The transmembrane domain of the p75 neurotrophin receptor stimulates phosphorylation of the TrkB tyrosine kinase receptor

The function of protein products generated from intramembranous cleavage by the γ-secretase complex is not well defined. The γ-secretase complex is responsible for the cleavage of several transmembrane proteins, most notably the amyloid precursor protein that results in Aβ, a transmembrane (TM) peptide. Another protein that undergoes very similar γ-secretase cleavage is the p75 neurotrophin receptor. However, the fate of the cleaved p75 TM domain is unknown. p75 neurotrophin receptor is highly expressed during early neuronal development and regulates survival and process formation of neurons. Here, we report that the p75 TM can stimulate the phosphorylation of TrkB (tyrosine kinase receptor B). In vitro phosphorylation experiments indicated that a peptide representing p75 TM increases TrkB phosphorylation in a dose- and time-dependent manner. Moreover, mutagenesis analyses revealed that a valine residue at position 264 in the rat p75 neurotrophin receptor is necessary for the ability of p75 TM to induce TrkB phosphorylation. Because this residue is just before the γ-secretase cleavage site, we then investigated whether the p75(αγ) peptide, which is a product of both α- and γ-cleavage events, could also induce TrkB phosphorylation. Experiments using TM domains from other receptors, EGFR and FGFR1, failed to stimulate TrkB phosphorylation. Co-immunoprecipitation and biochemical fractionation data suggested that p75 TM stimulates TrkB phosphorylation at the cell membrane. Altogether, our results suggest that TrkB activation by p75(αγ) peptide may be enhanced in situations where the levels of the p75 receptor are increased, such as during brain injury, Alzheimer’s disease, and epilepsy.

Intramembranous cleavage is mediated by γ-secretase, a multisubunit complex consisting of presenilin 1 or 2, nicastrin, anterior pharynx defective 1, and presenilin enhancer 2 (1). The p75 neurotrophin receptor undergoes sequential proteolytic cleavage first by metalloproteinase 17 (ADAM17), which generates an ectodomain (ECD)² and a carboxyl-terminal fragment (CTF) (2) similar to amyloid precursor protein (APP) and Notch-1. p75CTF is subsequently cleaved by γ-secretase and produces an intracellular domain, p75ICD, and a transmembrane domain, p75(αγ), that is predominately hydrophobic (3).

p75 is a type 1 membrane protein that is expressed in many neuronal cell types including stem cells, astrocytes, oligodendrocyte precursors, Schwann cells, and olfactory unsheathing glia. p75 is expressed during early development in many neural crest derivatives, such as sympathetic, sensory, enteric neurons, and Schwann cells (4). Significantly, later in development and in the adult, p75 expression is robustly up-regulated following injury, inflammation, or axotomy (5). The p75 receptor shares many common features with the tumor necrosis family of receptors, including extracellular cysteine repeats, a single transmembrane domain, and a cytoplasmic domain with a death domain motif (6). Although controversial, p75 can exist as a monomer, as a dimer (7,8), or in a trimeric form similar to other tumor necrosis family members (9).

There are many effects of p75 signaling because this receptor can bind pro- and mature neurotrophins to regulate a wide range of cellular functions, including programmed cell death, axonal growth and degeneration, cell proliferation, myelination, and synaptic plasticity through interaction with other receptors (10). For example, p75 modulates neurotrophin responsiveness through interactions with TrkA, TrkB, and TrkC (11). Moreover, p75 can modulate axonal guidance via association with the Nogo receptor and Lingo-1, as well as Ephrin A (12). The interaction between p75 and Sortilin facilitates pro NGF-induced neuronal apoptosis (13).

Studies on different regions of p75 suggested that the ECD binds to and sequesters pro-neurotrophins and other neurodegenerative ligands, thereby preventing neuronal death (14). The p75ICD has been found to exert an effect upon nuclear events and induce apoptosis through the transcription factor NFκB (15). Other in vitro experiments suggested that p75ICD can

²The abbreviations used are: ECD, ectodomain; CTF, carboxyl-terminal fragment; APP, amyloid precursor protein; TM, transmembrane; RTK, receptor tyrosine kinase; co-IP, co-immunoprecipitation; EEA, early endosome antigen; PMA, phorbol 12-myristate 13-acetate; DAPT, N-(N-3,5-difluorophenylacetyl)-L-alanyl-S-phenylglycine t-butyler ester; TBS, Tris-buffered saline.
influence gene transcription events in the nucleus (16). Although the intramembranous cleavage of APP has been associated with the development of Alzheimer’s disease (17, 18), the functional consequences of the transmembrane domain of p75, a similar product of γ-secretase cleavage, is not well understood.

The insulin receptor, which belongs to the receptor tyrosine kinase (RTK) family, also undergoes cleavage by γ-secretase (19), producing an insulin receptor transmembrane peptide that can facilitate activation of the insulin receptor (20). This suggests the transmembrane domain of a receptor may possess functions after cleavage.

The p75 TM is highly conserved across species, much more so than the ligand-binding cysteine repeats or the cytoplasmic death domains (21). This strong conservation implies that the TM domain possesses a vital function. Previous studies indicated that the cytoplasmic and transmembrane regions of p75 were responsible for regulating the affinity of binding of NGF to TrkA and potentiating Trk-mediated survival signaling (22). In addition, p75 was co-precipitated with Trk receptors, particularly the TrkB receptor (11). These results implied that there are interactions between p75 and Trk receptors (11, 22). Another reason for considering the cleavage of p75 is that this receptor is frequently elevated during traumatic brain injury, seizures, and several neurodegenerative diseases, such as Alzheimer’s dementia. Therefore, we sought to investigate whether the p75 TM, as well as the p75(αγ) peptide generated by intramembrane cleavage, can have a functional consequence, such as influencing TrkB activity.

In this study, we focused on the TrkB receptor, which is widely expressed in the CNS. In contrast, TrkA is expressed predominantly in sensory and sympathetic neurons in the peripheral nervous system and restricted to basal forebrain neurons in the brain (23). The TrkB receptor has been of considerable interest because of its involvement in many neurological and psychiatric disorders (24, 25). Consequently, there has been a concerted effort to understand how the TrkB receptor is regulated. It is known that p75 and the TrkB receptor interact (11); however, the mechanism of this interaction is not completely clear. Here, we provide evidence for an unexpected mechanism whereby p75 TM may associate with TrkB and influence its activation.

**Results**

**The transmembrane domain of p75, but not EGFR or FGFR1, enhances TrkB phosphorylation**

The TrkB receptor, a member of the tyrosine kinase family of transmembrane receptors, plays a prominent role in the development and maintenance of the vertebrate nervous system. TrkB is predominately expressed in many CNS regions, including the cerebellum, cortex, hippocampus, and the striatum. TrkB receptor signaling through intracellular tyrosine kinase phosphorylation plays a critical role in survival, plasticity, and long-term potentiation (26, 27), as well as cognitive and psychiatric functions (28). TrkB receptor signal transduction is required for neuronal differentiation, axonal growth, and synaptic transmission (29, 30). All these actions of TrkB require autophosphorylation stimulated by neurotrophin binding. In addition to BDNF, TrkB can also be activated by NT-3, by NT-4, and through transactivation of G-protein–coupled receptor ligands such as adenosine (31, 32).

The p75 receptor can directly bind to both mature and pro-forms of the neurotrophins. Several reports have indicated that p75 interactions with Trk receptors (11) enhance binding affinity of Trk to ligands when both receptors are co-expressed (33, 34). However, the nature of p75/TrkB interactions is not clear. To address this question, we established an *in vitro* phosphorylation assay to follow the activity of TrkB using a phosphospecific antibody against Tyr816 of TrkB (35). Based upon previous findings that p75 receptors lacking the extracellular domain could influence TrkB binding (22), we designed an experiment to test whether the p75 TM domain (Table 1) has any effect upon TrkB activation.

We incubated 600 ng (0.2 μl) of TrkB in 20 μg of solubilized S9-TrkB lysate (supplemental Fig. S1) with a peptide containing the transmembrane sequence of p75 (p75 TM). We observed increasing TrkB phosphorylation in response to increasing concentrations (0, 1.25, 2.5, 5, and 10 μM) of p75 TM (Fig. 1, B and C). According to our stoichiometric calculation, 0.2 μM TrkB incubated with 1.25, 2.5, 5, and 10 μM p75 TM used in our study had TrkB:p75 TM ratios of 1:6.25, 1:12.5, 1:25, and 1:50, respectively.

To determine the time course of this activation, we assayed the effect of 5 μM p75 TM upon TrkB phosphorylation. The results indicated that the phosphorylation of TrkB increased dramatically within 5 min and persisted up to 60 min (Fig. 1, D and E). This time course is similar to the activation of TrkB by BDNF (36).

To test the specificity of p75 TM effects on TrkB phosphorylation, we tested the TM domain from other tyrosine kinase receptors, in particular the TM domains from FGFR1 and EGFR (Table 1). Phosphorylation assay data showed that neither EGFR TM nor FGFR1 TM could induce TrkB phosphorylation under similar conditions as incubation with p75 TM (Fig.

| Peptide Name | Amino Acid Sequence (5′→3′) | Position |
|--------------|----------------------------|----------|
| TrkB TM      | LS V Y A V V Y A V V G F L L L E L L | 430-453  |
| p75 TM       | L I P Y C S I L A A A V G L V A Y A F | 252-273  |
| p75C257A     | L I P Y C S I L A A A V G L V A Y A F | 252-273  |
| p75/GRA      | L I P Y C S I L A A A V G L V A Y A F | 252-273  |
| p75V263A     | L I P Y C S I L A A A V G L V A Y A F | 252-273  |
| p75V264A     | L I P Y C S I L A A A V G L V A Y A F | 252-273  |
| p75 TM-HA     | L I P Y C S I L A A A V G L V A Y A F | 252-273  |
| p75V264A-HA   | L I P Y C S I L A A A V G L V A Y A F | 252-273  |
| p75(αγ)      | S Q S Q V Y A V V I A V V E I A V V | 239-264  |
| TAT-HA       | G R K K R Q R R P Q Y D P D Y A | N/A      |
| EGFR TM      | I A T G M V G A L L L L V L V A G I L G | 646-668  |
| FGFR1 TM     | I I Y C T G A F L I S C M V G S V I Y Y | 377-397  |
Because there is minimal homology between the TM domains of EGFR and FGFR1 with p75 TM, these results suggest that there is some specificity in the effects of p75 TM on TrkB activity.

The transmembrane domain of p75 does not enhance TrkA, TrkC, or FGFR1 phosphorylation

Next, to examine whether TrkB is the only member of the RTKs that can be activated by the p75 TM, we extended our in vitro phosphorylation assay to elucidate whether 5 and 10 μM p75 TM alters TrkA (Tyr816), TrkC (Tyr820), or FGFR1 (Tyr653/Tyr654) phosphorylation under the same conditions utilized for TrkB. The data suggest that none of these receptors are activated by the p75 TM, supporting the specificity of p75 TM on TrkB phosphorylation (Fig. 2).

The phosphorylation of reconstituted TrkB in brain lipids (TrkB proteoliposome) is enhanced upon treatment with p75 TM

The in vitro phosphorylation experiment was conducted in the presence of detergents to solubilize the TrkB protein. To assess the effect of p75 in the absence of detergent, we established a proteoliposome system to provide a more natural environment for the phosphorylation assay. To prepare the proteoliposomes, TrkB was reconstituted in brain lipids by using Bio-Beads SM2 to remove the detergent (37). Subsequently, the effect of 5 μM p75 TM on TrkB phosphorylation over 30 min was assessed using the in vitro phosphorylation assay and Western blot. Our results demonstrate that the p75 TM peptide is capable of inducing TrkB phosphorylation in the absence of detergent (Fig. 3). This suggests that p75 TM is capable of activating TrkB in the environment of a lipid bilayer, which may occur in vivo when p75 undergoes cleavage.

The transmembrane domain of p75 interacts with TrkB and co-localizes with TrkB in lipid raft regions of the plasma membrane in vitro

Previous studies showed that the activation of TrkB signal transduction pathways were initiated in lipid rafts (38). Furthermore, the mechanism by which the transmembrane domain of the insulin receptor triggers the activation of the insulin receptor was proposed to interact with and facilitate a dimerization of the holo-receptors at the plasma membrane (20). Therefore, we sought to examine whether there are similar interactions between p75 TM and TrkB and whether this interaction occurs at the cell membrane and lipid raft regions.

To test whether p75 TM interacts with TrkB, we performed a co-immunoprecipitation assay using a p75 TM peptide linked to an C-terminus epitope and solubilized TrkB protein. Mouse anti-HA antibody was used to immobilize p75 TM-HA or TAT-HA. TrkB was detected by a Western blot using rabbit
The transmembrane domain of p75 interacts with TrkB and co-localizes with TrkB in lipid raft regions of the plasma membrane in vitro. A, the interaction between p75 TM peptide and TrkB was detected in Sf9-TrkB cell lysates by co-IP. Mouse anti-HA antibody was used to immobilize p75 TM-HA or TAT-HA. TrkB was detected by Western blot using rabbit anti-TrkB (H-181). Mouse IgG was used as an IP control, and it did not pull down p75 TM-HA or TAT-HA. TAT-HA was used as a control for HA tag. B, sucrose subcellular fractionation assay was used to detect the distribution of p75 TM and TrkB receptor. The membrane was then probed with antibodies against Flotillin-1 (lipid raft marker), Na⁺/K⁺-ATPase (cell membrane marker), EEA1 (early endosome marker), and β-actin (a cytosolic marker) (n = 3 independent experiments). 

The transmembrane domain of TrkB enhances the phosphorylation of the TrkB receptor

It has been reported that the activation of the insulin receptor can be enhanced by its own transmembrane domain (20). Because TrkB and the insulin receptor both belong to the RTK family, we investigated whether TrkB TM is capable of triggering TrkB phosphorylation using an in vitro phosphorylation assay. Incubation of 0.2 μM solubilized TrkB with 1.25, 2.5, 5, and 10 μM TrkB TM (Table 1), in same ratio utilized for p75 TM dose-dependent experiments, stimulated TrkB phosphorylation in a dose-dependent manner within 30 min (Fig. 5, A and E).
To address the time course of the response, 5 μM TrkB TM was then incubated with TrkB over a 4-h time course. The Western blot demonstrated that the phosphorylation of TrkB could be enhanced within 15 min and persisted up to 60 min (Fig. 5, C and D). This suggests that TrkB phosphorylation can be enhanced by treatment with its own transmembrane domain. The interaction between the transmembrane domain of TrkB and the TrkB receptor may help provide insight into how p75 TM increases TrkB phosphorylation. Because p75 TM and TrkB TM both have more than four common amino acid residues implicated in their dimerization, including a cysteine and three valines in self-associative motifs (Table 1, blue), we propose that the self-associative motifs may play a role in TrkB activation (9, 39).

Mutagenesis analyses reveal that valine 264 is critical for p75 TM-induced TrkB phosphorylation

To characterize which regions in p75 TM are responsible for inducing TrkB phosphorylation, we tested the effects of several mutations in the transmembrane domain on stimulating TrkB activation. Each peptide is designated with the amino acid position according to the rat sequence (Table 1). A cysteine residue at position 257 in the transmembrane domain of human p75 is known to stabilize receptor dimerization through a disulfide bond and increase p75 activity (8). The cysteine residue was shown to induce constitutive p75 dimerization and activate the receptor even in the absence of NGF (40). These studies implied that the cysteine residue is crucial for p75 receptor activity. We therefore investigated whether a specific mutation of cysteine to alanine (C257A) in p75 TM influences TrkB activation. Incubation of the C257A p75 transmembrane peptide was still capable of inducing in vitro TrkB phosphorylation, suggesting that the cysteine residue may not participate in p75 TM-induced TrkB activation (Fig. 6, A and B).

In addition, a series of consecutive valine residues in the transmembrane domain of p75 have been proposed to act as a self-associative motif, AVVVG (Table 1, blue) (39, 41). This hydrophobic series of amino acids overlaps with the site of cleavage by the γ-secretase complex (3). Previously, a mutation of a valine residue in this motif was reported to change the motif orientation and prevent γ-secretase cleavage of the receptor (41).

To examine whether the AVVVG motif is involved in TrkB activation, we generated a peptide with a triple mutation replacing the valines in p75 TM to GRA (Table 1). The results using the in vitro TrkB phosphorylation assay demonstrated that the triple mutation prevented the effects of p75 TM upon TrkB phosphorylation (Fig. 6, C and D). We also explored the effects of an individual mutation of valine to alanine at position 263 and 264 (Table 1), which is adjacent to the γ-secretase cleavage site, on TrkB phosphorylation. The results showed that the phosphorylation of TrkB was significantly decreased after incubation with the p75V264A peptide, whereas the p75V263A induced the same levels of TrkB phosphorylation as p75 TM (Fig. 6, E and F). Because p75V264A, unlike p75 TM, does not stimulate TrkB phosphorylation, we ran a co-IP assay to see whether p75V264A interacts with TrkB. Our results showed that p75V264A did not interact with TrkB (supplemental Fig. S2).

Because p75GRA and p75V264A mutations failed to interact and stimulate TrkB phosphorylation, we sought to understand

![Figure 5. Transmembrane domain of TrkB enhances phosphorylation of the TrkB receptor.](image-url)

A and B, Western blot and its corresponding analyses demonstrate a dose-dependent effect of TrkB TM over 30 min on TrkB (Tyr816) phosphorylation using an in vitro phosphorylation assay. *, p < 0.05; **, p < 0.01 versus DMSO. 5 μM TrkB TM treatment in the absence of ATP was utilized as an experimental control. C and D, Western blot and its statistical analyses show the time-dependent effects of 5 μM TrkB TM on TrkB phosphorylation. The ratios of 0.2 μM TrkB incubated with 1.25, 2.5, 5, and 10 μM of TrkB TM were 1:6.25, 1:12.5, 1:25, and 1:50, respectively. *, p < 0.05; **, p < 0.01 versus DMSO (n = 3 individual experiments; means ± S.E.).
whether or not the mutations alter secondary structure of p75. To address this issue, we utilized the SPLIT 4.0 SERVER program (http://splitbioinf.pmfst.hr/split/4) to predict the α-helix preference of p75 neurotrophin receptor and the effect of mutations utilized in this study. According to the α-helix predictions made by the program, GRA slightly reduced α-helix preference; however, V264A mutation did not impact α-helix preference in the p75 neurotrophin receptor, which suggests that p75V264A does not disrupt the intermembrane region (supplemental Fig. S3).

Altogether, these data suggests that a valine 264 in p75 TM has consequences upon both intramembranous cleavage by γ-secretase (43) and activation of the TrkB receptor. Because the transmembrane domain of TrkB, like p75, induces TrkB phosphorylation and both TrkB and p75 possess a self-associative motif with shared triple valines, we hypothesize that the cluster of valines in p75 TM may be involved in triggering TrkB phosphorylation.

Discussion

In the current study, we found that the transmembrane domain of p75 could activate the TrkB receptor. In contrast, the transmembrane domain of other receptors failed to induce TrkB phosphorylation. We also tested whether other related the RTK members, including TrkA and TrkC, which have a high homology with TrkB (46, 47) and display similar structural domains, are affected by p75 TM. However, the p75 TM has a profound effect mainly on TrkB phosphorylation. Furthermore, we utilized co-immunoprecipitation and sucrose subcellular fractionation assays to explore how p75 TM may enhance TrkB phosphorylation. We found that p75 TM interacts with the TrkB receptor, and this association likely occurs in lipid rafts and the cell membrane. Moreover, mutagenesis revealed that a valine residue at position 264 in p75 TM was critical for TrkB activation in circumstances when these receptors are highly co-expressed during development or after neuronal injury.
across species, and it is known to influence NGF-binding kinetics (22).

Our in vitro assays results demonstrated that p75 TM enhances TrkB phosphorylation in a dose- and time-dependent manner. Neither the EGFR TM nor the FGFR1 TM influenced TrkB phosphorylation. Moreover, p75 TM did not alter the phosphorylation of TrkA, TrkC, or FGFR1 receptors. These results altogether suggest a level of specificity for TrkB activation by p75 TM.

To minimize any possible effects on TrkB phosphorylation by detergents, we removed detergents from the TrkB buffer and reconstituted the receptor in brain lipids to form proteoliposomes and provide a more natural context for the effect of p75 TM on TrkB phosphorylation. The data indicate that p75 TM is capable of triggering TrkB phosphorylation in proteoliposomes in the absence of detergent. Taken together, these experiments suggest that the TM domain of p75 may have a signaling function, in addition to anchoring the receptor in the membrane. The observed effect of the TrkB TM on TrkB phosphorylation and the recent report on the effect of insulin receptor TM on insulin receptor phosphorylation (20) suggest that there are likely transmembrane domains that have additional functions dependent upon sequence specific interactions.

Alterations of TM sequences in the p75 receptor revealed important amino acid residues in p75 TM important for stimulating TrkB phosphorylation. A structural study on p75 TM using nuclear magnetic resonance has shown that cysteine 257 in p75 TM participates in receptor dimerization through a disulfide bond and promotes p75 ligand-dependent signaling downstream, resulting in greater cell survival (39). However, our experiments using a mutation in p75 TM, p75C257A, suggested that cysteine 257 is not critical for p75 TM-induced TrkB phosphorylation, because the p75C257A peptide was still able to enhance TrkB activity. In contrast, we found that a specific mutation in p75V264A in the AVVVG motif, which has no predicted effect on β-helix preference, abolished p75 TM-induced TrkB phosphorylation. This suggests that valine 264, which is known to be adjacent to the site of intramembrane proteolysis of p75 catalyzed by the γ-secretase complex, plays a crucial role in inducing TrkB phosphorylation (3, 43).
Valine 264 is located in a p75 fragment, p75(αγ), which is released after intramembranous cleavage (3, 43), and the in vitro phosphorylation assay suggested that p75(αγ) peptide can alone stimulate TrkB phosphorylation; we then tested whether the activation of metalloprotease by PMA to promote sequential cleavage of p75 and generate the p75(αγ) fragment in cells could alter TrkB phosphorylation. Our data showed that the phosphorylation of TrkB in HEK293-TrkB cells expressing p75 was enhanced by 25% in response to PMA treatment, which is not a statistically significant increase. This could be due to low efficiency of the effects of PMA upon α-secretase cleavage and the lack of sufficient substrate required for γ-secretase activity. Differences in substrate specificity or cellular environment and characteristics, such as lipid composition, may also exert an effect upon the efficiency of cleavage (3). Furthermore, it is known that the p75 ICD peptide degrades rapidly; as such, it is likely that p75(αγ) does as well (45).

In conclusion, our study in vitro and within live cells suggest that p75 TM and p75(αγ) may impact TrkB activation in circumstances where these receptors’ expression levels are increased and when cleavage is maximal. Based upon these results, it is conceivable that small molecules that interact with transmembrane domains may possess agonistic qualities, as has been observed with the thyroid-stimulating hormone receptor (48). Additional in vivo studies will be needed to determine whether TrkB activation by small peptides can represent a therapeutic strategy.

**Experimental procedures**

**Cell culture and transfections**

The human embryonic kidney 293 cell line stably expressing human TrkB (HEK293-TrkB) (49) was maintained in DMEM containing 10% FBS supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, and 200 mg/ml G418 (Invitrogen). *Spodoptera frugiperda* 9 (Sf9) cells were grown in ESF921 insect cell culture medium (Expression Systems, Washington, DE) (Table 1). Rabbit anti-p75 (9992) (3), rabbit polyclonal anti-FGFR (Cell Signaling Technology), and mouse monoclonal anti-β-actin (Sigma) were used as primary antibodies. Normal mouse and rabbit IgG (Sigma), mouse, and rabbit HRP-conjugated secondary antibodies (Sigma) were used in this study. BDNF was purchased from Peprotech Inc. (Rocky Hill, NJ). Phorbol 12-myristate 13-acetate (PMA) and DAPT were purchased from Sigma-Aldrich (St. Louis, MO).

**Tyrosine kinase receptors expression and solubilization**

Rat TrkA, TrkB, TrkC (51), and human FGFR1 (from Prof. Dalibor Sames, Columbia University, New York, NY) with a carboxyl-terminal GFP were synthetized and subcloned in the Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer’s instructions using PCR and primers (Table 2).

**Peptides, antibodies, and reagents**

All peptides including rat p75 TM, p75C257A, p75GRA, p75V263A, p75V264A, p75 TM-HA, p75V264A-HA, p75(αγ), TAT-HA, rat p75 TM, human EGFR TM, and human FGFR1 TM with ≥70% purity, were synthesized by Biomatik (Wilmington, DE) (Table 1). Rabbit anti-p75 (9992) (3), rabbit polyclonal anti-phospho-TrkB (Y816) (50), rabbit polyclonal anti-phospho-TrkC (Y820) (ab79811) (Abcam), mouse monoclonal anti-phospho-TrkA (Y816) (50), sheep polyclonal anti-phospho-TrkC (Y820) (ab79811) (Abcam), mouse monoclonal anti-phospho-FGFR (Y653/Y654) (55H2) (Cell Signaling Technology, Beverly, MA), rabbit polyclonal TrkC (H-181) (Santa Cruz), rabbit polyclonal pan Trk (C-14) (Santa Cruz), rabbit polyclonal anti-FGFR (Cell Signaling Technology), mouse monoclonal anti-α-actinin (I2CA5) (Roche Diagnostics), mouse monoclonal anti-early endosome antigen 1 (EEA1) (Sigma), mouse monoclonal anti-flotillin-1 (BD Transduction Laboratories, Billerica, MA), rabbit polyclonal anti-Na⁺/K⁺-ATPase (Cell Signaling Technology), and mouse monoclonal anti-β-actin (Sigma) were used as primary antibodies. Normal mouse and rabbit IgG (Sigma), mouse, and rabbit HRP-conjugated secondary antibodies (Sigma) were used in this study. BDNF was purchased from Peprotech Inc. (Rocky Hill, NJ). Phorbol 12-myristate 13-acetate (PMA) and DAPT were purchased from Sigma-Aldrich (St. Louis, MO).

**In vitro phosphorylation assay**

The solubilization buffer of Sf9 lysates was dialyzed to phosphorylation buffer which contained 25 mM HEPEs, 15 mM MgCl₂, 10 mM MnCl₂, 150 mM NaCl, and 0.1% Triton X-100 by overnight dialysis at 4 °C using 10,000 molecular weight cutoff dialysis cassettes (Rockford). The total protein was measured by Bradford assay (Bio-Rad) and subsequently flash-frozen with liquid nitrogen. The samples were kept at −80 °C until use.

Because of high hydrophobicity, all peptides were dissolved in DMSO (Fisher Scientific) and were further diluted in distilled H₂O. To investigate whether the peptides could induce tyrosine kinase receptor phosphorylation, TM peptides were incubated with TrkA, TrkB, TrkC, and FGFR1 proteins in the presence of 25 μM ATP on a rotator at 37 °C. To stop phosphorylation reactions, SDS-loading buffer was added to the samples and boiled.

### Table 2

| Gene          | Forward (F) and reverse (R) primers | Annealing temp (°C) |
|---------------|-----------------------------------|---------------------|
| rat TrkA      | F: 5'-tgtagctcgaaggcaaggccagcagcagc -3' | 85.3                |
|               | R: 5'-gcccgcgegeccgcagcagcagcagc -3' |                     |
| rat TrkB      | F: 5'-tgtagctcgaaggcaaggccagcagcagc -3' | 79.5                |
|               | R: 5'-gcccgcgegeccgcagcagcagcagc -3' |                     |
| rat TrkC      | F: 5'-tgtagctcgaaggcaaggccagcagcagc -3' | 76.1                |
|               | R: 5'-gcccgcgegeccgcagcagcagcagc -3' |                     |
| human FGFR1   | F: 5'-tgtagctcgaaggcaaggccagcagcagc -3' | 79.2                |
|               | R: 5'-gcccgcgegeccgcagcagcagcagc -3' |                     |
p75NTR transmembrane domain stimulates TrkB phosphorylation

for 5 min. A dose-dependent and time course effect of TM peptides on receptor phosphorylation was detected by Western blot. To detect the total protein as a loading control for phosphorylated receptors, the membrane was stripped with 0.2 M glycine and 1% SDS for 15 min, blocked, and incubated with anti-TrkA, TrkB, TrkC, and FGFR1 antibodies (52, 53).

Proteoliposome assay

Porcine brain lipid in chloroform (Avanti Polar Lipids, Alabaster, AL) was transferred into a glass test tube and dried by N₂ until a lipid film appeared. The lipids were further dried under vacuum for 2 h. The lipids were rehydrated in phosphorylation buffer supplemented with 4% n-dodecyl β-D-maltoside and mixed with solubilized TrkB with a lipid protein ratio of 12.5 (w/w). Detergents were subsequently removed from the samples by overnight incubation with 600 mg of Bio-Beads SM2 resin (Bio-Rad) at 4°C (37). The proteoliposomes containing TrkB were isolated using high-speed centrifugation, 50,000 rpm for 1 h at 4°C. The proteoliposome pellet was resuspended in phosphorylation buffer without Triton X-100. The samples were flash-frozen in liquid nitrogen and stored at −80°C until analyzed. Electron microscopy of negatively stained samples (Fig. 3A) and SDS-PAGE with Coomassie Blue (R250) staining (Fig. 3B) were utilized to confirm TrkB proteoliposome formation (54, 55).

Co-immunoprecipitation

p75 TM-HA or p75V264A-HA or TAT-HA peptides (10 μg) were premixed with 250 μg of solubilized SF9-TrkB in 500 μl of Tris-buffered saline (TBS) at 4°C for 2 h before co-IP. The premixed p75 TM-HA/TrkB or p75V264A-HA/TrkB or TAT-HA/TrkB lysates were incubated with 1 μg of either mouse anti-HA or rabbit anti-TrkB (H-181) antibodies at 4°C overnight. Mouse and rabbit IgG served as negative controls for antibody pulldown in co-IP experiments. Subsequently, protein A-agarose beads (Sigma) were used to immobilize the antibodies and IgG. The beads were then washed four times with 0.01% Tween 20 in TBS (TBST), boiled in SDS loading buffer, and subjected to Western blot. 10 μg of sample lysate was loaded as a comparative (input) control.

Sucrose subcellular fractionation

HEK293-TrkB cells were mechanically lysed in TBS supplemented with protease inhibitor mixture and 2 mM PMSF by passing the sample through a 27-gauge needle six times (Sigma). The lysate was then subjected to centrifugation at 100 × g for 10 min at 4°C. The supernatant was isolated, and the total protein was measured by Bradford assay (Bio-Rad). 150 μg of HEK293-TrkB cell supernatants were premixed with 10 μg of p75 TM-HA at 4°C for 2 h prior to sucrose fractionation. The premixed sample was loaded on top of a continuous 12.5–50% sucrose/TBS gradient and centrifuged at 31,000 rpm for 3 h at 4°C using a SW60 rotor (Beckman). An equal volume of each fraction was taken and subjected to Western blot using 4–15% Tris-glycine and 16.5% Tris-Tricine precast gels (Bio-Rad). The membrane was probed with anti-TrkB (H-181), anti-HA (p75 TM-HA), anti-Flotilin-1 (protein marker for lipid raft), anti-Na⁺/K⁺-ATPase (cell membrane marker), anti-E1A (protein marker for early endosome), and anti-β-actin (a cytosolic marker) antibodies (56).

Western blot

The concentration of total protein isolated from Sf9, SF9-TrkB, and HEK293-TrkB was measured using the Bradford assay (Bio-Rad). 20–30 μg of the protein was separated by either 4–15% Tris-glycine precast gels (Bio-Rad) or 4–12% Bis-Tris precast gels (Invitrogen). 16.5% Tris-Tricine precast gels (Bio-Rad) were used to separate small peptides like p75 TM-HA, p75V264A-HA, and TAT-HA by electrophoresis. The proteins and peptides were transferred to a 0.2-μm nitrocellulose membrane (GE Healthcare). The membrane was blocked by 3% BSA in TBST for 1 h at room temperature (22°C) and then incubated with primary antibody diluted in 3% BSA in TBST (Sigma-Aldrich) overnight followed by incubation with HRP-conjugated secondary antibody in 3% BSA in TBST for 1 h at room temperature. The membrane was washed with TBST and developed with a Kodak X-OMAT 2000 machine (Rochester, NY) using chemiluminescence reagents (GE Healthcare) and X-ray film (GeneMate, Kaysville, UT).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 provided by New York University School of Medicine. Variables between groups were determined by either one-way repeated measures analysis of variance or Student’s t test. The values of p < 0.05 were considered statistically significant. The data are presented as means ± S.E.

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