THE EFFECTS OF TRANSMEMBRANE SEQUENCE AND DIMERIZATION ON CLEAVAGE OF THE p75 NEUROTROPHIN RECEPTOR BY γ-SECRETASE.

Alex M Sykes1#, Nickless Palstra 1, Daniel Abankwa2#, Justine M Hill3,4, Sune Skeldal1, Dusan Matusica1, Prahatha Venkatraman16, John F Hancock2# and Elizabeth J Coulson1*.

1 Queensland Brain Institute, 2 Institute for Molecular Bioscience, 3 School of Chemistry and Molecular Biosciences, 4 Centre for Advanced Imaging, The University of Queensland, Brisbane, Queensland 4072, Australia.

Running title: p75NTR transmembrane sequence regulates γ-secretase cleavage

#Current addresses:
AMS: Max Planck Institute for Molecular Cell Biology and Genetics, Dresden 01307, Germany.
DA: Turku Centre for Biotechnology, Abo Akademi University, 20520 Turku, Finland
PV: Department of Biological Sciences, Purdue University, Indiana 47906, USA
JFH: Integrative Biology and Pharmacology, University of Texas Health Science Center at Houston, Texas 77030 USA.

* Address correspondence to Elizabeth J Coulson, Queensland Brain Institute, The University of Queensland, Brisbane, Queensland 4072, Australia. Ph: +61 7 33466392, Fax: +61 7 33466301, email: e.coulson@uq.edu.au

Keywords: p75 neurotrophin receptor, RIP, γ-secretase, dimerization, FRET

Background: p75 neurotrophin receptor (p75NTR) signaling is modulated by dimerization and regulated intramembrane proteolysis (RIP).

Results: Transmembrane sequence and TrkA but not ligands regulate p75NTR homodimerization and γ-secretase cleavage.

Conclusion: Although γ-secretase does not require a dimeric substrate, p75NTR dimerization facilitates RIP.

Significance: Structural change mediated by homo- and heterodimerization is more important than ligand for inducing RIP of p75NTR.

SUMMARY

Cleavage of transmembrane receptors by γ-secretase is the final step in the process of regulated intramembrane proteolysis (RIP) and has a significant impact on receptor function. Although relatively little is known about the molecular mechanism of γ-secretase enzymatic activity, it is becoming clear that substrate dimerization and/or the substrate’s α-helical structure can regulate the site and rate of γ-secretase activity. Here we show that the transmembrane domain of the pan neurotrophin receptor p75NTR, best known for regulating neuronal death, is sufficient for its homodimerization. While the p75NTR ligands NGF and proNGF do not induce homodimerization or RIP, homodimers of p75NTR are γ-secretase substrates. However dimerization is not a requirement for p75NTR cleavage, suggesting that γ-secretase has the ability to recognize and cleave each receptor molecule independently. The transmembrane cysteine 257, which mediates covalent p75NTR interactions, is not crucial for homodimerization, but this residue is required for normal rates of γ-secretase cleavage. Similarly, mutation of the residues alanine 262 and glycine 266 of an AxxG dimerization motif flanking the γ-secretase cleavage site within the p75NTR transmembrane domain alters the orientation of the domain and inhibits γ-secretase cleavage of p75NTR. Nonetheless, heteromer interactions of p75NTR with TrkA increase full-length p75NTR homodimerization, which in turn potentiates the rate of γ-cleavage following TrkA activation independently of rates of α-cleavage. These results provide support for the idea that the helical structure of the p75NTR transmembrane domain, which may be affected by co-receptor interactions, is a key element in γ-secretase-catalyzed cleavage.

INTRODUCTION

The pan neurotrophin receptor p75 (p75NTR) is capable of regulating a range of neuronal functions. p75NTR is best known for its role in mediating neural cell death during development, as well as in the adult following injury, including that induced by pro-neurotrophins in conjunction...
with a co-receptor sortilin (1,2). However, p75NTR can also regulate cell proliferation, migration, axon guidance, and survival. In particular, when interacting with TrkA receptors, p75NTR can form high-affinity binding sites for NGF and enhance trophic signaling (2,3).

p75NTR is one of more than 60 transmembrane receptors known to undergo a two-step process of regulated intramembrane proteolysis (RIP) (4,5). As inhibition of this process influences some aspects of p75NTR-mediated signal transduction, including neuronal survival (6-9), understanding the normal regulation of p75NTR RIP could elucidate how specific p75NTR signals are modulated.

During RIP, transmembrane receptors are first cleaved by one or more metalloproteases (α- or β-cleavage) to release their extracellular domains, with p75NTR being cleaved five amino acids N-terminally above the membrane by TACE/ADAM17 (10,11), a process that can be induced by Trk activity (9). The remaining C-terminal fragments, containing the transmembrane and intracellular domains, are subsequently cleaved by γ-secretase, releasing the intracellular domain from its membrane tether. Removal of the extracellular domain of transmembrane proteins by α-secretase generates a free amine at the N-terminal stump that is recognized by one of the components of γ-secretase, nicastrin (12). This interaction enables γ-secretase to dock with its substrates and position the receptors for subsequent cleavage by the catalytic component, presenilin (12-16). Less is known about the regulation of this cleavage. However, the conformation of the transmembrane domain can exert a major influence on the site of γ-secretase cleavage in substrates other than p75NTR (14,15).

Transmembrane domains are α-helical in structure, and glycine and alanine residues are frequently found in contact with one another, forming the basis of helix interactions between the interfaces of two transmembrane domains (17-21). GxxxG motifs are found in about a third of γ-secretase substrates, with another 15% containing the homologous motifs AxxxA, GxxxxA, or AxxxxA (16,22). These motifs are thought to be involved in homodimerization and most γ-secretase substrates are also known to form homodimers (23-26). Although a number of studies have reported that GxxxG dimerization motifs play a role in regulating γ-secretase activity (17,27-32), there is currently no clear understanding of the relationship between dimerization through this motif and γ-secretase cleavage.

p75NTR has previously been reported to form dimers, most likely mediated through the transmembrane domain, as there is minimal contribution of the extra- and intracellular domains to its self-association (25,33,34). Furthermore, it has been suggested that the transmembrane A206xGGG266 sequence within p75NTR may contribute to its dimerization (24), and that dimers of p75NTR might be stabilized by an intermolecular cysteine 257 (C257) disulfide bridge within the membrane (24,35). In this study, we tested whether p75NTR uses its AxxxG motif as well as C257 to facilitate homodimerization, and whether these residues influence the rate of basal and NGF-induced γ-secretase cleavage.

**EXPERIMENTAL PROCEDURES**

**Construct design**
p75NTR expression constructs used a modified pCDNA3 (Invitrogen) backbone. The rat p75NTR signal peptide including a Kozak sequence (nucleotides -29 – +87) was inserted between the KpnI and EcoRV restriction sites, generating the vector pCDNA3-SP. To generate fluorescently tagged p75NTR, the fluorophores CFP and YFP were next amplified by PCR from peCFP-N1 and peYFP-N1 (Clontech), respectively, using primers incorporating 5’ EcoRV and NheI restriction sites and a 3’ stop codon and XhoI site. Fluorophores were cloned in frame between the EcoRV and XhoI restriction sites of pCDNA3-SP, generating the vectors pCDNA3-CFP and pCDNA3-YFP, respectively. p75NTR coding sequences were finally amplified under standard PCR conditions with 5’ EcoRV and 3’ NheI restriction sites incorporated into the respective primers. Subsequently, p75NTR coding sequences were cloned between the EcoRV and NheI restriction sites of pCDNA3-SP, generating the vectors pCDNA3-CFP and pCDNA3-YFP, respectively. p75NTR peptide coding sequences were finally amplified under standard PCR conditions with 5’ EcoRV and 3’ NheI restriction sites incorporated into the respective primers. Subsequently, p75NTR coding sequences were cloned between the EcoRV and NheI restriction sites of the pCDNA-CFP or pCDNA3-YFP vector to generate in-frame FRET-capable fusion proteins. Table 1 provides a list of p75NTR constructs used in this study, with the altered amino acids noted. The TrkA and kinase-inactive TrkA K538R constructs have been previously described (7).

**Cell culture and transfection**
HEK293 or HEK293T cells, which do not endogenously express p75NTR, were cultured in RPMI medium (Gibco) supplemented with 10% FCS (JRH Biosciences) at 37°C in a humidified
atmosphere with 5% CO₂. Unless otherwise noted, 40-50,000 cells were plated at a confluency of ~ 60% in a 24-well plate (Nunc) and transiently transfected using Fugene6 (Roche). 250-500 ng of DNA per well was used for cleavage experiments, and 125 ng of CFP DNA and 250 ng of YFP DNA was used for transfection for FRET experiments. For crosslinking and immunoprecipitation experiments, 6-well plates were used, with the cell number and transfections scaled up 4-fold. Wildtype and p75<sup>NTR</sup>-deficient PC12 cells were cultured in DMEM containing 1% non essential amino acids (Gibco), 1% Glutamax (Gibco), 10% horse serum and 5% FCS. p75<sup>NTR</sup>-deficient PC12 cells, stably transfected with shRNA against rat p75<sup>NTR</sup> and cultured under G418 selection, were kindly provided by Carlos Ibanez (Karolinska Institute). PC12 cells were transfected using the Amaxa system (Lonza; program U-029) and plated at a density of 1x10⁴ cells per well in a 96-well plate for acid phosphatase viability assay, and at 3x10⁵ cells in a 12-well plate for cleavage assays. To induce p75<sup>NTR</sup> expression, PC12 cells were treated for 15 minutes with a 30 Watt UV-C light bulb or 20 µM oligomeric human amyloid β peptide (36).

**Acid phosphatase experiments**

Survival of PC12 cells transfected with p75<sup>NTR</sup> expression constructs was measured by acid phosphatase viability assay. Cells were washed gently 3 times in PBS in the tissue culture plate. Cells were collected by centrifugation (360 RCF for 5 minutes) between washes to prevent cell loss. Following addition of 100 µl of acid phosphatase buffer (0.1M sodium acetate, 5mM p-(4) nitrophenyl phosphate (Sigma N 4645-1G), 0.1% Triton X-100, pH 5.0), plates were incubated for 30 minutes at 37°C before 10 µl of 1M NaOH was added per well to develop the reaction. Absorbance was measured at 405 nm on a POLARstar Optima plate reader.

**Cleavage experiments**

In cleavage experiments, cells were incubated for 4 hours with 5 µM of the proteasome inhibitor β-clasto-lactacystin (Calbiochem), 200 nM of phorbol-12 myristate-13-acetate (PMA; Sigma) or 100ng/ml NGF (Biosensis) to activate α-secretase cleavage, 20 µM of the TACE α-secretase inhibitor TAPI-2 (Calbiochem), or 200 nM of the γ-secretase inhibitor compound E (Calbiochem), as indicated, prior to cell lysis.

**Crosslinking and co-immunoprecipitation experiments**

Transiently transfected HEK293 cells were treated with the thiol-reducible protein crosslinker dithiobis succinimidylpropionate (DSP) or the non-thiol reducible crosslinker disuccinimidyldiisocyanate (DSS) (Pierce), according to the manufacturer’s instructions. After washing, cells were lysed on ice for 10 minutes in TNE buffer (20 mM Tris pH 8, 10 mM EDTA) supplemented with 1% NP40 and complete protease inhibitor cocktail (Roche Molecular Biochemicals). The results of immunoprecipitation experiments using cell lysates that had not been crosslinked with DSP prior to lysis were equivalent to those following crosslinking. For co-immunoprecipitation studies, samples were immunoprecipitated overnight at 4°C using Gammabind G-Sepharose beads (Amersham) coupled with anti-GFP (1181446001, Roche). Non-specific binding was removed by washing beads 5 times in Tris-buffered saline supplemented with 0.05% Tween-20, and proteins were eluted by boiling the samples in reducing sample buffer (20 mM DTT, 2.5% SDS). Protein samples were resolved under reducing conditions on 4-12% SDS-PAGE gels prior to Western blotting.

**Western blot analysis**

Western blots were performed by standard methods as previously described (11). Membranes were probed with rabbit anti-rat p75<sup>NTR</sup> intracellular domain (1:1000, 07-476, Millipore), rabbit-anti-human p75<sup>NTR</sup> intracellular domain (1:3000, Promega), rabbit/mouse anti-HIS (1:2000, BD Biosciences) or mouse-anti-GFP (1:2000, Roche) antibodies for 90 minutes at room temperature, followed by anti-rabbit Alexa680 (1:50,000, BioRad) secondary antibody for 45 minutes at room temperature. Membranes were imaged for protein visualization and quantification using an Odyssey (LI-COR) imaging system.

**Immunofluorescence and microscopic FRET analysis**

For immunofluorescence imaging, HEK293 cells were plated at ~ 25% confluency onto polyclornithine-coated 12 mm coverslips and transiently transfected with wildtype or YFP-tagged p75<sup>NTR</sup> expression constructs as described above. After 2 days, cells were fixed with 4% paraformaldehyde dissolved in PBS. The coverslips were mounted onto microscope slides
using Dako fluorescence mounting medium (Dako Corporation).

The FRET between p75\textsuperscript{NTR} proteins in single cells was measured as sensitized acceptor emission using the three-cube method, as previously described (37,38). Images in donor (excitation 405 nm, emission 470-500 nm), acceptor (excitation 514 nm, emission 530-600 nm) and FRET (excitation 405 nm, emission 530-600 nm) channels were acquired on a Zeiss LSM 510 Meta confocal microscope using a 63x oil immersion objective, with a numerical aperture of 1.4. Living HEK293T cells were imaged in PBS containing 1mM CaCl\textsubscript{2}. The 512 x 512 images recorded at 12-bit depth were batch converted from Zeiss LSM format into the tiff format using ImageJ and processed further in a custom-written procedure in IgorPro5 (Wavemetrics). This procedure performed background subtraction, crosstalk correction, shifting of correlated FRET channel images, if required, and thresholding. The FRET ratio (FR=\text{FAD}/\text{FA}, where F\text{AD} and F\text{A} are the fluorescence intensity of the acceptor in the presence and absence of the donor, respectively) was then calculated on a pixel-by-pixel basis. 

**Measurement of FRET in single cells by flow cytometry**

FRET analysis using flow cytometry was carried out as described previously (38,39). In brief, the FRET efficiency was calculated per HEK293 cell using an adapted sensitized acceptor emission method. Using custom-written algorithms in IgorPro5, calibration for approximate acceptor surface concentration (cA), FRET-efficiency and the donor-acceptor ratio were calculated. Only cells with a donor mole fraction \(x_D=0.5\pm0.1\), corresponding to a ~1:1 donor-acceptor ratio, were analyzed. The characteristic \(E_{\text{max}}\) value was determined by iterative fitting as previously described (38,39).

A mutant of the membrane targeting sequence of H-Ras, CTH-7A-L (39), was used as a control for random interactions (\(E_{\text{max}}\) was ~10%).

**Cell population FRET assay**

FRET between p75\textsuperscript{NTR} molecules within a population of transiently transfected HEK293T cells was determined 48 hours post-transfection of triplicate wells, using untransfected cells to determine background fluorescence. Cells from individual wells were washed once before being harvested in PBS (pH 7.4). Cell pellets were resuspended in 200 \(\mu\)l PBS and loaded into single wells of 96-well black microtiter plates (Greiner) for immediate analysis. Fluorescence was recorded simultaneously for the donor fluorophore (excitation 430±10 nm and emission 480±10 nm) and the acceptor fluorophore (excitation 485 nm and emission 530±10 nm), together with FRET (excitation 430±10 nm and emission 530±10 nm), on a POLARstar OPTIMA multidetection microplate reader (BMG Labtech). FRET was calculated as a FRET ratio (FR), which is the fractional increase in YFP emission due to FRET (37,40), according to the following formula.

\[
FR = \frac{A(f(S4-S1S5)-S2S4)}{A(S4-S1S5)}
\]

where \(D = \) donor filter set, \(A = \) acceptor filter set, \(F = \) FRET filter set, \(a = \) acceptor only, \(d = \) donor only, \(f = \) donor and acceptor, \(S_1 = Da/Fa\), \(S_2 = Ad/Dd\), \(S_3 = Ad/Fd\), \(S_4 = Fa/Aa\), \(S_5 = Da/Aa\).

**Primary neuronal cultures and FRET**

Experiments using animal tissue were approved by the Animal Ethics Committee at the University of Queensland and were conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Primary neuronal cultures were generated from dissociated dorsal root ganglia (DRG) derived from newborn p75\textsuperscript{NTR}-deficient (NGFR\textsuperscript{−/−}C57Bl6/J; 41) mice as previously described (7). Dissociated neurons (60,000) were transfected with 5 \(\mu\)g total plasmid DNA using a Basic Neuron Nucleofector Kit (Lonza), and electroporated with program G-013. Neurons were plated onto poly-ornithine (0.15%) and laminin (2.5 g/ml) coated glass bottom 3cm dishes (Matek) in MonoMed II medium (CSL) containing 1% FCS, 10\(^4\) units/ml leukemia inhibitory factor (Millipore) and 20 mM potassium chloride. After 36-48 hours, FRET images of neurons were captured on an inverted LSM510 confocal microscope (Zeiss) at 37\(^\circ\)C under 5% CO\textsubscript{2}, immediately before and after the addition of 5 ng/ml NGF. Where indicated, cultures were pretreated with compound E (200 nM) for 4 hours or TAPI-2 (20 \(\mu\)M) overnight as well as 4 hours prior to imaging.

**Modeling p75\textsuperscript{NTR} transmembrane domain dimerization**

The solution nuclear magnetic resonance (NMR) structure of glycophorin A (PDB ID 1AFO)(42) was used as a template to model the homodimeric transmembrane domain structure of p75\textsuperscript{NTR}. The structure of the transmembrane
domain was built as a canonical α-helix, with phi and psi angles of -57° and -47°, respectively. The sequences used for each helix were p75NTR residues 251-273 NLIPVYCSILA AVVG YIAF, and p75NTR-LxxL mutant NLIPVYCSI LA LVV VLLVAYIAF. The two helices in the homodimer were then aligned and oriented with a crossing angle of -40°, as observed experimentally for glycophorin A (42). The models were prepared using PyMOL (43).

Statistical analysis
Experimental results were analyzed using one-way ANOVA unless otherwise stated. When significant ANOVA was detected, planned comparisons were made to detect between-group differences. Alpha was controlled using a Bonferroni adjustment. p< 0.05 was considered significant.

RESULTS
p75NTR forms homodimers primarily at the cell surface
Formation of p75NTR protein dimers has been shown to have a strong regulatory effect on the activation of the receptor’s cell death signals (24,35). Similarly, the ability of p75NTR to undergo RIP has a major influence on p75NTR-mediated functions (2). To investigate the relationship between dimerization and RIP, we first assayed HEK293 cells transfected to express either full-length p75NTR (p75FL) or p75NTR with YFP fused to its C-terminus (p75FL-YFP; Table 1). p75NTR expression in these cells was compared to the levels, per µg protein, of p75NTR expressed endogenously in PC12 cells. Although the expression level of p75NTR in transfected cells was higher than basal PC12 cell expression (Figure 1a), it was of a similar level to that of cells under stress conditions known to induce p75NTR expression i.e., amyloid β treatment and UV exposure (44). Furthermore, overexpression of p75FL-YFP, like overexpressed wildtype p75FL (6,7), induced significant PC12 cell death compared to that obtained from control cells transfected with YFP (Figure 1b), demonstrating that the YFP motif does not affect the death signaling function of p75NTR.

Using our constructs we then measured the size of self-associating p75NTR oligomers in live HEK293 cells which do not express endogenous p75NTR or Trk co-receptors, and have negligible levels of the p75NTR co-receptor sortilin. Following crosslinking, the main complex size in both cases was double that of the monomer, indicating that the complexes were likely to be dimeric (Figure 1c).

To study the regulation of these homoassociations, we used FRET analysis, which allows the inference of a specific interaction between proteins based on a FRET signal (40). In the current study donor CFP and acceptor YFP proteins were fused to putative interaction partners. CFP and YFP give 50% FRET efficiency when they are within 4.92 nm of each other (45).
FRET methods which are not influenced by the intracellular domain.

The transmembrane domain mediates p75<sup>NTR</sup> dimerization.

To study the regulation of p75<sup>NTR</sup> dimerization, we next validated a high-throughput population-based FRET assay using a multimode plate reader. A construct of CFP fused to YFP served as a positive control for maximal protein-protein interaction (FR = 9.0±1.3; Figure 2a, column 1). The lower limit of the FRET ratio that was indicative of a non-specific interaction was established using a construct expressing only YFP together with a construct expressing p75<sub>FL</sub>-CFP. In this condition, FRET could only occur through non-specific interactions between the soluble acceptor fluorophore and the donor molecule tethered to p75<sup>NTR</sup>. This resulted in a FRET ratio close to 1 (FR = 1.2±0.2; Figure 2a, column 2), indicating that there was no energy transfer above background between the two fluorophores (37). In a second control experiment, p75<sub>FL</sub>-CFP was co-transfected with p75<sub>FL</sub> in which YFP was fused to the N-terminus of the receptor. Again, FRET was at background levels, as these fluorophores were located on opposite sides of the membrane (FR = 1.0±0.1; Figure 2a, column 3). By contrast, FRET between two p75<sub>FL</sub> receptors containing donor/acceptor fluorophores fused to their C-termini was significantly increased (FR = 2.4±0.1; Figure 2a, column 4), suggesting a significant protein-protein interaction.

As seen by microscopy, significant FRET ratios, equivalent to that of the wildtype p75<sup>NTR</sup> receptors, were obtained between two p75<sub>ECD</sub> in which YFP was fused to the N-terminus of the receptor. Again, FRET was at background levels, as these fluorophores were located on opposite sides of the membrane (FR = 1.0±0.1; Figure 2a, column 3). By contrast, FRET between two p75<sub>FL</sub> receptors containing donor/acceptor fluorophores fused to their C-termini was significantly increased (FR = 2.4±0.1; Figure 2a, column 4), suggesting a significant protein-protein interaction.

To determine whether the transmembrane domain is sufficient for p75<sup>NTR</sup> homodimerization, we first tested whether truncated p75<sub>ECD</sub>-YFP protein could associate with wildtype p75<sub>FL</sub>-CFP. A significant FRET ratio was measured for interactions between p75<sub>FL</sub> and p75<sub>ECD</sub> (Figure 2c, column 1). This somewhat surprising result suggested that the CFP linked to the 138 amino acid intracellular domain of p75<sub>FL</sub> was, nonetheless, within close proximity to the YFP located immediately adjacent to the plasma membrane due to its fusion to p75<sub>ECD</sub>. Based on this, we postulated that the C-terminus of the intracellular domain of p75<sup>NTR</sup> was normally oriented towards the plasma membrane. To investigate this further, we generated a construct in which the structurally ordered amino acids PPP<sub>295-297</sub> within the intracellular juxtamembrane region were mutated to AAA (p75<sub>AAA</sub>), in order to introduce conformational flexibility and allow movement of the intracellular domain e.g., away from the plasma membrane. This resulted in a significant decrease in p75<sup>NTR</sup> FRET, supporting our hypothesis that the C-terminus of the intracellular domain of p75<sup>NTR</sup> is normally oriented towards the membrane (Figure 2c, column 2).

We next used a p75<sup>NTR</sup> variant that contains the transmembrane sequence plus 14 amino acids of both the juxtamembrane extracellular and intracellular domains, which are 100% conserved in the vertebrate orthologs of p75<sup>NTR</sup> (p75<sub>TM</sub>). p75<sub>TM</sub>-YFP was appropriately trafficked to the plasma membrane of HEK293 cells (Figure 2d) and, when paired with p75<sub>FL</sub>-CFP for FRET experiments, a significant FRET ratio was recorded, suggesting a specific protein interaction (Figure 2c, column 3). Consistent with this, full-length p75<sup>NTR</sup> but not p75<sub>ECD</sub> was co-immunoprecipitated with p75<sub>TM</sub>-YFP (Figure 2e, lanes 2 and 1, respectively). These results indicate that the transmembrane domain of p75<sup>NTR</sup> is sufficient for dimer formation.

Dimeric p75<sup>NTR</sup> undergoes γ-secretase cleavage but this does not regulate dimerization

Based on the above results, we next asked whether monomers or dimers of p75<sup>NTR</sup> were being cleaved. This was achieved by determining whether the process of RIP, which occurs at low basal levels in HEK293 cells (Figure 3), decreased the extent of full-length p75<sup>NTR</sup> dimerization. We hypothesized that a fractional loss of receptor dimers due to γ-cleavage would produce fragments that do not undergo FRET, and thus a change in the FRET ratio would occur under conditions of altered rates of RIP. Firstly, α- and γ-cleavage of p75<sup>NTR</sup> were induced by treatment with the phorbol ester PMA, which resulted in a significant decrease in p75<sup>NTR</sup> FRET (Figure 3a, column 2). As the intracellular
domain of p75NTR is rapidly degraded, this was inhibited by treatment with the proteasome inhibitor β-clasto-lactacystin. This treatment did not rescue the extent of FRET change (Figure 3a, column 3), consistent with the soluble p75NTR fragments not producing a significant FRET signal (Figure 2b, column 3). By contrast, in the absence of PMA treatment but the presence of the α-secretase inhibitor TAPI-2, which prevents α- and thus γ-cleavage of p75NTR, a significant increase in the FRET ratio was recorded as compared to that observed in wildtype p75NTR-expressing cells undergoing low basal rates of RIP (Figure 3a, column 4). In order to confirm that a basal level of p75NTR proteolysis was responsible for reduced p75NTR:p75NTR FRET, we also used a non-cleavable mutant of p75NTR containing an N-glycosylation site (p75Ngly)(7), that co-immunoprecipitates with the p75NTR transmembrane domain (Figure 2e, lane 4) but which is not cleaved by α-secretase, thereby also preventing γ-secretase cleavage (Figure 3a, column 5). As with pharmacological α-cleavage inhibition, cells co-expressing full-length non-cleavable p75Ngly-YFP together with p75FL-CFP yielded a significantly higher FRET ratio (Figure 3a, column 5) than that recorded for cells expressing p75FL FRET pairs.

In order to explicitly test whether γ-secretase release of the intracellular domain of p75NTR to the cytoplasm was responsible for the decrease in p75NTR FRET during RIP, γ-secretase activity was blocked by adding a specific γ-secretase inhibitor, compound E. This treatment, which leads to an accumulation of the p75NTR C-terminal fragment, resulted in an increase in the FRET ratio equivalent to that resulting from α-cleavage inhibition (Figure 3a, column 6). These results indicate that dimeric p75NTR is capable of being processed by both α- and γ-secretase.

As the majority of known γ-secretase substrates are dimers (16) and the enzymatic component, presenilin, is also a dimer (46), we next tested whether γ-secretase requires a dimeric receptor substrate or is capable of cleaving only one component of a dimeric receptor complex at a time. To do this we took advantage of the requirement of α-cleavage for subsequent γ-cleavage (12,16) and the resistance to α-secretase cleavage of p75NTR containing the Ngly mutation; this modification did not affect the ability of p75NTR to form dimers when paired with wildtype p75NTR (Figure 3a column 5, and Figure 2e column 4). We therefore substituted the Ngly mutation into our minimal transmembrane protein (p75Ngly-TM).

The transmembrane domain construct, either with or without the Ngly substitution, was overexpressed at a 2:1 ratio with the p75FL construct to enhance formation of p75Ngly-TM:p75FL heterodimers. Use of this truncated construct ensured that any resulting cleavage fragments were derived from p75FL. No effect on the rate of intracellular domain fragment production from the full-length p75NTR protein was observed under either basal conditions or following stimulation with PMA (Figure 3b). This indicated that dimerization of p75NTR with a non-cleavable substrate had no discernable effect on its ability to undergo RIP. Therefore, either only monomers of p75NTR were being cleaved or one half of the mutant: wildtype p75NTR heterodimer was able to be cleaved. As the previous experiment had demonstrated that p75NTR dimers undergo RIP, these results suggest that a dimeric substrate (a dimer of p75NTR C-terminal fragments) is not a requirement for the γ-secretase complex to interact with and cleave p75NTR.

TrkA signaling-induced γ-secretase cleavage reduces p75NTR C-terminal fragment homooligomerization

We next investigated the influence of ligand and TrkA on p75NTR dimerization and RIP. The addition of 100ng/ml NGF had no effect on the extent of FRET between two p75NTR or two p75CTP proteins (Figure 4a,b). Similarly, sortilin co-expression with or without ligand had no effect on FRET levels (Figure 4b). However co-expression of TrkA either with or without NGF treatment resulted in a significant decrease in the FRET ratio compared to that obtained with only p75NTR-expressing cells (Figure 4b).

As overexpression of TrkA can result in autophosphorylation (7), and Trk signaling induces p75NTR RIP (9,11), we asked whether TrkA signaling was responsible for the reduction in p75NTR oligomerization. In contrast to the kinase-active TrkA, a kinase-inactive mutant (TrkAK538R) produced a significant increase in the FRET ratio compared to that observed in the absence of co-receptor (Figure 4b).

To determine whether the TrkA-induced decrease in FRET was due to conformational changes in the p75NTR intracellular domain, we used the truncated form of p75NTR in FRET
experiments performed on a per cell basis. Using this method we replicated the earlier finding that the non-cleavable form of p75NTR (p75ECD-Ngly) had a significantly increased homo-interaction (Emax = 25.0±0.6), and that TrkAL538R expression also significantly increased the FRET between p75ECD proteins (Emax = 25.3±1.0) to a level equivalent to the FRET measured between two non-cleavable p75ECD-Ngly proteins (Figure 4c). By contrast, TrkA again significantly decreased the FRET level of p75ECD homodimers (Emax = 19.1±0.3; Figure 4b). No significant change was detected between p75ECD-Ngly oligomers when TrkA was co-expressed (Emax = 24.9±0.8; Figure 4c). Importantly, neither TrkA dimerization induced by the addition of ligand, nor the ability of p75NTR to be cleaved, affected the extent of interaction between p75NTR and TrkA, as measured by p75ECD-CFP to TrkAECD-YFP FRET (Figure 4d).

These results indicated that TrkA-mediated signals were affecting the self-association of p75NTR, specifically C-terminal fragment dimerization, due to γ-secretase cleavage. We therefore next measured self-association of p75NTR proteins in the presence of TrkA. Analogous to cleavable p75FL this caused a significant decrease in the p75NTR:p75NTR FRET value, whereas TrkAL538R had no effect on this FRET ratio (Figure 4e). Furthermore, inhibition of γ-secretase cleavage prevented the significant decrease in p75NTR FRET observed due to TrkA co-expression (Figure 4f).

Endogenous TrkA activation by NGF similarly affected p75NTR dimerization. Activation of TrkA has previously been shown to induce p75NTR RIP within minutes (9). Therefore p75NTR constructs were transfected into DRG neurons and FRET was measured. Following the addition of NGF, a 25% reduction in the basal p75NTR FRET was observed within 5 minutes, which was prevented by the treatment of neurons with either TAPI-2 or compound E (Figure 4g). Indeed, when RIP was prevented, the resulting increased p75NTR FRET ratio was suggestive of an increase in the number of homodimers.

Together, these data suggest that the physical presence of TrkA promotes full-length p75NTR:p75NTR oligomerization, whereas TrkA activation not only activates α-cleavage (9,11), but also promotes the dissociation of p75NTR C-terminal fragment dimers through facilitation of γ-secretase cleavage.

Residues within the transmembrane domain of p75NTR are required for γ-secretase cleavage but not dimerization.

Given that many γ-secretase substrates contain transmembrane GxxxG or related dimerization motifs, we investigated whether the AxxxG motif of two p75NTR transmembrane sequences could self-associate by modeling the structure of the transmembrane domain. This was performed based on the NMR structure of a GxxxG-containing protein, glycoporphin A (GpA)(42). The GpA dimer interface utilizes the sequence G79VxxGV84 (Figure 5a). Therefore the model was based on p75NTR dimerizing through A262VxxGL267. Our model revealed that residues in the putative dimer interface were comparable in both the GpA and p75NTR structures (Figure 5). Specifically, the side-chains of A262 and G266 of one p75NTR monomer were able to pack against I259, V263 and L267 in the adjacent monomer (Figure 5b); the presence of small glycine and alanine residues in the AxxxG motif allows the helix backbones to reach close proximity and the larger side chains to pack in a “ridges into grooves” fashion (42).

In order to interfere with this interface, we substituted the A262 and G266 residues (residues 12 and 16 of the transmembrane domain) with leucine, generating a p75LxxxL expression construct. Modeling of the p75LxxxL mutant transmembrane revealed that the introduction of bulkier leucine residues at positions 262 and 266 would result in steric clashes between these residues and the three branched hydrophobic residues of the opposite monomer (Figure 5c). These mutations would therefore alter the dimerization face if it is mediated by the AxxxG domain.

Another transmembrane domain residue implicated in modulating dimerization of p75NTR is C257, which can mediate a disulfide bond between the p75NTR monomers (24). Surprisingly, our modeling highlighted that C257 was on the opposite face of the transmembrane domain to the AxxxG motif, indicating that the two possible modes of dimerization were mutually exclusive for a single pair of p75NTR molecules (Figure 5b). We therefore generated a construct in which the single transmembrane cysteine residue was changed to alanine (p75C257A), as well as a construct replacing the transmembrane domain with that of the tumor necrosis factor receptor transmembrane domain (p75TNFR), which contains cysteine residues but...
otherwise is not conserved compared to p75\textsuperscript{NTR} in terms of amino acid sequence (Figure 5d).

We then measured the ability of these mutant p75\textsuperscript{NTR} variants to form covalent dimers. As previously reported, under non-reducing conditions (i.e., in the absence of DTT), a p75\textsuperscript{NTR}-immunopositive band was observed at \( \sim 160 \text{kDa} \) (Figure 6a, asterisk). This complex is likely to be mediated by the disulfide bond via the transmembrane C\textsubscript{257} as it was not detected in lysates of HEK293 cells expressing p75\textsuperscript{C257A} (Figure 6a, arrowhead). However, p75\textsuperscript{LxxxL} and p75\textsuperscript{TNFR} proteins formed covalent complexes to a similar extent and size as wildtype p75\textsuperscript{NTR} (Figure 6a, asterisk). By contrast, although p75\textsuperscript{C257A} retained the ability to FRET with p75\textsuperscript{FL} (Figure 6b, column 2), the ability of the p75\textsuperscript{LxxxL} and p75\textsuperscript{TNFR} proteins to undergo FRET with wildtype p75\textsuperscript{NTR} was significantly reduced (Figure 6b, columns 3 and 4; FRET ratios: 1.08 ± 0.27 and 1.52 ± 0.03, respectively). p75\textsuperscript{LxxxL} was nonetheless able to undergo significant FRET when paired with another p75\textsuperscript{LxxxL} protein (FRET ratio: 1.97 ± 0.04; \( N=3, p<0.001 \), c.f. column 4), indicating that the reduction in FRET between p75\textsuperscript{FL} and p75\textsuperscript{LxxxL} proteins may be due to a change in the relative position of the two C-terminal fluorophores rather than a disruption to dimerization itself.

To test this, variant p75\textsuperscript{NTR} proteins (lacking YFP) were crosslinked. All p75\textsuperscript{NTR} variants were found within a complex with combined molecular weight of \( \sim 140 \text{kDa} \) (Figure 6a, hash), twice the molecular weight of monomeric p75\textsuperscript{NTR}, but less than the weight of the complex observed in non-reducing conditions (Figure 6a, asterisk). Similarly, despite their altered sequences, proteins from all three constructs (lacking YFP), and their C-terminal fragments, were able to be pulled-down by p75\textsuperscript{TM}-YFP (Figure 6c), indicating that they retained the ability to associate and form dimers with the wildtype p75\textsuperscript{NTR} transmembrane domain.

Finally, the rate of \( \gamma \)-secretase cleavage of the p75\textsuperscript{NTR} proteins that contained transmembrane domain mutations was examined. In HEK293 cells, \( \gamma \)-secretase cleavage of p75\textsuperscript{NTR} proteins in which the transmembrane domain cysteine (p75\textsuperscript{C257A}) had been substituted was significantly reduced rate of under both basal conditions and, more obviously, following stimulation with PMA (Figure 6d, \( p<0.01, N=4 \)). This indicated that, although covalent dimerization is not a requirement for cleavage to occur, C\textsubscript{257} facilitates this cleavage event. The level of the intracellular domain fragment in cells expressing either p75\textsuperscript{TNFR} or p75\textsuperscript{LxxxL} was negligible in both basal and stimulated conditions, indicating that \( \gamma \)-secretase cleavage (but not \( \alpha \)-cleavage) of these proteins was significantly impaired (Figure 6d). However, when transfected into TrkA-expressing PC12 cells, intracellular domain fragments were produced from each of the p75\textsuperscript{NTR} mutant constructs except p75\textsuperscript{Ngly} (Figure 6e). For all constructs, on average, PMA induced a 2 fold increase, and NGF a 1.2-1.5 fold increase, in the rate of basal cleavage. Although the rate of \( \gamma \)-cleavage remained significantly impaired for p75\textsuperscript{C257A}, p75\textsuperscript{LxxxL} and p75\textsuperscript{TNFR}, accumulating up to 4 fold higher levels of C-terminal fragment (Figure 6e), the amount of intracellular domain fragment generated in the mutants mirrored the level of C-terminal fragment. Therefore, the ability of p75\textsuperscript{NTR} to heterodimerize with TrkA, in addition to the p75\textsuperscript{NTR} transmembrane sequence and/or structure, appears to affect the ability of \( \gamma \)-secretase to dock with and/or cleave the p75\textsuperscript{NTR} C-terminal fragment.

**DISCUSSION**

Here we show that p75\textsuperscript{NTR} forms dimers via its transmembrane domain. Neither the extracellular domain (27,36) nor ligand, nor the intracellular domain fragment of p75\textsuperscript{NTR} released following \( \gamma \)-secretase cleavage, contributed to self-association to a substantial degree. Although p75\textsuperscript{NTR} dimers were capable of being processed by both \( \alpha \)- and \( \gamma \)-secretases, a dimeric p75\textsuperscript{NTR} substrate was not required for RIP. Mutation of amino acids within the transmembrane sequence that have been implicated in p75\textsuperscript{NTR} dimerization had only subtle effects on homodimerization but significantly affected normal rates of \( \gamma \)-secretase cleavage. Although ligand activation of p75\textsuperscript{NTR} did not significantly affect rates of RIP in the cells examined here, signals mediated by TrkA activation induced the cleavage of p75\textsuperscript{NTR}, thereby reducing the self-association of the intracellular domain fragments. Furthermore, TrkA expression alone increased full-length p75\textsuperscript{NTR} homodimerization, which appeared to facilitate subsequent \( \gamma \)-secretase cleavage.

The transmembrane domain of p75\textsuperscript{NTR} mediates self-association. A number of lines of evidence now suggest that both exogenously and endogenously expressed p75\textsuperscript{NTR} is capable of self-associating in the
absence of a ligand (24,25,33). Such interactions were measured here through crosslinking, immunoprecipitation and FRET analyses in live cells. The extracellular domains of p75\textsuperscript{NTR} do not dimerize in the absence of a ligand (25,33), and our studies revealed that, once cleaved by γ-secretase, the intracellular domain of p75\textsuperscript{NTR} also does not self-associate. In addition, the transmembrane domain alone is sufficient for self-association, consistent with reports that p75\textsuperscript{NTR} dimers are stabilized by an intermolecular disulfide bond mediated by C\textsubscript{257} within the transmembrane domain (35). Although covalent p75\textsuperscript{NTR}-immunoreactive complexes required C\textsubscript{257} (Figure 5a), we found that self-association, close enough for FRET, crosslinking and co-immunoprecipitation to occur (Figure 5a-d), remains possible when the cysteine is mutated to an alanine, indicating that other residues in the transmembrane domain also or alternatively mediate dimerization. The AxxxG motif within the transmembrane domain has also been implicated in mediating dimerization of p75\textsuperscript{NTR} (35). However, substitution of the AxxxG with LxxXL did not prevent p75\textsuperscript{NTR} transmembrane co-immunoprecipitation or the formation of higher molecular weight covalent and chemically crosslinked complexes. Nonetheless, we and others (35) found that mutation of this motif reduces the FRET signal between the mutant p75\textsuperscript{NTR} and a wildtype p75\textsuperscript{NTR} molecule. While such a finding can be indicative of loss of dimer association, FRET signals can also be reduced if the fluorophores are no longer in alignment i.e. if their orientation is rotated such that the emission dipole of the donor forms a 90º angle with the absorption dipole of the acceptor, preventing energy transfer (40). As our modeling predicted that the LxxXL mutations would change the crossing angle of the helices (Figure 4c), this would be likely to alter the relative orientation of both the transmembrane and intracellular domains, an explanation for the change in FRET (Figure 6b). We therefore interpret the reduced FRET signal in this scenario as indicating a change in the orientation of the transmembrane structure of p75-LxxXL relative to that of wildtype p75\textsuperscript{NTR}. While not necessarily preventing receptor dimerization, it is likely that these mutations change the interactions which make up the dimer interface.

Two modes of p75\textsuperscript{NTR} dimerization?

Our modeling of the transmembrane domain revealed that the AxxxG motif and the C\textsubscript{257} residues are on opposite faces of the transmembrane domain helix. However, it is possible that the disulfide bond and the AxxxG motif can mediate self-association independently. Rather than participating in the formation of a single dimer, p75\textsuperscript{NTR} might form a tetramer or larger complex through an alternating series of non-covalent AxxxG interactions and disulfide bonds (e.g. see ref 47). Indeed, in non-reducing conditions, minor p75\textsuperscript{NTR}-immunopositive complexes were observed at sizes greater than 200kDa (Figure 6a). Indeed, TrkA co-expression increased p75\textsuperscript{NTR} homodimerization, but this did not preclude Trk-p75\textsuperscript{NTR} FRET, indicating that larger heteromer complexes are possible. Furthermore, the size difference in the crosslinked and the disulfide bond-mediated p75\textsuperscript{NTR} immunoreactive complexes (see also 24), indicates that they may contain different constituents, i.e., that either the AxxxG motif or the transmembrane cysteine of p75\textsuperscript{NTR} facilitates interactions with a protein other than a second p75\textsuperscript{NTR}. The larger size of the DTT-reducible complex (greater than twice 75kDa) suggests that p75\textsuperscript{NTR} dimerizes through the AxxxG domain and covalently bonds to another larger protein (neither Trk nor sortilin have transmembrane cysteines, making them unlikely candidates). Alternatively, both the AxxxG domain and C\textsubscript{257} mediate structurally different homodimers, with formation of the C\textsubscript{257} covalent p75\textsuperscript{NTR} homodimer being permissive for its interaction with a third, smaller protein.

γ-secretase recognizes only half of a dimeric substrate at a time

Our experiments revealed that when p75\textsuperscript{NTR} could not be linked to a partner protein by a disulfide bond, the rate of γ-secretase cleavage was significantly reduced, suggesting the residue normally facilitates this cleavage event. There is emerging evidence that components of the γ-secretase complex (i.e., presenilin and APH-1) form dimers (16,46,48), and that it can recognize and cleave dimeric substrates (27,35), suggesting that the entire enzyme-substrate complex may be dimeric. As recognition of the N-terminal stump of the C-terminal fragment is a requirement for nicastrin to dock the protein substrate (12), we tested whether preventing α-cleavage and thus γ-cleavage of one p75\textsuperscript{NTR} monomer within the dimer substrate had an impact on γ-secretase processing of the other p75\textsuperscript{NTR} monomer. Little if
any effect on total levels of intracellular domain production was observed in this experiment (Figure 3b), demonstrating that α-secretase can recognize p75NTR monomers within homodimers. Interestingly, forced dimerization of p75NTR (through the addition of cysteines within its transmembrane domain), also has no discernable influence on rates of γ-secretase cleavage (35), and dimerization in its self is insufficient to promote γ-secretase cleavage of a widely studied γ-secretase substrate, amyloid protein precursor (APP)(31). This highlights an assumption in the field that when a dimeric receptor undergoes RIP, the entire dimer is processed by metalloprotease to generate a dimeric C-terminal fragment, with both fragments subsequently being cleaved by γ-secretases to release two intracellular domain fragments. Our work raises the possibility that only one half of a dimer receptor complex is processed by RIP at a time. Indeed, as inactive TrkA co-expression increased p75NTR FRET to a level equivalent to that between p75NTR receptors, inactive TrkA:p75NTR heterodimers might physically inhibit p75NTR RIP.

By contrast, a decrease in p75NTR FRET in neurons occurred within minutes of TrkA activation following the addition of NGF. This corresponds to the increased activity of the α-cleavage enzyme ADAM17 and production of the intracellular domain fragment (9), supporting our conclusion that γ-secretase is primarily responsible for the observation. However, it remains possible that TrkA dimerization and activation causes a conformational change in the bound homomonomer p75CTF fragments, facilitating cleavage by γ-secretase.

Mutations that alter transmembrane structure prevent γ-secretase cleavage of p75NTR

Our work indicates that transmembrane conformation, in particular changes induced in p75LxxxL and p75FxxxL proteins, is important for cleavage of p75NTR by γ-secretase. The molecular mechanism of RIP is not well understood; however, unraveling of the secondary structure of the helical transmembrane domain to expose the backbone carbonyl for γ-cleavage, rather than sequence conservation (49) or dimerization (31,35), is emerging as a key event in APP cleavage. It has been proposed that transmembrane glycine residues may enable destabilization of the α-helix to promote unraveling in the presence of the γ-secretase complex (50). Extending the length of the α-helical structure of the transmembrane domain, in exchange for the wildtype structure that becomes a random coil at the cleavage site, leads to a decrease in the rate of γ-secretase cleavage of APP (30), supporting the idea that the ability of the helices to uncoil is important. The LxxxL mutation in p75NTR may similarly stabilize the α-helix and thus prevent the transmembrane domain from unraveling to allow γ-secretase access to its substrate.

Alternatively, alterations to the transmembrane helical structure through mutation have the potential to shift the site of enzyme docking and thus introduce mismatched interactions between the γ-secretase site and the γ-secretase enzyme, significantly reducing its ability to catalyze intracellular domain release (32). It is also theoretically possible that GxxxG motifs within γ-secretase components (48) facilitate docking between the enzyme and its substrate. The AxxxxG motif within p75NTR directly flanks the γ-secretase cleavage site (AV/VVG), and disruption of this domain may therefore prevent its heterodimerization with enzyme components. One or a combination of these events would explain the reduced ability of γ-secretase to cleave the mutant proteins.

Functional significance of p75NTR cleavage

p75NTR has been identified as an important regulator of cell death and survival in the nervous system, including during neurodegeneration, with processing by RIP and disulfide bond formation a crucial step in the activation of cell death signaling (7,36,51,52). Here we show that modification of the transmembrane structure of p75NTR affects its cleavage by γ-secretase, in agreement with findings reported for APP (27-32). Our results indicate that merely inhibiting dimerization would not directly prevent RIP of p75NTR because each substrate receptor may be processed as a monomer. However receptor dimerization or structural changes caused or permitted by the transmembrane disulfide bond, which is necessary for cell death signaling, was shown here to facilitate γ-secretase cleavage, consistent with the generation of the intracellular domain fragment being required for transcription-dependent cell death (8). TrkA activation also promoted γ-secretase cleavage of both wildtype and mutant p75NTR, subsequent to increasing p75NTR self-association. Sortilin can
also regulate p75 NTR RIP (Skeldal et al., *JBC* MI# 2012/344710). Whether these influences are primarily due to physical interaction (herein), activation of secretases (9,11) and/or movements within membrane compartments (7) remains to be determined. Other heterodimer interactions might also alter the rate of $\gamma$-secretase cleavage, and impact on the ability of p75 NTR to signal (36,53), because they modulate the p75 NTR transmembrane structure. Further investigation of the effect of proteins that can associate with p75 NTR and modulate RIP are therefore warranted.

**REFERENCES**

1. Nykjaer, A., Lee, R., Teng, K. K., Jansen, P., Madsen, P., Nielsen, M. S., Jacobsen, C., Kliemannel, M., Schwarz, E., Willnow, T. E., Hempstead, B. L., and Petersen, C. M. (2004) Sortilin is essential for proNGF-induced neuronal cell death. *Nature* **427**, 843-848
2. Skeldal, S., Matusica, D., Nykjaer, A., and Coulson, E. J. (2011) Proteolytic processing of the p75 neurotrophin receptor: A prerequisite for signalling? *BioEssays* **33**, 614-625
3. Reichardt, L. F. (2006) Neurotrophin-regulated signalling pathways. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **361**, 1545-1564
4. Kanning, K. C., Hudson, M., Amieux, P. S., Wiley, J. C., Bothwell, M., and Schecterson, L. C. (2003) Proteolytic processing of the p75 neurotrophin receptor and two homologs generates C-terminal fragments with signaling capability. *J. Neurosci.* **23**, 5425-5436
5. Jung, K. M., Tan, S., Landman, N., Petrova, K., Murray, S., Lewis, R., Kim, P. K., Kim, D. S., Ryu, S. H., Chao, M. V., and Kim, T. W. (2003) Regulated intramembrane proteolysis of the p75 neurotrophin receptor modulates its association with the TrkA receptor. *J. Biol. Chem.* **278**, 42161-42169
6. Coulson, E. J., Reid, K., Baca, M., Shipham, K., Hulett, S. M., Kilpatrick, T. J., and Bartlett, P. F. (2000) Chopper, a new death domain of the p75 neurotrophin receptor which mediates rapid neuronal cell death. *J. Biol. Chem.* **275**, 30537-30545
7. Underwood, C. K., Reid, K., May, L. M., Bartlett, P. F., and Coulson, E. J. (2008) Palmitoylation of the C-terminal fragment of p75 NTR regulates death signaling and is required for subsequent cleavage by $\gamma$-secretase. *Mol. Cell. Neurosci.* **37**, 346-358
8. Kenchappa, R. S., Zampieri, N., Chao, M. V., Barker, P. A., Teng, H. K., Hempstead, B. L., and Carter, B. D. (2006) Ligand-dependent cleavage of the p75 neurotrophin receptor is necessary for NRIF nuclear translocation and apoptosis in sympathetic neurons. *Neuron* **50**, 219-232
9. Ceni, C., Kommaddi, R. P., Thomas, R., Vereker, E., Liu, X., McPherson, P. S., Ritter, B., and Barker, P. A. (2010) The p75 NTR intracellular domain generated by neurotrophin-induced receptor cleavage potentiates Trk signaling. *J. Cell Sci.* **123**, 2299-2307
10. Weskamp, G., Schlondorff, J., Lum, L., Becherer, J. D., Kim, T. W., Saftig, P., Hartmann, D., Murphy, G., and Blobel, C. P. (2004) Evidence for a critical role of the tumor necrosis factor alpha convertase (TACE) in ectodomain shedding of the p75 neurotrophin receptor (p75 NTR). *J. Biol. Chem.* **279**, 4241-4249
11. Kommaddi, R. P., Thomas, R., Ceni, C., Daigneault, K., and Barker, P. A. (2011) Trk-dependent ADAM17 activation facilitates neurotrophin survival signaling. *FASEB J* **25**, 2061-2070
12. Shah, S., Lee, S. F., Tabuchi, K., Hao, Y. H., Yu, C., LaPlant, Q., Ball, H., Dann, C. E., 3rd, Sudhof, T., and Yu, G. (2005) Nicastrin functions as a $\gamma$-secretase-substrate receptor. *Cell* **122**, 435-447
13. De Strooper, B., and Annaert, W. Novel research horizons for presenilins and gamma-secretases in cell biology and disease. *Annu. Rev. Cell Dev. Biol.* **26**, 235-260
14. Struhl, G., and Adachi, A. (2000) Requirements for presenilin-dependent cleavage of notch and other transmembrane proteins. *Mol. Cell* **6**, 625-636
15. Zhang, J., Ye, W., Wang, R., Wolfe, M. S., Greenberg, B. D., and Selkoe, D. J. (2002) Proteolysis of chimeric $\beta$-amyloid precursor proteins containing the Notch transmembrane domain yields amyloid $\beta$-like peptides. *J. Biol. Chem.* **277**, 15069-15075
16. Beel, A. J., and Sanders, C. R. (2008) Substrate specificity of $\gamma$-secretase and other intramembrane proteases. *Cell. Mol. Life Sci.* **65**, 1311-1334
17. Gorman, P. M., Kim, S., Guo, M., Melnyk, R. A., McLaurin, J., Fraser, P. E., Bowie, J. U., and Chakrabarty, A. (2008) Dimerization of the transmembrane domain of amyloid precursor proteins and familial Alzheimer's disease mutants. *BMC Neurosci* **9**, 17

18. Choi, S., Lee, E., Kwon, S., Park, H., Yi, J. Y., Kim, S., Han, I. O., Yun, Y., and Oh, E. S. (2005) Transmembrane domain-induced oligomerization is crucial for the functions of syndecan-2 and syndecan-4. *J. Biol. Chem.* **280**, 42573-42579

19. Dewes, I., and Mackenzie, K. R. (2007) Transmembrane domains of the syndecan family of growth factor coreceptors display a hierarchy of homotypic and heterotypic interactions. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 20782-20787

20. Liu, W., Crocker, E., Zhang, W., Elliott, J. I., Luy, B., Li, H., Aimoto, S., and Smith, S. O. (2005) Structural role of glycine in amyloid fibrils formed from transmembrane alpha-helices. *Biochemistry (Mosc)*. **44**, 3591-3597

21. Abad, C., Martinez-Gil, L., Tamborero, S., and Mingarro, I. (2009) Membrane topology of gp41 and amyloid precursor protein: interfering transmembrane interactions as potential targets for HIV and Alzheimer treatment. *Biochim. Biophys. Acta* **1788**, 2132-2141

22. Kleiger, G., Grothe, R., Mallick, P., and Eisenberg, D. (2002) GXXXG and AXXAX: common alpha-helical interaction motifs in proteins, particularly in extremophiles. *Biochemistry (Mosc)*. **41**, 5990-5997

23. Sakamoto, K., Chao, W. S., Katsube, K., and Yamaguchi, A. (2005) Distinct roles of EGF repeats for the Notch signaling system. *Exp. Cell Res.* **302**, 281-291

24. Vilar, M., Charalamopoulos, I., Kenchappa, R. S., Simi, A., Karacea, E., Reversi, A., Choi, S., Bothwell, M., Mingarro, I., Friedman, W. J., Schiavo, G., Bastiaens, P. I., Verveer, P. J., Carter, B. D., and Ibanez, C. F. (2009) Activation of the p75 neurotrophin receptor through conformational rearrangement of disulphide-linked receptor dimers. *Neuron* **62**, 72-83

25. Scheuermann, S., Hambsch, B., Hesse, L., Stumm, J., Schmidt, C., Beher, D., Bayer, T. A., Beyreuther, K., and Multhaup, G. (2001) Homodimerization of amyloid precursor protein and its implication in the amyloidogenic pathway of Alzheimer's disease. *J. Biol. Chem.* **276**, 33923-33929

26. Munter, L. M., Botev, A., Richter, L., Hildebrand, P. W., Althoff, V., Weiße, C., Kaden, D., and Multhaup, G. (2007) GXXXG motifs within the amyloid precursor protein (APP) transmembrane sequence are critical for the etiology of Aβ42. *EMBO J.* **26**, 1702-1712

27. Miyashita, N., Straub, J. E., Thirumalai, D., and Sugita, Y. (2009) Transmembrane structures of amyloid precursor protein dimer predicted by replica-exchange molecular dynamics simulations. *J. Am. Chem. Soc.* **131**, 3438-3439
33. 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

34. Gong, Y., Cao, P., Yu, H. J., and Jiang, T. (2008) Crystal structure of the neurotrophin-3 and p75<sup>NTR</sup> symmetrical complex. *Nature* **454**, 789-793

35. Vilar, M., Charalampopoulos, I., Kenchappa, R. S., Reversi, A., Klos-Applequist, J. M., Karaca, E., Simi, A., Spuch, C., Choi, S., Friedman, W. J., Ericson, J., Schiavo, G., Carter, B. D., and Ibanez, C. F. (2009) Ligand-independent signaling by disulfide-crosslinked dimers of the p75 neurotrophin receptor. *J. Cell Sci.* **122**, 3351-3357

36. Sotthibundhu, A., Sykes, A. M., Fox, B., Underwood, C. K., Thangnipon, W., and Coulson, E. J. (2008) β-amyloid<sub>42</sub> induces neuronal death through the p75 neurotrophin receptor. *J. Neurosci.* **28**, 3941-3946

37. Erickson, M. G., Alseikhan, B. A., Peterson, B. Z., and Yue, D. T. (2001) Preassociation of calmodulin with voltage-gated Ca<sup>2+</sup> channels revealed by FRET in single living cells. *Neuron* **31**, 973-985

38. Abankwa, D., and Vogel, H. (2007) A FRET map of membrane anchors suggests distinct microdomains of heterotrimeric G proteins. *J. Cell Sci.* **120**, 2953-2962

39. Abankwa, D., Hanzal-Bayer, M., Ariotti, N., Plowman, S. J., Gorfe, A. A., Parton, R. G., McCammon, J. A., and Hancock, J. F. (2008) A novel switch region regulates H-ras membrane orientation and signal output. *EMBO J.* **27**, 727-735

40. Meyer, B. H., Segura, J. M., Martinez, K. L., Hovius, R., George, N., Johnsson, K., and Vogel, H. (2006) FRET imaging reveals that functional neurokinin-1 receptors are monomeric and reside in membrane microdomains of live cells. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 2138-2143

41. Lee, K. F., Li, E., Huber, L. J., Landis, S. C., Sharpe, A. H., Chao, M. V., and Jaenisch, R. (1992) Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. *Cell* **69**, 737-749

42. Mackenzie, K. R., Prestegard, J. H., and Engelman, D. M. (1997) A transmembrane helix dimer: structure and implications. *Science* **261**, 131-133

43. DeLano, W. L. (2002) The PyMOL molecular graphics system. in *DeLano Scientific, San Carlos, CA, USA*

44. Chakravarthy, B., Gaudet, C., Menard, M., Atkinson, T., Brown, L., Laferla, F. M., Armato, U., and Whitfield, J. (2010) Amyloid-β peptides stimulate the expression of the p75<sup>NTR</sup> neurotrophin receptor in SHSY5Y human neuroblastoma cells and AD transgenic mice. *J Alzheimer’s Dis* **19**, 915-925

45. Patterson, G. H., Piston, D. W., and Barisas, B. G. (2000) Forster distances between green fluorescent protein pairs. *Anal. Biochem.* **284**, 438-440

46. Schroeter, E. H., Ilagan, M. X., Brunkan, A. L., Hecimovic, S., Li, Y. M., Xu, M., Lewis, H. D., Saxena, M. T., De Strooper, B., Coonrod, A., Tomita, T., Iwatsubo, T., Moore, C. L., Goate, A., Wolfe, M. S., Shearman, M., and Kopan, R. (2003) A presenilin dimer at the core of the γ-secretase enzyme: insights from parallel analysis of Notch 1 and APP proteolysis. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 13075-13080

47. Lee, S. F., Shah, S., Yu, C., Wigley, W. C., Li, H., Lim, M., Pedersen, K., Han, W., Thomas, P., Lundkvist, J., Hao, Y. H., and Yu, G. (2004) A conserved GXXXG motif in APH-1 is critical for assembly and activity of the gamma-secretase complex. *J. Biol. Chem.* **279**, 4144-4152

48. Ye, J., Dave, U. P., Grishin, N. V., Goldstein, J. L., and Brown, M. S. (2000) Asparagine-proline sequence within membrane-spanning segment of SREBP triggers intramembrane cleavage by site-2 protease. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 5123-5128

49. Li, S. C., and Deber, C. M. (1992) Glycine and beta-branched residues support and modulate peptide helicity in membrane environments. *FEBS Lett.* **311**, 217-220
51. Geetha, T., Kenchappa, R. S., Wooten, M. W., and Carter, B. D. (2005) TRAF6-mediated ubiquitination regulates nuclear translocation of NRIF, the p75 receptor interactor. *EMBO J.* **24**, 3859-3868

52. Del Valle, J., Duran-Vilaregut, J., Manich, G., Casadesus, G., Smith, M. A., Camins, A., Pallas, M., Pelegri, C., and Vilaplana, J. (2010) Early amyloid accumulation in the hippocampus of SAMP8 mice. *J. Alzheimers Dis* **19**, 1303-1315

53. Fombonne, J., Rabizadeh, S., Banwait, S., Mehlen, P., and Bredesen, D. E. (2009) Selective vulnerability in Alzheimer's disease: amyloid precursor protein and p75<sup>NTR</sup> interaction. *Ann. Neurol.* **65**, 294-303

**FOOTNOTES**

This work was supported by the National Health and Medical Research Council of Australia (NHMRC) project grant funding (569507 and 10012610). EJC and JMH were supported by NHMRC Career Development (RD Wright) Fellowships (569601 and 401748 respectively). We thank Rowan Tweedale for critical reading of the manuscript and helpful discussions.

The abbreviations used are: APP, amyloid precursor protein; CTF, C-terminal fragment; DRG, dorsal root ganglia; DSP, dithiobis succinimidylpropionate; DSS, disuccinimidyl suberate; ECD, extracellular domain; FL: full-length; ICD, intracellular domain; p75<sup>NTR</sup>, p75 neurotrophin receptor; PMA, phorbol-12-myristate-13-acetate; RIP, regulated intramembrane proteolysis; TM, transmembrane domain; TNFR, tumor necrosis factor receptor.
**FIGURE LEGENDS**

**Figure 1. p75\textsuperscript{NTR} forms homodimers primarily at the cell surface**

(a) Western Blot of lysates of PC12 cells expressing endogenous p75\textsuperscript{NTR} or HEK293 cells expressing transfected p75\textsuperscript{FL}. Expression of p75\textsuperscript{NTR} is increased to a similar extent to that in transfected HEK293 cells when subjected to treatments mimicking pathological treatment with amyloid β (Aβ) or exposure to UV (b) Extent of cell survival, as measured by an acid phosphatase (AP) viability assay, of p75\textsuperscript{NTR}-deficient PC12 cells overexpressing p75\textsuperscript{FL} or p75\textsuperscript{FL-YFP}, 24 hours after transfection. *** p<0.001 N=2 experiments in triplicate. (c) Western blots of lysates of HEK293 cells expressing either p75\textsuperscript{FL} or p75\textsuperscript{FL-YFP}. Where indicated, cells were treated with DSS to crosslink proteins prior to cell lysis. In each case, p75\textsuperscript{NTR}-immunopositive crosslinked complexes are twice the size of the monomeric species. (d) Confocal images of transfected HEK293 cells expressing p75\textsuperscript{FL-YFP} or p75\textsuperscript{ECD-YFP}. p75\textsuperscript{NTR} (green) is predominantly located in the plasma membrane. (e) Pixel-based FRET analysis of a confocal slice through two HEK293 cells transiently transfected with full-length p75\textsuperscript{NTR} (p75\textsuperscript{FL-CFP} and p75\textsuperscript{FL-YFP}) constructs, generating proteins fused to donor/acceptor fluorophores at the p75\textsuperscript{NTR} C-terminus. Heat map indicates the intensity of the FRET signal: black/red indicates where FRET is maximal (at the plasma membrane, arrows) and yellow/white indicates where FRET is occurring at a lower level/frequency. (f) Pixel-based FRET analysis of a confocal slice through HEK293 cells transiently transfected with truncated p75\textsuperscript{NTR} (p75\textsuperscript{ECD-CFP} and p75\textsuperscript{ECD-YFP}) constructs. Heat map indicates the intensity of the FRET signal. Arrows indicate higher FRET frequency occurring at the plasma membrane. (g) FRET analysis as determined by E\textsubscript{max} calculation of p75\textsuperscript{NTR} extracellular domain constructs (p75\textsuperscript{ECD}) fused to CFP or YFP. Non-specific interactions were standardized to the membrane-tethered control marker (CTH-7A-L, see Methods), demonstrating significant p75\textsuperscript{ECD} self-association. *** p<0.001 (mean ± SEM, N = 8 experiments).

**Figure 2. The transmembrane domain of p75\textsuperscript{NTR} mediates self-association**

(a) FRET ratio of HEK293T cells expressing either control proteins (CFP-YFP; YFP) and/or p75\textsuperscript{FL} as outlined in the figure (blue represents the CFP donor, yellow represents the YFP acceptor). FRET ratios for each p75\textsuperscript{NTR} pair were compared (ANOVA) to those of the negative control (column 2) *** p<0.001 (mean ± SEM, N = 5 experiments). (b) FRET ratio of interactions between truncated forms of p75\textsuperscript{NTR} (p75\textsuperscript{ECD}, p75\textsuperscript{CTR}, p75\textsuperscript{ICD}) with each other, as outlined in the figure. FRET ratios for each p75\textsuperscript{NTR} pair were compared (ANOVA) to those of the negative control (panel a, column 2) *** p<0.001 (mean ± SEM, N = 4 experiments; ANOVA). The intracellular domain fragments of p75\textsuperscript{NTR} had a FRET ratio that was not significantly different from that of random interactions. (c) The FRET ratio calculated for p75\textsuperscript{NTR} truncation and p75\textsuperscript{AAA} constructs fused to YFP when co-transfected with p75\textsuperscript{FL-CFP}. FRET ratios for p75\textsuperscript{ECD} and p75\textsuperscript{TM} paired with p75\textsuperscript{FL} were compared (ANOVA) to those of the negative control (panel a, column 2). p75\textsuperscript{AAA} paired with p75\textsuperscript{FL} had a FRET ratio that was significantly lower than that of p75\textsuperscript{FL}:p75\textsuperscript{FL} (panel a column 4) *** p<0.001 (mean ± SEM; N = 4 experiments; ANOVA). (d) Confocal micrograph of a HEK293 cell expressing p75\textsuperscript{TM-YFP}, showing its location at the plasma membrane of the cell. (e) Western blots of immunoprecipitates and lysates of HEK293 cells transfected with either p75\textsuperscript{FL}, p75\textsuperscript{Ngly} or p75\textsuperscript{ICD} constructs and co-transfected with p75\textsuperscript{TM-YFP}. p75\textsuperscript{FL} and p75\textsuperscript{Ngly} but not p75\textsuperscript{ICD} was immunoprecipitated by the minimal p75\textsuperscript{NTR} transmembrane domain protein (representative of N=3 experiments). FL: full-length p75\textsuperscript{NTR}; CTF: C-terminal fragment; ICD: intracellular domain fragment; IP: immunoprecipitation; WB: Western blot.

**Figure 3. p75\textsuperscript{NTR} undergoes γ-secretase cleavage which does not require dimerization**

(a) The FRET ratio between p75\textsuperscript{FL-CFP} and p75\textsuperscript{FL-YFP} in transfected HEK293T cells calculated under conditions designed to manipulate p75\textsuperscript{NTR} proteolysis, compared with the FRET ratio of p75\textsuperscript{NTR}-transfected cells under basal conditions (top panel, column 1; ANOVA). Effects of the manipulations are illustrated in the Western blot of p75\textsuperscript{NTR} lacking the YFP epitope (lower panels). Overexposure of the Western blot illustrates that basal cleavage occurs in untreated cells (bottom; see also panel b, in which all conditions contain β-clasto-lactacystin). α- and γ-cleavage were activated with PMA, intracellular domain degradation was inhibited with β-clasto-lactacystin (CL), α-cleavage was inhibited with TAPI-2 or the p75\textsuperscript{Ngly} mutation, and γ-secretase was inhibited with compound E (Cmp E). Inhibition of α- and γ-secretases by TAPI-2, the non-cleavable Ngly mutation or compound E significantly increased the p75\textsuperscript{NTR} FRET ratio, whereas release of the p75\textsuperscript{NTR} intracellular domain...
promoted by PMA significantly reduced the FRET ratio. ** p<0.01; *** p<0.001 (mean ± SEM, N = 4-8 experiments). CTF: C-terminal fragment, ICD: intracellular domain fragment. (b) Western blots of cell lysates from HEK293 transfected with p75FL either alone or together with p75TM or p75Ngly-TM (not detectable by the anti-p75NTR antibody) and treated with PMA to induce α- and γ-cleavage and compound E to inhibit γ-cleavage (as marked; all conditions contain the proteasome inhibitor, CL). Co-expression of these constructs had no appreciable effect on the rate at which full-length p75NTR underwent RIP and there was no change in the CTF:ICD ratios.

Figure 4. TrkA regulates p75NTR dimerization and RIP
(a) The FRET ratios measured live between p75CTF and p75FL pairs in transfected HEK293 cells following the addition of 100ng/ml NGF. (b) The FRET ratio of p75FL constructs in transfected HEK293 cells 24 hours after the addition of 100ng/ml NGF or 40ng/ml proNGF. Co-transfection with sortilin, TrkA or kinase-inactive TrkAK538R is as indicated. (c) FRET analysis as determined by the E_{max} of cleavable p75NTR extracellular domain constructs (p75ECD) and non-cleavable extracellular domain constructs (p75ECD-Ngly) fused to CFP or YFP. Non-specific interactions were standardized to the membrane marker CTH-7A-L (column 1). By comparison, there was significant p75ECD self-association (# p<0.001). TrkA co-expression significantly decreased whereas TrkAK538R significantly increased p75ECD FRET. TrkA co-expression had no significant effect on p75ECD-Ngly FRET. p75ECD FRET E_{max} values were compared to the E_{max} values of p75ECD without co-receptor (mean ± SEM, N = 7-8 experiments). (d) Levels of FRET between TrkA ECD-YFP and p75ECD-CFP or p75ECD-Ngly-CFP constructs. E_{max} values were compared to the E_{max} values of p75ECD-YFP co-transfected with control CTH-7A-L. p75NTR-TrkA FRET was not significantly altered after the application of NGF or introduction of the uncleavable N-glycosylation mutation (mean ± SEM, N = 4 experiments; significance is relative to column 2, except where indicated by # which is non-significant compared to column 3). (e) Co-expression of TrkA, but not TrkAK538R, significantly reduced the FRET ratio of two p75CTF proteins (mean ± SEM, N = 3 experiments). (f) The TrkA activity-induced decrease in p75NTR FRET was prevented by treatment of cells with the γ-secretase inhibitor compound E (Cmp E). (g) FRET ratios calculated for DRG neurons isolated from p75NTR-/- animals expressing p75FL FRET constructs in the presence or absence of TAPI-2 or compound E as measured live over a period of 9 minutes following the administration of 5 ng/ml NGF (mean ± SEM; n >5 neurons per condition from 3 experiments). * p<0.05, **p<0.01, *** p<0.001

Figure 5. Model of the p75NTR transmembrane domain dimer
(a) The NMR structure of the glycoprophorin A (GpA) dimer (PDB ID 1AFO)(42) compared with the predicted transmembrane domain structures of wildtype p75NTR (b) and the p75-LxxxL mutant (c). The GpA dimer contains residues GpVxxGV together with I_{Gp} as the central packing contacts, whereas the p75NTR dimer utilizes A_{p75NTR}VxxGL and I_{p75NTR}. Glycine and alanine residues comprising the GxxxG or AxxxG motif are shown in orange for one monomer and the branched hydrophobic residues in the opposite monomer are shown in blue. C_{p75NTR} proposed to form a disulfide bond is shown in red. The introduction of leucine residues at positions 262 and 266 of the p75LxxxL mutant would result in steric clashes (indicated by circles) that would alter helix packing. (d) Sequence alignment of the transmembrane domains of GpA, p75NTR and TNFR, with glycine and alanine residues that putatively facilitate helix-helix interaction highlighted in orange, and cysteine residues in red.

Figure 6. Residues within the transmembrane domain of p75NTR are required for γ-secretase cleavage but not dimerization
(a) Western blots of lysates of HEK293 cells expressing either p75FL, p75C257A, p75TNFR or p75LxxxL. Where indicated, lysates did not contain DTT (+DTT), or, prior to lysis, cells were treated with DSS to crosslink proteins (+DSS). p75NTR-immunopositive DTT-reducible (*) or crosslinked (#) complexes are indicated. p75C257A did not form a covalent complex (>). (b) The FRET ratio calculated for p75NTR mutant constructs fused to YFP when co-transfected with p75FL-CFP. The FRET ratio for each set of p75NTR mutant constructs was compared to that for wildtype p75FL homo-oligomers (column 1). Equivalent FRET ratios were observed between two full-length p75NTR proteins and p75FL:p75C257A, whereas the p75FL:p75TNFR and p75FL:p75LxxxL FRET ratios were significantly reduced. *** p<0.001 (mean ± SEM, N = 4-7 experiments). (c) Western blots of immunoprecipitates and lysates of HEK293 cells transfected with p75NTR mutant constructs and co-transfected with p75TM-YFP, p75C257A, p75TNFR
and p75LxxxL (and their C-terminal fragments) were co-immunoprecipitated by the minimal p75NTR transmembrane domain protein. (d) Western blot of HEK293 cells transfected with p75FL, p75C257A, p75TNFR or p75LxxxL undergoing basal rates of cleavage or enhanced rates of cleavage following stimulation with PMA (+) for 3 hours. All conditions contain a proteasome inhibitor so as to visualize the intracellular domain fragment. p75C257A had a significant reduction in the rate of γ-cleavage following PMA stimulation compared to the rate of p75NTR cleavage. A comparison of the ratio of densitometry measurements of C-terminal fragments to intracellular domain bands in lysates of cells transfected with p75FL-YFP and p75C257A-YFP following PMA treatment (mean ± SEM; N=4 experiments) revealed that p75C257A produces significantly less intracellular domain fragment relative to wildtype p75NTR (p<0.01, t-test). Increased levels of the C-terminal fragment were detected in cells transfected with p75TNFR or p75LxxxL. However, even following PMA stimulation no intracellular domain fragment was detected. Blots are representative of 4 experiments.

(e) Western blot of p75NTR-deficient PC12 cells transfected with p75FL, p75C257A, p75TNFR, p75LxxxL or p75Ngly undergoing basal rates of cleavage or cleavage stimulated by treatment with PMA or NGF for 4 hours. All conditions contain a proteasome inhibitor. While all mutant constructs except p75Ngly produced intracellular domain fragments, the rates of γ-secretase cleavage were significantly reduced, resulting in significant accumulation of the C-terminal fragments (C-terminal fragment intensity was normalized to the intensity of full-length p75NTR in each lane and averaged for 3 experiments before being compared to the level of C-terminal fragment in unstimulated p75FL-transfected cells). Blots are representative of 3 experiments.
Table 1. *Description of expression constructs used in the study.*

The p75NTR signal peptide is residues 1-28. α-secretase cleaves between V242 and V243, the transmembrane domain is between residues D250 and K275-R276, and γ-secretase cleaves between V263 and V264.

| Vector name | Amino acid sequence | Description of expressed protein |
|-------------|---------------------|----------------------------------|
| p75FL       | 1-28, 29-425        | Full-length rat p75NTR           |
| p75ECD      | 1-28, 29-287        | Intracellular domain deletion    |
| p75CTF      | 1-31, 228-425       | Extracellular domain deletion mimicking the C-terminal fragment of p75NTR |
| p75ICD      | 1-28, 274-425       | Extracellular and transmembrane domain deletion |
| p75Ngly     | V243N               | N-linked glycan attachment site (NVT) is generated which prevents α-cleavage (7) |
| p75AAA      | PPP295-297AAA       | Prolines 295-297 exchanged for three alanine residues |
| p75TM       | 1-28, 228-287       | Extracellular and intracellular domain deletion |
| p75Ngly-TM  | 1-28, 228-287; V243N| Extracellular and intracellular domain deletion containing N-linked glycan attachment site. |
| p75TNFR     | 251-273 exchanged for transmembrane domain of TNFR | Chimera of p75NTR with the rat TNFR transmembrane domain (amino acids 212-234 of TNFR; see Fig. 4d) |
| p75LxxxL    | A262L and G266L mutations | Mutates putative AxxxG dimerization motif to LxxxL, where x is any amino acid. |
Figure 1

a. PC12

PC12 + 20 μM Aβ + UV

HEK293/p75-FL

WB: p75^imm

WB: tubulin

b. Relative cell survival

Control CFP + p75-ECD-YFP

p75-ECD-CFP + p75-ECD-YFP

p75-FL + UV

p75-FL-YFP

---

Relative cell survival (AP assay; OD 405nm)

blank

untransfected control

transfection

p75-FL

p75-FL-YFP

---


c. p75-FL p75-FL-YFP

d. p75-FL-YFP

p75-ECD-YFP

---

FRET ratio

2 4 6 8 10

2 4 6 8 10

2 4 6 8 10

p75:FL:p75FL

p75-ECD:p75-ECD

---

E_max (%) 30

Control CFP + p75-ECD-YFP

p75-ECD-CFP + p75-ECD-YFP

---

by guest on March 23, 2020
Figure 3

(a) FRET ratio

- untreated
- PMA
- PMA + CL
- TAPI-2
- p75-Ngly
- Cmp E

(b) Western blot analysis

- FL
- CTF
- ICD

Longer exposure
Figure 4

a) % change in FRET vs Time after NGF administration (mins)

b) FRET ratio vs Ligand: - NGF pro - NGF pro - NGF pro

p75-FL: p75-FL
p75-ECD: p75-ECD
p75NTR+ sortilin p75 NTR + TrkA
p75NTR+ TrkAK538R

---

c) E_{max} (%) vs Ligand: +TrkA +TrkAK538R +TrkA

---

d) E_{max} (%) vs Ligand: - NGF - NGF - NGF

---

e) FRET ratio vs Ligand: +TrkA +TrkAK538R

---

f) FRET ratio vs Ligand: Cmp E : - - + +

---

g) % change in FRET vs Time after NGF administration (mins)

Legend:
- NGF
- NGF + TAPI-2
- NGF + CmpE
Figure 5

d
|     |     | ITLIIFGVMAGVIGTILLISYG | NLIIPVYCSILAAVVVGLYAYIAF | VLLPLVIFLGLCILLLICSLLC |
|-----|-----|-------------------------|---------------------------|-------------------------|
| GpA | 73  | I76 V80 G79 G83         | I259 V263 A262 G266      | L262 L266               |
| p75 NTR | 251 | L262 G266 I259 V263   | L267 C257 C257           |                         |
| TNFR | 213 |                         |                           |                         |
Figure 6

**a**

[Image showing a gel with bands labeled FL, CTF, and ICD for FLp75, p75C257A, p75-LxxxL, and p75-TNFR.]

**b**

[Bar graph showing FRET ratio with DTT and DSS treatments, with asterisks indicating statistical significance.]

**c**

[Image showing immunoprecipitation (IP) and Western blot (WB) analysis for p75C257A and p75-TNFR with and without FL, CTF, and ICD.]

**d**

[Graph showing CTF relative to ICD in HEK293 cells with PMA stimulation and NGF treatment, with ratios and standard deviations indicated.]
The effects of transmembrane sequence and dimerization on cleavage of the p75 neurotrophin receptor by \( \gamma \)-secretase

Alex M. Sykes, Nickless Palstra, Daniel Abankwa, Justine M. Hill, Sune Skeldal, Dusan Matusica, Prahatha Venkatraman, John F. Hancock and Elizabeth J. Coulson

*J. Biol. Chem.* published online October 26, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.382903

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts