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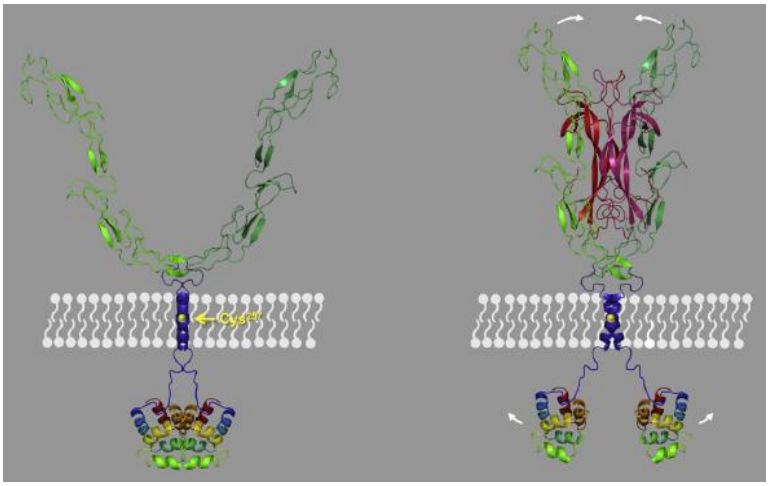
"The Molecular Basis of Human Disease" Graduate Program

The role of p75 neurotrophin receptor in the nervous system: a structure-function study



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Περίληψη

Ο p75^{NTR} ανήκει στην υπεροικογένεια των υποδοχέων του TNF (TNFRSF) και είναι γνωστό πως έχει την ικανότητα να προσδένει όλες τις έως τώρα χαρακτηρισμένες νευροτροφίνες (BDNF,NGF, NT-3, NT-4/ 5) αλλά και τις πρόδρομες μορφές τους (proneurotrophins). Γνωρίζουμε επίσης ότι είναι σε θέση να αλληλεπιδρά με ένα πλήθος άλλων μορίων, μεταξύ των οποίων συγκαταλέγονται οι υποδοχείς Trk (A, B, C) και πολλές ενδοκυτταρικές πρωτεΐνες (Sortilin, Nogo66, TRAF-4 και 6, RIP-2, Bex-1, Rho-GDI κ.α.). Ο p75^{NTR} συμμετέχει στα μονοπάτια που οδηγούν στον κυτταρικό θάνατο αλλά και την προαγωγή της επιβίωσης του κυττάρου, ενώ φαίνεται πως διαδραματίζει σημαντικό ρόλο σε φυσιολογικές διαδικασίες όπως η κυτταρική διαφοροποίηση και αύξηση. Η πρωτεϊνική δομή του p75^{NTR} αποτελείται από μια εξωκυτταρική περιοχή (Extracellular Domain, ECD), μια μικρή διαμεμβρανική περιοχή και την ενδοκυταρρική περιοχή (Intracellular Domain, ICD), η οποία αποτελείται από μια υποπεριοχή 60 αμινοξικών καταλοίπων ακολουθούμενη από μια δεύτερη υποπεριοχήη οποία σχετίζεται με την ικανότητα του p75^{NTR} να προκαλεί απόπτωση (Death Domain, DD), και καταλήγει σε 6 επιπλέον αμινοξικά κατάλοιπα. Σκοπός της παρούσας μεταπτυχιακής εργασίας είναι η ταυτοποίηση συγκεκριμένων καταλοίπων της DD περιοχής του p75^{NTR} όσον αφορά την ενεργοποίηση συγκεκριμένων και διακριτών σηματοδοτικών μονοπατιών, καθώς και η σύνδεση τους με βιολογικές δράσεις του κυττάρου όπως ο κυτταρικός θάνατος. Τα αποτελέσματα αυτά θα διασαφηνίσουν τις πολύπλοκες κυτταρικές οδούς οι οποίες εκκινούν απο διαφορετικές περιοχές του υποδοχέα, παρέχοντας τις απαιτούμενες πληροφορίες για εξειδικευμένη φαρμακολογική στόχευση του υποδοχέα.

Abstract

The p75 neurotrophin receptor ($p75^{NTR}$) was the first neurotrophin receptor discovered which can essentially bind to all neurotrophins with equal affinity. Recent studies have shown that it can also bind to the pro-form of NGF with a higher affinity than its mature form. $p75^{NTR}$ is capable of interacting with an increasing list of co-receptors (including Trk, Nogo and Lingo) and endocytoplasmic proteins, apparently by a variety of different mechanisms. Many of the effectors identified have been implicated in cell cycle regulation, apoptosis, or both, suggesting pleiotropic roles for $p75^{NTR}$ during development. By using genetic analysis of distinct sets of exposed residues in the intracellular domain that can selectively interfere with the activation of $p75^{NTR}$ signaling pathways, we tried to identify the exact receptor domains capable of activating some pathways but not others, resulting in distinguishing the multiple functions of the receptor in the Nervous System.

1. INTRODUCTION

1.1. Neurotrophins

The p75 neurotrophin receptor ($p75^{NTR}$), which is a member of the Tumor Necrosis Factor receptor (TNF-R) superfamily, has been identified as a transmembrane protein that can bind with approximately equal affinity to each of the neutrophins (*Roux & Barker, 2002*).

Neurotrophins are important regulators of neural survival, development, function and plasticity (*Eide et al., 1993; Segal & Greenberg 1996; McAllister et al., 1999; Sofroniew et al., 2001*). There are known four neurotrophins characterized in mammals, named Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), and Neurotrophin-4 (NT-4). All four are derived from a common ancestral gene and they are similar in sequence and structure (*Hallbook, 1999*). The mature forms of neurotrophins interact with a member of the Trk receptor tyrosine kinase family and $p75^{NTR}$ (*Bothwell et al., 1995; Friedman et al., 1999; Lee et al., 2001*). In addition, unprocessed neurotrophins (pro-neurotrophins) have been found to display selectivity for $p75^{NTR}$ over Trk receptors (*Lee et al., 2001*).

1.2 $p75^{NTR}$ structure

$p75^{NTR}$ is not widely expressed in the normal adult brain. However, $p75^{NTR}$ is expressed in the nervous system during development or after injury (*Chao, 2003*) and it has been primarily studied as regulator of survival and apoptosis in neurons and glia cells (*Dechant & Barde, 2002*).

$p75^{NTR}$ is a transmembrane protein, with its molecular weight (75 kDa) determined by glycosylation through both N- and O-linkages in the extracellular domain (*Grob et al., 1985; Johnson et al., 1986*). It consists of intracellular, transmembrane and extracellular domains which are different from other TNF receptors (**Figure 1**).

The crystal structure of the ligand binding domain (CRDs 1 to 4) of $p75^{NTR}$ in complex with NGF has recently been solved (*He et al., 2004*). The complex was shown to be composed of an NGF homodimer asymmetrically bound to a single $p75^{NTR}$ molecule. $p75^{NTR}$ was found to bind along the homodimeric interface of NGF, and to disable NGF's symmetry-related second binding site through an allosteric conformational change (*He et al., 2004*). Neurotrophins bind to the second and third CRD in $p75^{NTR}$ (*He et al., 2004; Welcher et al., 1991; Yan et al., 1991; Baldwin et al., 1995*).

Ligand-mediated dimerization has emerged as a universal mechanism of growth factor receptor activation. Despite the recently described crystal structure of the complex of NGF with the $p75^{NTR}$, we now know that neurotrophins can interact with dimers of the p75 neurotrophin receptor, but the mechanism of receptor activation has remained elusive. Recently studies have shown that $p75^{NTR}$ forms disulphide-linked dimers independently of neurotrophin binding through the highly conserved Cys257 in its transmembrane domain (*Vilar et al., 2009*).

The three-dimensional structure of the intracellular domain of $p75^{NTR}$ ($p75^{ICD}$) has been investigated by nuclear magnetic resonance (NMR) (*Liepinsh et al., 1997*). The $p75^{ICD}$ was found to contain a 60 residue long and highly flexible juxtamembrane segment ($p75^{JUX}$) of undefined structure, followed by a globular domain of 84 residues known as the death domain, and a short unstructured C-terminal tail of 6 residues. The $p75^{NTR}$ death domain ($p75^{DD}$) consists of six conserved α-helices connected by loop regions packed together into a small globular structure of about 25 to 30 Å in diameter. The intracellular domain of $p75^{NTR}$ interacts with a number of death-signalling binding partners (*Coulson et al., 2004; Frade, 2000; Roux & Barker, 2002*) and its C-terminal tail may bind to a PDZ domain containing proteins known for protein trafficking and receptor complex association (**Figure 1**).

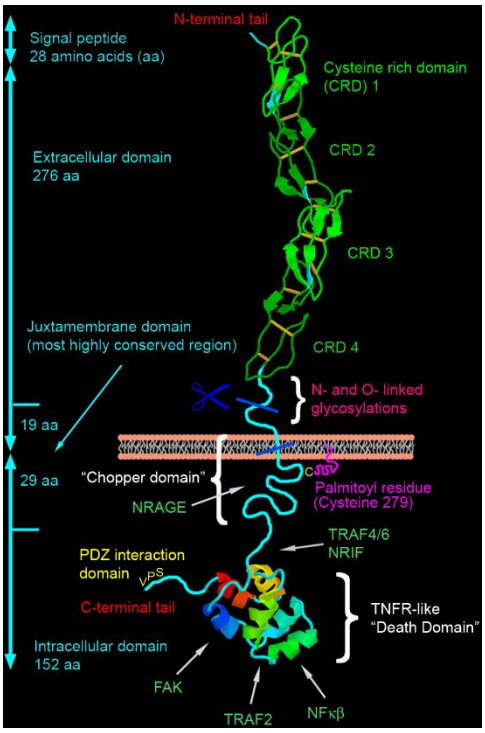

Figure 1.

The structure and the interactions of $p75^{NTR}$.

(*Underwood & Coulson, IJBCB, 2007*)

1.3. The Biological Function of $p75^{NTR}$

$p75^{NTR}$ can interact with tyrosin kinase receptors (TrkA, TrkB and TrkC constitute the high-affine receptors for NGF, BDNF and NT-3 respectively) to facilitate neuronal survival and differentiation in response to neurotrophins (*Hempstead, 2002*), and can mediate an apoptotic response to pro-neurotrophins (the immature form of neurotrophins) by interacting with sortilin as a co-receptor (*Nykjaer et al., 2004; Teng et al., 2005; Volosin et al., 2006*). In addition, $p75^{NTR}$ is part of the Nogo receptor signaling complex that is activated by myelin proteins to inhibit axonal growth (*Wang et al., 2002; Wong et al., 2002*). Therefore, $p75^{NTR}$ may promote cell survival or death, and facilitate or inhibit axonal growth, depending on its receptor partners.

$p75^{NTR}$ has no kinase activity nor is it linked to a G-protein-coupled pathway. Instead, $p75^{NTR}$ interacts with different intracellular factors by recruiting intracellular binding proteins to mediate specific functions. Different binding proteins maybe recruited to the receptor in distinct cell types (*Barker, 2004; Yamashita et al., 2005; Arevalo & Wu, 2006; Blochl, 2007; Hennigan et al., 2007*). The $p75^{NTR}$-mediated signaling pathways are only now becoming defined, in part due to the identification of proteins that bind the $p75^{NTR}$ intracellular domain upon neurotrophin activation of $p75^{NTR}$. Many of the effectors identified have been implicated in cell cycle regulation, apoptosis, or both, suggesting pleiotropic roles for $p75^{NTR}$ during development (*Dechant & Barde, 2002; Hempstead, 2002; Lopez-Sanchez & Frade; 2002; Roux & Barker, 2002*).

An important pro-survival signaling pathway activated by NGF, but not BDNF or NT-3, through $p75^{NTR}$ is the NF-κB pathway (Carter et al., 1996). The activation of NF-κB requires several proteins, including TRAF6, p62, interleukin-1 receptor-associated kinase (IRAK), and receptor-interacting protein-2 (RIP-2) (*Kuruvilla et al., 2000; Yeiser et al., 2004; Casademunt et al., 1999; Mamidipudi et al., 2002; Khursigara et al., 2001*). Upon activation in response to neurotrophins, NF-κB translocates to the nucleus and triggers the expression of Hes1/5 to modulate dendritic growth (*Salama-Cohen et al., 2005*). This pro-survival pathway, unlike JNK activation, is not abolished by the expression of TrkA and is more likely to be activated when the cells have been previously exposed to stress conditions, as with addition of TNF or serum deprivation (*Bhakar et al., 1999; Cosgaya et al., 2001*).

Among other functions, $p75^{NTR}$ can also modulate axonal growth with different outcomes depending on the molecule that binds to the receptor. Neurotrophin binding leads to axonal growth whereas myelin-derived growth inhibitors (MDGIs) evoke growth-cone collapse. These opposite effects are obtained by the regulation of the small GTPase RhoA, a member of the Rho family of proteins that have been shown to control the organization of the actin cytoskeleton in many cell types. In the absence of neurotrophins, a constitutive interaction between $p75^{NTR}$ and Rho-GDI (inhibitor of RhoA) maintains RhoA activation and inhibition of axonal growth. Neurotrophin binding to $p75^{NTR}$ causes dissociation of RhoGDI from the receptor, blocking RhoA activity and leading to axonal growth (*Yamashita et al., 1999, 2003*).

Modulation of axonal growth through $p75^{NTR}$-RhoA pathways is not exclusive to neurotrophins. In the last few years several reports have implicated $p75^{NTR}$ as a part of a receptor complex with the Nogo receptor (NgR) that mediates the axonal outgrowth inhibitory signals of myelin derivatives, such as Nogo66, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) (*Wang et al., 2002; Wong et al.,2002*). LINGO-1, a nervous system-specific transmembrane protein, also binds NgR1 and $p75^{NTR}$ and is a functional component of the NgR1/$p75^{NTR}$ signaling complex.

1.4. The function of $p75^{NTR}$ in glia

The $p75^{NTR}$ is expressed additionally to neurons in a variety of other cell types like glia population. Among glial cells, Schwann cells have been the most extensively studied for $p75^{NTR}$ signaling (**Figure 2**). Schwann cells express high levels of $p75^{NTR}$ in developing and regenerating peripheral nerves, although the specific role of $p75^{NTR}$ in mediating myelination is unclear. *Cosgaya and his colleagues* demonstrated that the enhancement of myelin formation in co-cultures of dorsal root ganglia neurons and Schwann cells by endogenous BDNF is mediated by $p75^{NTR}$ receptors (*Cosgaya et al., 2002*). Developmentally, the myelin sheaths of p75-/- mice were significantly thinner than those of wild-type mice (*Cosgaya et al., 2002*). The induction of myelination by BDNF depends on the recruitment of the polarity protein Par-3 to interact with $p75^{NTR}$ at the axon–glial junction in Schwann cells (*Chan et al., 2006*). To elucidate the *in vivo* function of $p75^{NTR}$ in Schwann cells during remyelination after peripheral nerve injury, Tomita and his colleagues studied the effects of $p75^{NTR}$-deficient Schwann cells after their transplantation in nude mice. They found that the mice that received the p75–/– Schwann

cells showed impaired myelination and lower numbers of the retrograde labeled motor neurons at 6 and 10 weeks after injury (*Tomita et al., 2007*). In addition to its role in remyelination, $p75^{NTR}$ may also contribute to extracellular matrix remodelling. $p75^{NTR}$, which was upregulated after tissue injury, blocked fibrinolysis by down-regulating the serine protease, tissue plasminogen activator, and by up-regulating plasminogen activator inhibitor-1 (*Sachs et al., 2007*).

Oligodendrocytes were among the earliest cell populations shown to undergo $p75^{NTR}$-mediated apoptosis *in vitro* (*Casaccia et al., 1996*), mediated by Rac GTPase activity, JNK phosphorylation (*Harrington et al., 2002*) and caspase activation (*Gu et al., 1999*) (**Figure 2**).

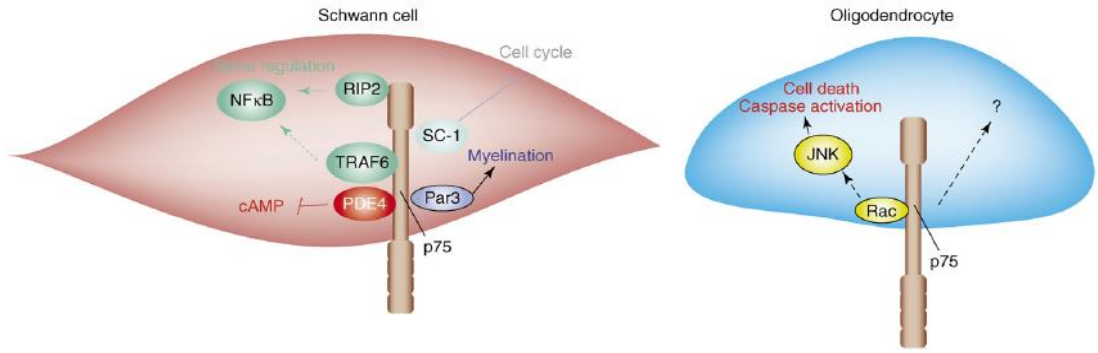

Figure 2.

Schematic diagram showing some of the identified $p75^{NTR}$ binding proteins and their suggested functions in Schwann cells and oligodendrocytes.

(Friedman W.J., Trends in Neurosciences, 2007)

In vivo, $p75^{NTR}$-mediated oligodendrocyte apoptosis occurs after spinal cord injury (*Casha et al., 2001; Beattie et al., 2002*). However, $p75^{NTR}$ does not always mediate the death of oligodendrocytes (*Ladiwala et al., 1998*) and evidence for $p75^{NTR}$-mediated oligodendrocyte differentiation has also been found (*Copray et al., 2005*). Recently, *Sha Mi and his colleagues* have demonstrated that LINGO-1 is a key regulator of oligodendrocyte differentiation and myelination (*Mi et al., 2004; Mi et al., 2005*). Furthermore, diminished LINGO-1 function in mice and rats caused by Lingo-1 knockout or treatment with LINGO-1 antagonist antibody alleviates the symptoms of clinical neuropathology associated with MOG-induced encephalomyelitis (EAE) (*Mi et al., 2007*).

Other studies have demonstrated that the signal transduction mechanism may involve the displacement of Rho-GDI from RhoA, resulting in its activation and the consequent axonal growth inhibition (*Yamashita et al., 2003*). Additionally, a positive feedback loop occurs upon spinal cord injury where $p75^{NTR}$ expression is upregulated through a RhoA-dependent mechanism, with a subsequent further increase in RhoA activation (*Dubreuil et al., 2003*).

Numerous studies have demonstrated that astrocytes express neurotrophin receptors in primary culture (*Hutton et al., 1992*) and NGF treatment upregulates $p75^{NTR}$ mRNA expression (*Hutton et al., 1992*). There is uncertainty about the role of $p75^{NTR}$ in pathological conditions associated with astrocytes, although one possibility may be that $p75^{NTR}$ regulates the proliferation of astrocytes to restrict glial scar formation after CNS injury.

1.5. Experimental Approach

Different regions of the $p75^{ICD}$ bind to different intracellular effector molecules. These are in turn responsible for downstream propagation of different $p75^{NTR}$ signals. The most important intracellular domain of this receptor is the Death Domain (DD), because we know that it possess a major role in all interactions and propagation of the molecular signaling pathways that originate from the $p75^{NTR}$. By using point mutagenesis analysis we now identify regions in the DD of $p75^{NTR}$ receptor without disrupt its 3-dimensional structure, involved in the activation of different signaling pathways and biological effects. These genetically manipulated mutants will be a useful tool for separating the multiple signaling and functional capabilities originated from the $p75^{NTR}$ receptor.

For this purpose, we explore the interaction between the mutant variants of $p75^{DD}$ and receptor-interacting protein 2 (RIP-2) that may accounts for the ability of neurotrophins to choose between a survival versus death pathway. RIP-2 is an adaptor protein with a serine threonine kinase and a caspase recruitment domain (CARD) and is highly expressed in dissociated Schwann cells and displays an endogenous association with $p75^{NTR}$. RIP-2 binds to the death domain of $p75^{NTR}$ via its CARD domain in an NGF-dependent manner (*Khursigara et al., 2001*).

In addition, we describe the interaction between the mutant variants of $p75^{DD}$ and Rho-GDP dissociation inhibitor (Rho-GDI) by using neutrophins as ligands. Rho-GDI interacts directly with $p75^{NTR}$ and initiates the activation of RhoA and this interaction is

strengthed by MAG or Nogo. In its active form, Rho rigidifies the actin cytoskeleton, thereby inhibiting axonal elongation and causing growth cone collapse. In contrast, neutrophin binding to $p75^{NTR}$ inactivates RhoA by releasing Rho-GDI from the death domain of the receptor (*Yamashita et al., 1999, 2003, 2005*).

In order to investigate the ability of these mutant isoforms of the receptor to bind to intracellular interactors such as RIP-2 and RGDI, HEK293 cells which not express $p75^{NTR}$ endogenously, co-tranfected with $p75^{NTR}$-wild type or mutant variants of p75DD and with RIP-2 or RhoGDI expression vectors and then treated with different kind of neurotrophins. Cell extracts of the transfected cells immunoprecipitated with anti-p75 antibody and the presence of either RIP-2 or RhoGDI in the precipitates will be detected in Western Blots using anti-RIP-2 (RICK) or anti-RhoGDI antibodies.

In addition, HEK293 cells first transfected with $p75^{NTR}$-wild type or the $p75^{NTR}$ mutant expression vectors and then treated with the $p75^{NTR}$ ligands such as (pro)NGF and (pro)BDNF in order to measure the cell death by using the TUNEL method.

1.3. The Aim of this Study

These studies aim at elucidating a structure-function map of the $p75^{ICD}$, a prerequisite for understanding the contribution of its different downstream effectors and signaling pathways to the biological activities of $p75^{NTR}$.

2. MATERIALS AND METHODS

2.1. Plasmids

Rat $p75^{NTR}$ was expressed from the pCDNA3 vector backbone (Invitrogen) using a full length coding sequence. $p75^{DD}$ mutants from the laboratory of Dr. Carlos Ibáñez at the Karolinska Institute, Stockholm, Sweden. Mutations were introduced using QuickChange (Stratagene) and verified by DNA sequencing. Plasmids to express RIP-2 and Rho-GDI and $shRNA^{p75NTR}$ expression vectors that down-regulate only the endogenous expression of $p75^{NTR}$.

2.2. Antibodies, proteins and chemicals

The origin of antibodies was as follows: MC192 anti-p75NTR from Chemicon, anti-RhoGDI from Cell Signaling, anti-RIP2 from Santa Cruz. NGF was purchased from Millipore and proBDNF was purchased from Alomone. NGF and proBDNF was typically applied at 100 ng/ml and 10μg/ml respectively for 20 minutes.

Lipofectamine 2000 was purchased from InVitrogen, TUNEL was purchased from Roche and APOpercentage Apoptosis Assay from Biocolor.

2.3. Cell cultures

Human Embryonic Kidney 293 cells (HEK293) are a specific cell line originally derived from human embryonic kidney cells grown in tissue culture. HEK-293 cells were obtained from LGC Promochem (LGC Standards GmbH, Germany). Cells were grown in DMEM medium containing 10% Fetal Bovine Serum (charcoalstripped for removing endogenous steroids), 100 units/ml penicillin and 0.1 mg/ml streptomycin, at 5% CO2 and 37°C.

PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla. PC12 cells were obtained from LGC Promochem. Cells were grown in RPMI 1640 containing 2mM L-glutamine, 15mM HEPES, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% horse serum, 5% fetal calf serum (both charcoal-stripped for removing endogenous steroids) at 5% CO2 and 37°C.

HEK293 cells do not express p75 or TrkA proteins endogenously in contrast to PC12 cells.

2.4. Molecular cloning and site-directed mutagenesis

E. coli strains DH5a were used as hosts in cloning plasmids ($p75^{NTR}$, $p75^{NTR}$ death domain mutants, RIP-2 and Rho-GDI). Column filters for lysate clarification were purchased from Macherey-Nagel.

The laboratory of Dr. Carlos Ibáñez at the Karolinska Institute, Stockholm, Sweden has initiated an alanine-scanning mutagenesis survey of the $p75^{DD}$. They have selected 30 amino acid residues in this region displaying ≥ 50% solvent accessibility, representing likely candidate sites for interaction with downstream effectors. Each of these residues was substituted for Ala, alone or in combinations, in the full-length sequence of the $p75^{NTR}$. Compound $p75^{DD}$ mutants were generated by combining Ala substitutions of residues which appear in close proximity in the 3-dimensional structure of the $p75^{DD}$.

2.5. Cell transfection

HEK-293 and PC12 cells were transfected with Lipofectamine 2000 according to manufacturer's instructions. Transfected cells were typically used on the 2nd day after transfection

2.6. Co-immunoprecipitation

HEK293 cells were transfected with the appropriate plasmids ($p75^{NTR}$ or $p75^{NTR}$ death domain mutants, RIP2 and RhoGDI) by using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 hours after transfection, suspended in lysis buffer (50 mM Tris-HCl, 0.15 M NaCl, 1% Triton-X100, pH 7.4) supplemented with protease inhibitors. Lysates were precleared for 1h with Protein A-Sepharose beads (Amersham) and immunoprecipitated with the appropriate antibody (MC192 anti-$p75^{NTR}$) overnight at 4°C. Protein A- Sepharose beads were incubated with the lysates for 4h at 4°C with gentle shaking. Beads were collected by centrifugation, washed four times with lysis buffer, and resuspended in SDS loading buffer. Proteins were separated by SDS/PAGE, followed by immunoblotting with specific antibodies.

2.7. Western Blot Analysis

HEK293 cells lysates were electrophoresed through a 10% SDS-polyacrylamide gel, and then proteins were transferred to nitrocellulose membranes, which were processed according to standard Western blotting procedures. To detect protein levels, membranes were incubated with the appropriate antibodies: p75NTR (dilution 1:500), RIP-2 (dilution 1:1000) and Rho-GDI (dilution 1:1000) Proteins were visualized using the ECL Western blotting kit (ECL Amersham Biosciences, UK) and blots were exposed to Kodak X-Omat AR films. A PC-based Image Analysis program was used to quantify the intensity of each band (Image Analysis, Inc., Ontario, Canada).

To normalize for protein content the blots were stripped and stained with GAPDH or Actin antibodies (dilution 1:1000); the concentration of each target protein was normalized versus GAPDH or Actin.

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2.8. Cell death assays.

Cell death was assessed by the TUNEL method using kit from Roche. NGF/proBDNF were added 2 days after transfection and left for another day in serum free-medium prior to assay of cell death. Cell death was also assessed by APOpercentage Apoptosis Assay from Biocolor.

2.9. Flow cytometry

HEK293 cells were cultured in 12-well plates, 24 hours later they were transfected with the $p75^{NTR}$ wild type or $p75^{NTR}$ death domain mutants. Staining was performed 48h later. Transfectants (5x105 cells) were peleted and incubated with 20µl of the primary antibodies against $p75^{NTR}$ receptors (mouse, Millipore MAB365R, dilution 1:500). Afterwards, transfectants were washed three times with PBS and 20µl of the secondary antibodies, anti-mouse-fluorescein conjugated were added at 1/1000 dilution for 30min at 4°C. Transfectants were washed twice with PBS, resuspended in 500µl of PBS, and were analyzed by a Beckton-Dickinson FACSArray apparatus and the CELLQuest software (Beckton-Dickinson, Franklin Lakes, NJ).

3. RESULTS

3.1. Identification of residues in the intracellular domain of p75^{NTR} (p75^{ICD}) that are involved in the activation of different signaling pathways.

3.1.1. Recruitment of RIP-2 to p75 ^{NTR} variants carrying mutations on the intracellular domain in transfected HEK293 cells.

The association between RIP-2 and p75 ^{NTR} was tested by co-immunoprecipitation in transfected HEK293 cells. As shown in **Fig. 3**, wild type p75 ^{NTR} could be immunoprecipitated together with RIP-2 after stimulation with NGF, indicating a ligand dependent interaction in agreement with previous observations (*Khursigara et al, 2001*).

As a prelude to a more detailed structure-function analysis of the p75^{ICD}, we first tested p75^{NTR} molecules lacking the death domain (mutantΔDD). As we expected mutantΔDD was unable to interact with RIP-2 in the presence or absence of ligand stimulation.

MutantDD2 and mutantDD3 co-immunoprecipitated together with RIP-2 in the presence of NGF treatment, indicating that these mutations mimicked wild type p75^{NTR}. On the contrary, mutants DD1, DD5 and DD7 showed that they could interact with RIP-2 even in the absence of the ligand, and thus behaving like constitutively-active receptors. MutantDD4 and DD6 were the only mutants that represent a new category of effects because it seems that they can recruit RIP-2 in the absence of NGF, and this interactation is abolished after the stimulation with the neurotrophin. However, further investigation is required in order to clarify the aforementioned results and provide statistically significant experiments.

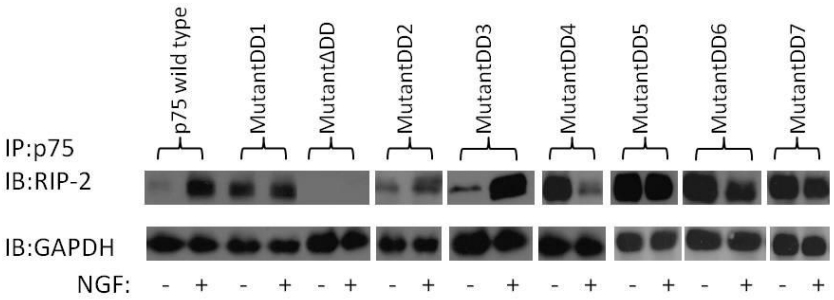

Figure 3.
Differential activities of p75^{DD} mutants in recruitment of RIP-2.

3.1.2. Association between Rho-GDI and mutants of $p75^{DD}$ in transfected HEK293 cells.

In addition, we describe the interaction between the mutant variants of $p75^{DD}$ and Rho-GDP dissociation inhibitor (Rho-GDI) by using neutrophins as ligands. The association between Rho-GDI and mutants of $p75^{DD}$ was tested by co-immunoprecipitation in transfected HEK293 cells.

As shown in **Fig. 4**, neutrophin (NGF) binding to $p75^{NTR}$ wild type release Rho-GDI from the death domain of the receptor (*Yamashita et al., 1999, 2003, 2005*). $p75^{NTR}$ molecules lacking the death domain (mutantΔDD) and mutantDD4 were unable to interact with Rho-GDI in the presence or absence of ligand stimulation. However, all the other mutants were unable to release Rho-GDI after ligand stimulation, indicating that these death domains mutations were important for this interactor release and subsequently inactivation of RhoA.

The problem that we have to figure out in the future is the non-specific bands that arise from the primary antibody Rho-GDI. As shown in **Fig.4** the bands that represent Rho-GDI protein are not as clear as the bands of RIP-2 protein.

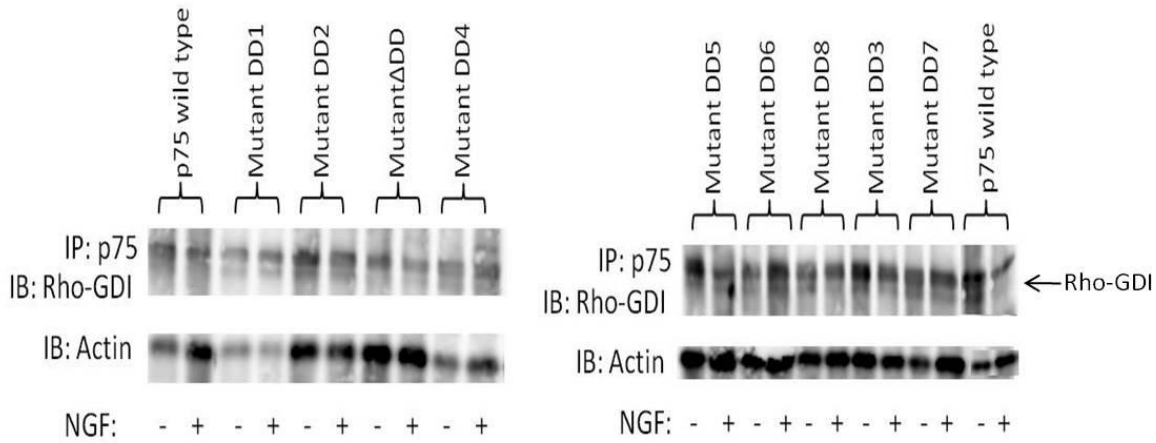

Figure 4.
The interaction between the $p75^{DD}$ mutants and Rho-GDI.

3.2. The role of $p75^{DD}$ mutants in the induction of cell death after (pro)neurotrophin stimulation.

We firstly examined the ability of $p75^{NTR}$ wild type to mediate cell death in response to NGF (*Rabizadeh, 1993*) and proBDNF (*Lee et al., 2001*) in transfected HEK293 cells. Cells were transfected with the $p75^{NTR}$ wild type or the empty vector. The treatment with NGF and proBDNF lasted 24hours. As shown in **Fig.5**, FACS analysis of apoptotic cells (stained with TUNEL) has shown that NGF increase the number of apoptotic cells in serum deprived HEK293 cell cultures transfected with $p75^{NTR}$ wild type in comparison to serum free condition (control). However, the pro-neurotrophin (proBDNF) did not significantly alter the apoptotic effect as expected. Perhaps this action requires the co-receptor complex of $p75^{NTR}$ and sortilin (*Teng et al., 2005*) or the specific proneurotrophin was not effective due to its construction.

Next, we are planning to transfect HEK293 cells with $p75^{DD}$ mutants and treat these cells with the $p75^{NTR}$ ligands such as NGF and (pro)BDNF in order to measure the cell death by using the TUNEL method.

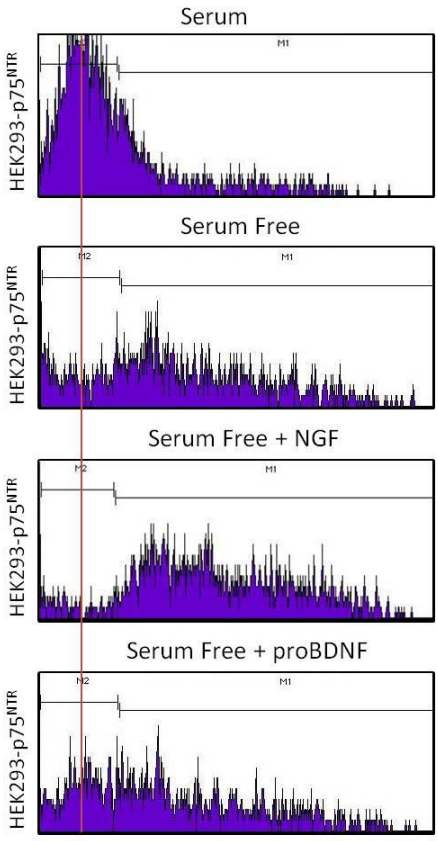

Figure 5.
Cell death assay in HEK293 cells transfected with $p75^{NTR}$ wild type construct in response to NGF and proBDNF. Cell death was assessed by the TUNEL method.

The results could also be confirmed by using the APOpercentage Apoptosis Assay. Phosphatidylserine transmembrane movement, as produced by the *flip-flop* mechanism, results in the uptake of the APOPercentage Dye by apoptotic committed cells. The APOPercentage Dye enters the cells following this event and dye uptake continues until blebbing occurs. No further dye can then enter the now defunct cell and the dye that has accumulated within the cell is not released. Exposure of phosphatidylserine to the exterior surface of the membrane has been linked to the onset of the execution phase of apoptosis, experimentally supported by annexin-V binding to phosphatidylserine. As shown in **Fig.6**, HEK293 cells transfected with $p75^{NTR}$ wild type and treated with NGF (24h) induce apoptosis in comparison to serum free conditions (control).

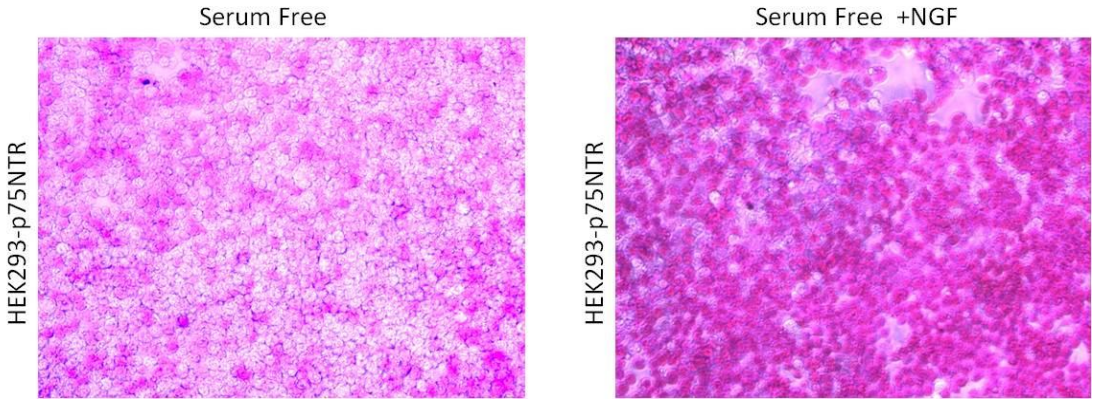

Figure 6.

APOpercentage Apoptosis Assay in HEK293 cells transfected with $p75^{NTR}$ wild type construct in response to NGF.

Conclusively, the structure-function map of the $p75^{DD}$ would allow us not only to molecularly dissect the different pathways activated by this receptor but also to study their physiological importance in cellular physiology and to design new pharmacological drugs with specific targets on the receptor's functions, minimizing side effects from the multiple mediators of receptor's signaling.

3.3. The role of $p75^{DD}$ mutants in PC12 cells after neurotrophin stimulation.

In order to probe the functional importance of $p75^{DD}$ mutants for $p75^{NTR}$ signaling in a more physiological context, PC12 cells will be co-transfected with $p75^{NTR}$ wild type or the $p75^{DD}$ mutants and with $shRNA^{p75NTR}$ (down-regulate only endogenous $p75^{NTR}$ expression) expression vectors and then treated with the $p75^{NTR}$ ligands such as NGF and (pro)BDNF and apoptosis will be measured. As shown to **Fig. 7**, when introduced together into PC12 these shRNAs effectively suppressed endogenous $p75^{NTR}$ expression, while a control shRNA had no effect.

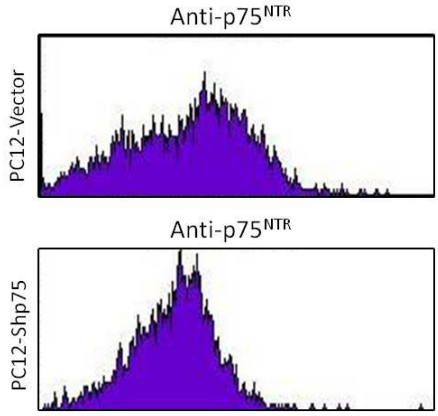

Figure 7.
Downregulation of $p75^{NTR}$ expression in PC12 cells following transfection of $p75^{NTR}$ shRNAs.

4. DISCUSSION

The p75 neurotrophin receptor ($p75^{NTR}$) is at the cross-roads of several biological processes important for the development, maintenance and function of the nervous system, including neuron survival/death decisions, axonal growth and differentiation, neuronal responses to environmental stimuli and nerve regeneration. Moreover, new experimental and clinical results are implicating this receptor to an increasing list of actions in other tissues like the immune system or epithelial cells, or in various disease's development like cancer and autoimmune diseases.

Although the number of known $p75^{NTR}$ interacting partners has increased steadily in recent years, this knowledge has not yet resulted in a better understanding of the physiological functions of this receptor or its different signaling pathways. By using genetically manipulated mutants of the Death Domain of $p75^{NTR}$, we will be able to test the ability of these molecules to bind to intracellular interactors and to elicit activation of downstream signaling pathways and biological effects after stimulation with neurotrophins. This methodological approach will allow to separate the multiple signaling pathways that are mediated from $p75^{NTR}$, and thus providing more specific explanations about its biological role.

As it was mentioned above, neutrophin binding to $p75^{NTR}$ inactivates RhoA by releasing RhoA-inhibitor named Rho-GDI from the death domain of the receptor. In contrast, mutantDD3 was unable to release Rho-GDI in the presence of NGF without losing its capacity to recruit RIP-2 (**Table 1**). Taken together, these results suggest that primarily mutantDD3 cannot mediate Rho-GDI signaling and subsequently inactivate RhoA (promoting axonal inhibition), while RIP-2 recruitment is not affected.

Mutants DD1, DD5 and DD7 showed that they could interact with RIP-2 even in the absence of the ligand, and thus behaving like constitutively-active receptors. Taking into consideration that $p75^{NTR}$ activation by NGF involves the separation of intracellular domains (*Vilar et al., 2009*), those results suggest that these mutants provoke conformational rearrangements of $p75^{DD}$ at the level of Death Domain that mimics activation elicited by neurotrophins.

Our deletion analysis revealed that MutantDD6 can recruit RIP-2 in the absence of NGF, and this interaction is abolished after the stimulation with the neurotrophin. On the other hand, mutantDD6 was unable to release Rho-GDI in the presence or absence of ligand stimulation (**Table 1**). These results suggest that Rho-GDI and RIP-2 may compete directly for the same binding site on the Death Domain of $p75^{NTR}$.

MutantDD4 could not interact with Rho-GDI even in the absence of NGF, but it could recruit RIP-2 in the same condition (absence of NGF) and it cannot continue to bind RIP-2 in the presence of NGF (**Table 1**). This would imply that other interactors may compete for the same binding site except RIP-2 and Rho-GDI.

However, there are many problems that we have to figure out in the future. One of these problems is the non-specific bands that arise from the primary antibody Rho-GDI. As shown in **Fig.4** the bands that represent Rho-GDI protein are not as clear as the bands of RIP-2 protein are. In addition, the bands that represent RIP-2 protein were also observed in the absence of ligand stimulation. Perhaps we have to reduce the plasmid concentration of RIP-2 in transfected HEK293 cells in order to lower the levels of expression of RIP-2 similar to those seen in wild type $p75^{NTR}$. By solving these problems we could conclude for the mutants that have not been clarified yet.

	RIP2 (NGF)	Rho-GDI (NGF)
p75 wild type	++	--
MutantDD1	--/?	++
MutantDD2	--/?	++
MutantDD3	++	++
MutantDD4	--	--
MutantDD5	?	++
MutantDD6	--	++
MutantDD7	?	++

Table 1.
Differential activities of $p75^{DD}$ mutants.

++ means binding of RIP2, RhoGDI,
-- means release of RhoGDI,
? means no clarified yet

The structure-function map of the $p75^{DD}$ would allow us not only to molecularly dissect the different pathways activated by this receptor but also to study their physiological importance. Having setup the control experiment by using HEK293 cells transfected with $p75^{NTR}$-wild type and treated with NGF and proBDNF, we are able to transfect HEK293 cells with $P75^{DD}$ mutants and treat these cells with the $p75^{NTR}$ ligands such as NGF and proBDNF in order to measure the cell death by using the TUNEL method.

In addition, PC12 cells that express endogenously all neurotrophins receptors will be used as model for studying apoptotic machinery. In order to specify the input of its receptor to certain signaling pathways and cellular phenotypes, shRNAs that down-regulate only endogenous $p75^{NTR}$ expression will be used.

Our long-term goal is to generate novel *in vivo* models for studying physiological functions of $p75^{NTR}$ by knocking-in in the mouse germ line a loss-of-function mutant that described above. These animal models will help to clarify the consequences of $p75^{NTR}$ signaling within neurons, an issue that has still not been adequately addressed partly due to the paucity of neuronal systems in which activation of $p75^{NTR}$ signaling can be studied. The full consequences of these mutations are unknown and could reveal unexpected functions. In conclusion, these animals are likely to provide novel, unexpected insights into $p75^{NTR}$ biology.

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