fig. 4, d and e).

To test whether other forms of p75NTR also had this neurotrophic effect, we used a number of p75NTR variant constructs. PC12 cells overexpressing full-length p75NTR (p75FL) or a protein mimicking p75CTF were subject to increased rates of cell death within 48 h of transfection (data not shown), as has been reported previously (29, 31, 46). However, cells overexpressing a non-cleavable variant of p75NTR (p75NC8) that prevents formation of both p75CTF and p75ICP or a variant with a deletion in the juxtamembrane region corresponding to c29 (p75JUX) remained viable. Cells expressing these variants, in contrast to those expressing p75ICD, had significantly reduced neurite outgrowth and Erk1/2 signaling when cultured in the presence of NGF (Fig. 4, a and b). This suggested that the neurotrophic effect of p75NTR was limited to the p75ICD fragment containing the c29 sequence.

We next examined whether the generation of endogenous p75ICD fragments generated by intramembrane proteolysis of p75NTR was required for neurite outgrowth. NGF has previously been shown to induce cleavage of p75NTR to generate p75ICD (24, 25, 27). In agreement with these previous studies, treatment of PC12 cells with 1, 10, or 100 ng/ml NGF resulted in the dose-dependent generation of p75ICD (Fig. 5a). However, in the presence of inhibitors of p75NTR proteolysis (TAPI-2, a metalloprotease inhibitor, and compound E, a γ-secretase inhibitor), NGF-stimulated p75ICD generation was inhibited (Fig. 5b). These compounds also markedly reduced NGF-stimulated PC12 cell neurite outgrowth (Fig. 5c) and Erk1/2 activation (Fig. 5d). In contrast, neither the α- nor the γ-secretase inhibitor had any significant effect on EGF-induced neurite outgrowth (Fig. 5e) or signaling (Fig. 5f).

SCG explants treated with compound E in the presence of NGF also had clearly reduced neurite outgrowth compared with that observed in control cultures (Figs. 5, g and h). Importantly, the inhibitory effect of compound E on pErk1/2 production (Fig. 5f) and neurite growth of SCG explants was rescued by co-treatment with c29 (Fig. 5, g and h), mimicking the results obtained using p75NTR-deficient SCG explants (Fig. 3, a–c). These observations demonstrate that the γ-secretase cleavage of endogenous p75NTR specifically enhances TrkA-mediated neurite outgrowth, a function that can be mimicked by c29. Thus, these data are consistent with the idea that generation of p75ICD by p75NTR proteolysis is required for TrkA-mediated responses at low concentrations of NGF.

c29 Interacts with TrkA and Facilitates Increased NGF Binding—To understand the mechanism by which p75ICD and c29 facilitate TrkA signaling, we investigated whether a p75NTR-TrkA complex (47, 48) could be retained following p75ICD proteolysis. TrkA-p75NTR interactions were examined via co-immunoprecipitation following transfection of HEK293 cells. Full-length and truncated p75NTR constructs were transfected together with either kinase-active TrkA or kinase-inactive TrkAK538A. Both full-length p75NTR (Fig. 6a) and its C-terminal fragment (Fig. 6a) were able to co-immunoprecipitate TrkA and TrkAK538A, indicating that the interaction was not mediated via the extracellular domain of p75NTR. Consistent with this, neither TrkA activation nor dimerization by NGF significantly influenced the amount of TrkA that was co-immunoprecipitated with p75ICD (Fig. 6a). Importantly, p75ICD was also able to co-immunoprecipitate TrkA (Fig. 6a), whereas the p75A-JUX protein (lacking 33 amino acids of the intracellular juxtamembrane region) was not (Fig. 6a). Moreover, c29 but not a control peptide (LC1), was able to pull down overexpressed (Fig. 6b) as well as endogenously expressed TrkA (Fig. 6c). Neither peptide pulled down the EGF receptor (Fig. 6d). These results indicate that the 29-amino acid intracellular jux-
tamembrane sequence is sufficient and necessary for p75NTR to interact with TrkA.

Based on a report that the juxtamembrane domain of p75NTR is required for the generation of high-affinity NGF receptors (19), we next tested whether c29 was acting by affecting binding of NGF to TrkA. To investigate this, we used fluorescently labeled NGF (NGF-FITC) and flow cytometry analysis of ligand binding. The advantage of using flow cytometry, rather than the more traditional method based on radiolabeled NGF, is the ability to assess ligand binding in real time, and on a per cell as well as total population basis (49, 50).

c29-treated HEK293 cells transfected with TrkAK538R and PC12 cells bound significantly more NGF per cell than untreated or scrambled peptide-treated cultures (Fig. 7a and supplemental Table 1). To determine whether this increase in NGF binding in the presence of c29 was due to an increased rate of association of NGF for its receptors, we measured the real-time binding rate of NGF in HEK293 cells 24 h after treatment with NGF (n = 3 experiments). f, quantification of neurite length of wild-type PC12 cells transfected with p75ICD and either wild-type TrkA or dominant-negative kinase-dead TrkAK538R 5 days after NGF treatment (n > 50 cells per condition, from three experiments; median ± S.E.; *, p < 0.05; **, p < 0.001; ANOVA). g, representative Western blots of pErk1/2 in lysates of p75NTR-transfected p75ICD PC12 cells 24 h after treatment with NGF or EGF. Although both NGF and EGF increase pErk1/2, p75ICD potentiated this effect in the presence of NGF but not EGF, whereas full-length p75NTR had no effect on pErk1/2 levels in any condition.

cells expressing only p75NTR was unaffected by c29 treatment (Fig. 7c).

Cells expressing p75ICD together with TrkA also bound significantly more NGF-FITC (Fig. 7e). In contrast, cells expressing TrkA and either p75N-Gly (Fig. 7e) or p75/H9004-JUX (Fig. 7f and supplemental Table 1) did not have significantly enhanced NGF binding, with a trend toward reduced NGF binding capacity despite increased numbers of NGF-binding receptors expressed by these cells compared with those transfected with TrkA alone. These results are consistent with our finding that cells treated with c29 and p75ICD displayed enhanced responses to low concentrations of NGF in neurite outgrowth and signaling assays, whereas full-length p75NTR failed to mediate these effects.

c29 Alters Ligand Accessibility Not TrkA Receptor Levels—To determine whether c29 facilitates TrkA ligand binding and function by altering TrkA or p75NTR expression, we measured the cell surface expression of these receptors in PC12 cells. The presence of c29, scrambled peptide, or NGF had no effect on the levels of surface TrkA or p75NTR (Fig. 8a). Similarly, although NGF induced the formation of TrkA dimers, the number of

FIGURE 4. p75ICD but not full-length p75NTR promotes an enhanced response to NGF. a, photomicrographs of PC12 cells transfected with control YFP, full-length wild-type (p75FL), or mutant p75NL-YFP plasmids 5 days after transfection and treatment with 100 ng/ml NGF. Scale bars are 50 μm. Shown is quantification of neurite length of wild-type (b) and p75ICD (c) PC12 cells transfected with p75NTR variants 5 days after NGF treatment (n > 50 cells per condition from three experiments; median ± S.E.; **, p < 0.01; *** , p < 0.001; ANOVA, compared with YFP control). d, representative Western blots of pErk1/2 and total Erk1/2 (tErk) in lysates of p75NTR-transfected PC12 cells 24 h after treatment with NGF. e, quantification of Western blots for pErk1/2 relative to total Erk1/2 (tErk) in lysates of p75NTR-transfected PC12 cells 24 h after treatment with NGF (n = 3 experiments). f, quantification of neurite length of wild-type PC12 cells transfected with p75ICD and either wild-type TrkA or dominant-negative kinase-dead TrkAK538R 5 days after NGF treatment (n > 50 cells per condition, from three experiments; median ± S.E.; *, p < 0.05; **, p < 0.001; ANOVA). g, representative Western blots of pErk1/2 in lysates of p75NTR-transfected p75ICD PC12 cells 24 h after treatment with NGF or EGF. Although both NGF and EGF increase pErk1/2, p75ICD potentiated this effect in the presence of NGF but not EGF, whereas full-length p75NTR had no effect on pErk1/2 levels in any condition.
TrkA dimers (basal or NGF-induced) was unchanged in the presence of c29, despite the markedly increased level of TrkA phosphorylation compared with that of cells treated with NGF alone (Fig. 8b).

One mechanism by which receptor ligand-binding capacity can be increased without changing receptor numbers is through structural modulation of the ligand-binding site (32, 51). To investigate whether c29 might be causing a conformational change to facilitate the access of ligands to their binding site within the extracellular ligand-binding domain of TrkA.

Finally, we examined the ability of c29 to modulate NGF binding to a TrkA variant (TrkA<sup>P203A</sup>), which has a constitutively increased binding affinity for NGF due to a mutation within the extracellular flexible linker region (32). c29 had a negligible effect on the rate of NGF binding to HEK293 cells expressing TrkA<sup>P203A</sup> (Fig. 8d), suggesting that c29 mediates a conformational change within wild-type TrkA, an effect that is constrained in the TrkA<sup>P203A</sup> mutant.

### FIGURE 6. p75<sup>ICD</sup> and c29 interact with TrkA.

a, co-immunoprecipitation (IP) of kinase-active TrkA or kinase-inactive TrkA<sup>K538A</sup> following precipitation by anti-YFP antibodies in lysates from cells co-expressing full-length p75<sup>NTR</sup> (p75<sup>FL,YFP</sup>), p75<sup>ICD,YFP</sup>, p75<sup>CTF,YFP</sup>, or p75NTR lacking the juxtamembrane c29 domain (p75<sup>-JUX,YFP</sup>) proteins with or without NGF. Western blot (WB) of TrkA (b) or EGF receptor (EGFR-YFP) (d) from lysate of transfected HEK293 cells or PC12 cells (c) following pulldown with biotinylated c29 or scrambled peptide (representative figure for n = 3).

b, representative blots of phosphorylated and total Erk1/2 (tErk) in lysates of PC12 cells treated with cleavage inhibitors, c29, and either NGF (d) or EGF (f). g, representative photomicrographs of individual mouse SCG explants cultured in 10 ng/ml NGF, compound E, and 1 μM of c29 or scrambled peptide and stained for βIII-tubulin (scale bar, 500 μm). h, quantification of neurite outgrowth from SCG explants cultured with compound E and c29 (n = 6 explants per condition; mean ± S.E.; ***, p < 0.001; ANOVA). c29 treatment rescues the compound E-induced reduction in pErk1/2 and neurite outgrowth.
Here, we show that the juxtamembrane intracellular domain of p75NTR is required and sufficient to potentiate TrkA-mediated signaling and functional outcomes at nanomolar concentrations of neurotrophins. This domain mediates interactions between p75NTR and TrkA but does not associate with the EGF receptor. Moreover, it modulates the binding of NGF to TrkA, significantly increasing the amount of NGF (or an agonist ligand-mimicking TrkA antibody) bound to TrkA-expressing cells, with no change in the surface expression of either p75NTR or TrkA receptors. Together these results suggest that this small p75ICD juxtamembrane domain acts as a modulator of Trk receptor function.

**DISCUSSION**

Here, we show that the juxtamembrane intracellular domain of p75NTR is required and sufficient to potentiate TrkA-mediated signaling and functional outcomes at nanomolar concentrations of neurotrophins. This domain mediates interactions between p75NTR and TrkA but does not associate with the EGF receptor. Moreover, it modulates the binding of NGF to TrkA, significantly increasing the amount of NGF (or an agonist ligand-mimicking TrkA antibody) bound to TrkA-expressing cells, with no change in the surface expression of either p75NTR or TrkA receptors. Together these results suggest that this small p75ICD juxtamembrane domain acts as a modulator of Trk receptor function.

**c29 and p75ICD Potentiate Trk-mediated Trophic Signaling Pathways and Functions**—Our previous work (29) as well as studies assessing the role of p75NTR in high-affinity NGF binding (19, 20) have implicated the proximal-intracellular juxtamembrane 29-amino acid fragment of p75ICD, termed the Chopper domain, in potentiating neurotrophic actions. Here, we established that a peptide comprising the Chopper fragment lacking a transmembrane linker (c29) enables PC12 cells and SCG neurons to respond to a 10-fold lower neurotrophin concentration in terms of neurite outgrowth and activation of trophic Erk1/2 and Akt signal cascades. A consistent finding from these results was that the trophic actions of c29 did not occur in the absence of Trk activation and did not require expression of full-length p75NTR. This strongly suggests that the effect of the peptide does not occur solely via inhibition of p75NTR death signaling cascades (52).

The generation of p75ICD has been reported as necessary for many functions of p75NTR (23), including activation of trophic signaling pathways (25, 26) and cell death in the absence of trophic support (53). In this and our previous studies (29–31), the p75ICD fragment did not mediate death. Rather, we have reported that the p75CTF promotes neuronal death (29, 31, 54, 55) and here have observed that p75ICD enhanced TrkA-dependent neurite outgrowth and Erk1/2 signaling. Importantly, in these assays, other forms of p75NTR did not have the same trophic effects as p75ICD. Non-cleavable p75N-Gly and p75/JUX inhibited the neurotrophic effects of NGF, reducing rather than enhancing NGF binding to TrkA-expressing cells. Full-length p75NTR or p75CTF again promoted cell death in PC12 cells. Our findings suggest that the generation of a membrane-free intracellular domain fragment containing the c29 sequence is critical for endogenous p75NTR to mediate these trophic actions. Both cleavage inhibitors, which prevent the generation of p75ICD from endogenous p75NTR, and p75NTR gene deletion curtailed NGF-induced neurite outgrowth. These results, together with the rescue of neurite outgrowth by c29, illustrate that the production of this p75ICD fragment is required for the trophic response of the cells to NGF via TrkA.
p75ICD Enhances Trk Receptor Function

Our experiments demonstrate that the c29 sequence is necessary for and sufficient to increase the amount and association rate of NGF binding to TrkA-expressing cells, with no measurable effect on NGF binding to p75NTR. Whether this observation represents high-affinity NGF receptor activity has not been addressed. Nonetheless, our results are consistent with previous reports demonstrating that the intracellular domain of p75NTR, in particular the c29-encompassing region of the intracellular domain, is required to reconstitute NGF binding sites with a faster association rate than that of cells expressing TrkA alone (19, 20). The p75NTR transmembrane domain was also shown to be necessary for high-affinity NGF binding as a chimeric p75NTR protein containing the EGF receptor transmembrane and intracellular domains failed to generate high-affinity receptor sites (20). We recently reported that the p75NTR transmembrane sequence and structure have a significant effect on the rate of γ-secretase cleavage (27). Therefore, the p75NTR-EGF receptor chimeric protein may have failed to recapitulate high-affinity NGF binding sites because generation of the p75ICD fragment was hindered.

Our observation of enhanced NGF binding per cell following the application of c29 or overexpression of p75ICD is also consistent with the idea that the ratio of p75NTR to TrkA affects NGF binding affinity (19, 56) and that only a small fraction of surface Trk receptors participate in high-affinity binding (6). Indeed, our finding that c29 and expressed p75ICD could potentiate the neurotrophic activity in cells already expressing endogenous p75NTR indicates that the response of the cells was not yet maximal. A limiting step in the endogenous response to low neurotrophin levels may not be the ratio of Trk to p75NTR in itself but rather the amount of available p75ICD relative to TrkA. Jung et al. (21) concluded that p75ICD did not interact with TrkA as the majority of p75ICD generated following phorbol esters treatment of transfected cells was not found in the same size exclusion fractions as TrkA. However, the small proportion of p75ICD in the TrkA fraction observed in their experiments may be sufficient for enhanced function under physiological conditions with the rate or location of p75ICD generation being a limiting factor. An estimation of the amount of c29 within cells relative to the amount of p75ICD generated after phorbol esters treatment suggests that c29 would be present in cells at a much higher concentration than endogenous p75ICD (supplemental Fig. 2). Therefore, in our experiments, application of excess c29 or overexpression of p75ICD may have eliminated this barrier.

Inside-out Modulation of Trk by p75ICD?—Our results indicate that c29 and p75ICD can interact with TrkA but do not significantly affect surface receptor levels or dimerization of the receptors. Rather, our data are consistent with these fragments altering the ability of the extracellular domain of TrkA to interact with and bind its ligands. We speculate that this is by an inside-out structural modulation similar to that which occurs during activation of the integrin receptor (57).

Neurotrophins bind to the IgG-C2 domains of Trk receptors (16, 51). Structural and biochemical data indicate that ligand binding to TrkA at these sites can be allosterically modulated and facilitated by p75NTR through the additional surface exposure of the more N-terminally located IgG-C1/LRM domains (51). Although this may occur due to mutation of residues within these N-terminal domains, such as occurs in the TrkA<sup>AP203A</sup> mutant used herein (32), the extracellular domain of wild-type TrkA is considered to be rigid. Thus, the structural change required to enable this modulation in vivo is probably mediated via the TrkA intracellular and/or transmembrane domains (16). We therefore propose that c29 and p75ICD act to cause and/or stabilize such a change, thereby resulting in the formation of receptors with enhanced ligand-binding abilities.

An in vivo trigger for such an inside-out structural change could be the release of p75NTR from its transmembrane domain following γ-secretase generation of the p75ICD (24–26). The p75ICD juxtamembrane domain is considered to be structurally flexible (58), which means that it may form a different configuration when membrane-bound compared with its structure following γ-secretase cleavage. Structural change within the juxtamembrane region could, in turn, be permissive for altered association with the Trk receptor, thereby mediating allosteric modulation of the Trk receptor. Additional structural studies will be required to determine whether our proposed model is viable.

In summary, we have demonstrated that a specific fragment of p75NTR, p75ICD, can interact with TrkA and is required for its enhanced function. Furthermore, our results show that a c29 amino acid fragment of p75ICD is sufficient to increase the binding of NGF to TrkA-expressing cells, promote NGF trophic signaling, and enhance neurite outgrowth. Our work suggests...
that the interaction of this 29-amino acid region of p75ICD with TrkA triggers a conformational change within the extracellular domain of TrkA, resulting in increased receptor binding site availability and receptor activation. These results provide evidence for a new model of interaction between TrkA and the p75ICD fragment and identify the 29-amino acid functional moiety of this p75NTR fragment as being responsible for facilitating enhanced TrkA activation.

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