

# Activation of the p75 neurotrophin receptor through conformational rearrangement of disulphide-linked receptor dimers

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## SUMMARY

Ligand-mediated dimerization has emerged as a universal mechanism of growth factor receptor activation. Neurotrophins interact with dimers of the p75 neurotrophin receptor (p75<sup>NTR</sup>), but the mechanism of receptor activation has remained elusive. Here we show that p75<sup>NTR</sup> forms disulphide-linked dimers independently of neurotrophin binding through the highly conserved Cys<sup>257</sup> in its transmembrane domain. Mutation of Cys<sup>257</sup> abolished neurotrophin-dependent receptor activity but did not affect downstream signaling by the p75<sup>NTR</sup>/NgR/Lingo-1 complex in response to MAG, indicating the existence of distinct, ligand-specific activation mechanisms for p75<sup>NTR</sup>. FRET experiments revealed a close association of p75<sup>NTR</sup> intracellular domains that was transiently disrupted by conformational changes induced upon NGF binding. Although mutation of Cys<sup>257</sup> did not alter the oligomeric state of p75<sup>NTR</sup>, the mutant receptor was no longer able to propagate conformational changes to the cytoplasmic domain upon ligand binding. We propose that neurotrophins activate p75<sup>NTR</sup> by a mechanism involving rearrangement of disulphide-linked receptor subunits.

## INTRODUCTION

The neurotrophins are a family of neurotrophic factors that control multiple aspects of nervous system development and function, including neurogenesis, neuronal differentiation, cell survival, neurite outgrowth, target innervation and synaptic plasticity (Bibel and Barde, 2000). Four neurotrophins are present in mammals: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). The neurotrophins are synthesized as a pre-pro-peptide from which the mature portion is released by proteolytic cleavage. The mature forms of neurotrophins interact with two distinct receptors, a cognate member of the Trk receptor tyrosine kinase family and the common p75 neurotrophin receptor (p75<sup>NTR</sup>), a member of the Tumor Necrosis Factor Receptor (TNFR) superfamily of death receptors (Friedman and Greene, 1999; Kaplan and Miller, 2000; Lee et al., 2001a; Patapoutian and Reichardt, 2001). On the other hand, unprocessed neurotrophins (pro-neurotrophins) are thought to display selectivity for p75<sup>NTR</sup> over Trk receptors (Lee et al., 2001b) with the aid of the co-receptor sortilin (Nykjaer et al., 2004). While this interaction is thought to preferentially lead to cell death (Lee et al., 2001b), cell survival is mediated by Trk signaling (Patapoutian and Reichardt, 2001). A key issue in neurotrophin research is the elucidation of the molecular mechanisms underlying each of the physiological actions of the neurotrophins in different cell types, throughout development, and in pathological situations.

Unlike Trk receptors, p75<sup>NTR</sup> lacks catalytic activity. The intracellular region of p75<sup>NTR</sup> contains a flexible juxtamembrane segment followed by a globular domain known as the death domain (Liepinsh et al., 1997). Signal transduction by p75<sup>NTR</sup> is thought to proceed via ligand-dependent recruitment and release of cytoplasmic effectors to and from the receptor. Over 20 different intracellular interactors of p75<sup>NTR</sup> have been identified to date (Barker, 2004; Bronfman and Fainzilber, 2004; Dechant and Barde, 2002; Roux and Barker, 2002), but this wealth of interactions has not translated in a comparable understanding of receptor function. Some of the major downstream

signaling events triggered by p75<sup>NTR</sup> in response to neurotrophins include activation of NF- $\kappa$ B (Carter et al., 1996), c-jun kinase (JNK) (Friedman, 2000; Yoon et al., 1998), and caspases (Troy et al., 2002). In addition, p75<sup>NTR</sup> can also activate the small GTPase RhoA (Yamashita et al., 1999), but this requires a different set of ligands derived from myelin, such as myelin-associated glycoprotein (MAG) and Nogo (Wang et al., 2002; Wong et al., 2002; Yamashita et al., 2002), and two different co-receptors: a lipid-anchored ligand-binding subunit known as the Nogo receptor (NgR) (Fournier et al., 2001) and Lingo-1 (Mi et al., 2004). In addition, p75<sup>NTR</sup> is also known to undergo proteolytic cleavage upon activation by several of its ligands or induction of membrane metalloproteases with phorbol esters (Jung et al., 2003; Kanning et al., 2003). The release of the p75<sup>NTR</sup> intracellular domain following intramembrane cleavage by  $\gamma$ -secretase is thought to be required for activation of some signaling pathways, such as NRIF-mediated neuronal death of sympathetic neurons (Kenchappa et al., 2006).

Receptor oligomerization has been recognized as a crucial step in the activation of many plasma membrane receptors. In receptors carrying intrinsic kinase activity, ligand-mediated dimerization stabilizes the active conformation of the kinase domain by receptor *trans*-phosphorylation (Schlessinger, 2002). In non-catalytic receptors, such as cytokine and death receptors, oligomerization of intracellular domains results in recruitment of adaptors or activation of intracellularly bound effectors (Ashkenazi and Dixit, 1999; Constantinescu et al., 1999). However, while necessary, receptor oligomerization may not always be sufficient for signaling. Several studies have indicated that some cytokine and growth factor receptors exist in a pre-assembled dimeric complex prior to ligand stimulation, and that receptor activation results from a ligand-mediated change in the relative orientation of the two receptor protomers (Jiang and Hunter, 1999; Seubert et al., 2003).

Although p75<sup>NTR</sup> was discovered over 20 years ago, and has since been intensively studied, its mechanism of activation has remained mysterious. Numerous studies have reported crosslinking of radiolabeled neurotrophins to p75<sup>NTR</sup> dimers and higher-order



oligomers (Hempstead et al., 1991; Mahadeo et al., 1994; Rydén et al., 1997b), demonstrating the ability of these ligands to directly bind oligomeric forms of this receptor. Based on this evidence, and the dimeric nature of neurotrophins, it has been assumed that these ligands activate p75<sup>NTR</sup> by classical ligand-mediated dimerization. A recent study has brought support to this notion by solving the x-ray crystal structure of a 2:2 complex between NT-3 and p75<sup>NTR</sup> (Gong et al., 2008). In the same work, a 2:2 complex between NGF and p75<sup>NTR</sup> could also be purified, and it was shown that the glycosylated extracellular domain of p75<sup>NTR</sup> is monomeric in solution in the absence of ligand, suggesting a general model of p75<sup>NTR</sup> dimerization by all neurotrophins. In the present study, we investigated and characterized endogenous determinants of receptor dimerization. We discovered that full-length p75<sup>NTR</sup> forms disulphide-linked dimers in the absence of neurotrophins through the highly conserved Cys<sup>257</sup> in its transmembrane domain. Functional analysis of a p75<sup>NTR</sup> mutant lacking Cys<sup>257</sup> revealed the functional importance of disulphide-mediated receptor dimerization and suggested a novel mechanism of p75<sup>NTR</sup> activation by neurotrophins.

## RESULTS

### **Transmembrane Cys<sup>257</sup> mediates formation of disulphide-linked p75<sup>NTR</sup> dimers**

Surface expression of p75<sup>NTR</sup> was assessed in transfected cells following biotinylation of cell surface proteins, immunoprecipitation and gel electrophoresis under non-reducing and reducing conditions. This analysis revealed that wild type p75<sup>NTR</sup> forms disulphide-linked dimers in the plasma membrane of transfected cells (Figure 1A). At high levels of expression in COS-7 cells, comparable amounts of cell surface p75<sup>NTR</sup> monomers and disulphide-linked dimers could be detected under non-reducing conditions (Figure 1A). However, the proportion of disulphide-linked p75<sup>NTR</sup> dimers in transfected cells depended upon the level of expression of the receptor. At moderate overexpression levels, less than 10% of wild type p75<sup>NTR</sup> was disulphide-linked (Figure 1B). NGF treatment had no effect on the proportion of disulphide-linked p75<sup>NTR</sup> dimers

(data not shown). Since neither the extracellular (Gong et al., 2008; He and Garcia, 2004) or intracellular (Liepinsh et al., 1997) domains of p75<sup>NTR</sup> have been reported to form disulphide-linked dimers, we turned our attention to the transmembrane domain of the receptor, which contains a Cys residue (Cys<sup>257</sup>) that is absolutely conserved in all p75<sup>NTR</sup> orthologs known to date, from echinoderms to mammals (Bothwell, 2006) (Figure 1C). Mutation of Cys<sup>257</sup> to Alanine (C257A) abolished the ability of p75<sup>NTR</sup> to form disulphide-linked dimers (Figure 1A), indicating that such dimers are generated by disulphide bonding of two p75<sup>NTR</sup> molecules through Cys<sup>257</sup> in the transmembrane domain. Disulphide-linked dimers could also be detected to varying degrees on the surface of cells endogenously expressing p75<sup>NTR</sup>, such as PC12 pheochromocytoma, RN22 Schwannoma and sympathetic neurons from the rat superior cervical ganglion (SCG) (Figure 1D), as well as in total extracts of newborn cortex, hippocampus and cerebellum (Figure 1E). Interestingly, in the latter cases the majority of p75<sup>NTR</sup> was disulphide-linked. Of note, a previous study found high levels of disulphide-linked p75<sup>NTR</sup> dimers in one melanoma cell line (A875) but not in another (HS294) (Grob et al., 1985). Importantly, the C257A mutation did not affect the ability of p75<sup>NTR</sup> to bind NGF (Figure 1F), nor its capacity to undergo  $\gamma$ -secretase-dependent intramembrane cleavage upon stimulation with the phorbol ester PMA (Figure 1G).

### **Cys<sup>257</sup> is essential for p75<sup>NTR</sup> signaling in response to neurotrophins**

The ability of native p75<sup>NTR</sup> to form disulphide-linked dimers prompted the question of its possible significance to p75<sup>NTR</sup> activation and downstream signaling. We therefore investigated the effects of the C257A mutation on the ability of the receptor to recruit intracellular effectors and activate downstream pathways in response to NGF. Unlike wild type p75<sup>NTR</sup>, the C257A mutant was unable to interact with NRIF, either in the presence or absence of NGF (Figure 2A). Although a weak interaction could be observed between the C257A mutant and TRAF6, this was not increased by NGF treatment, which on the other hand readily promoted TRAF6 recruitment to wild type receptors (Figure 2B). These data suggested that, although wild type p75<sup>NTR</sup> is present as both monomers and disulphide-linked dimers at the cell membrane, only the latter

appear to be capable of recruiting downstream effectors such as NRIF and TRAF6 in a ligand-dependent manner. In order to address this directly, we tested the ability of NRIF and TRAF6 to differentially interact with monomers and disulphide-linked dimers of wild type p75<sup>NTR</sup> by analyzing pull-down products under reducing and non-reducing conditions. We found that NRIF interacted exclusively with disulphide-linked p75<sup>NTR</sup> dimers, but not at all with monomers, and NGF treatment could readily increase this interaction (Figure 2C). On the other hand, although TRAF6 could pull-down both p75<sup>NTR</sup> monomers and dimers, NGF treatment only stimulated TRAF6 binding to p75<sup>NTR</sup> dimers, but not monomers (Figure 2D). Also in sympathetic neurons from rat SCG, NRIF interacted only with p75<sup>NTR</sup> disulphide-linked dimers, not with monomers, and BDNF treatment strongly stimulated NRIF recruitment to the receptor (Figure 2E).

We also examined three different downstream readouts of p75<sup>NTR</sup> activity in transfected fibroblasts and 293 cells, including activation of NF- $\kappa$ B (Figure 3A), caspase-3 (Figure 3B), and induction of cell death (Figure 3C) in response to NGF. In all cases, the C257A mutant was unresponsive to stimulation, indicating that Cys<sup>257</sup> is required for neurotrophin-dependent p75<sup>NTR</sup> signaling and downstream biological effects.

In order to probe the functional importance of Cys<sup>257</sup> for p75<sup>NTR</sup> signaling in a more physiological context, we developed two shRNA constructs directed to 3' UTR sequences of the rat p75<sup>NTR</sup> mRNA to knock-down endogenous p75<sup>NTR</sup> expression in neurons. When introduced together into primary cultures of rat SCG neurons, these shRNAs effectively suppressed endogenous p75<sup>NTR</sup> expression, while a control shRNA had no effect (Figure 4A). As expected, control shRNA did not affect the ability of BDNF to induce JNK phosphorylation (Figure 4B) and cell death (Figure 4C) in SCG neurons, which express p75<sup>NTR</sup> and TrkA but not TrkB. In contrast, shRNAs directed against p75<sup>NTR</sup> abolished both responses, indicating their dependence on p75<sup>NTR</sup> signaling (Figures 4B and 4C). Under those conditions, BDNF-mediated JNK phosphorylation and cell death could be restored by introduction of a wild type p75<sup>NTR</sup> expression construct that is insensitive to our p75<sup>NTR</sup> shRNAs (Figures 4B and 4C). In

contrast to the wild type construct, the C257A mutant was unable to restore either JNK phosphorylation or cell death in response to BDNF in SCG neurons (Figures 4B and 4C). Together, these results demonstrate that Cys<sup>257</sup> is essential for p75<sup>NTR</sup> signaling in response to neurotrophins, and support the notion that disulphide-linked p75<sup>NTR</sup> dimers are the active, neurotrophin-sensitive receptor species in neurons.

### **Cys<sup>257</sup> is not required for downstream signaling by the p75<sup>NTR</sup>/NgR/Lingo-1 complex in response to MAG**

The importance of Cys<sup>257</sup> in neurotrophin-dependent p75<sup>NTR</sup> signaling prompted the question of its role in the activation of p75<sup>NTR</sup> by other types of ligands. We therefore tested the ability of wild type and mutant p75<sup>NTR</sup> to recruit RhoGDI and to increase RhoA activity in response to the myelin-derived ligand MAG. These activities require the presence of two additional receptor subunits, NgR and Lingo-1. Interestingly, cells expressing p75<sup>NTR</sup>, NgR and Lingo-1 responded equally well to stimulation with MAG in both RhoGDI recruitment to p75<sup>NTR</sup> (Figure 5A) and RhoA activity (Figure 5B) regardless of whether they received wild type or the C257A mutant. Thus, and in contrast to activation by neurotrophins, Cys<sup>257</sup> is not required for the ability of p75<sup>NTR</sup> to signal in response to the non-neurotrophin ligand MAG, suggesting the existence of mechanistic differences in the activation of p75<sup>NTR</sup> by different ligands.

### **Cys<sup>257</sup> allows disulphide-linked p75<sup>NTR</sup> dimers to propagate conformational changes to intracellular domains following NGF binding**

How does Cys<sup>257</sup> contribute to p75<sup>NTR</sup> signaling in response to neurotrophins? Its localization in the transmembrane domain suggested that it may play a role in the mechanism of receptor activation, either by regulating p75<sup>NTR</sup> oligomerization, or by allowing the propagation of conformational changes induced by neurotrophin binding. Although Cys<sup>257</sup> clearly contributes to the formation of disulphide-linked p75<sup>NTR</sup> dimers, it remained unclear whether this was the primary determinant of receptor oligomerization. In particular, the ability of the C257A mutant to bind NGF at normal levels (Figure 1E) suggested that the mutation may not have disrupted the oligomeric

state of p75<sup>NTR</sup> at the cell surface, as this would have been expected to affect ligand binding affinity. In order to examine the possible role of non-covalent interactions in p75<sup>NTR</sup> oligomerization we performed chemical crosslinking studies of wild type and mutant cell surface receptors in living COS-7 cells. Following chemical crosslinking of cell surface proteins, p75<sup>NTR</sup> was immunoprecipitated from cell lysates, and then subjected to SDS/PAGE under reducing conditions, so that only oligomeric complexes stabilized by chemical crosslinking would be visualized. As expected, chemical crosslinking allowed the detection of wild type receptor dimers (Figure 6A), demonstrating that p75<sup>NTR</sup> indeed forms dimers at the surface of living cells in the absence of ligand. As chemical crosslinkers have only a limited efficiency, the actual proportion of receptor dimers and monomers at the cell surface can not be determined with this method. NGF binding did not alter the proportion of p75<sup>NTR</sup> dimers detected by chemical crosslinking in transfected cells (Figure 6A). Surprisingly, however, mutation of Cys<sup>257</sup> did not affect the proportion of p75<sup>NTR</sup> dimers detected after chemical crosslinking, either in the absence or presence of NGF (Figure 6A). This indicated that p75<sup>NTR</sup> may still oligomerize in the absence of Cys<sup>257</sup> through non-covalent interactions.

Inspection of the p75<sup>NTR</sup> transmembrane domain, revealed the presence of a relatively well conserved AxxxG<sup>266</sup> motif (Figure 6B), which is characteristic of self-associating transmembrane domains, including those from integrins and glycoporphin A (GpA) (Kubatzky et al., 2001). Interactions between isolated transmembrane domains can be studied in a biological membrane using the bacterial ToxCAT system (Russ and Engelman, 1999) (see Experimental Procedures for further details). Dimerization of wild type and mutant p75<sup>NTR</sup> transmembrane domains was assessed along with wild type GpA transmembrane domain, which is known for its strong self-association, and a GpA point mutant (G83I) that disrupts homodimerization, as positive and negative controls, respectively. The transmembrane domain of p75<sup>NTR</sup> was found to homodimerize in this system to about 60% of the level shown by GpA (Figure 6C). Mutation of Cys<sup>257</sup> had only a small effect on transmembrane homodimerization (Figure 6C). In contrast,

mutation of Gly<sup>266</sup> to Ile (G266I), which is analogous to the G83I in GpA, abolished self-association of the p75<sup>NTR</sup> transmembrane domain (Figure 6C). When introduced in full-length p75<sup>NTR</sup> together with C257A, the G266I mutation diminished the formation of p75<sup>NTR</sup> dimers at the plasma membrane (Figure 6D). Together, these data indicate that p75<sup>NTR</sup> homodimers can be stabilized at the cell surface by both covalent and non-covalent interactions between transmembrane domains, and that the primary function of Cys<sup>257</sup> is not in the formation of receptor dimers.

We then considered the possibility that Cys<sup>257</sup> may instead contribute to p75<sup>NTR</sup> signaling by mediating the propagation of conformational changes induced by neurotrophin binding. We reasoned that conformational changes involving alterations in the relative positions of p75<sup>NTR</sup> intracellular domains may be detected by fluorescence resonance energy transfer (FRET). Unlike methods based on enzyme complementation, in FRET, the transfer of energy between two chromophores depends both on the distance between them and their relative orientation, and it is therefore more suitable to quantitatively assess conformational rearrangements of receptor subunits in a complex. We chose homo-FRET over other more conventional FRET techniques because of its greater sensitivity and the advantage of using a single spectral variant to detect interactions between identical molecules (Squire et al., 2004). Homo-FRET can be detected as a decrease in steady-state fluorescence anisotropy that results from energy transfer between identical fluorophores. Monomeric Enhanced Green Fluorescent Protein (EGFP1) displays high anisotropy (i.e. low FRET), while a concatenated EGFP trimer (EGFP3) shows very low anisotropy (i.e. very high FRET), and they were used as negative and positive controls, respectively (Figures 7A, 7B and 7E). Wild type and C257A p75<sup>NTR</sup> were tagged at the C-terminus with monomeric EGFP (see Experimental Procedures) and transfected in COS-7 cells. Anisotropy and total fluorescence were measured at different locations in the plasma membrane of transfected cells. At basal conditions, wild type p75<sup>NTR</sup>-EGFP displayed lower anisotropy than EGFP1 (i.e. higher FRET) at the plasma membrane (Figures 7C and 7E), in agreement with its oligomeric state in living cells. Using the anisotropy value of

EGFP1 as baseline, the FRET level of wild type p75<sup>NTR</sup>-EGFP could be estimated to about 25% that of EGFP3 (Figure 7E), which gives a very strong FRET signal. Mutant C257A p75<sup>NTR</sup>-EGFP also showed lower anisotropy than EGFP1 at the cell surface, albeit not as much as wild type p75<sup>NTR</sup> (Figures 7D and 7E), which is in accordance with our crosslinking studies showing that this mutant retains the ability to form dimers at the plasma membrane. Interestingly, both p75<sup>NTR</sup>-EGFP constructs appeared mainly monomeric at intracellular locations (Figures 7C and 7D), suggesting that oligomerization occurs upon transit to the plasma membrane.

NGF was added to cells expressing wild type and C257A p75<sup>NTR</sup>-EGFP and changes in anisotropy were evaluated by time-lapse microscopy. Acute addition of NGF produced an increase in anisotropy (i.e. decrease in FRET) at the plasma membrane of cells expressing wild type p75<sup>NTR</sup>-EGFP (Figure 7E). This anisotropy rise was observed in all cells examined (n=30), showed a consistent peak 2-3 minutes after NGF addition, and lasted for 3 to 10 minutes, waning towards the end of the recording (15 min) (Figures 7F and 7G). In contrast, NGF had no effect on cell surface anisotropy in cells expressing the mutant C257A p75<sup>NTR</sup>-EGFP (n=22 cells examined) (Figures 7F and 7G). These results indicate that NGF induces a decrease in FRET in wild type p75<sup>NTR</sup>-EGFP at the plasma membrane, indicating that receptor activation by neurotrophins involves conformational changes in the relative position or orientation of p75<sup>NTR</sup> intracellular domains. The inability of NGF to induce any change in FRET in cells expressing C257A p75<sup>NTR</sup>-EGFP correlates with the loss of neurotrophin-dependent p75<sup>NTR</sup> signaling in this mutant, and supports a role for Cys<sup>257</sup> in the propagation of conformational changes induced by neurotrophin binding.

## DISCUSSION

In this study, we have investigated the mechanism of activation of the p75 neurotrophin receptor. The first major finding of this work is the ability of native p75<sup>NTR</sup>

to form disulphide-linked dimers through the conserved Cys<sup>257</sup> in the transmembrane domain. The second is the unexpected requirement of this cysteine residue for the recruitment of intracellular effectors and downstream signaling by p75<sup>NTR</sup> in response to neurotrophins, but not in response to other ligands, such as MAG. The third is the ability of the p75<sup>NTR</sup> dimer to undergo a conformational change in response to NGF, and the essential role of Cys<sup>257</sup> in this process. Based on these observations, we propose a novel mechanism of receptor activation involving ligand-induced rearrangement of disulphide-linked receptor subunits.

While it is relatively straightforward to visualize how receptor kinases may become activated upon ligand binding (Schlessinger, 2002), this is less obvious for non-catalytic receptors. Since these signal by selective interactions with intracellular effectors, the question that arises is how ligand binding regulates those interactions. Clearly, something ought to change in the receptor after ligand binding that makes its intracellular domain more or less prone to interact with downstream effectors. Ligand binding may change the oligomerization state of the receptor —by, for example, inducing dimers— leading to cooperative binding or release of downstream effectors. Although this has been the prevalent model for p75<sup>NTR</sup> activation to date, our results show that the active p75<sup>NTR</sup> species pre-exists as a disulphide-linked dimer which is essential for downstream signaling in response to neurotrophins, obviating a classical ligand-mediated dimerization mechanism. Although an earlier study using artificially deglycosylated p75<sup>NTR</sup> extracellular domain had suggested that NGF may activate a preformed receptor dimer by inducing its dissociation into monomers (He and Garcia, 2004), more recent work has clearly shown that glycosylated p75<sup>NTR</sup> extracellular domain is monomeric in the absence of ligand, and that NGF interacts with p75<sup>NTR</sup> dimers, not monomers (Gong et al., 2008). Another possible mechanism for receptor activation is that ligand binding changes the relative orientation of receptor subunits in a dimeric or multimeric receptor complex. In some cytokine and growth factor receptors, this is achieved through the relative rotation of their transmembrane domains within the plane of the membrane (Moriki et al., 2001; Seubert et al., 2003). In



the case of p75<sup>NTR</sup>, however, the disulphide link formed by Cys<sup>257</sup> in the transmembrane domain clearly restricts the possibilities for relative movement of receptor subunits.

Our FRET experiments indicate that the two intracellular domains of the p75<sup>NTR</sup> dimer are in close proximity under basal conditions. This is in agreement with data from a recent study using a  $\beta$ -gal complementation strategy (Wehrman et al., 2007). Unlike FRET, however, enzyme complementation gives an all-or-none response at the single molecule level, and is hence less suited to reveal subtle conformational changes that do not alter the oligomeric state of the receptor. In our experiments, we observed a consistent decrease in FRET upon NGF binding to wild type p75<sup>NTR</sup>. This could either result from a separation of p75<sup>NTR</sup> intracellular domains or changes in their relative orientation due to rotation of receptor subunits. Although these two possibilities are not mutually exclusive, it is difficult to envision how changes in the relative orientation of intracellular domains can be brought about by ligand binding to the extracellular region of a dimeric receptor that is covalently crosslinked at the plasma membrane. As explained above, a disulphide bridge linking the transmembrane regions of two receptor subunits would clearly prevent the propagation of rotational movements from the extracellular to the intracellular domains. Thus, we believe that the decrease in FRET observed after NGF binding most likely reflects the separation of p75<sup>NTR</sup> intracellular domains. Importantly, and in contrast to the wild type receptor, we found no evidence of NGF-mediated conformational changes in the intracellular domain of the C257A p75<sup>NTR</sup> mutant. Based on these observations, we propose that neurotrophin binding produces a scissors-like movement of disulphide-linked p75<sup>NTR</sup> subunits with the Cys<sup>257</sup>-Cys<sup>257</sup> disulphide link acting as the fulcrum, thereby altering the relative proximity of intracellular domains (Figure 8). In this model, Cys<sup>257</sup> would function as the pin in the scissors: in its absence, relative movements at one end can not be propagated to the other. Unlike normal scissors, however, our results suggest that as the extracellular domains come closer together on binding to the neurotrophin dimer, the intracellular domains separate, not unlike a “snail-tong” mechanism (Figure 8). This type of movement could be achieved if the p75<sup>NTR</sup> protein were to be kinked, as

opposed to straight, relative to the plane of the plasma membrane, in the vicinity of Cys<sup>257</sup>. Proline residues are known to introduce kinks that may vary from 5 to 50 degrees, and are common in transmembrane helices (Yohannan et al., 2004). Thus, the highly conserved Pro<sup>254</sup>, three residues upstream of Cys<sup>257</sup> (see Figure 1C), seems like a good candidate for such function. Although not as well conserved, an unusual Pro triad is also present in the intracellular juxtamembrane region of p75<sup>NTR</sup>, 20 residues away from the plasma membrane (Pro<sup>295</sup>-Pro<sup>296</sup>-Pro<sup>297</sup>). This portion of p75<sup>NTR</sup> is not known to be helical *in vivo*, and has been characterized by nuclear magnetic resonance as unstructured in solution (Liepinsh et al., 1997). The conformational changes inferred from our FRET experiments require a relatively rigid structure connecting the extra and intracellular domains, and suggest that the juxtamembrane region of p75<sup>NTR</sup> may be stabilized *in vivo* by interaction with other intracellular components.

The conformational changes observed upon NGF binding showed a characteristic maximum at 2-3 minutes after NGF addition and lasted for an additional 3 to 10 minutes. This kinetics is consistent with the onset of p75<sup>NTR</sup> internalization and downstream signaling, which requires at least 15 min after ligand stimulation (Bronfman et al., 2003). The separation of intracellular domains induced by NGF binding may facilitate the recruitment of downstream effectors by exposing determinants important for their interaction with the receptor. This may be particularly important for receptors with relatively small intracellular domains, such as p75<sup>NTR</sup>. Weak homotypic interactions between these domains may keep the receptor in a closed, inactive state, and could potentially explain the residual amount of dimers observed after simultaneous mutation of Cys<sup>257</sup> and Gly<sup>266</sup>. Although the p75<sup>NTR</sup> death domain was found to be monomeric in solution up to 2.5 mM (Liepinsh et al., 1997), such interactions might be detected in crystallization experiments. Even though it is unclear at present why the conformational change observed is not sustained for longer periods of time, the variation observed in its duration could reflect interactions with different effector molecules or activation of different downstream signaling events. That

p75<sup>NTR</sup> activation involves separation of intracellular domains is consistent with a previous study using chimeric intracellular domains artificially tethered to the plasma membrane that were induced to dimerize by addition of a dimeric drug (Wang et al., 2000). In this work, dimerization inhibited the proapoptotic effect of the constructs, from which the authors inferred that neurotrophin-mediated receptor dimerization should likewise silence p75<sup>NTR</sup> activity, a conclusion that was at odds with the bulk of studies in the field. Our results indicating that p75<sup>NTR</sup> is already dimeric before ligand binding and that p75<sup>NTR</sup> activation by NGF involves the separation of intracellular domains reconcile those results and suggest that this is in fact the way in which neurotrophins activate this receptor.

Previous work has shown that p75<sup>NTR</sup> can enhance Trk responses to neurotrophins, particularly when these are present at limiting concentrations. For example, sensory and sympathetic neurons from p75<sup>NTR</sup> knock-out mice show reduced responsiveness to NGF (Lee et al., 1994), and a mutant NGF unable to bind p75<sup>NTR</sup> displays lower binding affinity and reduced biological activity in PC12 cells and sensory and sympathetic neurons (Rydén et al., 1997a). This positive modulatory role of p75<sup>NTR</sup> on neurotrophin activities mediated by Trk receptors has been attributed to receptor-receptor interactions and/or ligand concentration or presentation effects. It is unclear at present whether these activities require disulphide-linked dimerization of p75<sup>NTR</sup>. Given the proposed role for the TrkA and p75<sup>NTR</sup> transmembrane domains in their functional interaction (Esposito et al., 2001), it is possible that formation of high-affinity NGF binding sites may be sensitive to conformational changes mediated by Cys<sup>257</sup>.

The fact that p75<sup>NTR</sup> signaling in response to MAG does not require Cys<sup>257</sup> suggests that myelin-derived ligands activate p75<sup>NTR</sup> by a different mechanism. Although our results indicate that disulphide-linked dimerization is important for p75<sup>NTR</sup> activation by neurotrophins, the stoichiometry of the complex formed by p75<sup>NTR</sup>, NgR and Lingo-1 needs to be further investigated. The lack of involvement of Cys<sup>257</sup> in signaling by MAG opens an opportunity to specifically disrupt neurotrophin-specific p75<sup>NTR</sup> effects in

loss-of-function studies. Intriguingly, we found that the proportion of cell surface disulphide-linked p75<sup>NTR</sup> dimers varied in different cell types and with different levels of receptor expression. As only such dimers are able to respond to neurotrophins, changes in their proportion or abundance at the cell surface, e.g. between different cell types or in response to external stimuli or redox states, could determine the relative degree of p75<sup>NTR</sup> responsiveness to different ligands.

Cys<sup>257</sup> represents a critical element in the activation mechanism of p75<sup>NTR</sup> by neurotrophins and is absolutely conserved in all vertebrate and invertebrate p75<sup>NTR</sup> molecules isolated so far (Bothwell, 2006). We propose that neurotrophins neither activate p75<sup>NTR</sup> by inducing receptor dimers or disassembly into monomers, as previously suggested, but through a novel mechanism involving the rearrangement of disulphide-linked receptor subunits. Many receptors in the TNFR superfamily –to which p75<sup>NTR</sup> belongs– bear intramembrane cysteines, including Fas, DR6, RANK, RELT, BCM, TACI, CD30, CD40 and receptors for TNF, lymphotoxin beta and TRAIL. The “snail-tong” mechanism described here could therefore represent a general way by which receptors bearing intramembrane cysteines are activated.

## **EXPERIMENTAL PROCEDURES**

### **Plasmids, antibodies , proteins and chemicals**

Rat p75<sup>NTR</sup> was expressed from the pCDNA3 vector backbone (Invitrogen) using a full-length coding sequence flanked by an N-terminal hemagglutinin (HA) epitope tag. Mutations were introduced using QuickChange (Stratagene) and verified by DNA sequencing. Plasmids to express RIP2, TRAF6, NRIF, sortilin, Lingo-1 and RhoGDI were previously described. EGFP plasmid was from Clontech. Luciferase reporter plasmid for NF- $\kappa$ B was from Promega. The origin of antibodies were as follows: MC192 anti-p75<sup>NTR</sup> from Phil Barker; anti-HA from Roche; anti-myc, anti-phospho and -total JNK, cleavage-specific anti-caspase-3, anti-RhoA and anti-RhoGDI from Cell

Signaling; anti-tubulin from Sigma; anti-RIP2 from Santa Cruz. NGF was purchased from Alomone Labs, MAG-Fc and Nogo peptide from R&D. NGF was typically applied at 100 ng/ml for 30 minutes unless otherwise indicated. MAG-Fc was used at 25  $\mu$ g/ml for 30 minutes. PMA was used at 200 nM for 1 h. Epoxomycin (1  $\mu$ M) and DAPT (2  $\mu$ M) were applied 1.5 h prior to PMA. All compounds were from Sigma.

### **Cell transfection and tissue culture**

COS-7 cells were transfected with polyethylenimine (PEI). HEK293 and M23 cells were transfected with Lipofectamine 2000 (Gibco). M23 is a clonal derivative of MG87 cells (Eketjäll et al., 1999), originally derived from mouse NIH3T3 fibroblasts. Cells were typically used on the 2<sup>nd</sup> day after transfection for short-term signaling assays, at which point cell death was still low or undetectable. We found that different signaling assays worked best in different cell lines: RhoA and RhoGDI in COS-7, NF- $\kappa$ B in M23 and P-JNK and caspase-3 in HEK293 cells. This may be related to the specific complement of downstream effectors expressed by each cell type. Cell lines were cultured under standard conditions and primary neurons in serum-free, N2-supplemented DMEM:F12 medium (Gibco).

### **Sympathetic neuron culture and p75<sup>NTR</sup> shRNAs**

Sympathetic neurons from P4 rat SCGs were cultured and transfected with control or p75<sup>NTR</sup> shRNAs alone or co-transfected with wild type or mutant p75<sup>NTR</sup> as previously described (Kenchappa et al., 2006; Palmada et al., 2002). The target sequence of the first p75<sup>NTR</sup> shRNA was ACGGACCTATCTGAGCTGAAA, the second was ATGGCGTGACTTTCAGGGAAA. Control shRNA was targeted against EGFP sequences. Three days after transfection neurons were treated with 200 ng/ml of BDNF for 1 hr and lysed in NP-40 buffer (10% glycerol, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mg/ml leupeptin and aprotinin) to assess JNK activation. For cell survival assay the cells were fixed in 4% paraformaldehyde 48 hrs after BDNF treatment and scored for apoptosis as previously described (Kenchappa et al., 2006).

### **Biotinylation, immunoprecipitation, immunoblotting and chemical crosslinking**

Cell surface proteins were biotinylated with Sulfo-NHS-LC-Biotin (Pierce). Cells were lysed in buffer containing 1% Triton X-100, 60 mM octyl-glucosyde, 10 mM iodoacetamide and protease inhibitors (Roche). For reducing conditions, immunoprecipitates were boiled in sample buffer containing 1M DTT. Gels were blotted to PVDF membranes (Amersham). Biotinylated proteins were detected using Neutravidin conjugated to Alkaline Phosphatase (Sigma). Filters were developed by chemifluorescence (Amersham) and scanned on a STORM840 fluorimager (MolDynamics). Radioiodination of NGF was done with lactoperoxidase (Sigma), and EDAC/SulfoNHS (Pierce) was used as chemical crosslinker. Autoradiography was done on a STORM840 phosphorimager. Quantifications of immunoblots and autoradiograms were done with ImageQuant software (MolDynamics).

### **Assays of NF- $\kappa$ B, RhoA and cell death**

NF- $\kappa$ B activity was assayed using a luciferase reporter kit (Promega). NGF was added 2 days after transfection and left for 24 h prior to cell lysis. RhoA activity was evaluated using a kit from Cytoskeleton. Cell death was assessed by the TUNEL method using kits from Roche and Biocolor. NGF was added 2 days after transfection and left for another day in serum free-medium prior to assay of cell death.

### **ToxCAT assay of self-association of transmembrane domains**

The reporter system exploits the ability of the ToxR transcription activator of the *Vibrio Cholerae* pathogen to bind the cholera toxin (*ctx*) gene promoter only when dimerized. A transmembrane segment of interest fused to ToxR is delivered to the bacterial inner membrane by fusion to maltose-binding protein (MBP). Varying amounts of ToxR dimers will be formed in the cytosol in direct proportion to the oligomerization ability of the TM domain. Binding of dimerized ToxR to the *ctx* DNA element triggers expression of a chloramphenicol transferase (*cat*) gene reporter and production of CAT protein, which can then be quantified by a CAT-ELISA kit (Roche Diagnostics) as described by McClain et al. (McClain et al., 2003). Expression levels of different ToxR-TM-MBP

chimeras were determined by Western blotting. The ToxR and ToxCAT systems have been previously applied to demonstrate self-association of a range of TM domains from glycoporphin A (GpA) (Kubatzky et al., 2001), integrin  $\alpha_{IIb}$  (Li et al., 2004), ErbB1 to 4 (Mendrola et al., 2002), and the Epo receptor (Kubatzky et al., 2001).

### **Anisotropy microscopy and FRET**

Anisotropy microscopy was done as described by Squire et al. (Squire et al., 2004) in transiently transfected COS-7 cells. Images were acquired 15-24 h post-transfection, using a Olympus IX81 inverted microscope (Olympus, Germany) equipped with a MT20 illumination system. A linear dichroic polarizer (Meadowlark Optics, Frederick, Colorado, US) was placed in the illumination path of the microscope, and two identical polarizers were placed in an external filter wheel at orientations parallel and perpendicular to the polarization of the excitation light. The fluorescence was collected via a 20X 0.7 NA air objective, and parallel and polarized emission images were acquired sequentially on an Orca CCD camera (Hamamatsu Photonics, Japan). Data acquisition was controlled by the CellR software supplied by the microscope manufacturer. NGF (or vehicle) was added 1 min after the start of the time-lapse at a concentration of 100 ng/ml. Anisotropy values were extracted from image stacks of 30 images acquired in both parallel and perpendicular emission modes every 30 sec for a time period of 15 min after NGF addition. For each construct, 2 areas per field of view were measured in 4 independent transfections performed in duplicate or triplicate. Fluorescence intensity and anisotropy images were calculated as described by Squire et al. (Squire et al., 2004). Wild type and C257A p75<sup>NTR</sup> constructs were tagged at the C-terminus with a monomeric version of EGFP (Clontech) carrying the A207K mutation that disrupts EGFP dimerization. A cytosolic EGFP monomer (EGFP1) gave a mean anisotropy value of  $0.295 \pm 0.005$  (no FRET), and a concatenated EGFP trimer (EGFP3) gave a value of  $0.2369 \pm 0.013$  (maximal FRET), so thresholds for image analysis (done with Image J software) were set between 0.2 and 0.35 (Figure 7A to D).

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## FIGURE LEGENDS

### **Figure 1. Transmembrane Cys<sup>257</sup> mediates formation of disulphide-linked p75<sup>NTR</sup> dimers**

(A) Cell surface expression of disulphide-linked p75<sup>NTR</sup> dimers (dim) and monomers (mon) in transfected COS-7 cells visualized by Neutravidin probing of p75<sup>NTR</sup> immunoprecipitates under non-reducing (-DTT) and reducing (+DTT) conditions. In non-reducing conditions, p75<sup>NTR</sup> dimers run somewhat higher, and monomers lower, than their predicted molecular weights.

(B) Cell surface expression of disulphide-linked p75<sup>NTR</sup> dimers (dim) and monomers (mon) in COS-7 cells transfected with different amounts of wild type p75<sup>NTR</sup>. Immunoprecipitates were electrophoresed under non-reducing conditions and p75<sup>NTR</sup> was visualized by Neutravidin probing of p75<sup>NTR</sup> immunoprecipitates. Results are expressed as mean dimer/monomer ratio.

(C) Alignment of p75<sup>NTR</sup> transmembrane domain sequences from vertebrate and invertebrate species.

(D) Cell surface expression of endogenous disulphide-linked p75<sup>NTR</sup> dimers (dim) and monomers (mon) in PC12 cells, RN22 Schwannoma cells and SCG sympathetic neurons visualized by Neutravidin probing of p75<sup>NTR</sup> immunoprecipitates. Control, untransfected COS cells.

(E) Expression of endogenous disulphide-linked p75<sup>NTR</sup> dimers (dim) and monomers (mon) in extracts of newborn rat cerebellum (cblm), cortex (ctx) and hippocampus (hc) visualized by immunoblotting of p75<sup>NTR</sup> immunoprecipitates under reducing and non-reducing conditions. The control lane represents a mock immunoprecipitate of the cblm extract.

(F) Binding of <sup>125</sup>I-NGF to wild type and C257A p75<sup>NTR</sup> analyzed by chemical crosslinking. Samples were run under reducing conditions. For each construct, binding counts were normalized to levels of expression as assessed by immunoblotting (IB). Results are expressed as mean ± SD of three independent determinations.

(G)  $\gamma$ -secretase-dependent intramembrane cleavage of C257A p75<sup>NTR</sup> following stimulation with PMA in transfected COS cells. The proteasome inhibitor epoxomicin was used to prevent degradation of CTF and ICD fragments. Mutant and wild type (wt) p75<sup>NTR</sup> molecules were recovered by immunoprecipitation and visualized by immunoblotting. The  $\gamma$ -secretase inhibitor DAPT blocked the generation of p75<sup>NTR</sup> intracellular domain (ICD). CTF, carboxy terminal fragment.

**Figure 2. Cys<sup>257</sup> is essential for recruitment of NRIF and TRAF6 to p75<sup>NTR</sup> in response to NGF**

(A) Binding of NRIF to wild type and mutant p75<sup>NTR</sup> in transfected HEK293 cells assayed by immunoprecipitation (IP) and immunoblotting (IB).

(B) Binding of TRAF6 to wild type and mutant p75<sup>NTR</sup> in transfected HEK293 cells.

(C) Binding of NRIF to wild type and mutant p75<sup>NTR</sup> in transfected HEK293 cells. Prior to gel electrophoresis, the pull-down sample was splitted in two equal parts, one was boiled in sample buffer, the other was treated with 1M DTT prior to boiling. The migration of p75<sup>NTR</sup> dimers and monomers is indicated.

(D) Binding of TRAF6 to wild type and mutant p75<sup>NTR</sup> in transfected HEK293 cells analyzed as in panel (C).

(E) Binding of NRIF to wild type and mutant p75<sup>NTR</sup> in rat SCG neurons. NRIF only interacts with p75<sup>NTR</sup> disulphide-linked dimers, not monomers.

**Figure 3. Cys<sup>257</sup> is essential for p75<sup>NTR</sup> signaling to NF- $\kappa$ B, caspase-3 and cell death in response to NGF**

(A) NF- $\kappa$ B activity in transfected M23 fibroblasts in the presence and absence of NGF.

(B) Activation of caspase-3 visualized with a cleavage-specific antibody in HEK293 cells transfected with p75<sup>NTR</sup> constructs in response to NGF. Reprobing controls for p75<sup>NTR</sup> and GAPDH are shown. Independent experiments confirmed comparable expression of transfected p75<sup>NTR</sup> constructs.

(C) Cell death assay in HEK293 cells transfected with p75<sup>NTR</sup> constructs in response to NGF. Results are expressed as mean  $\pm$  SD of three independent experiments, each performed in duplicate.

**Figure 4. Cys<sup>257</sup> is essential for p75<sup>NTR</sup> signaling in SCG neurons**

(A) Downregulation of p75<sup>NTR</sup> expression in rat SCG neurons following transfection of p75<sup>NTR</sup> shRNAs.

(B) Assay of JNK phosphorylation in SCG neurons in response to BDNF. SCG neurons were transfected with the indicated shRNA constructs. In rescue experiments, wild type p75<sup>NTR</sup> or C257A p75<sup>NTR</sup> constructs insensitive to p75<sup>NTR</sup> shRNAs were also introduced by DNA transfection. JNK phosphorylation was assessed by immunoblotting of total cell lysates.

(C) Cell death in SCG neurons transfected with p75<sup>NTR</sup> shRNAs in response to BDNF. Rescue experiments with wild type and C257A p75<sup>NTR</sup> constructs were performed as above. \*,  $p < 0.05$  vs. wild type,  $n = 3$ .

**Figure 5. Cys<sup>257</sup> is not required for downstream signaling by the p75<sup>NTR</sup>/NgR/Lingo-1 complex in response to MAG**

(A) Binding of RhoGDI to wild type and C257A p75<sup>NTR</sup> in COS-7 cells co-transfected with NgR and Lingo-1 and stimulated with MAG-Fc.

(B) RhoA activity in COS-7 cells transfected with wild type and mutant p75<sup>NTR</sup> in the presence of NgR and Lingo-1 after stimulation with MAG-Fc for 30 min. Results are expressed as mean  $\pm$  SD relative to wild type without MAG treatment. \*,  $p < 0.05$  vs. control,  $n = 3$ .



**Figure 6. Cell surface p75<sup>NTR</sup> dimerization is mediated by covalent and non-covalent interactions between transmembrane domains**

(A) Wild type and C257A p75<sup>NTR</sup> dimers analyzed by cell surface chemical crosslinking under reducing conditions. Cell lysates were immunoprecipitated with anti-p75<sup>NTR</sup> antibodies; immunoblot was probed with anti-HA antibodies.

(B) Alignment of p75<sup>NTR</sup> transmembrane domains highlighting the AxxxG self-association motif and Gly<sup>266</sup>.

(C) ToxCAT assay of self-association of wild type and mutant p75<sup>NTR</sup> transmembrane domains. Wild type and mutant transmembrane domains from glycoporphin A (GpATM) were used as positive and negative controls, respectively.

(D) Wild type and mutant p75<sup>NTR</sup> dimers analyzed by cell surface chemical crosslinking as above.

**Figure 7. Analysis of conformational changes in p75<sup>NTR</sup> intracellular domains by anisotropy microscopy**

(A-D) Steady-state anisotropy in transfected cells. Examples of areas used for anisotropy measurements are boxed and shown as high magnification insets. Monomeric EGFP (EGFP1, high anisotropy, low FRET) and a concatenated EGFP trimer (EGFP3, low anisotropy, high FRET) were used as controls. The calibration bar of the look-up table is shown below.

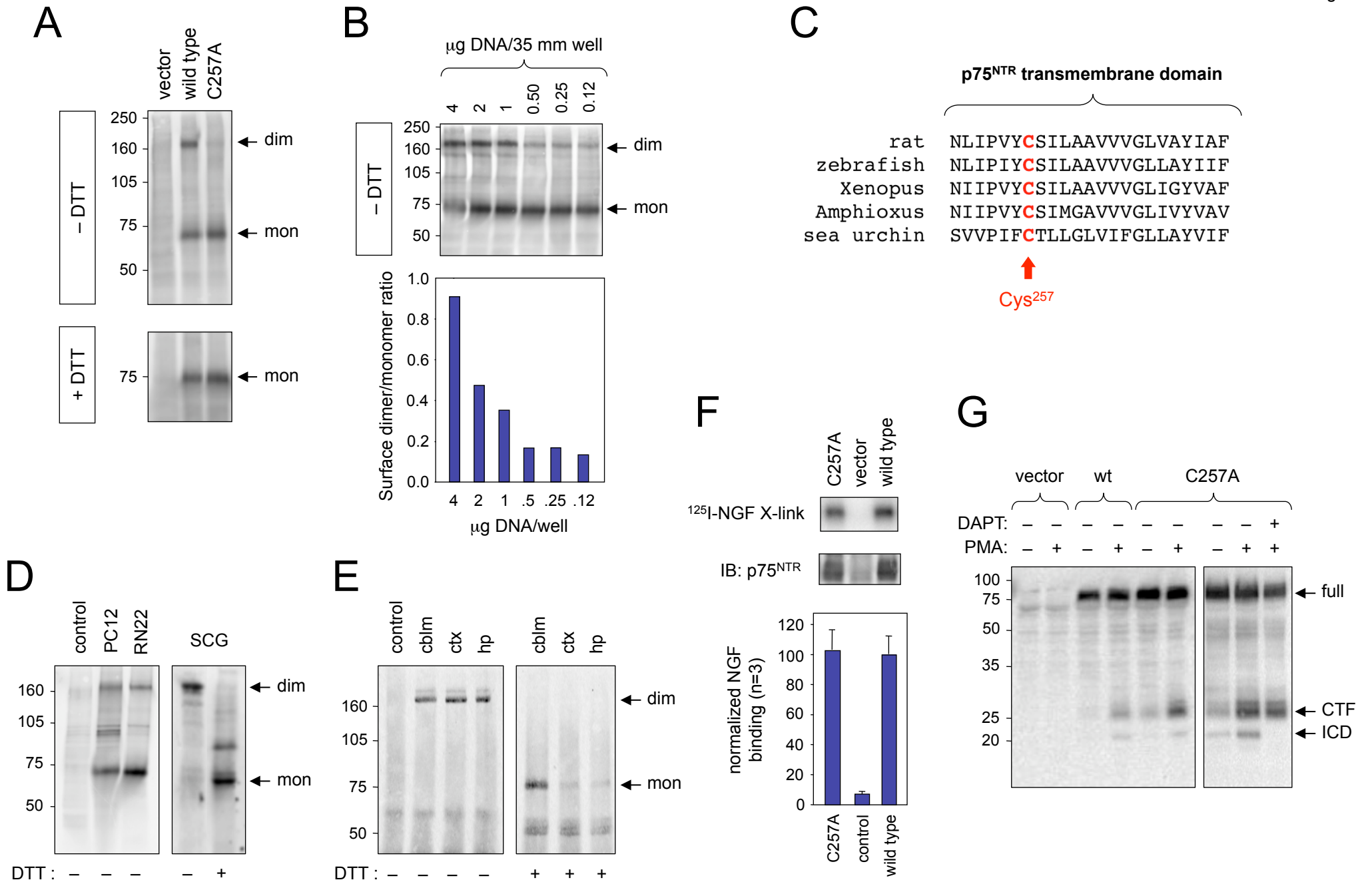
(E) Steady-state anisotropy of wild type and C257A p75<sup>NTR</sup>-EGFP in COS-7 cells. The anisotropy value of EGFP1 was arbitrarily set to zero and used as baseline for the histogram. Bars show average  $\pm$  SD (n=8-11 cells for EGFP and 22-30 cells for p75<sup>NTR</sup>).

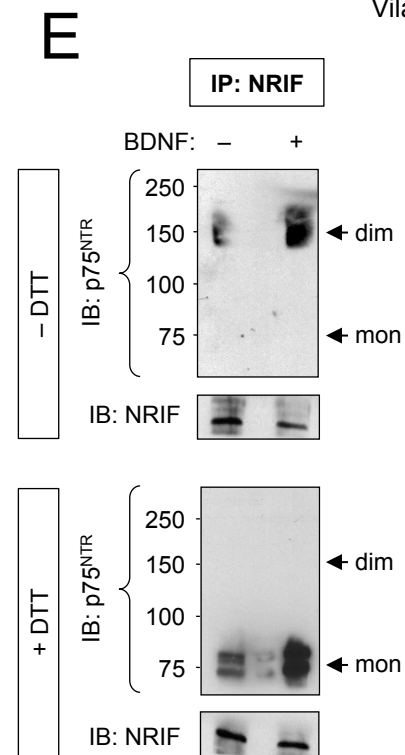
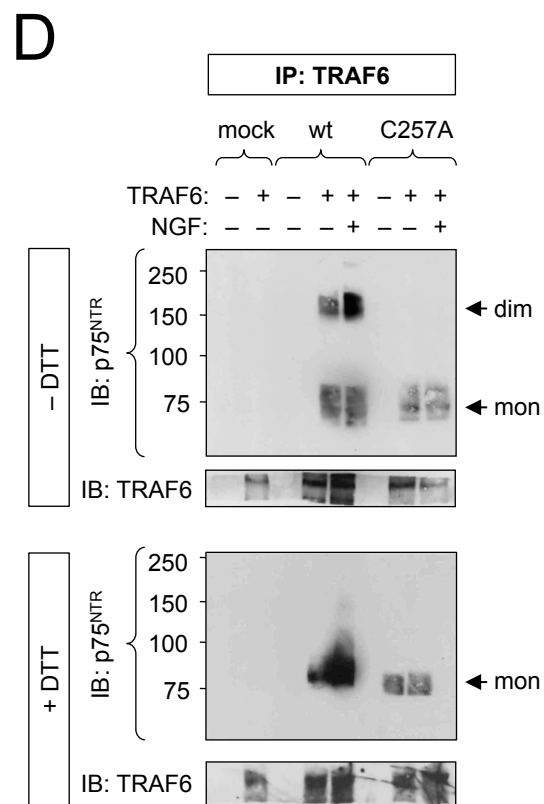
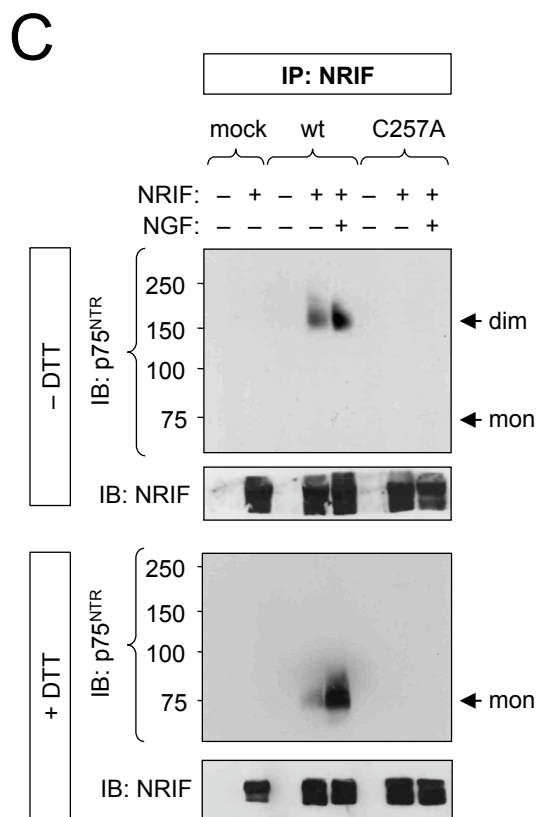
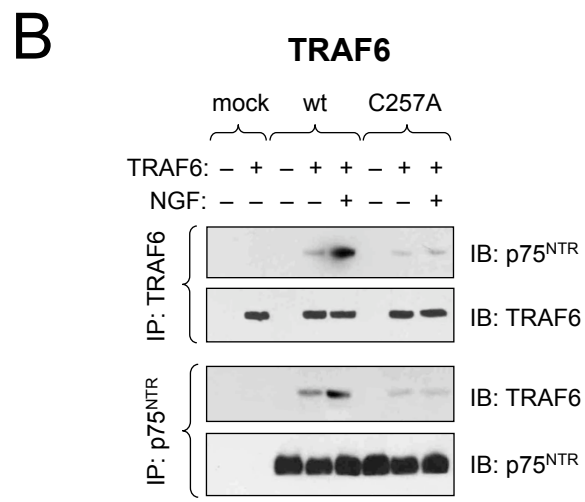
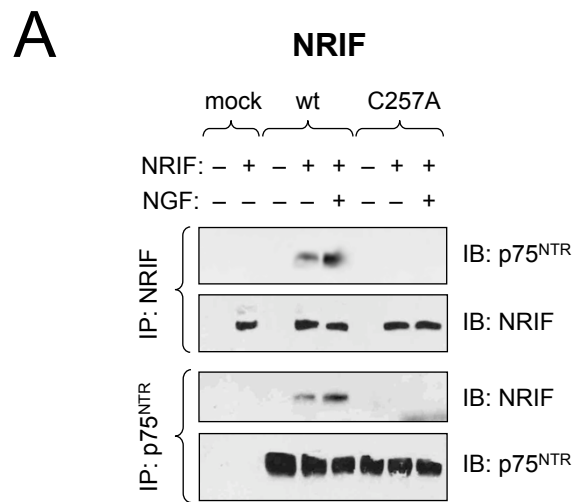
(F) Representative examples of anisotropy traces after addition of NGF or medium in cells expressing wild type or C257A p75<sup>NTR</sup>-EGFP. A peak in anisotropy was observed after NGF addition in all cells expressing wild type p75<sup>NTR</sup> that were examined (n=30) regardless of their initial baseline anisotropy level.

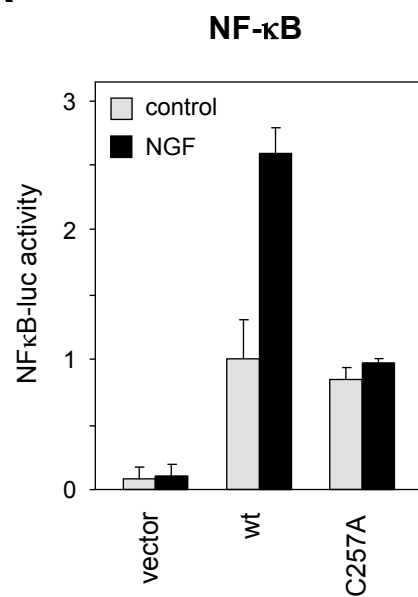
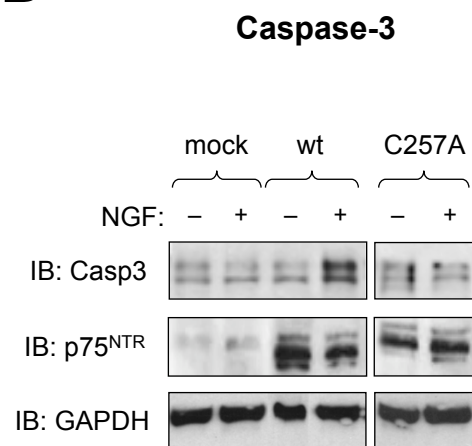
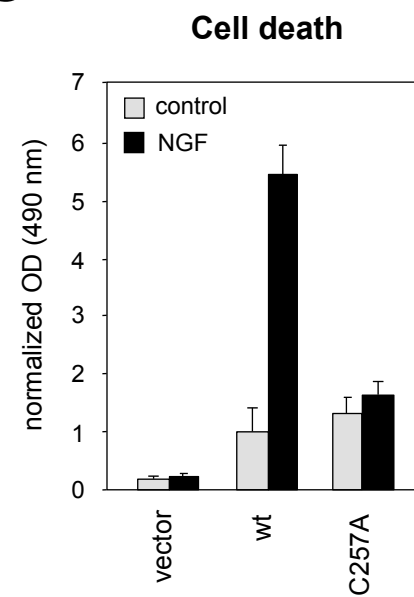
(G) Anisotropy change after addition of NGF or medium (control). The difference in anisotropy before and after addition of NGF (i.e. peak minus baseline value) or medium was calculated for wild type and C257A p75<sup>NTR</sup>-EGFP. Results are expressed as average  $\pm$  SD (n=15 to 17 cells examined). \*, p<0.0001 vs. C257A.

**Figure 8. The “snail-tong” mechanism of p75<sup>NTR</sup> activation in response to neurotrophins**

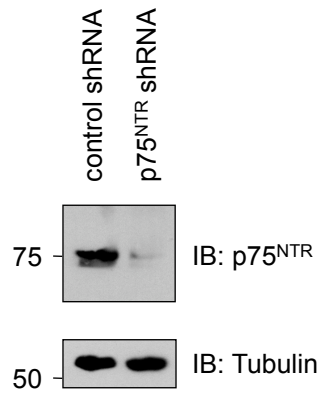
Hypothetical schematic of p75<sup>NTR</sup> in the cell membrane before and after neurotrophin binding (adapted from Gong et al. (2008) and Liepinsh et al. (1997)). The approximate position of Cys<sup>257</sup> is indicated. Arrows denote the postulated “snail-tong” movement of p75<sup>NTR</sup> subunits initiated by ligand binding: closing onto the neurotrophin dimer in the outside, opening in the inside.



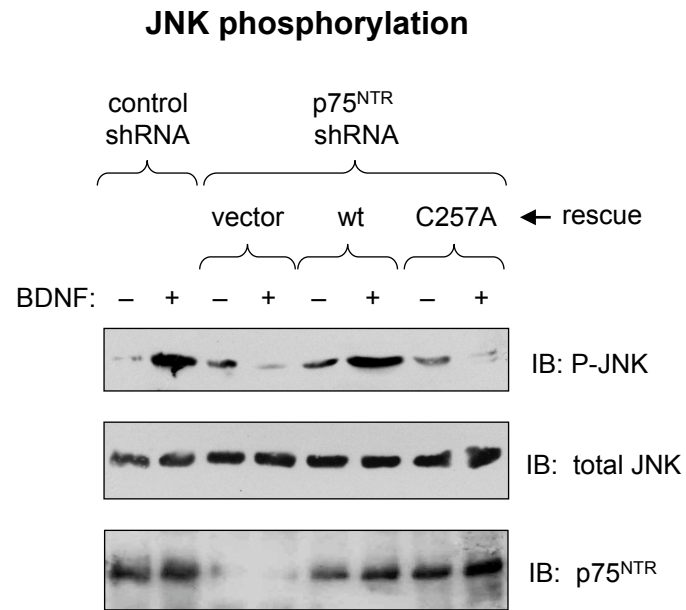


**A****B****C**

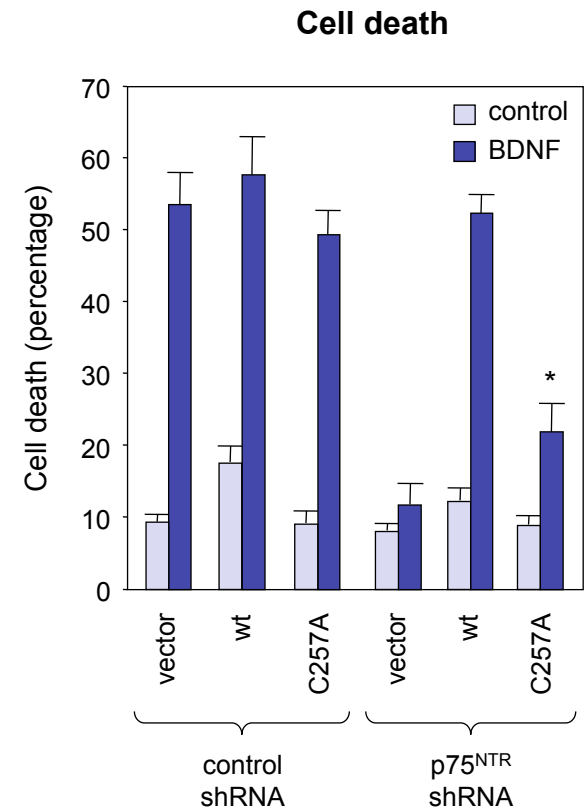
**A**



**B**

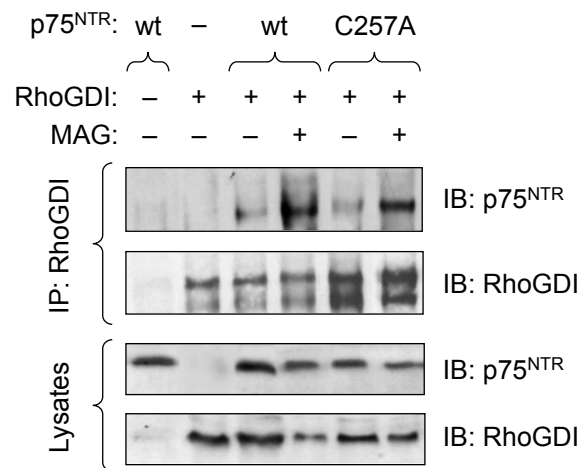


**C**



**A**

**RhoGDI**



**B**

**RhoA**

