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ANALISI FUNZIONALE DEI RECETTORI PER LE NEUROTROFINE p75NTR E Trka IN NEUROBLASTOMA

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INDEX

Introduction

Growth factor receptors define the linkage point between extracellular events and the intracellular responses, activating a plethora of signalling pathways. Transmembrane receptors proteins play critical roles in either development and maintenance of the cellular biology and are strongly correlated with the occurrence of disease (Shor NF, 2005). More than other, the brain tissue is the most sensitive to the presence of growth factors and, the differential expression of their specific receptors, amplify the complexity of the system. Neurotrophin are the specific growth factors in the nervous system. They were first identified as survival factors for sympathetic and sensory neurons but it is appreciated that they regulate many aspects of neuronal development and function, including synapse formation and synaptic plasticity (Lewin & Barde 1996; Bibel & Barde 2000; Kaplan & Miller 2000; Huang et al. 2001; Poo 2001; Shooter 2001; Sofroniew et al. 2001; Dechant & Barde 2002; Chao 2003; Huang & Reichardt 2003; Lu et al. 2005). The first Neurotrophin identified was the Nerve Growth Factor (NGF), searching for survival factors that could explain the deleterious effects of deletion of target tissues on the subsequent survival of motor and sensory neurons (Levi-Montalcini 1987; Shooter 2001). The second neurotrophin to be characterized was the Brain-Derived Neurotrophic Factor (BDNF) purified from pig brain as a survival factor for several neuronal populations not responsive to NGF (Barde et al. 1982). Given the conserved features of the sequences, it has been easier to clone the other members of the family. Together with the NGF (Nerve Growth Factor) and BDNF (Brain Derived Neurotrophin Factor) other two factors are reported so far in mammals, the NT-3 and the NT-4 (Neurotrophin-3 and 4). It is known that neurothrophins have an important role in the development and function of neurons either in the central and in the peripheral nervous systems, including precursor proliferation and commitment, cell survival, axon and dendrite growth, membrane trafficking, synapse formation and function, as well as glial differentiation and interactions with neurons. Biological effects of each of the four mammalian neurotrophins are mediated through activation of one or more of the three members of the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases (TrkA, TrkB and TrkC). In addition, all neurotrophins activate the p75 neurotrophin receptor p75NTR, a member of the tumour necrosis factor receptor superfamily.

NGF is the best characterised member of the family and is known to signal for opposite effects through interaction with two distinct families of receptors: activation of cell survival by TrkA and induction of cell death by binding to p75NTR. Engagement of Trk receptors leads to activation of well characterized pathway like Ras, phosphatidylinositol 3-kinase, phospholipase C-gamma1 and the mitogen-activated protein kinases. On the other side, the molecular mechanisms mediated by p75NTR still remain to define even though the JNK, NF-κB and ceramide have been implicated (Huang and Reichardt, 2003). Preclinical studies point to the therapeutic potential of neurotrophic factors in preventing or slowing the progression of neurodegenerative conditions. Given the difficulties inherent with a protein therapeutic approach to treating central nervous system disorders, increasing attention has turned to the development of alternative strategies related to the signalling pathways involved in the neurodegenaration (Reichardt LF, 2006).

1.0 Neurotrophins and Neurotrophin Receptors

The neurotrophins and their genes share homologies in sequence and structure and the organization of the genomic segments adjacent to these genes also resulted to be similar. Together, these observations provide evidence that the neurotrophin genes have arisen through successive duplications of a portion of the genome derived from an ancestral chordate (Hallbook 1999). Their genes share many similarities, including the existence of multiple promoters. The protein product of each gene includes a signal sequence and a prodomain, followed by the mature Neurotrophin sequence (Hempstead B. L. 2006). Thus, each gene product must be processed by proteolysis to form a mature protein. A recent work has demonstrated that regulation of their maturation is an important post-transcriptional control point that limits and adds specificity to their actions (Lee et al. 2001). The mature neurotrophin proteins are non-covalently associated homodimers. Although some neurotrophin monomers are able to form heterodimers with other neurotrophin monomers *in vitro*, there is no evidence that these heterodimers exist at significant concentrations *in vivo.* Each of these four proteins shares a highly homologous structure with features tertiary fold and cysteine knot that are present in several other growth factors, including transforming growth factor-β (TGF-β) and platelet derived growth factor (PDGF). Additional neurotrophins have been isolated from fish, where NT-6 and NT-7 have been characterized but they do not have orthologues in mammals or birds. The biological effect of the neurotrophins is mediated by interaction with two distinct classes of receptors:

- **Trk receptors** *(tropomyosin- related kinase)*
- **p75NTR** (p75Neurotrophin Receptor)

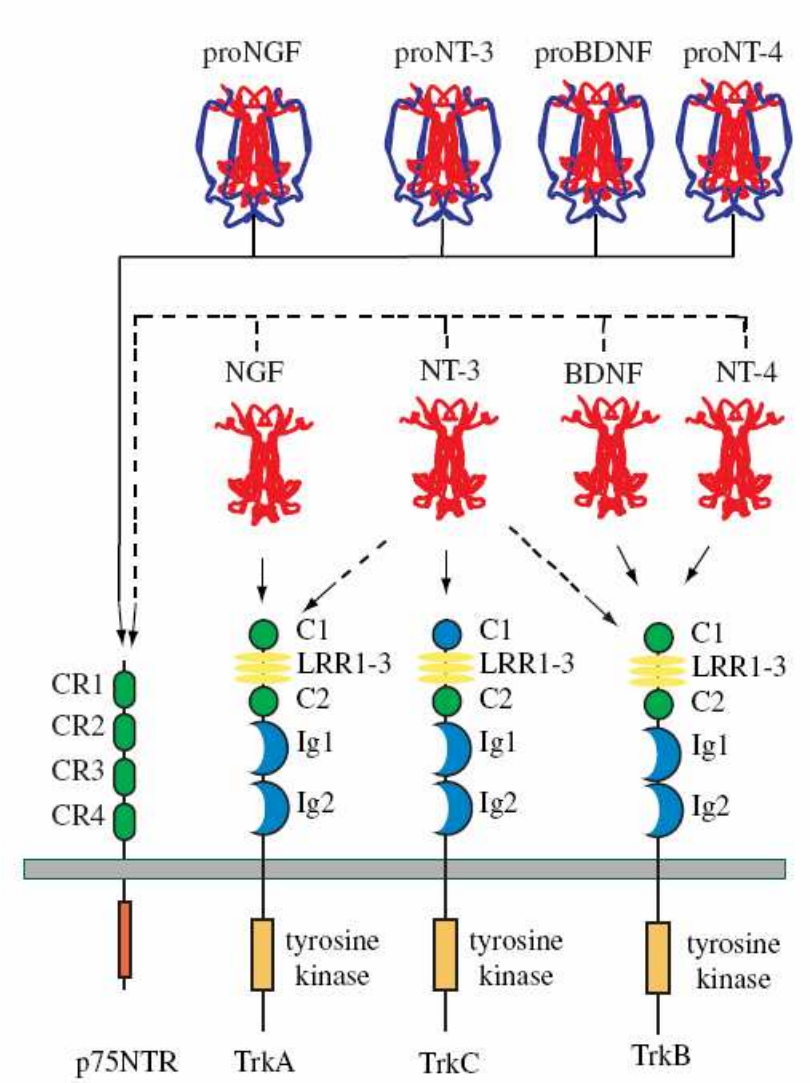


Fig. 1. Neurotrophin and Neurotrophin receptors. This illustrates the major interactions of each of the four mammalian neurotrophins. Each proneurotrophin binds p75NTR, but not the Trk receptors. Following maturation through proteolysis of the proneurotrophins, each mature Neurotrophin is able to bind and activate p75NTR, but exhibits more specific interactions with the three Trk receptors. NGF binds specifically TrkA; BDNF and NT4 recognize TrkB; NT3 activates TrkC. In some cellular contexts, NT3 is also able to activate TrkA and TrkB with less efficiency. Differential splicing generates isoforms of TrkB and TrkC that have truncated cytoplasmic domains lacking a tyrosine kinase motif. Splicing also generates an isoform of TrkC with a small insert in the kinase domain that affects substrate specificity. Splicing of exons that generate the extracellular domain of each Trk receptor results in the expression of receptors with small peptide inserts between the second immunoglobin and transmembrane domains that affect ligand-binding specificity. Ligand-binding specificity is also affected by the presence of p75NTR.

1.1 The Trk receptors

TrkA was originally characterized as a transforming oncogene in which tropomyosin was fused to an unknown tyrosine kinase (Martin-Zanca et al., 1989). The corresponding proto-oncogene was shown to be a member of a highly related family of transmembrane tyrosine kinases which were expressed in discrete neuronal populations and which bound and were activated by specific neurotrophins, with TrkA preferentially binding NGF, TrkB preferring BDNF and NT-4/5, and TrkC interacting with NT-3 (Klein et al., 1990, 1991a,b; Cordon-Cardo et al., 1991; Kaplan et al.,1991a,b; Lamballe et al., 1991; Soppet et al., 1991; Squinto et al., 1991; Ip et al., 1993b). In the absence of p75NTR, high concentrations of NT-4/5 can activate TrkA and likewise, NT-3 can activate TrkA and TrkB. NT-3 is therefore a non-preferred ligand for TrkA and TrkB, and NT-4 is a non-preferred ligand for TrkA (Segal and Greenberg, 1996). All Trk receptors are Type I transmembrane proteins that are members of the receptor tyrosine kinase superfamily (Martin-Zanca et al., 1989; reviewed in Ip and Yancopoulos, 1994; Barbacid, 1995). The extracellular domains (ECDs) of the Trk receptors contain two cysteine-rich regions (domains 1 and 3) flanking a leucine-rich repeat (domain 2), followed by two immunoglobulin (IgG)-like domains in the juxtamembrane region (domains 4 and 5; Windisch et al., 1995). Binding and deletion studies on TrkA, TrkB and TrkC indicate that domain 5 is responsible for neurotrophin binding (Urfer et al., 1995, 1998; Perez et al., 1995; Ultsch et al., 1999), with the second leucine-rich domain having a modulatory, perhaps indirect, role in ligand interaction (Windisch et al., 1995).

1.2 *The low affinity Neurotrophin receptor p75NTR*

The first Neurotrophin receptor discovered was p75 neurotrophin receptor (p75NTR), initially identified as a low-affinity receptor for NGF, subsequently has been shown to bind each of the neurotrophins with a similar affinity (Rodriguez-Tebar et al. 1990; Frade and Barde 1998; Chao and Hempstead, 1995). Based on the primary sequence and secondary structure, p75NTR belongs to the Fas-tumor necrosis factor (TNF) receptor superfamily with an extracellular domain that includes four cysteine-rich motifs, a single transmembrane domain and a cytoplasmic domain that includes a juxtamembrane domain and a 'death' domain similar to those present in other members of this family (Liepinsh et al. 1997; He & Garcia 2004).

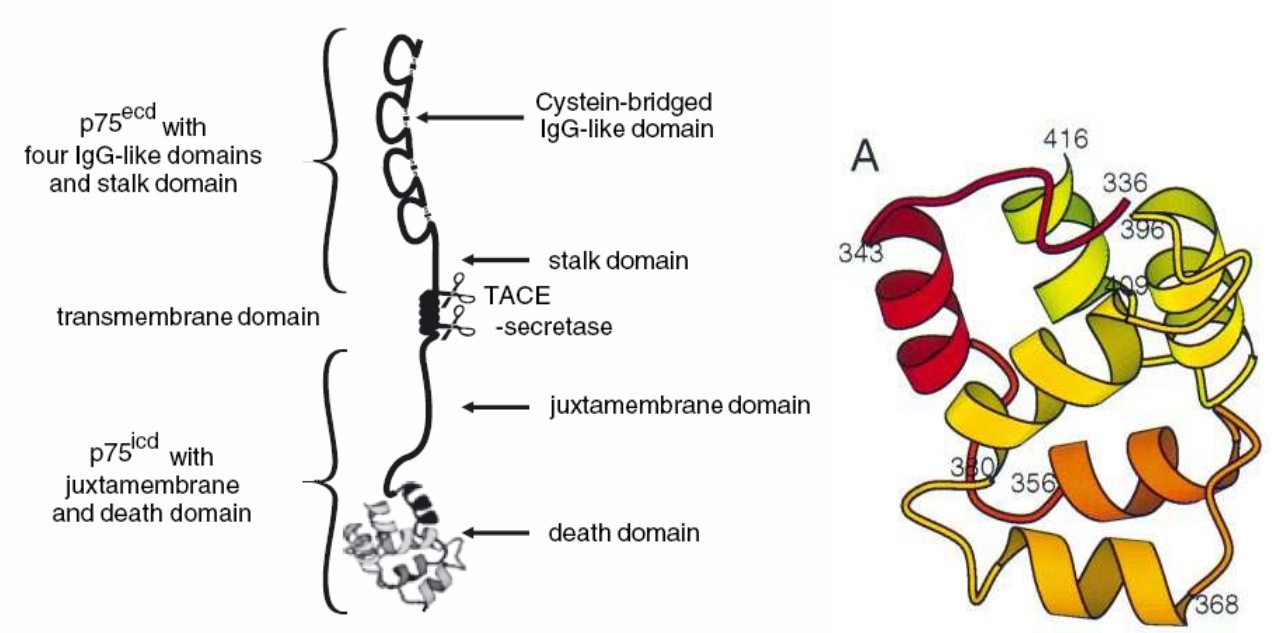


Fig. 2. Schematic representation of p75NTR. Extracellular, transmembrane and intracellular domains of the receptor on the left. Solution structure of the death domain of rat p75ICD on the right. Ribbon representation of residues 336–416. The positions of the N-terminal residues of the helices are labelled. (Blochl and Blochl, 2007; Liepinsh et al, 1997)

Recent studies have identified new mechanisms able to process the receptor with subsequent generation of soluble peptides supposed to exert new cytoplasmic or nuclear functions (Kanning KC et al, 2003; Podlesniy et al, 2006).

Since p75NTR does not contain a catalytic motif, the signal mediation depends on the interaction with cytoplasmic proteins important for regulating neuronal survival and

differentiation as well as synaptic plasticity. The complex signalling of p75NTR makes it difficult to formulate a unified functional model for this receptor. Indeed, many trophic and apoptotic activities are integrated with that of other receptors and mechanisms. Synergistic and antagonistic interactions of p75NTR and Trk receptors such as complex formation, mutual inhibition, or negative control of Trk effects by p75NTR have been extensively investigated without defining a general model (Dechant 2001; Huang and Reichardt 2003; Teng and Hempstead 2004).

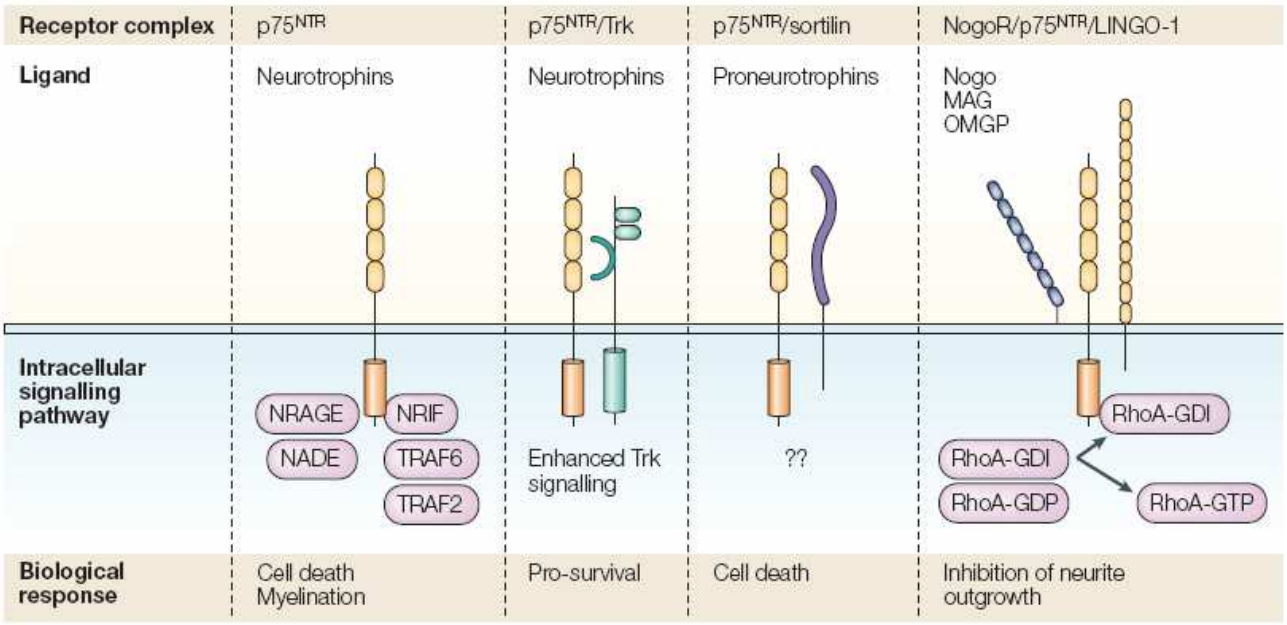


Fig. 3. p75 neurotrophin receptor and interactor receptors. P75NTR has been referred to as a 'low-affinity' Neurotrophin receptor, but this definition should be avoided because proNGF binds p75NTR with an affinity similar to that of nerve growth factor (NGF) binding to TrkA. Although it lacks a kinase domain, p75NTR can cooperate with many different protein partners and form multimeric receptor complexes to produce a number of cellular responses, including apoptosis, neurite outgrowth and myelination111–113. So far, sortilin61, LINGO-1, Nogo-66 (NgR)114 and Trk receptors115 have been identified as co-receptors. In addition to extracellular interactions that yield multimeric receptor complexes, the intracellular domain of p75NTR can also interact with many different adaptor and signalling proteins. These include neurotrophin-receptor-interacting MAGE (melanoma-associated antigen) homologue (NRAGE)116, neurotrophin-associated cell death executor (NADE)117, TNF (tumour necrosis factor)-receptor-associated factors 2 and 6 (TRAF2 and TRAF6)118,119, and neurotrophin-receptor-interacting factor (NRIF)120,121. GDI, guanine-nucleotide dissociation inhibitor; MAG, myelin-associated glycoprotein; OMGP, oligodendrocyte myelin glycoprotein; RhoA, small G protein.

Beside the more characterized interaction with the Trk receptors, p75NTR can also interact with other different cytoplasmic receptors. p75NTR can be influenced by co-receptors like NgR and Lingo1, which cooperate to prevent p75NTR activation, or sortilin, which shares the pro-neurotrophin ligands with p75NTR and directs p75NTR activity towards apoptosis (Nykjaer et al. 2004; reviews: Bronfman and Fainzilber 2004; Ceni and Barker 2005).

Taking together, all these studies suggest that rather than being just a co-receptor, all the identified interactors for p75NTR can modulate, inhibit or enhance its activities. The results is that the actions of the receptor may be explained by considering three different biological outcomes:

1. positive regulation of cell survival and differentiation
2. constitutive pro-apoptotic activation
3. ligand dependent p75NTR cell death

1.1.1 p75NTR as a positive regulator of cell survival and differentiation

In neurotrophin-responsive neuronal populations, p75NTR and Trk receptor members are frequently co-expressed, particularly in the vertebrate peripheral nervous system. P75NTR provides a positive modulatory influence on TrkA function by increasing the number of high affinity binding sites (Mahadeo et al, 1994). Regulation of high-affinity site formation by co-expression of TrkA and p75NTR provides an explanation for how these receptors may cooperate to increase Neurotrophin responsiveness during development. Indeed, neuronal cell lines express both receptors have an enhanced autophosphorylation of TrkA, leading to a faster differentiative response with NGF (Verdi et al, 1994). The general feeling proposes that the cell survival is enhanced by a higher ratio of p75NTR to Trk receptors. Indeed, considering p75NTR null mice it has been found a selective losses in sensory and sympathetic innervations. From neonatal p75NTR null mice, sympathetic neurons required higher concentrations of NGF to survive than neurons from normal mice at earlier developmental stages (Lee et al, 1994). To explain how p75NTR can modulate Trk receptors functions two models have been proposed so far. The first is a ligand passing mechanism, which predicts that the high-affinity state is the result of ligand presentation by p75NTR to the TrkA receptor (Barker and Shooter, 1994). The second model predicts that p75NTR and TrkA are capable of a ligand-independent association, which produces a high affinity binding interaction. (Chao and Hempstead, 1995). The second model would predict that conformational changes would occur to facilitate ligand binding. (Ross et al, 1996) All of these observations are consistent with the hypothesis that p75NTR can serve as a positive influence upon TrkA function.

1.1.2. p75NTR as a constitutively active pro-apoptotic receptor

In the1993 Bredesen and colleagues hypothesized that p75NTR was a death receptor. Based on the observation that immortalized neural cells overexpressing p75NTR display an higher rate of apoptosis in response to serum withdrawal, they proposed a mechanism of ligand-indipendent death (Rabizadeh et al, 1993). According to this model, apoptosis promoted by p75NTR can be rescued after binding to NGF. How NGF gives a survival signal through binding to p75NTR has not been established. However, the correlation between high levels of p75NTR expression and susceptibility to apoptosis after growth factor withdrawal has been proved also in PC12 cells supporting this mechanism of cell death (Barrett and Georgiou, 1996). Furthermore, down-regulation of p75NTR expression in neonatal dorsal root sensory neurons, using an antisense strategy, reveals enhanced survival (Barrett and Bartlett, 1994). The phenotype of the p75NTR null mice supports a role in neuronal survival (Davies et al., 1993; Lee et al., 1994a,b) but an apoptotic function as well. Analysis of mice has revealed a significantly higher number of cholinergic neurons in the basal forebrain in p75NTR -/- mice compared to wild type controls (Van der Zee et al., 1996; Yeo et al., 1997). These observations indicate that the absence of p75NTR resulted in enhanced survival, similar to the antisense effects observed in postnatal sensory neurons (Barrett and Bartlett, 1994).

1.1.2. Ligand-dependent p75NTR cell death

Several lines of evidence now firmly support that neurotrophins can actively kill cells through direct engagement of its p75NTR receptor. Both in vitro and in vivo evidence has indicated that neurotrophins and p75NTR are required for apoptosis of selective cell populations. In contrast to the model of NGF rescue through p75NTR, other studies demonstrate that cultured trigeminal neurons at embryonic age E10 are killed by NGF through binding to p75NTR (Davey and Davies, 1998). The cell death role of p75NTR is elicited upon injury or traumatic conditions. Similarly, cultured glial cells, such as fully differentiated oligodendrocytes, express elevated levels of p75NTR are effectively killed by NGF (Casaccia-Bonnefil et al., 1996). The apoptotic effects of p75NTR are not only enhanced by NGF binding but, in certain conditions, also by high concentrations of BDNF and other neurotrophins. Postnatal sympathetic neurons express p75NTR and TrkA, but are killed by BDNF-mediated activation of p75 (Bamji et al., 1998). BDNF for survival are killed by NT-4 through binding to p75NTR (Agerman et al., 1999). Primary cell culture experiments demonstrated that p75NTR was necessary for neuronal cell survival promoted by BDNF, but NT-4 binding to p75 induced cell death. This remarkable set of observations indicates that p75NTR and Trk receptor can simultaneously influence life-and-death decisions in neurons depending upon which ligands are available. An issue raised by these experiments is that although all neurotrophins (NGF, BDNF, NT-3, and NT-4) bind to p75NTR with similar affinity, each neurotrophin may exert different effects on cell function and viability through p75NTR. For example, the effect of NGF on oligodendrocyte cultures could not be reproduced by similar concentrations of BDNF or NT-3. Furthermore, in PC12 cells treated with antisense oligonucleotides to downregulate TrkA expression, BDNF but not NGF can rescue cells from serum-withdrawal (Taglialatela et al., 1996). A very likely explanation for the effect of distinct neurotrophins in the oligodendrocyte system is the presence of TrkB and TrkC receptors in these cells (Cohen et al., 1996).An alternative explanation for cells not expressing other Trk receptors is the differential ability of neurotrophins to activate distinct signal transduction pathways (Carter et al., 1996; Carter and Lewin, 1997). This hypothesis is also supported by striking differences in the kinetics of binding and the degree of positive cooperativity of each neurotrophin to p75NTR

(Rodriguez-Tebar et al., 1990, 1992). A related explanation may involve different adaptor molecules that are associated with the receptor.

2. Downstream cell death signalling of p75NTR

Since the many effects modulated by p75NTR, it is reasonable the identification of as such high number of interactors that bind the intracellular domain of the receptor. Several different p75NTR interacting molecules, with and without catalytic activity, have been identified to date (Gentry et al, 2004). Non-catalytic interactors include a series of scaffolding and adaptor-like molecules, such as caveolin-1 (Bilderback et al, 1997), Bex3/NADE (Mukai et al, 2000) and TRAF6 (Khursigara et al, 1999; Ye et al, 1999); larger proteins containing zinc-finger domains with some degree of nuclear localization, such as NRIF1/2 (Casademunt et al, 1999) and SC-1 (Chittka and Chao, 1999); and members of the MAGE homology domain family, such as NRAGE (Salehi et al, 2000) and necdin (Tcherpakov et al, 2002) with proposed roles in the regulation of apoptosis. p75NTR interactors with catalytic activity include serine–threonine kinases involved in interleukin and NF-kB signaling, such as IRAK (Mamidipudi et al, 2002) and RIP2 (Khursigara et al, 2001); a protein tyrosine phosphatase (FAP-1) (Irie et al, 1999); and the small GTPase RhoA (Yamashita et al, 1999). How these p75NTR-interacting proteins connect to downstream signaling pathways and cellular responses is still not clear. Some of the principal downstream events characterized in p75NTR signalling include ceramide production (Dobrowsky et al, 1994) and activation of the transcription factor NF-kB (Carter et al, 1996) and the c-Jun kinases JNK1–3 (Casaccia-Bonnefil et al, 1996; Friedman, 2000; Harrington et al, 2002; Costantini et al, 2005).

3. p75NTR and the neurodegeneration

Alzheimer's Disease (AD) is the most common form of dementia in the elderly that affects more that 20 million people world wide . It is characterized by progressive memory loss and confusion depending on neuropathological changes that include loss of neurons, extracellular deposition of amyloid peptide in the neuritic plaques and intracellular deposition of hyperphosphorylated tau protein ('tangles'). One pathological feature of AD is the senile plaque, whose major component is the amyloid β-peptide (Aβ). Accumulation of a 37-43 aminoacid peptide, amyloid peptide (Aβ), that derives from multiple proteolytic cleavage of a large transmembrane precursor, amyloid precursor protein (APP), by specific enzymes belonging to the secretase family.

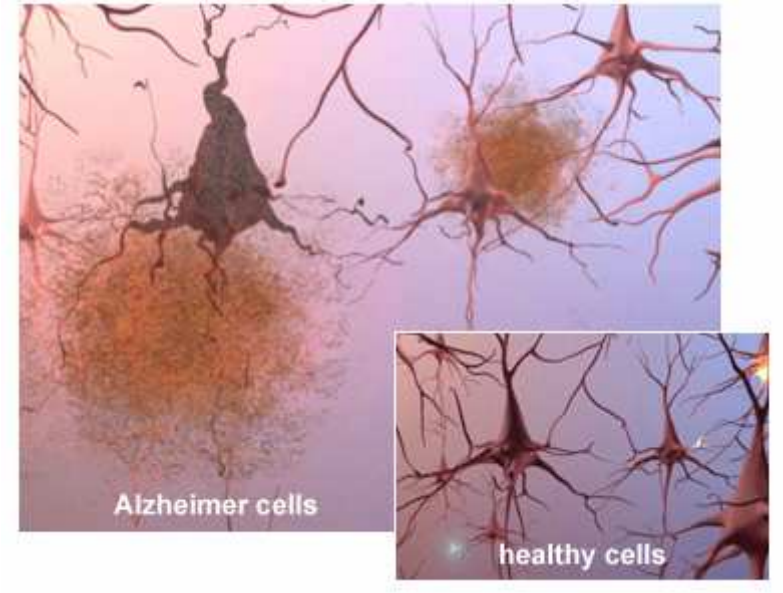


Fig. 4. Representation of senile plaques in AD brain

The generation of Aβ peptide is a physiological peptide derives from an amyloid precursor protein that can be processed in different ways by different sets of enzymes. One pathway leads to amyloid plaque formation (amyloidogenic), while another does not (non-amyloidogenic). Usually about 90% of APP enters the non-amyloidogenic pathway, and 10% the amyloidogenic one, but these ratios can change due to mutations, environmental factors, as well as the age of the individual. Cleavage products from both these pathways may play important roles in neural development and function.

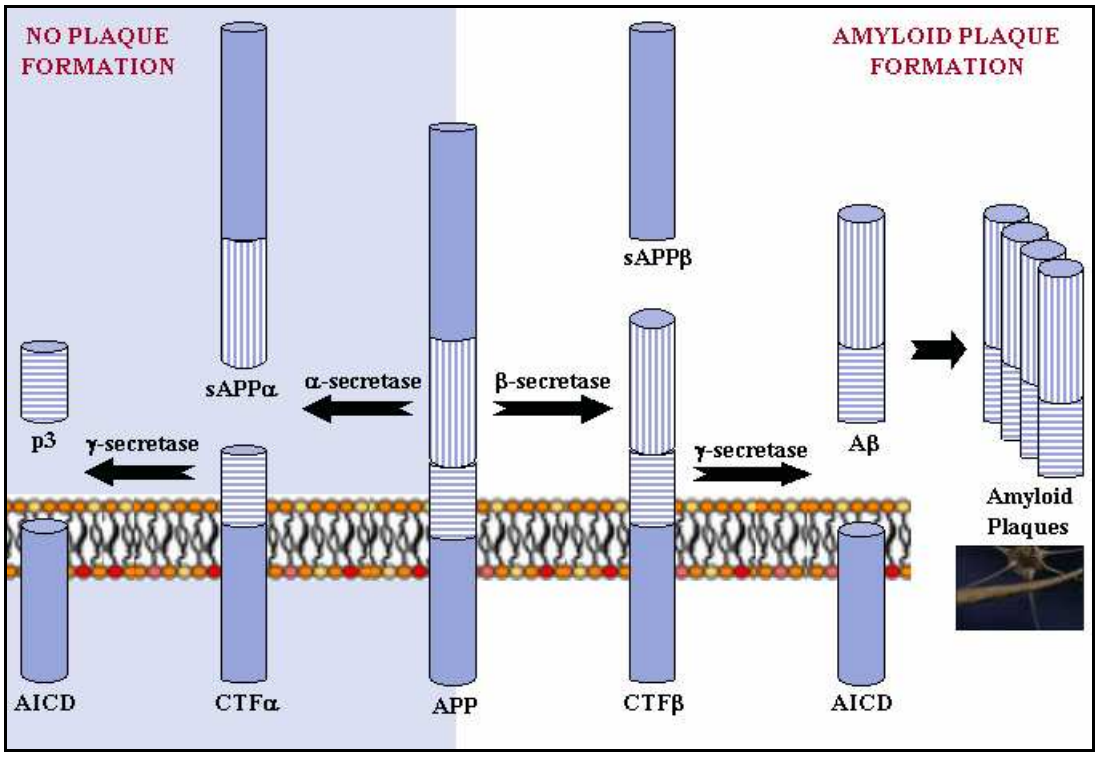


Fig. 5. APP Processing α-secretase and γ-secretase produce non-plaque forming p3, while β-secretase and γ-secretase produce amyloid plaque-forming Aβ. The different regions of the APP protein are indicated.

In the non-plaque-forming pathway, APP is cleaved first by α-secretase to yield a soluble N-terminal fragment (sAPPα) and a C-terminal fragment (CTFa). sAPPα may be involved in the enhancement of synaptogenesis, neurite outgrowth and neuronal survival, and are considered to be neuroprotective. CTFα is retained in the membrane, where it is acted upon by presenilin-containing γ-secretase to yield a soluble N-terminal fragment (p3) and a membrane-bound C-terminal fragment (AICD, or APP intracellular domain). AICD may be involved in nuclear signalling via transcriptional regulation as well as axonal transport through its ability to associate with a host of different proteins.

In the plaque-forming pathway, APP is cleaved first by a different enzyme, β-secretase (a transmembrane aspartic protease), yielding a soluble N-terminal fragment (sAPPβ) and a membrane-bound C-terminal fragment (CTFβ). This cut is made closer to the N-terminal end of APP than the cut with α-secretase, making CTFβ longer than CTFα. CTFβ is then acted upon by γ-secretase (as occurred in the previous pathway), yielding a membrane-bound C-terminal fragment (AICD) the same as before, and a soluble N-terminal fragment (amyloid-β, or Aβ) that is longer than p3.

Aβ can be accumulated in the extracellular cortex neurons and basal forebrain nuclei where it can aggregate to form amyloid plaques exerting deleterious effects on neuronal and synaptic function, ultimately causing neuronal cell death. Major part of AD cases are sporadic without a known aetiology connection. However, some cases are associated with mutations of genes that are involved in the APP expression, proteolytic processing, or in the changes in Aβ stability or aggregation. These in turn result in a chronic imbalance between Ab production and clearance. The gradual accumulation of aggregated Aβ may initiate a complex, multistep cascade that includes gliosis, inflammatory changes and formation of neurofibrillary tangles.

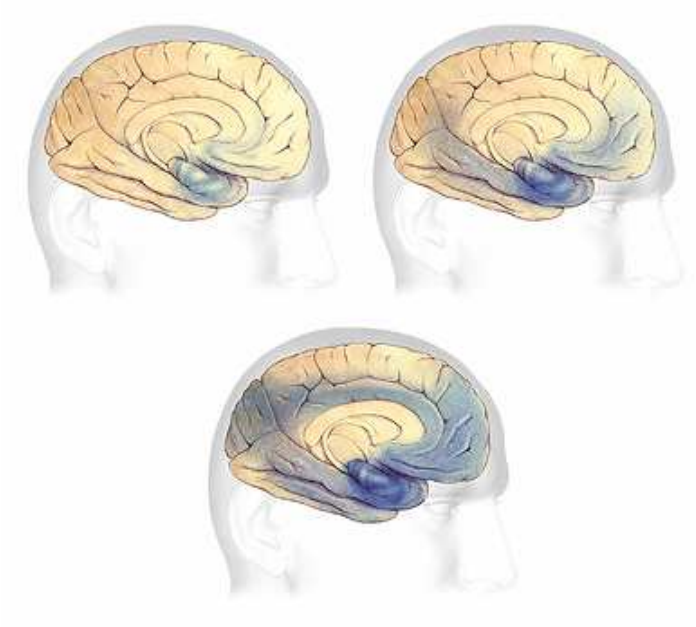


Fig. 6. Progression of neurodegeneration in brain in AD

In this scenario, an hypothesis that has been put forward to explain the aetiology of AD arises the connection between the occurance of the pathology with the Neurotrophic factors, suggesting a causal link (Rabizadeh et al, 1994).

An early indicator of Alzheimer's disease is the degeneration of the cholinergic basal forebrain neurons, which express the highest levels of the pan neurotrophin receptor (p75NTR) in the adult brain (Gibbs et al. 1989). These neurons are dependent on NGF-mediated survival signalling through TrkA which, unlike p75NTR, is reduced in Alzheimer's disease patients. In addition, p75NTR is also expressed in the Trk-negative, degenerating cortical neurons of Alzheimer's disease sufferers, a situation which is not reflected in healthy elderly subjects (Mufson et al. 1992). Furthermore, the neurotrophins, acting differentially through Trk receptors and p75NTR, appear to regulate the expression and cleavage of the amyloid protein precursor (APP; Costantini et al. 2005a), potentially regulating the generation of Aβ with ageing. Given that up-regulation and ligand activation of p75NTR have been widely shown to mediate neural cell death in animal models of neurodegenerative disease (Coulson et al. 2000a; Dechant and Barde 2002; Roux and

Barker 2002) and that the pro-form of NGF, which selectively binds to p75NTR to promote neuronal death (Lee et al. 2001), is increased in Alzheimer's disease (Fahnestock et al. 2001; Peng et al. 2004), the receptor is a strong candidate for independently mediating the degeneration occurring in Alzheimer's disease.

3.1 Aβ binds to p75NTR

Further support to the hypothesis that p75NTR could be a mediator of the cytotoxic effect induced by deposition of Aβ derives from the study of Mina Yaar in which they proved the direct binding. In 1997 Yaar et al. reported that Aβ 1–40 binds to and immunoprecipitates with p75NTR (Yaar et al. 1997). In the brain, this protein is expressed at the highest level by the cholinergic neurons of the basal nuclear complex, which are sensitive to Aβ neurotoxicity, and undergo degeneration in AD. In contrast, the neurons of other cholinergic complexes in the brain (pedunculoponine and lateral tegmental nuclei) neither express p75NTR nor undergo degeneration in AD, suggesting that the vulnerability of basal nuclear neurons and their projections may be related to their high-level expression of p75NTR (Rabizadeh et al,1994). The use of rat cortical neurons and a cell line engineered to express p75NTR has demonstrated that P75NTR binds specifically fAβ, and that this binding is followed by apoptosis (Yaar et al, 1997; 2002). The binding of Aβ to P75NTR activates NF-kB in a time- and dose-dependent manner. Blockade of the interaction between Aβ and p75NTR with nerve growth factor or inhibition of NFKB activation by curcumin or NF-κB SN50 attenuated or abolished Aβ-induced apoptotic cell death (Kuner et al, 1998). Other studies have shown that P75NTR may be present in a trimer form that binds Aβ to induce receptor activation, and that Aβ binds to both the p75NTR trimer and the P75NTR monomers. In neuronal hybrid cells, it has been confirmed that p75NT mediates Aβ toxicity, and that the p75NTR-mediated Aβ neurotoxicity involves Go, c-jun kinase, reduced nicotinamide adenine dinucleotide oxidase and caspases 9/3 (Tsukamoto et al, 2003). However, it has been reported that, in human primary neurons in culture, p75NTR protects against extracellular Aβ-mediated apoptosis. This neuroprotection might occur through a P13Kdependent pathway. The reason for this difference may be explained by differential activation of a signal transduction pathway in primary neurons versus tumour

cell lines, a cell-type or species-specific effect of Aβ, or a differential expression of the other neurotrophic receptors (Zhang et al, 2003). Along with this hypothesis, other studies focused their attention on the possible mechanism that can be activated after binding of Aβ to p75NTR. In the 2002 Perini et al identified a specific cytoplasmic domain that resulted to be essential for the mediation of the Aβ cell death (Perini et al, 2002). To this purpose, Aβ has been tested for the neuronal toxicity on a SK-N-BE neuroblastoma cell line devoid of all Neurotrophin receptors, and on several SK-N-BE derived cell clones either expressing the full-length or truncated forms of p75NTR . It has been found that p75NTR plays a direct role in Aβ cell death through the signalling function of the death domain (DD). Furthermore, it has been showed that the cell death is caspase dependent, in particular through activation of caspase-8 and oxidative stress.

Results

p75NTR signals cell-death upon binding to the Aβ peptide and recruiting TNFR1–associated death domain (TRADD)

1.1 $p75^{NTR}$/TNFR1 conserved death domain aminoacidic residues are required to trigger Abeta peptides induced cell death

In the past years it has been shown that p75NTR signals cell death upon binding to the β-amyloid peptide (Aβ) (Yaar et al,1997; Kuner et al,1998; Costantini et al,2005).

In a previous study published from this laboratory, it has been proposed that p75NTR activates apoptosis through the Death Domain (DD) that characterizes the cytoplamic sequence of the receptor (Perini et al,2002). In this further study, we deeper analyze the mechanism by which p75NTRDD signals cell death putting forward a more detailed map of the domain. Furthermore, we describe one of the possible initial steps of the cytotoxic effects induced by Aβ correlating p75NTR with the changes that occur in Alzheimer's disease.

In our studies the cell system of reference is a Neuroblastoma cell line (SK-N-BE) that does not express any neurotrophic receptors at detectable level and that allow us to better define the unique role of the p75NTR in the signal transduction induced by Aβ (Bunone et al., 1997)

Using derived Neuroblastoma cell pools of SK-N-BE engineered to express full-length or various truncated forms of p75NTR, we have already demonstrated that p75NTR is involved in the direct signaling of cell death A□□□dependent through activation of caspases-8 and 3, inducing the production of reactive oxygen intermediates and formation of oxidative stress (Perini et al,2002).

To better understand the molecular mechanism by which p75NTR mediates this cell death, we reasoned on the assumption that p75NTR is devoid of any intrinsic enzymatic activity so it should signal, across the cell membrane, through protein-protein interaction, likely involving aminoacidic residues placed on the surface of the intracellular p75NTR-DD. Starting from this observation, the first aim of our experiments was to characterize which residues were more responsible or more involved in the transduction of the Aβ dependent cell death. Previous NMR and solvent accessibility studies have analyzed the conformation of the p75NTR DD highlighting the amino acidic residues putatively located on the outer

surface. (*Liepinsh et al,1997*) Knowing the tridimensional structure, we decided to mutagenize the identified outer residue by site-specific mutagenesis through Alanine-substitution. (*Cunningham & Wells,1989*).
We targeted different residues listed as follow:

- **D355** (Interhelical loop I)
- **H359** and **E363** (Helix II) **Q367** (Interhelical loop II)
- **P368** and **E369** (Helix II) **A378** (Interhelical loop III)
- **D392** and **S393** (Interhelical loop IV)
- **D397** (Helix V)
- **R404, R405, Q407** and **R408** (Interhelical loop VI)
- **D410** and **E413** (Helix VI)

The specific residues are numbered following the order in the original paper (Liepinsh et al,1997)
All the residues found by NMR to be located at the surface of the p75NTR DD have been substituted to A but the A378 substituted to D. (Residues in the helix I have been excluded from this analysis because they were not deleted in the $p75^{NTR}\Delta DD$ truncated mutant used in the previous study *Perini et al,2002*). For all the PCR-mediated site-specific mutagenesis we used the rat p75NTR cDNA cloned in the pcDNA3 expression vector ($p75^{NTR}$/pcDNA). The mutated plasmids have been used to generate stable pools of SK-N-BE. Subsequentially, cells expressing wild-type or mutant p75NTR, carrying either single or multiple substitutions (Table 1 and Fig 1A) have been analyzed to determine if and in which way they could be involved in the mediation of cell death induced by Aβ. Pools of SK-N-BE have been exposed to the aggregated Aβ(25-35) as well as to the Aβ(35-25) reverse peptides as negative control. The treatment has been performed for 48 hours the levels of cell death have been evaluated by microscopic analysis after TUNEL labeling.
Analyzing the cell death levels after Aβ treatment, it was evident that not all the substituted residues were involved at the same way in mediating cytotoxicity. Expression of the $Rp75^{NTR}$-C, $Rp75^{NTR}$-E and $Rp75^{NTR}$-F mutant receptors decreases the level of Aβ peptide induced cytotoxicity (fig 1B) whereas the $Rp75^{NTR}$-A, B, D and G did not show any changes

in the levels of cell death. Therefore, our first conclusion was that only the most C-terminal half of p75NTR-DD (between interhelical region III and the helix VI) is required for the cell death induced by Aβ.

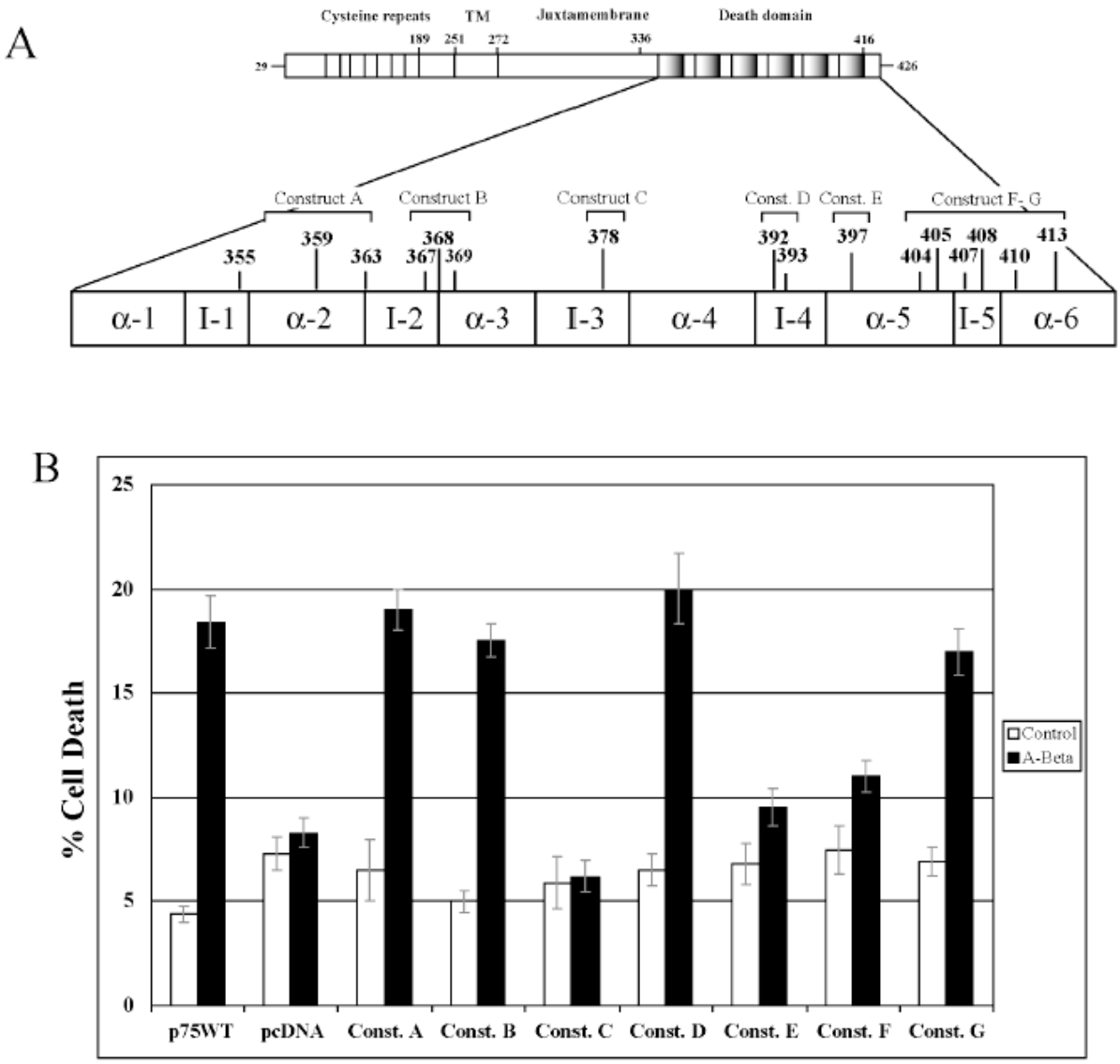


Fig. 1. Role of the p75^{NTR} death domain outer aminoacidic residues in Aβ–induced cytotoxicity. (A) Schematic representation of the rat p75^{NTR} receptor with details of the death domain. The aminoacidic residues target of the site directed mutagenesis in the different mutant p75^{NTR} expression vectors (constructs A to G) are indicated (construct A: aa 355-359-363; construct B: aa 367-368-369; construct C: aa 378; construct D: aa 392-393; construct E: aa 397; construct F: aa 404-405-407-408-410-413; construct G: aa 407). The mutated amino acid are putatively located at the death domain surface and their position in the death domain alpha-helix (α) or interhelical loop (I) are indicated. The different expression vectors has been transfected in neuroblastoma SK-N-BE cells and stably transfected pools have been selected. (B) Aβ cytotoxicity analysis by TUNEL labelling and Ethidium Bromide nuclear counterstaining. SK-N-BE cell pools expressing either wild type p75^{NTR} (p75WT) or the indicated mutant p75^{NTR} were exposed to A□(25-35) 20 □M (A-Beta) or A□□(35-25) 20 □M (control) for 48 hrs, then cell death level has been evaluated. Plotted cell death results are shown. SK-N-BE cells stably transfected with the empty pcDNA vector are used as negative control. Data are means + ES of three experiments.

These first data gave us a new insights and new interests to better define the protein conformation of the p75NTRDD. Indeed, we considered that, since its primary sequence

and secondary structure, p75NTR belongs to the Superfamily of the Tumor Necrosis Factor Receptors, which contains also the TNFRI itself and Fas receptors.

The similarity of the structures and the homology of the sequences between the death domains of p75NTR, TNFRI and Fas receptor have been previously analyzed in a paper by Barbara Chapman (Chapman et al, 1995). In that study the comparison of DDs sequences from $p75^{NTR}$, TNFR1 and Fas from human, mouse and rat using a matrix procedure to detect low level of aminoacid identity, has let to evaluate 32,8% identity between $p75^{NTR}$ and TNFR1 and 25,4% between TNFR1 and Fas (*Chapman,1995*). Notably, some specific residues were found almost completely conserved and more importantly four of them have been previously demonstrated to be necessary for TNFα-induced TNFR1-mediated cell death (*Tartaglia et al,1993*). In detail, the residues E369, W378, D390, I408 are highly conserved between the DD of p75NTR and TNFRI and correspond to the following $p75^{NTR}$ residues: E377, W388, D397 and I411. (figure Rasmol!)

Interestingly, our first screening revealed that mutations in conserved residues or very close to them (A378D, D397A, and residues substituted in the construct $p75^{NTR}$-F) are sufficient to block almost completely the cytotoxic signal (Fig.1B).

To extend our analysis we also mutated the E377, W388 and I411 residues into Ala (Fig. 2A). Interestingly, as shown for D397 (Fig. 1B) also E377, W388 and I411 seem to be crucial for Aβ triggered cytotoxicity.

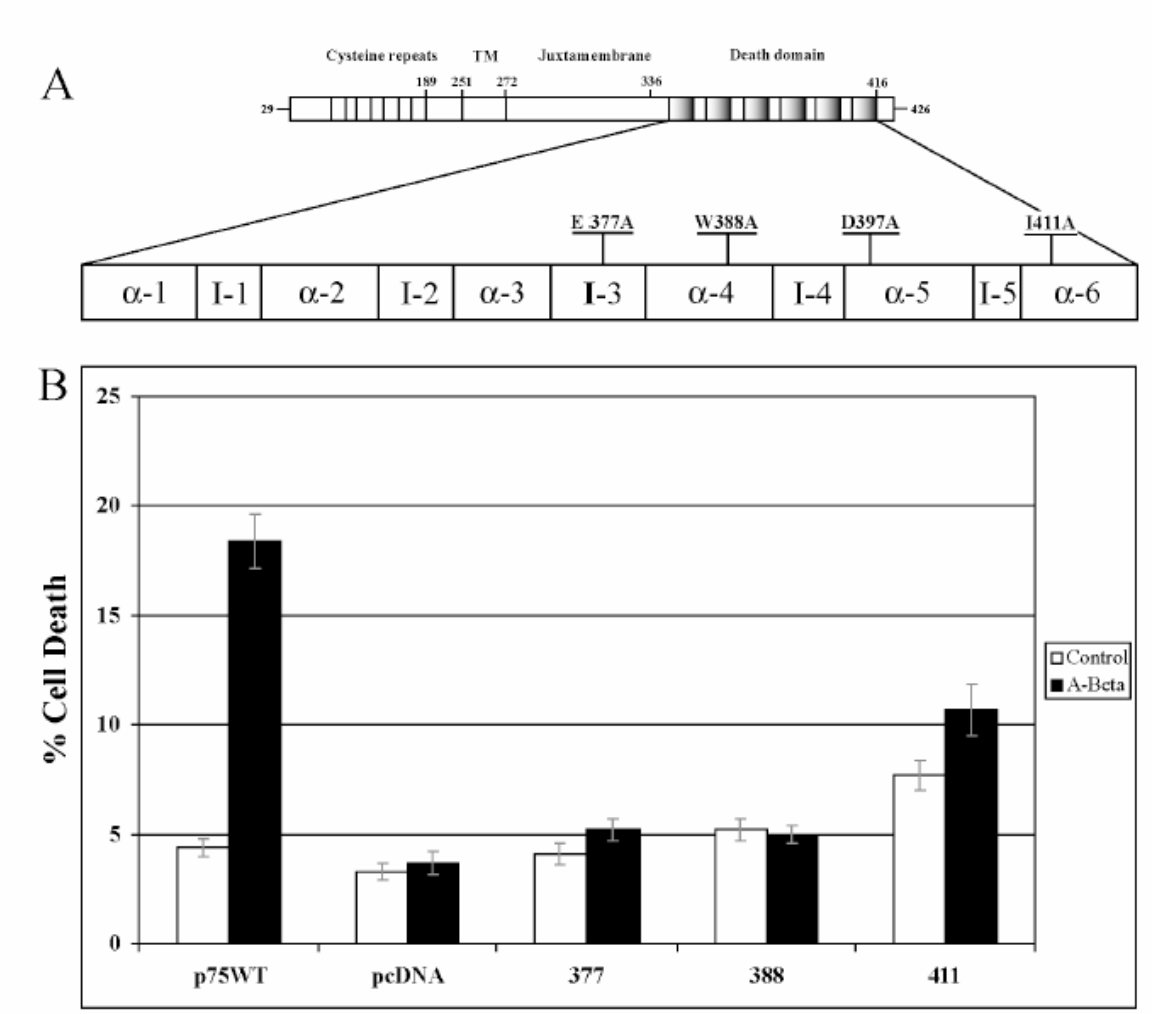


Fig. 2 Role of the p75^{NTR}/TNFR1 conserved Aminoacidic residues in Aβ–induced cytotoxicity (A) Schematic representation of the rat p75^{NTR} receptor with details of the death domain. Mutant of p75^{NTR} cDNA were generated by PCR introducing substitution to A of the indicated amino acid residues conserved with TNFR1 (aa E377, W388, and I411). The different expression vectors has been transfected in neuroblastoma SK-N-BE cells and stably transfected pools have been selected. (B) Aβ cytotoxicity analysis by TUNEL labelling and Ethidium Bromide nuclear counterstaining. SK-N-BE cell pools expressing either wild type p75^{NTR} (p75WT) or the indicated mutants p75^{NTR} (377, 388, 411) were exposed to A□□(25-35) 20 □M (A-Beta) or A□□(35-25) 20 □M (control) for 48 hrs, then cell death has been evaluated. SK-N-BE cells stably transfected with the empty pcDNA vector are used as negative control. Data are means + ES of three experiments.

We found that indeed single point mutations are enough to abate almost to the background level the percentage of TUNEL positive nuclei after 48 hours of Aβ exposure.

All together these data suggest that, upon Aβ peptide activation, p75NTR can signal cell death through some specific areas of its DD and importantly these areas include conserved residues shared with TNFR1 receptor.

1.2 TRADD dominant negative is able to interfere with p75NTR-dependent Aβ– peptide induced cell death.

The first set of experiments we performed clarified that, after exposure to the A□ peptide, p75NTR signals cell death in Neuroblastoma cell line and we identified specific Death Domain residues required in mediating cell death.

The identification of the conserved residues between p75NTR and TNFR1 represented a moment of discussion that allow us to speculate about the possible mechanisms by which p75NTR can transduce the signal into the cell. Since the involvement of the shared residues seemed to be crucial for Aβ-induced cell death, we postulated that at least in this contest, p75NTR could signal cell death through a pathway similar to the one already characterized for TNFR1.

In support of our idea many reports have showed that Aβ is able to trigger death in different cell types in which p75NTR is expressed: rat primary cortical neurons, NIH-3T3 and neuroblastoma cell stably expressing p75NTR. It is also known that Aβ activates different signaling pathways involving JNKs, p38/SAPK and the transcription factor NFkB (Kuner et al,1998;Yaar et al,2002;Costantini et al,2005; Yao et al,2005). Interestingly, all of these pathways are known to be activated in various cell context through the TNFR1-DD (PER JNK: De Smaele et al,2001: Tang et al,2001; Deng et al,2003; Li et al,2005; Kamata et al, 2005). Furthermore, it has been recently reported that in MCF7 breast cancer cells the Tumor Necrosis Factor Receptor1-associated Death Domain protein (TRADD), the most proximal TNFR1 cytoplasmic adaptor protein, is able to interact with the p75NTR-DD (*El Yazidi-Belkoura et al,2003*).

To address the question if TRADD can have a role in Aβ induced p75NTR-mediated cytotoxicity, we generated a dominant negative (DN) for TRADD. The HA-TRADDΔDD mutant lacks the Death Domain of the protein at the C-terminal end and is HA-tagged at the N-terminal end.

A TRADD deleted of the C-terminal domain cannot anymore interact with the TNFRI DD but can still interact with downstream factors through the N-terminal region. A similar mutant has been successfully used to block the activation of TNFR1- or p75NTR-dependent signaling pathways such as the Nf-κB, JNKs and p38/SAPK pathways through a dominant negative effect most likely elicited by titration of downstream factors, such as TNFR-

associated factor-2 (TRAF2) (*Kieser et al,1999; El Yazidi-Belkoura et al,2003*). To validate the effectiveness of this reagent, we proceeded verifying if the TRADDΔDD mutant was actually able to interfere with a TNFR1-dependent cell death process.

To do so we induced TNFRI overexpression in HEK293 cells, leading to the formation of trimeric receptor complex that mimic the activation induced by ligand. Thus, we performed experiments of co-transfection to study the role of the dominant negative, TRADDΔDD. To label the transfected cells we included a vector to express GFP in every transfection experiment.

24 hours after transfection, cells have been fixed and stained with Hoechst 33342 and observed at the microscope to evaluate the number of transfected cell showing normal or apoptotic morphology. The transfected cells have been scored for the general cell morphology made evident by the ubiquitous expressed GFP, looking at the presence of the typical membrane blebbings and for the nuclear morphology, looking at the bright and condensed Hoechst staining. Indeed, overexpressed TNFR1 was able to trigger high level of cell death in HEK293 whereas co-transfection with the TRADDΔDD vector significantly reduced the number of dead cells. (fig. 2A) This experiment indicates that the TRADDΔDD is able to interfere with a TNFR1/TRADD dependent cell death process making it a suitable reagent to test if TRADD is also involved in Aβ induced p75NTR-mediated cytotoxicity in Neuroblastoma SK-N-BE cells.

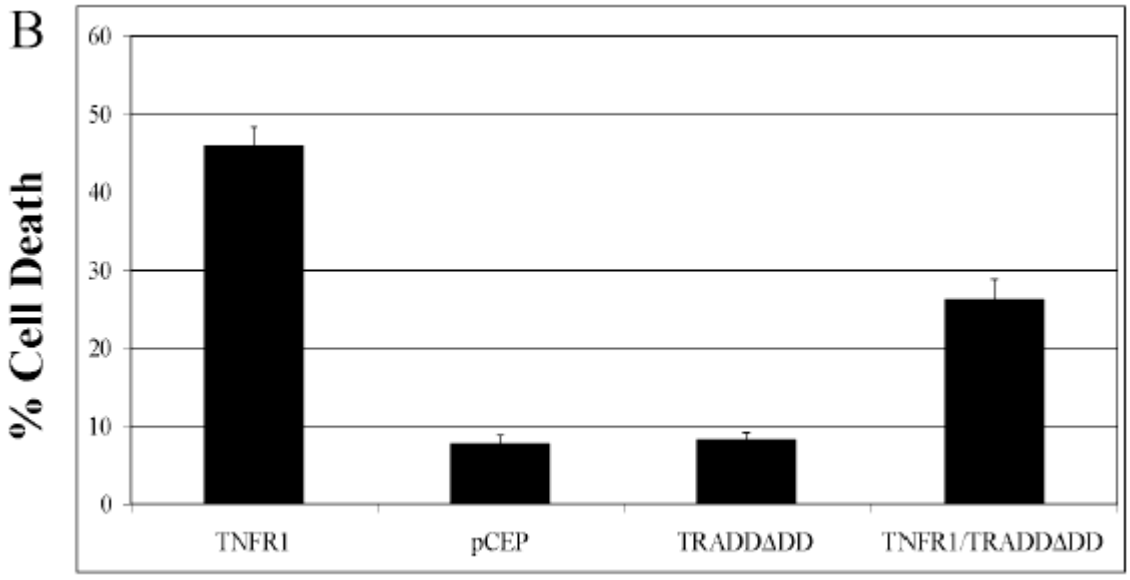


Fig. 3A Role of cytoplasmic adaptor TRADD in mediation of TNFRI depending cell death. The dominant negative effect of the TRADD truncated mutant (TRADDΔDD) has been validated in a known cell-death paradigm as TNFR1 overexpression in transiently transfected HEK293 cells. HEK293 cells have been transiently co-transfected with a

plasmid to express EGFP to label transfected cells and an empty vector (pCEP) or plasmids to express TRNFRI alone (TNFR1), TRADDΔDD alone (TRADDΔDD) or TNFR1 and TRADDΔDD (TNFR1/TRADDΔDD). After 24 hrs cells have been fixed and stained with Hoechst 33342 and cell death has been assessed looking for nuclear condensation in GFP positive cells displaying round shape and membrane blebbing. Data are means + ES of three experiments.

After validation of our dominant negative, we tested whether we could reproduce the same effect observed with TNFRI/TRADD□DD also co-transfecting TRADDΔDD in cells expressing p75NTR. To this purpose SK-N-BE derived pools, selected after transfection of either $p75^{NTR}$/pcDNA or pcDNA empty vectors, were further transfected with either TRADDΔDD/pCEP4 or pCEP4, the latter one used as negative control. After Hygromycin selection, pools of double transfected cells were collected, characterized both for $p75^{NTR}$ and for TRADDΔDD expression and cell pools expressing comparable level were analyzed for the cell death. No changes in the TRADD expression level have been detect with the overexpression of p75NTR. (Figure2B)

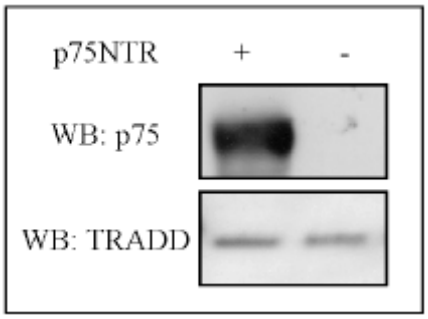


Fig. 3B Expression level of SK-N-BE derived pools. Western-blotting analysis of total protein extracts obtained from SKNBE cell pools stably transfected with a pCDNA empty vector or a $p75^{NTR}$/pCDNA vector to express wild type $p75^{NTR}$. After SDS-PAGE of equal amount of total protein extract and transfer to nitrocellulose membrane, the blot has been probed with anti-$p75^{NTR}$ or anti-TRADD antibody as indicated. The panel shows that both cells expressing $p75^{NTR}$ and control cells express equal amount of endogenous TRADD protein.

As described above, cells were exposed to either Aβ peptide or to the control peptide for 48 hours and cell death has been evaluated after TUNEL labeling. As shown by the histogram in figure 3C, the treatment with Aβ in SK-N-BE expressing wild type p75NTR arose a 22% of dead cell.

Expression of TRADDΔDD by itself does not affect in any way the level of cell death. The co-transfection p75NTR WT/TRADDΔDD shows a level of cytotoxicity comparable with the one observed in the control.

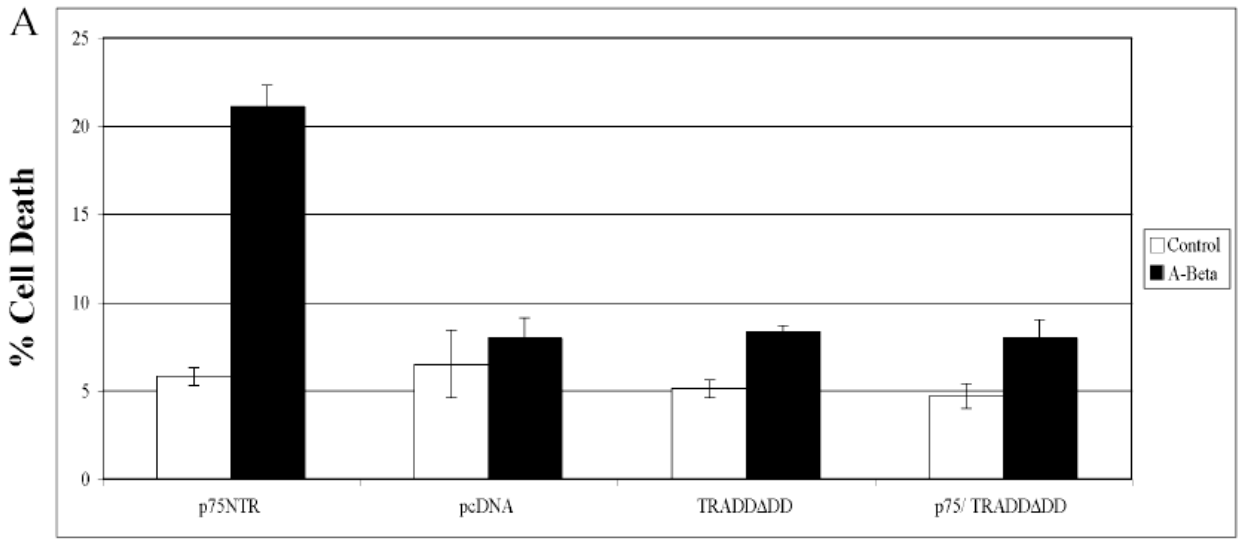


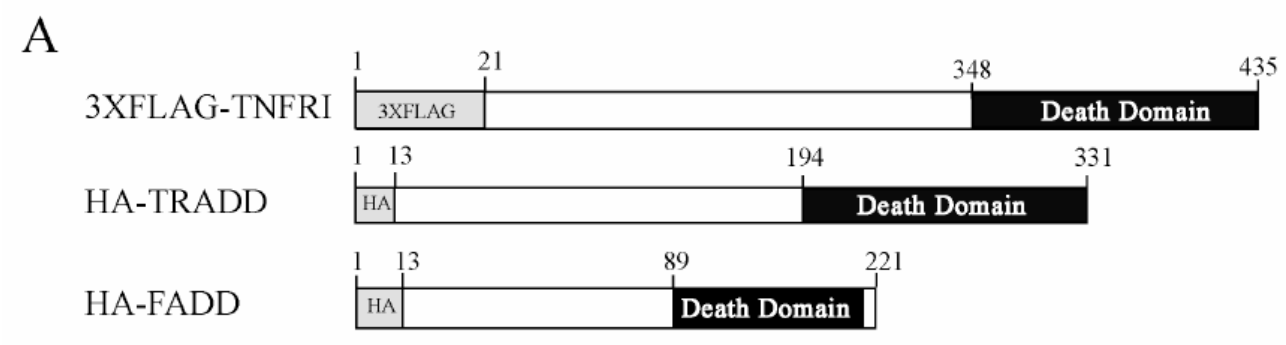
Fig. 3C Role of the cytoplasmic adaptor TRADD in Aβ–induced $p75^{NTR}$ mediated cytotoxicity. A TRADD truncated mutant lacking the death domain (TRADDΔDD)generate a dominant negative effect in neuroblastoma cell line. A-SKNBE derived cell pools stably transfected with empty vectors (pcDNA), or plasmids to express $p75^{NTR}$ alone ($p75^{NTR}$), TRADDΔDD alone (TRADDΔDD), $p75^{NTR}$ and TRADDΔDD (p75/TRADDΔDD), were selected and exposed to Aβ 25-35 (A-Beta) or to the control peptide A 35-25 (control). After 48 hrs cells were analyzed to assess cell death using TUNEL labelling and Ethidium Bromide nuclear counterstaining. Data are means + ES of three experiments.

Indeed, TRADDΔDD is able to interfere with the cytotoxic signal and almost to block Aβ-induced p75NTR-mediated cell death. (fig 3C) These data suggest that, at least upon Aβ binding, p75NTR could signal cell death through a protein complex that involves a p75NTR/TRADD pathway, resembling in this way the pathway used by TNFR1.

1.3 p75NTR and TRADD interact mainly through the p75NTR/TNFR1 conserved residues required for Aβ-induced cell death..

The reduced cell death levels observed in SK-N-BE pools expressing p75NTR-WT and TRADDΔDD, pushed us to verify whether TRADD could also be a direct interactor for p75NTR. The best way to address this question was to set up a Co-Immunoprecipitation Assay after transient transfection of HEK293 cells. Since it is known that TRADD is able to interact directly with TNFRI Death Domain but not with Fas-associating protein with death domain (FADD), the direct Fas cytoplasmic adaptor protein, we used these two death domain containing receptors respectively as positive and negative control.

We generated expression vectors to express HA tagged versions of FADD (HA-FADD) and TRADD (HA-TRADD) and a FLAG tagged version of TNFR1 (FLAG-TNFR1) (Fig. 3A).



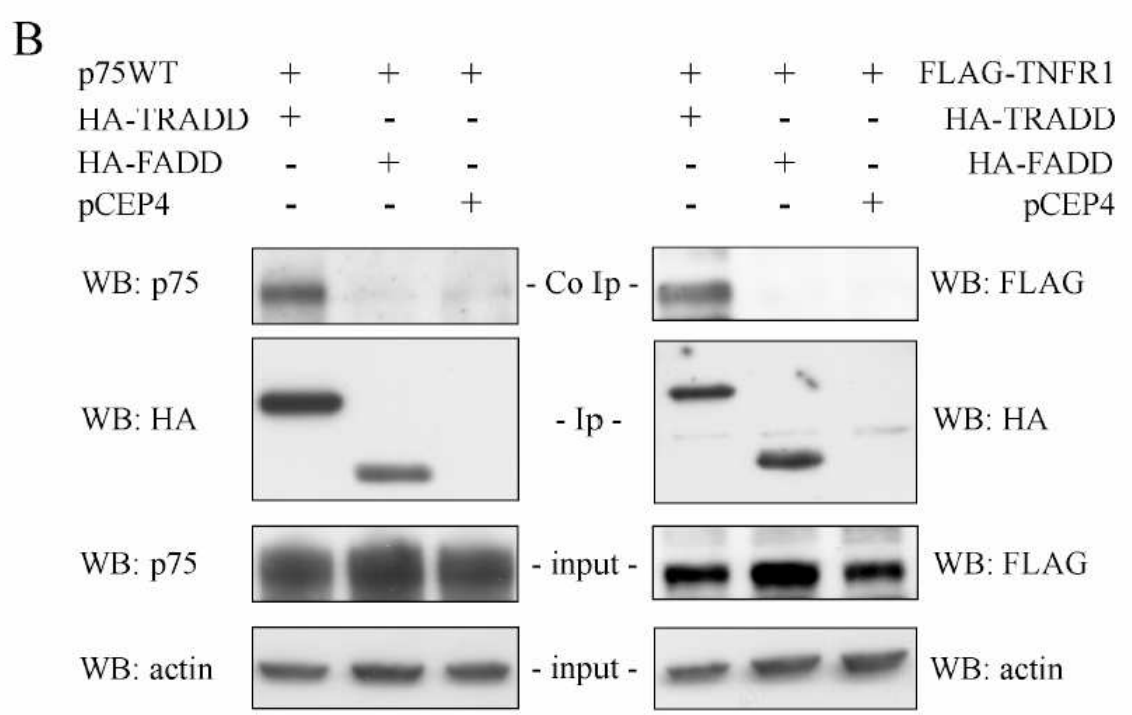


Fig. 4 Physical interaction between p75^{NTR} and TRADD. Co-Immunoprecipitation Assays after transient transfection of HEK293 cells have been performed to compare the ability of p75^{NTR} and TNFR1 to interact with the cytoplasmic adaptors: TRADD and FADD. (A) Schematic drawings of the transfected tagged-proteins: 3XFLAG-TNFR1, HA-TRADD and HA-FADD. (B) After 24 hrs of transfection of indicated vectors, 1.5 mg of total protein extract has been used to immunoprecipitate HA-tagged TRADD or FADD. Western-blotting analysis has been done probing film with antibodies raised against the indicated epitopes: i) p75NTR antibody against p75^{NTR}, flag antibody against FLAG-TNFR1; HA antibody against HA-TRADD and HA-FADD. The shown autoradiographs are representative of at least three independent experiments.

Indeed, the Co-Immunoprecipitation Assay showed that in the double transfection p75NTR/TRADD experiment, pulling down TRADD by HA antibody, we could co-immunoprecipitate p75NTR, reproducing for p75NTR the same interaction that occurs between TNFR1 and TRADD.

Notably, the importance and specificity of this results was highlighted by the absence of coimmunoprecipitation signal from the cotransfection of p75NTR and FADD expressing vectors.

As already known and reproduced by our experiment in Fig. 4B, FADD does not interact with TNFRI even if it possesses a Death Domain. The fact that we did not find interaction between FADD and p75NTR gives more specificity and relevance to the observation of a phisical interaction between p75NTR and TRADD. In other words, the presence of a Death Domain is a required condition but not necessary to guarantee a protein-protein interaction

through Death Domain containig proteins and so the Co-Immunopracipitation TRADD/p75NTR likely reflects the existence of a real protein complex.

Indeed, to investigate the possible role of a p75NTR/TRADD interaction in mediating the cytotoxic signal of Aβ, we tested if the p75NTR single substitution mutants could affect in some way the complex assembly.

HEK293 cells have been co-transfected with plasmid to express HA-TRADD alone or together with either wild type $p75^{NTR}$ or the mutants p75E377A and p75W388A. As shown in Fig. 5, the p75E377A and p75W388A mutants have not been efficiently co-immunoprecipitated by TRADD-HA as we observed for p75NTR WT. This could suggest that p75NTR mutants could fail to transduce the cytotoxic signal most likely because they cannot interact efficiently with TRADD. In agreement with our results, Telliez and collegues showed that the mutation E369A in TNFR1-DD (corresponding to $p75^{NTR}$ E377A) not only blocks TNFR1-dependent cell-death but also affects TNFR1-TRADD interaction (*Telliez et al,2000*). All together these data suggest that a $p75^{NTR}$/TRADD protein complex could mediate the Aβ cytotoxic signal.

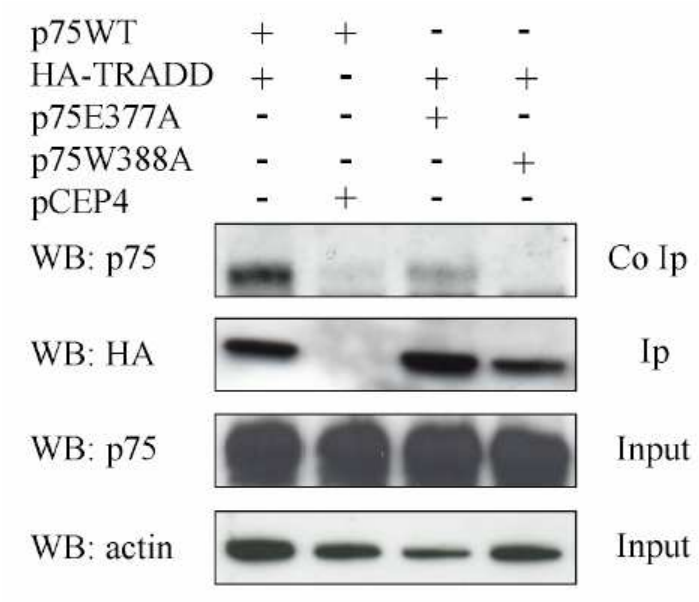


Fig. 5. The p75NTR mutants that do not signal cytotoxicity have reduced ability to co-immunoprecipitate with TRADD. Transient transfection of HEK293 cells has been performed to compare the level of the Co-Immunoprecipitation efficiency between wild type $p75^{NTR}$ (p75WT) and $p75^{NTR}$ mutants (p75E377A, p75W388A). After 24 hrs transfection of above indicated vectors, 1.5 mg of total protein extract has been used to immunoprecipitate HA-tagged TRADD followed by Western-blotting probed with antibodies raised against the indicated epitopes: i) p75NTR antibody against $p75^{NTR}$; HA antibody against HA-TRADD. The shown autoradiographs are representative of at least three independent experiments.

1.4 Aβ peptides modulates p75^{NTR}-TRADD interaction.

To assess the role of the p75NTR/TRADD interaction in a more physiologic setting, we investigated if the protein complex formation could be modulated by Aβ peptide exposure. We engineered SK-N-BE neuroblastoma cells to express both p75NTR and HA-tagged full length TRADD (HA-TRADD). Transfected cells have been selected to generate pools that we characterized for the p75NTR and HA-TRADD expression levels (data not shown). The selected cell pool has been exposed to Aβ peptides, or to control reverse peptides, for 1, 2, 3, 4, 7, 8 hours. The time course has been done trying to reproduce the possible kinetic required to allow the binding of the Aβ to the receptor. After exposures, cells have been scraped and equal amount of crude cell protein extracts have been incubated on anti-HA agarose-beads. Following the Co-Immunoprecipitation protocol, complex have been eluted from the beads and separated on SDS-PAGE then transferred to a membrane that has been probed sequentially with anti-p75^{NTR} and anti-HA antibodies to quantify the co-immunoprecipitated p75^{NTR} and the immunoprecipitated TRADD. As results from Fig 6, after 1 hour of treatment we could not detect p75NTR protein pulled down by HA-TRADD. Proceeding with a longer exposure of 2 and 4 hours, we noticed an increased amount of p75NTR co-immunoprecipitated with HA-TRADD. Prolonged treatments at 6-8 hours do not show anymore interaction between the proteins.

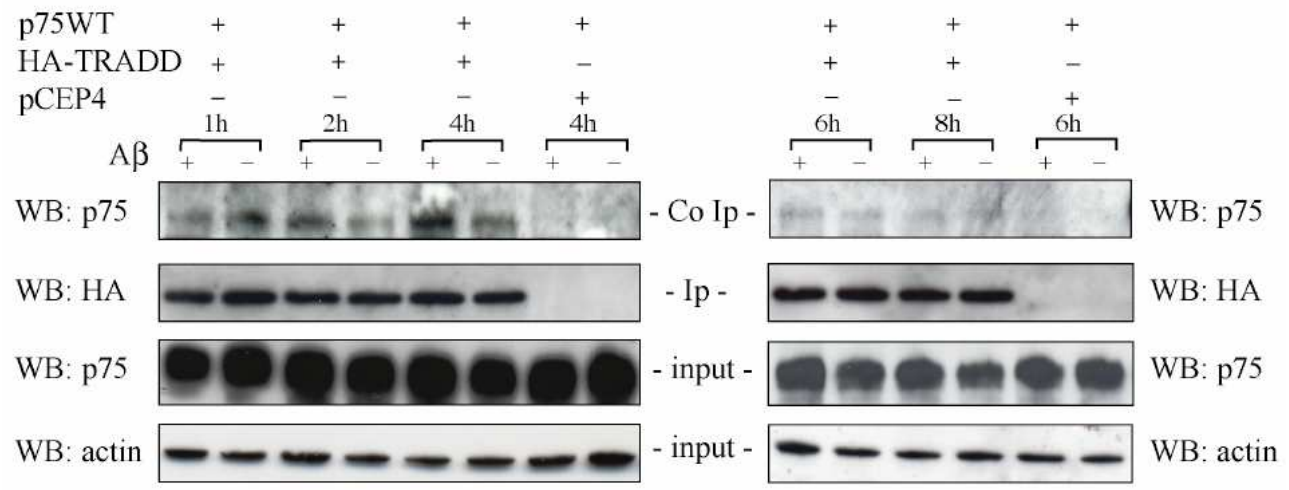


Fig 4A

Fig. 6. Aβ peptide modulates p75^{NTR}/TRADD interaction. Time course of p75^{NTR}/TRADD interaction: SKNBE cell pools stably transfected to express p75^{NTR} and HA-TRADD or stably transfected with a pCEP4 empty vector have been exposed to Amyloid Beta peptide (Aβ) for 1, 2, 4, 6, 8 hrs. 1,5 mg total protein extract for each time course point has been used to immunoprecipitate HA-TRADD followed by Western-blotting probed with antibodies raised against the

indicated epitopes: i) $p75^{NTR}$ and HA-TRADD. The shown autoradiographs are representative of at least three independent experiments.

Indeed, this result indicates that Aβ peptides can modulate and activate the interaction between p75NTR and TRADD. As showed, this activation occurs in a specific time-dependent manner that peaks after 4 hours of exposure.

Finally, we define a possible mechanism by which p75NTR can mediate cell death in an Aβ dependent manner, recruiting TRADD at the level of the membrane and transferring the signal to the cytoplasm.

Discussion

In our study we discuss the role of the Neurotrophin Receptor p75NTR in mediating cell death induced by exposure to the Aβ peptide. The aim of our project is to investigate the molecular mechanisms that follow the binding of the peptide to the receptor peptide and mainly to identify the cytoplasmic pathway that are involved in the cell death signal. By deleting specific sequences in the intracellular domain of p75NTR, we already defined the essential role of the death domain (DD) as responsible for the signal transduction (Perini et al., 2002). Furthermore, in the present work we address in details why the DD is essential and in which way mediates cell death. We show for the first time that the membrane receptor p75NTR, upon binding to β-Amyloid (Aβ) peptide, is able to transduce a cytotoxic signal through a mechanism very similar to the one adopted by Tumor Necrosis Factor Receptor 1 (TNFR1), when activated by TNFα.

Our first approach was based on the analysis of the tridimensional conformation of the receptor. This allowed us to identified specific residues in the DD that, when mutated to A, are no longer able to signal cell death. p75NTR belongs to the family of Tumor Necrosis Factors Receptor 1, this suggested us the possibility of a common mechanism to signal cell death. To test our hypothesis we considered the sequence homology between the death domains of p75NTR and TNFR1 and we identified few almost complete identical residues. By exposing to Aβ peptide neuroblastoma cell stably expressing p75NTR mutated versions we could indeed verify that those aminoacids (E377, W388, A397 and I411) are involved in mediating cell death. Since the residues shared between p75NTR and TNFRI seemed to be important for both the receptors for transducing the death signal (Tartaglia et al 1993) we further speculated about the putative cytoplasmic interactors involved in this process. The Neurotrophin receptor p75NTR is known to mediate different effects, from cell survival to cell death, into the cell most likely depending on which is the extracellular stimulus and on which are, among the many identified, the cytoplasmic interactors expressed in the considered cell type . A recent paper reported that in MCF7 cells, p75NTR binds the Tumor Necrosis Factor Receptor1-associated Death Domain protein (TRADD), one of the first player in the TNFRI depending pathway. (Kieser et al,1999; El Yazidi-Belkoura et al,2003). Considering all together these insights, we decided to test if TRADD could be involved in

mediating Aβ-induced p75NTR-dependent cell death. For this purpose we generated a TRADD dominant negative mutant lacking the death domain required for the interaction with the receptor. Coexpressing p75NTR and TRADDΔDD in Neuroblastoma cells we assessed that also in this contest, TRADD is involved in the mediating the cell death upon Aβ exposure. To confirm these preliminary indications we set up a Co-Immunoprecipitation assays in transiently transfected HEK293 cells that indeed proved the direct interaction between p75NTR and TRADD. The p75NTR and TRADD forming complex resembles the same initial step of TNFR1 dependent signal transduction pathway even though in a different contest. Furthermore, we verified that the Fas-associating protein with death domain (FADD), the direct Fas cytoplasmic adaptor protein, does not interact with p75NTR, although displaying a death domain. This can suggest the presence of a death domain could be a feature necessary but not sufficient to generate a protein-protein interaction. So we proved that TRADD is a good candidate to initiate the p75NTR-dependent death signal transduction into the cytoplasm.

Once assessed the involvement of TRADD in the p75NTR dependent signaling pathway, we tried to clarify whether it could also be an important player in mediating Aβ induced cell death. As first approach, we challenged by Co-Ip the efficiency of the interaction between TRADD and two p75NTR mutants: p75E377A and p75W388A. In this case, we found that these two p75NTR mutants, that more than the others failed to signal Aβ cell death, have been co-immunoprecipitated with a lower efficiency compared to wild type p75NTR. This suggests that p75NTR mutants could be less efficient to transduce the cytotoxic signal most likely because they cannot interact efficiently with TRADD. These findings arise a possible involvement of TRADD as a mediator of the cell death signal induced by Aβ peptide. To deeper prove that TRADD contributes to the Aβ induced cell death of neuronal cells, we performed a time-course treatment on SK-N-BE pools expressing p75NTR to see if the Aβ peptide binding to p75NTR can modulate p75NTR physical interaction to TRADD.

To this purpose, we monitored the efficiency of the interaction between p75NTR and TRADD at different time point after exposures to Aβ and we could assess that the complex formation is indeed modulated by the peptide since we could observe a time-dependent kinetic.

We observed that TRADD interacts with p75NTR raising a peak between 2 to 4 hrs after peptide exposure. This observation possibly correlates with the kinetic of the Aβ sedimentation and the activation of the receptor. Indeed, the relatively slow kinetic of the $p75^{NTR}$/TRADD interaction we observed can be explained by the low solubility level of aggregated Aβ peptide and is consistent with the activation of the downstream signaling pathways, JNK and p38/SAPK, previously described using our cell system (*Costantini et al,2005*). Collectively, our study proposes a new mechanism that underlies the neurotoxicity induced by Aβ peptide and mediated by the Neurotrophin receptor p75NTR. In this contest, multiple proteins have been so far claimed to be candidates for the Aβ depending cell death (Hashimoto Y et al., 2004), perhaps depending on the different neuronal cell populations considered. In this respect, it has been reported that neuronal cell death in AD is mediated in part by the interaction of Aβ with p75NTR. (Rabizadeh et al 1994). Aβ binding to p75NTR results in c- JUN N-terminal kinase (JNK) activation and apoptotic cell death in p75NTR expressing cells (Yaar et al 1997; Bhakar et al,2003; Becker et al, 2004; Ham et al,2005). Aβ activates nuclear factor- kB (Nf-κB) by binding to p75NTR in neuroblastoma cells (Kuner and Hertel, 1998). Furthermore, we proved that the p75NTR DD is responsible for the mediation of Aβ neurotoxicity (Perini et al, 2002). Finally, reflecting some similarity with the TNFR1, we map specific area in the DD of p75NTR responsible for the mediation of the Aβ cell death. This signal transduction recruits the new player, TRADD, to the membrane and activates the apoptotic pathway. In this study, we confirm the essential role of p75NTR in the mediation of the neurotoxicity depending on Aβ, even though we still have to verify the downstream pathway that ultimately activates the programmed cell death (Costantini et al, 2005).

With our study we try to elucidate the role of p75NTR in the Aβ neurotoxicity, suggesting a possible molecular mechanism. In this respect, still a lot remains to be proved since the even higher complexity of the neurodegeneration observed in the Alzheimer's Disease.

Functional cooperation between TrkA and p75NTR accelerates neuronal differentiation by increased transcription of GAP-43 and p21(CIP/WAF) genes via ERK1/2 and AP-1 activities

2.1 p75NTR cooperates with TrkA to accelerate NGF-mediated neuronal differentiation

To investigate how the expression of both p75NTR and TrkA can affect neuronal differentiation, we took advantage of SK-N-BE, a human neuroblastoma cell line which expresses neither receptors [67] although it can be committed to differentiate into sympathetic neurons upon treatment with retinoic acid or TPA [68,69]. SK-N-BE cells represent an interesting neuronal model in which it is possible to re-establish the expression of both receptors and study their reciprocal influence on the activation of specific signal transduction pathways. In the specific case, SK-N-BE cells were transfected with the appropriate vectors and stable cell pools (BEp75, BETrkA and BEp75/TrkA) were isolated and maintained in the appropriate selective medium (see Materials and methods for further details). As a control, cell pools carrying the empty vector (BE vector) were also generated. Expression of either TrkA or p75NTR was monitored by immunoblotting (Fig. 1) and receptor membrane localization was verified by fluorescent immunolabeling with specific antibodies (data not shown). Cell pools expressing comparable levels of the receptors were chosen for further analyses.

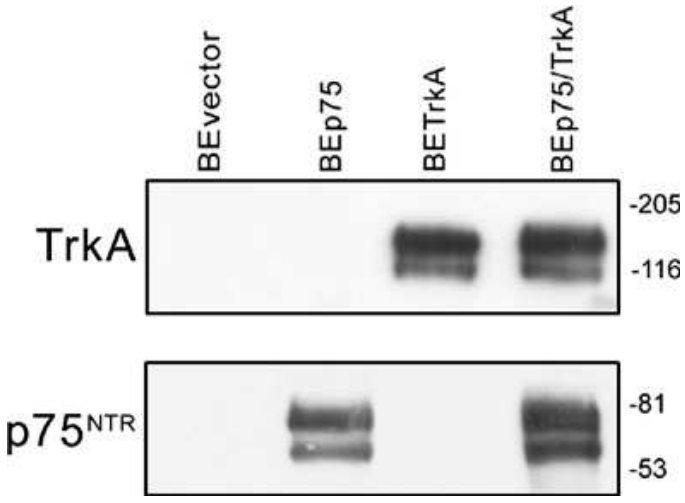


Fig. 2.1 – Expression of TrkA and p75NTR in SK-N-BE derived cells. Total protein extracts from each cell pool (50 μg) were separated by SDS-PAGE, transferred onto a nylon membrane and

probed with either anti-Trk (C-14, Santa Cruz) or anti-p75NTR antibodies (9992). The position of protein molecular weight markers (kDa) is indicated.

First, we studied whether NGF can induce neuronal differentiation in the selected cell pools. BEvector, BEp75, BETrkA and BEp75/TrkA cells were seeded at a density of 2.5×104/cm2 and treated with NGF. Neurite outgrowth, taken as a marker of neuronal differentiation, was monitored at different time points (0, 24, 48, 72 h). As shown in Fig. 2A, 100 ng/ml NGF can only induce differentiation of cells expressing the TrkA receptor. A similar experiment was also conducted using 10 ng/ml NGF to show that NGF, even at very low concentrations, can stimulate neuronal differentiation (data not shown).

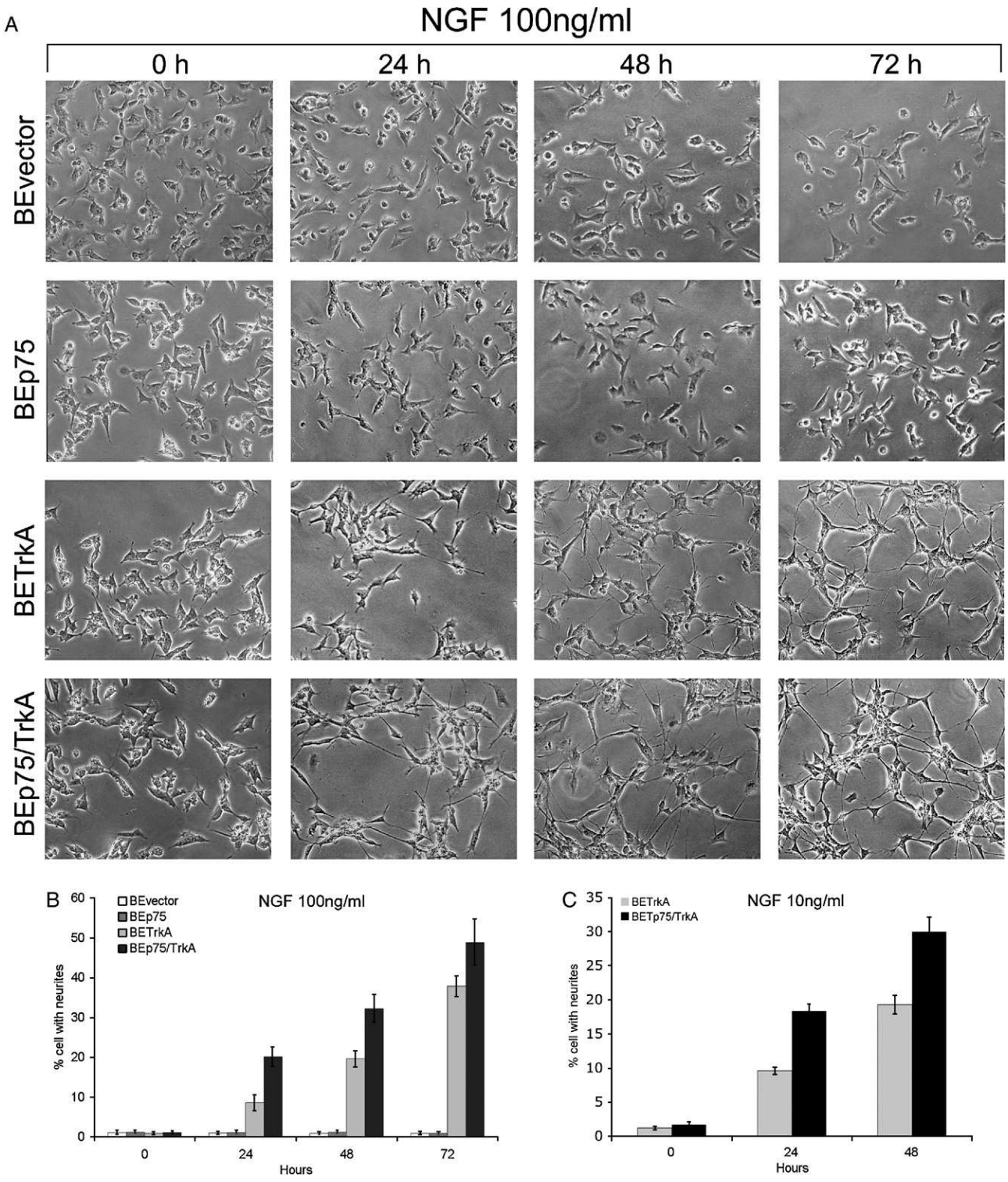


Fig. 2 – NGF dependent differentiation of the cells. Cells were seeded at a density of 2.5×104/cm2 in complete medium. (A) After 24 h cells were washed with 1× PBS and maintained in a 0.1% FBS medium containing 100 ng/ml of NGF. Pictures (200× magnification) of the differentiating cells were taken at an interval of 24 h. (B, C) Quantification of the rate of differentiation is expressed as the percentage of cells that display neurite outgrowth upon exposure to 100 ng/ml NGF (B) or 10 ng/ml NGF (C) Specifically neurite outgrowth was considered positive when the length of the neuritis was at least twice the cell body diameter. For each tested condition, 500 cells from 10 independent fields were counted and the standard error was calculated.

Quantification of the differentiation state, expressed as a percentage of cells displaying neurite outgrowth, is described in Fig. 2B (100 ng/ml NGF) and Fig. 2C (10 ng/ml NGF). Neurite outgrowth is significantly more pronounced in BEp75/TrkA than in BETrkA cells at 24 and 48 h after NGF exposure (Figs. 2B, C), suggesting that coexpression of both receptors may contribute to increase and accelerate the rate of NGF-mediated neuronal differentiation.

2.2 p75NTR enhances TrkA autophosphorylation

To investigate the molecular basis of this phenomenon, we have initially studied whether p75NTR may affect the autophosphorylation state of TrkA. In fact, previous studies showed that p75NTR can modify TrkA function by increasing TrkA phosphorylation [26,27,70,71]. Therefore, we analyzed the degree of TrkA phosphorylation as a function of NGF treatment. Specifically, both BETrkA and BEp75/TrkA cells were treated with NGF for 10 min and then lysed to prepare cell extracts. TrkA was immunoprecipitated with anti-TrkA antibodies and analyzed by Western blotting with an antiphospho-tyrosine antibody. Results of Fig. 3 show that TrkA phosphorylation is higher in cells expressing both receptors than in cells expressing TrkA alone. This observation, which is consistent with the idea that p75NTR can potentiate TrkA kinase activity, supports the view that a higher TrkA autophosphorylation may be causally related to an increased rate of neuronal differentiation.

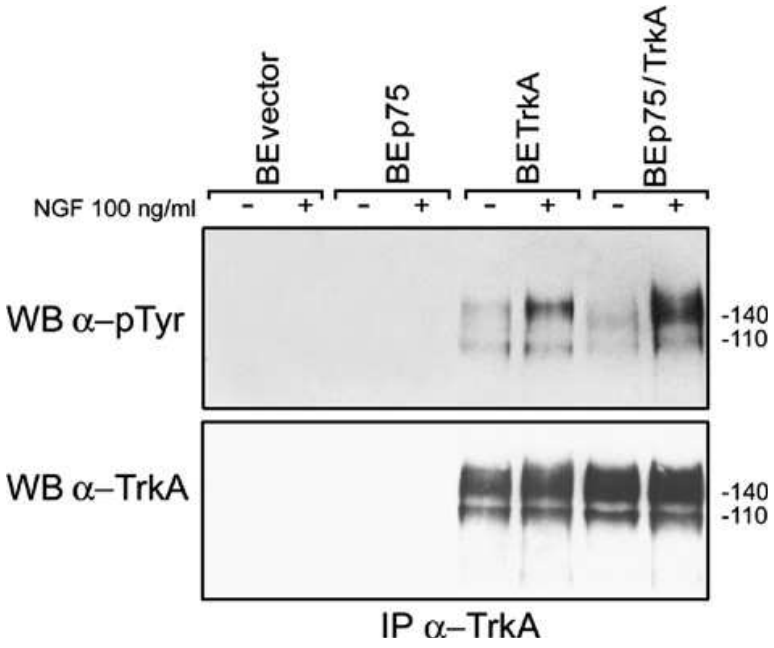


Fig. 2.3 p75NTR stimulates TrkA autophosphorylation. BETrkA and BEp75/TrkA cell pools were analyzed for NGF-mediated autophosphorylation of TrkA. Cells, where indicated, were treated for 10 min with NGF (100 ng/ml) and then lysed. Total protein extracts were subjected to immunoprecipitation with specific anti-Trk antibodies. Immunoprecipitated complexes were separated by SDS-PAGE and transferred onto a nylon membrane. The filter was first probed with anti-phospho-tyrosine antibodies to establish the level of TrkA phosphorylation and then reprobed with anti-Trk antibodies to determine the total level of TrkA present in the immunoprecipitated samples. The molecular weight (kDa) of the most abundant TrkA forms is indicated.

2.3 p75NTR contributes to TrkA signaling by prolonging ERK1/2 activation

Since it has been shown that ERK1/2 are involved in cell differentiation [37,40], we investigated how p75NTR may affect TrkA downstream signaling, with respect to the activation of the ERK1/2 pathway. Cells were serum-starved for 8 h and then treated with 100 ng/ml NGF for 30 min. Afterward, cells were processed and protein extracts were analyzed by immunoblotting with anti-phospho-ERK1/2 antibodies.

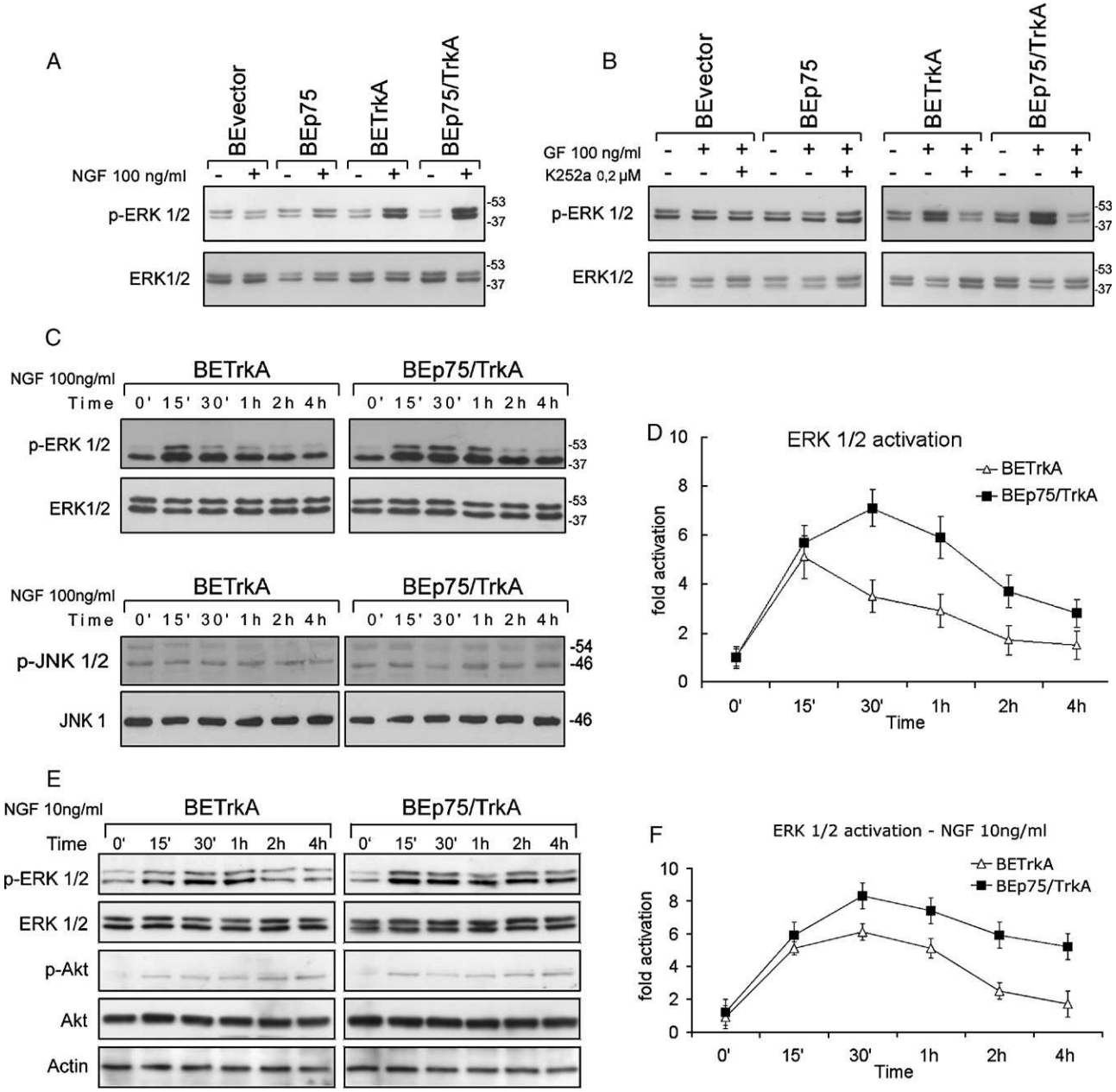


Fig. 2.4 Effect of the combined expression of p75NTR and TrkA on ERKs activation. (A) Cells were serum starved for 8 h and then treated with NGF (100 ng/ml) for 30 min. Protein extracts were analyzed by Western blotting by using antibodies against phospho-ERK1/2. Total amount of loaded ERK1/2 was determined by re-probing the filter with anti-

ERK1/2. (B) ERK1/2 activation of BEp75/TrkA cells completely depends on the TrkA kinase activity. In addition to NGF (100 ng/ml), cells were pre-treated with K252a (0.2 μM), a specific TrkA kinase inhibitor. ERK1/2 activation was monitored as described above. (C) ERK1/2 activation is sustained in BEp75/TrkA cells. BETrkA and BEp75/TrkA cells were serum starved for 8 h and then treated with NGF (100 ng/ml). Phosphorylated and total ERK1/2 were revealed by Western blotting. JNK1/2 are not activated by NGF in BETrkA and BEp75/TrkA cells. JNK1/2 activation was detected by Western blotting using specific anti-phospho-JNK1/2 antibodies. Cell extracts prepared at different time points were analyzed. The filter was reprobed with anti-ERK1/2 or anti-JNK1 antibodies to determine the total amount of ERK1/2 or JNK1 protein in each lane, respectively. (D) Quantification of phosphorylated ERK1/2 in both BETrkA and BEp75/TrkA was determined by densitometric analysis. Results are plotted as fold activation vs. the time points of NGF treatment. (E) ERK1/2 activation is sustained even in BEp75/TrkA cells exposed to 10 ng/ml NGF. The experiment has been performed as described for panel C. ERKs and Akt activity was monitored at different time points. Akt is activated at low level already at 15′ compared to 0′ both in BETrkA and BEp75/TrkA cells. (F) Quantification of phosphorylated ERK1/2 in both BETrkA and BEp75/TrkA cells was determined as described above. The diagram summarizes the results of three independent experiments. The standard error is indicated.

As expected, NGF activates ERK1/2 only in TrkA expressing cells (Fig. 4A). Notably, the activation is significantly increased in cells carrying both receptors (Fig. 4A). The fact that NGF did not activate ERK1/2 in BEp75 cells may indicate that the enhancement of ERK1/2 phosphorylation in BETrkA and BEp75/TrkA cells occurs through TrkA signalling exclusively. To address this point, we analyzed the effect of the K252a compound, a specific TrkA kinase inhibitor [72,73], on the activation of ERK1/2. Fig. 4B shows that the K252a completely blocks the phosphorylation of ERK1/2 in both BETrkA and BEp75/TrkA cells. Overall, these results indicate that in BEp75/TrkA cells, p75NTR cooperates with TrkA to stimulate ERK1/2 activation. To better understand the dynamics of how this occurs, we performed a time course experiment in which the level of ERK1/2 phosphorylation was monitored byWestern blotting at different time points after NGF treatment. The experiment was performed at two different concentrations of NGF (100 ng/ ml and 10 ng/ml, Figs. 4C and E, respectively). Quantification of results shows that phosphorylation of ERK1/2 peaks between 15 and 30 min in TrkA expressing cells and then quickly decays at basal levels (Figs. 4D, F). On the contrary, the level of phosphorylation of ERK1/2 in BEp75/TrkA cells is higher than that of BETrkA and is sustained for more than 1 h after NGF treatment. We have also analyzed the NGF-mediated activation of JNK1/2 in both BETrkA and BEp75/TrkA cells. Results of Fig. 4C indicate that JNK1/2 are not activated by NGF. Finally we have tested the effect of NGF (10 ng/ml) on Akt to verify whether Akt might also be involved in the differentiation process. Results of Fig. 4E show that Akt becomes weakly phosphorylated upon NGF treatment although we could not observe any difference in terms of intensity and kinetic of phosphorylation between BETrkA and BEp75/TrkA cells. Therefore, this result excludes that the AKT pathway may be primarily involved in the accelerated neuronal differentiation of BEp75/TrkA cells. Overall our findings suggest that,

at least, one of the downstream effects caused by the cooperation of p75NTR with TrkA is that of specifically enhancing and prolonging ERK1/2 activation.

2.4 Sustained ERKs activation correlates with an increased AP-1 transcriptional activity

To understand how sustained ERKs activation may affect differentiation, we studied the activity profile of three transcription factors: CREB, NF-kB and AP-1, which under diverse circumstances were shown to be regulated by TrkA [45,49,74]. To this purpose, the in vivo activity of CREB, NF-KB and AP-1 was determined by using luciferase reporters designed to monitor the specific transcriptional function of each factor. Definite amounts of reporter vectors were transfected into BETrkA and BEp75/TrkA cells either stimulated or not with NGF. Twenty-four hours later, luciferase activity was measured.

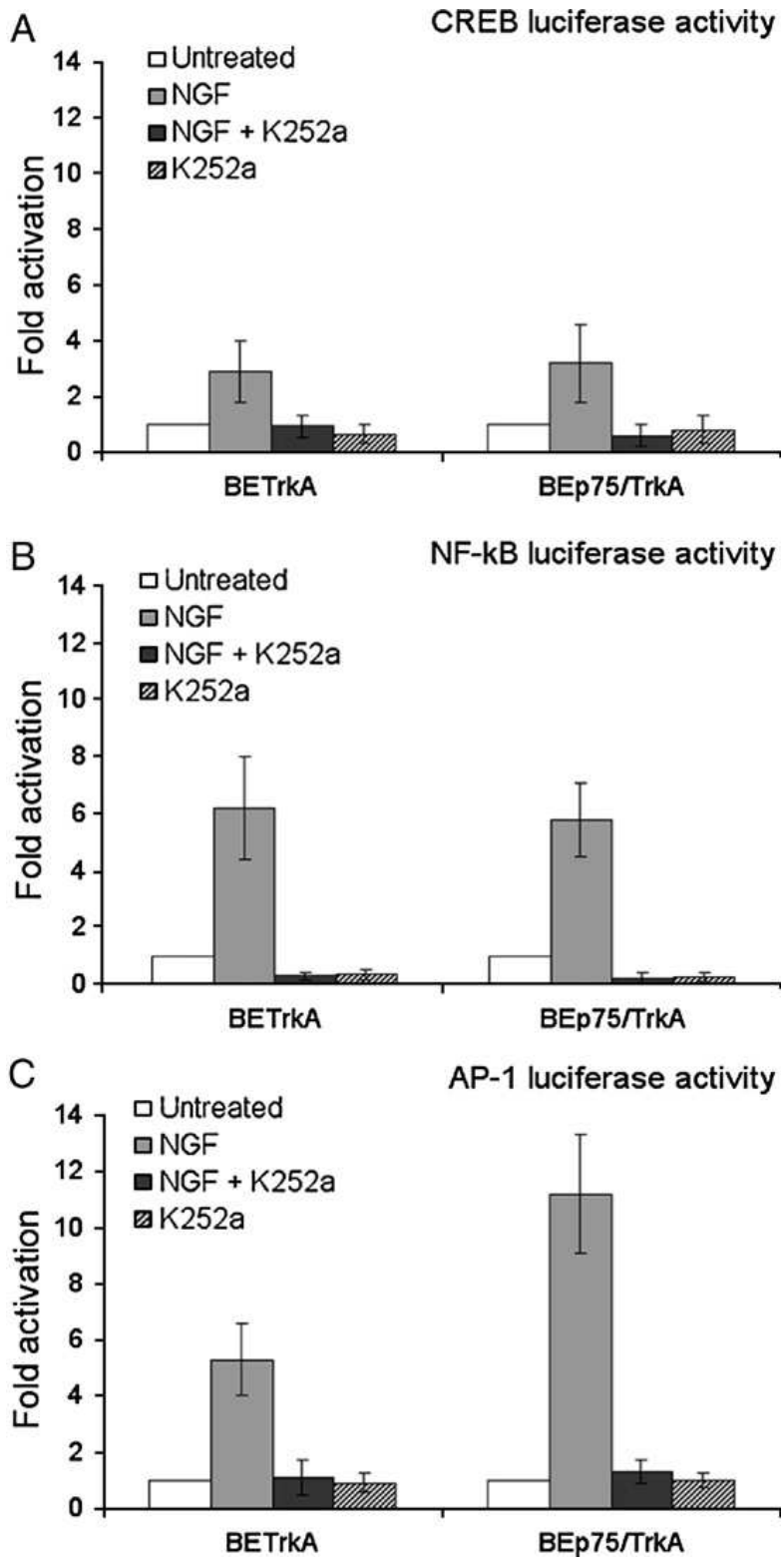


Fig. 5 Analysis of NGF-mediated transcription regulation in BETrkA and BEp75/TrkA cells. Transcriptional activity of CREB, NF-kB and AP-1 factors was monitored through a luciferase assay. BETrkA and BEp75/TrkA cells were independently transfected with 500 ng of CREB-Luc, NF-kB-Luc and AP-1-Luc reporter plasmids and 24 h later were treated with either BSA (0.1%) or NGF (100 ng/ml). Cell protein extracts were generated to measure the enzymatic activity of the luciferase. Fold induction of the transcriptional activity of the reporter is expressed as the ratio between the luciferase activity of treated and untreated cells. The diagram relative to CREB, NF-kB and AP-1 summarizes the results of four independent experiments. Standard error is indicated.

Fig. 5 shows that NGF could induce transcription of NF-kB-Luc, CREB-Luc and AP-1-Luc reporters in both cell pools. However, NGF-mediated transcription of the AP-1-Luc reporter resulted significantly stronger in cells expressing both receptors than in cells expressing TrkA alone, whereas no relevant difference was observed with regard to NF-kB-Luc and CREB-Luc reporters [75]. Interestingly, NGF-mediated transcription of all reporter vectors was completely abolished by the K252a inhibitor, supporting the view that TrkA kinase activity is required for transcriptional activity of all tested transcription factors.

2.5 Activation of ERKs is required for NGF-mediated neuronal differentiation

To confirm that the activation of ERKs is necessary to increase the AP-1 transcriptional activity of both BETrkA and BEp75/TrkA cells, U0126, a specific MEK1/2 inhibitor, which blocks ERKs activationwas used [76]. Three different concentrations of U0126 were employed tomonitor both neuronal differentiation and the luciferase activity of the AP-1-Luc vector when transfected into the BETrkA or BEp75/TrkA cells.

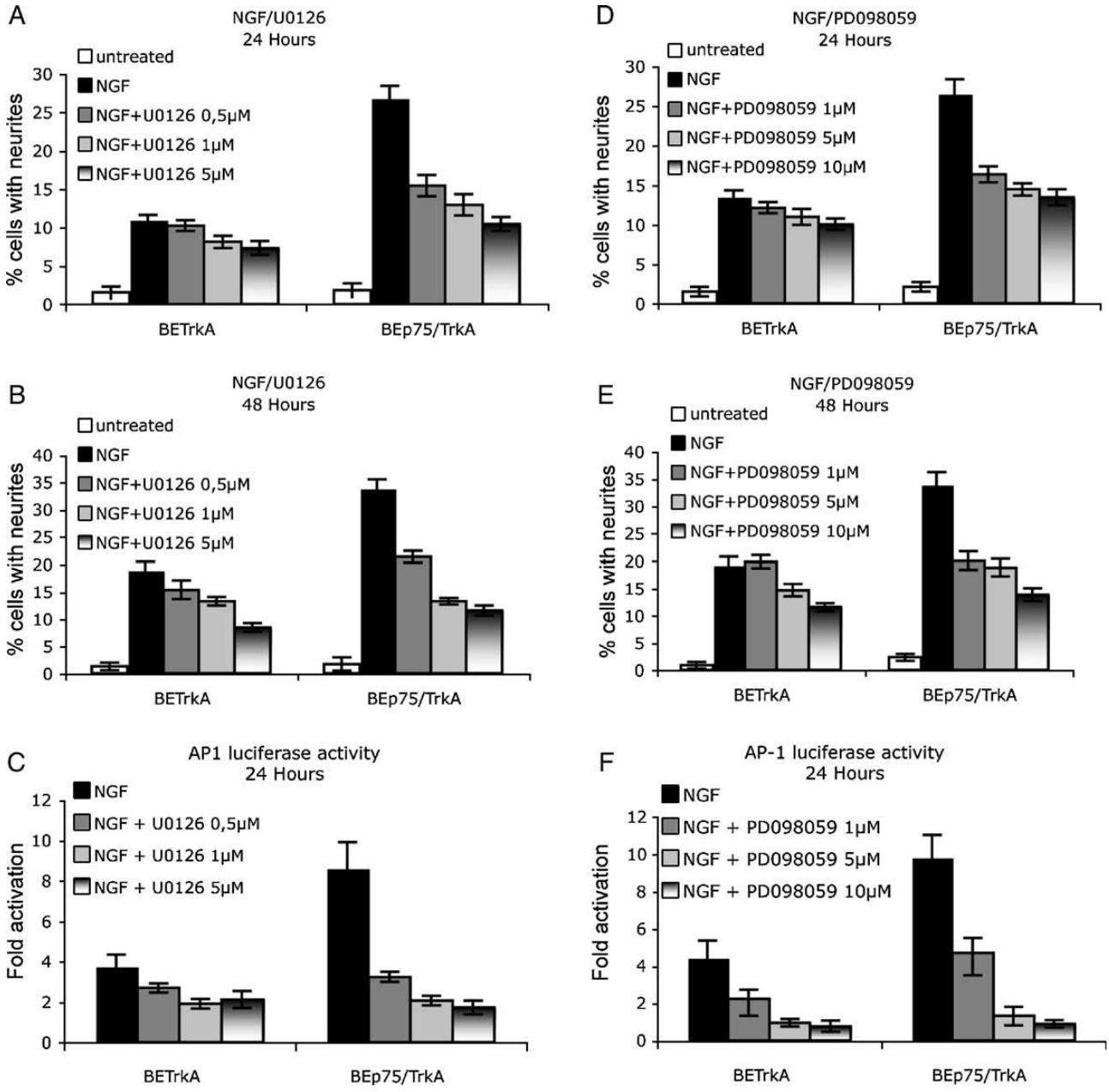


Fig. 2.6 NGF-mediated differentiation and AP-1 transcriptional activity require the activation of ERK1/2 in BETrkA and BEp75/TrkA cells. (A, B, D, E) Degree of differentiation of BETrkA and BEp75/TrkA cells was determined as a function of NGF (10 ng/ml) following pretreatments with either U0126 (0.5 μM, 1 μM, 5 μM) or PD098059 (1 μM, 5 μM, 10 μM) at 24 h (A, D) and 48 h (B, E) with U0126 and PD098059 being two specific MEK1/2

inhibitors. Quantitation of neurite outgrowth was assessed as described in Fig. 2B. (C, F) AP-1 driven luciferase activity was tested in BETrkA and BEp75/TrkA cells in response to NGF (10 ng/ml) and either U0126 (0.5 μM, 1 μM, 5 μM) (C), or PD098059 (1 μM, 5 μM, 10 μM) (F). Fold activation of the AP-1 reporter transcription was measured as described in Fig. 5.

Results show that U0126 could blocks neuronal differentiation more effectively in BEp75/TrKA than in BETrkA cells (Figs. 6A, B). Moreover, AP-1-Luc activity was drastically reduced in BEp75/TrkA as a function of increasing concentrations of U0126 (Fig. 6C). To confirm U0126 results, we have also tested the effect of PD098059, another MEK1/2 inhibitor [77,78]. We designed an experiment similar to that described for U0126 in which we monitored neuronal differentiation and AP-1-Luc activity as a function of increasing doses of PD098059. Results show that PD098059 has a stronger inhibitory effect on BEp75/TrkA than on BETrkA cells (Figs. 6D–F). Overall, these results indicate that accelerated NGF induced differentiation and potentiation of the AP-1 factor transcriptional activity in BEp75/TrKA cells require ERK1/2 activation. To demonstrate that AP-1 activity is required for accelerated BEp75/TrkA cells differentiation, these cells were pretreated with NDGA (Nordihydroguaiaretic Acid) a specific AP-1 inhibitor [79,80]. Again, as for the other inhibitors employed in this study, NDGA could inhibits neuronal differentiation more effectively in BEp75/TrkA than in BETrkA cells (Figs. 7A, B) and could also drastically abate the luciferase activity of the AP-1- Luc vector (Fig. 7C).

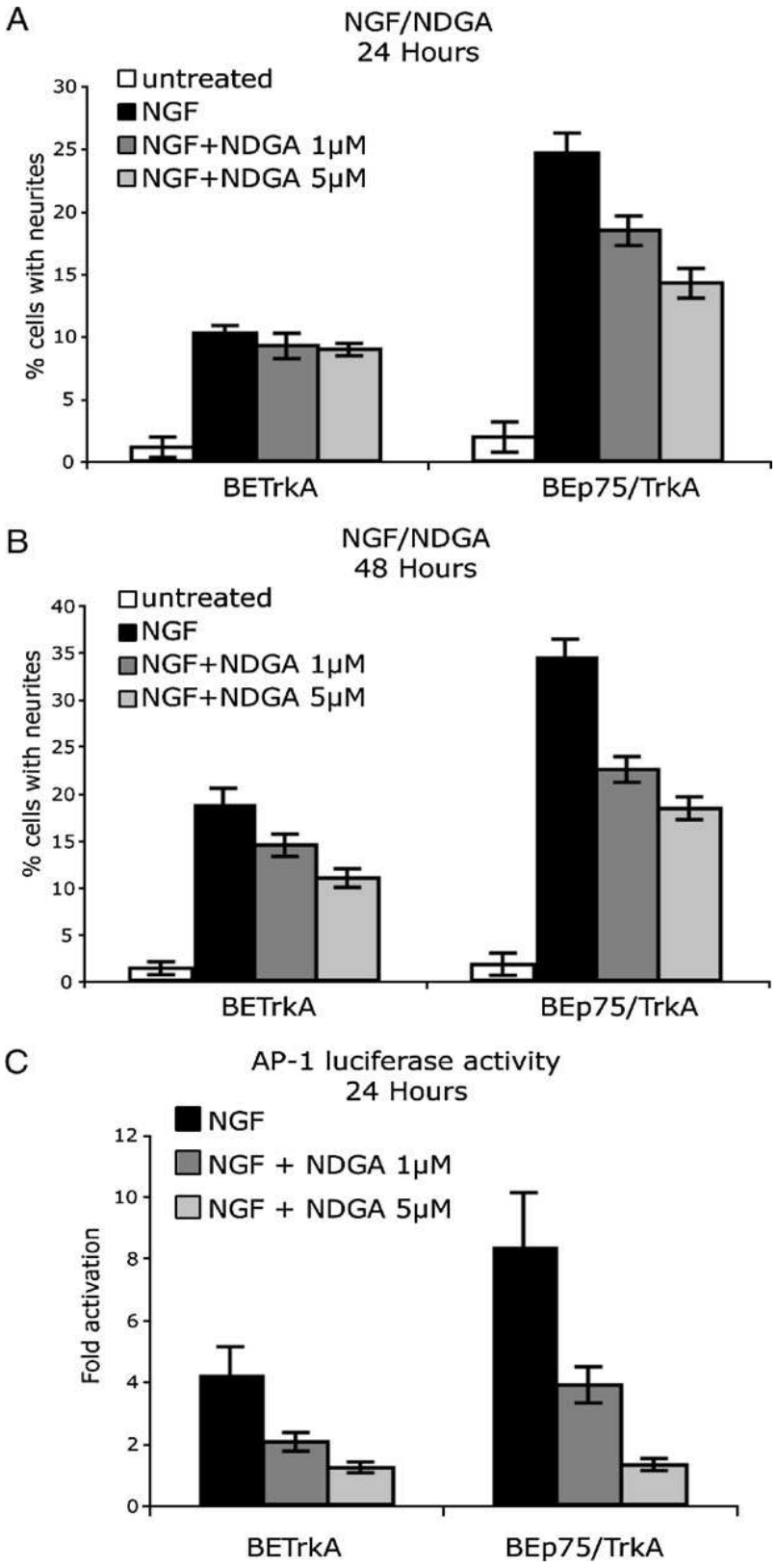


Fig. 2.7 – NGF-mediated differentiation requires AP-1 transcriptional activity. (A, B) Degree of differentiation of BETrkA and BEp75/TrkA cells was determined as a function of either NGF (10 ng/ml) or NGF plus NDGA (1 μM, 5 μM), a specific AP-1 activity inhibitor, at 24 h (A) and 48 h (B). Quantification of neurite outgrowth was determined as described in Fig. 2B. (C) As a control of the NDGA effect, AP-1 driven luciferase activity was tested in BETrkA and BEp75/TrkA cells in response to NGF (10 ng/ml) and/or NDGA (1 μM, 5 μM). Fold activation of the AP-1 reporter transcription was measured as described in Fig. 5.

Taken together these results support the model in which a specific AP-1 activity is promoted in BEp75/TrkA cells upon NGF treatment and that such an activity is required for accelerated neuronal differentiation.

Previous studies have shown that activation of the AP-1 factor by MAPK correlates with the increased transcription of GAP-43 and p21(CIP/WAF), two genes that participate to neuronal differentiation and cell growth arrest, respectively [59–63]. To understand whether the cooperative action of both NGF receptors may affect the expression of GAP-43 and p21 (CIP/WAF) genes, we monitored the mRNA expression of the two genes in both BETrkA and BEp75/TrkA cells as a function of NGF treatment. Cells treated with NGF for 0, 24, 48 and 72 h were processed in order to purify total RNA. The relative amount of both transcripts was determined by quantitative RT-PCR and normalized to that of the Glucuronidase Beta (GUSB) housekeeping gene.

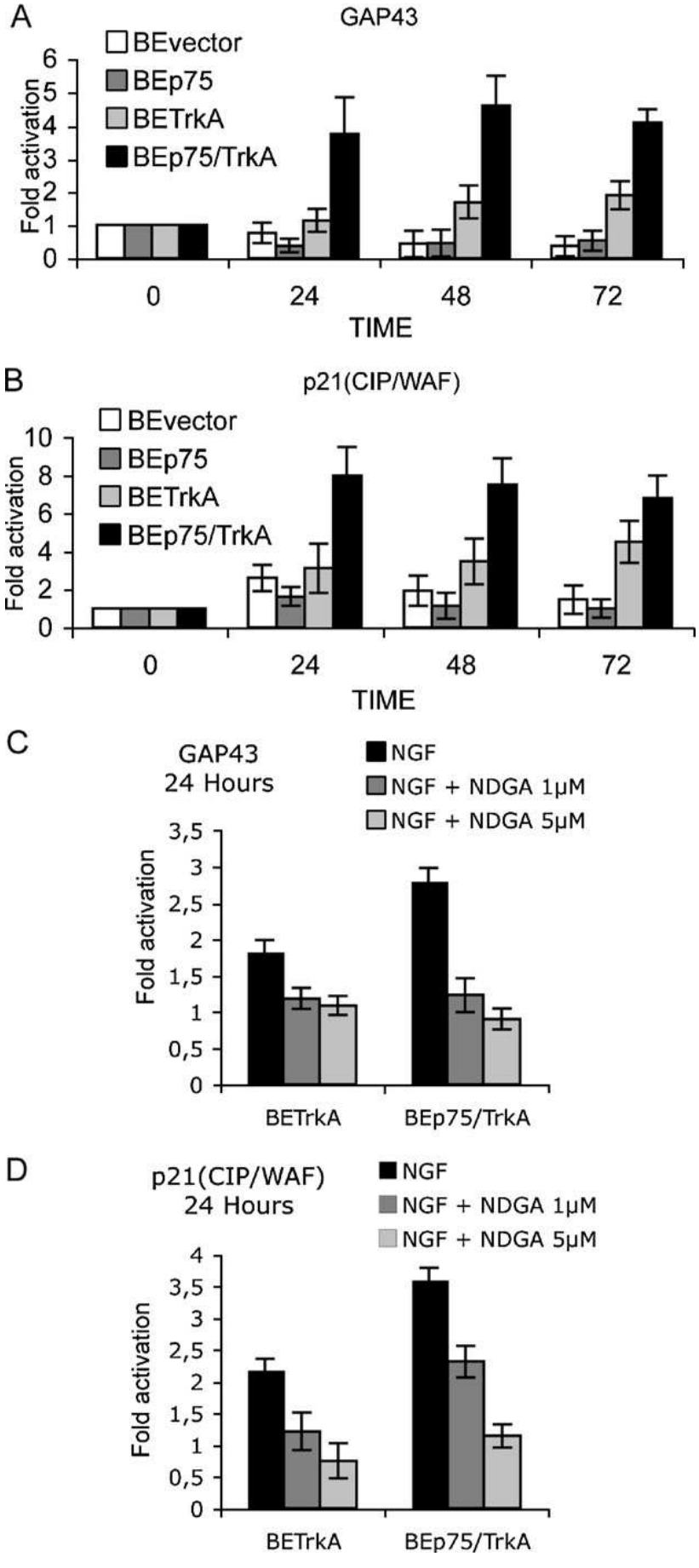

A
GAP43
BEvector
BEp75
BETrkA
BEp75/TrkA
Fold activation
0
1
2
3
4
5
6
0
24
48
72
TIME
B
p21(CIP/WAF)
BEvector
BEp75
BETrkA
BEp75/TrkA
Fold activation
0
2
4
6
8
10
0
24
48
72
TIME
C
GAP43
24 Hours
NGF
NGF + NDGA 1μM
NGF + NDGA 5μM
Fold activation
0
0,5
1
1,5
2
2,5
3
3,5
BETrkA
BEp75/TrkA
D
p21(CIP/WAF)
24 Hours
NGF
NGF + NDGA 1μM
NGF + NDGA 5μM
Fold activation
0
0,5
1
1,5
2
2,5
3
3,5
4
BETrkA
BEp75/TrkA

Fig. 2.8 – NGF stimulation of GAP-43 and p21(CIP/WAF) gene transcription requires AP-1 activity. (A, B) SK-N-BE derived cells were treated with NGF (100 ng/ml) for 0, 24, 48 and 72 h. Relative mRNA expression of GAP-43 (A) and p21(CIP/WAF) (B) genes was determined by quantitative real-time PCR. GAP-43 and p21(CIP/WAF) mRNAs levels were normalized to that of the GUSB housekeeping gene and expressed as fold induction with respect to them RNA level detected before NGF treatment, which has been set to 1. (C, D) Relative mRNA expression of GAP-43 (C) and p21(CIP/WAF) (D) genes was determined as a function of either NGF (10 ng/ml) or NGF plus NDGA (1 μM, 5 μM) at 24 h. The results are an average of three independent experiments. Standard errors are indicated.

Results of Figs. 8A and B show that mRNA expression of both genes is significantly higher in BEp75/TrkA cells than in cells expressing either one of the receptors. To further confirm a direct role of AP-1 in neuronal differentiation we quantified GAP-43 and p21(CIP/WAF)mRNA expression in BETrkA and BEp75/TrkA cells when, in addition to NGF, these cells were pretreated with NDGA. The results of this experiment (Figs. 8C, D) show that transcription reduction of both genes is significantly more pronounced in BEp75/TrkA than in BETrkA cells. Overall, our data support a model in which the cooperative action of both receptor eventually results in increased expression of GAP-43 and p21(CIP/WAF), possibly by the action of the AP-1 transcription factor.

Discussion

In this study, we have focused our interest on how p75NTR may affect TrkA downstream signalling with respect to neuronal differentiation. Here, we have shown that p75NTR can cooperate with TrkA by modulating the activation of ERK1/2 in response to NGF and thus leading to an accelerated neurite outgrowth. We have analyzed this process at different levels. First, we have determined the degree of autophosphorylation of TrkA in the presence or absence of p75NTR and found that p75NTR increases the overall levels of TrkA phosphorylation. Second, we have monitored the activation of ERK1/2 as a function of the specific contribution of each Neurotrophin receptors and found that the combined expression of the receptors leads to a persistent ERK1/2 activation. Prolonged activation of ERK1/2 in BEp75/TrkA is dependent on TrkA autophosphorylation in that it is completely abated by the K252a a specific inhibitor of the TrkA kinase. This result indicates that the functional cooperation between p75NTR and TrkA is not achieved through the activation of parallel signal pathways that may eventually merge within the cell, but it is rather the result of a specific enhancement of TrkA signaling. Moreover, these findings suggest that the enhancement of TrkA phosphorylation may be causally related to the sustained activity of ERKs in BEp75/TrkA cells. Although it is clear that the exact mechanism remains to be elucidated, our results reveal that the NGF dependent ERK activity can be finely modulated by controlling the relative expression of TrkA and p75NTR. The persistent activation of ERK1/2 has been correlated to neuronal differentiation [37,40]. Moreover, recent reports have shown that NGF-mediated ubiquitination of TrkA can be enhanced by p75NTR causing prolonged and increased ERKs activation [81,82]. Our results are consistent with this view and show that sustained ERKs activity may play a specific role in accelerating the differentiation process. In addition, our findings point to a critical function of the AP-1 transcription factor in this process. Comparative analysis of three distinct transcription factors CREB, NF-kB and AP-1, often studied as major effectors of p75NTR and TrkA signal pathways, reveals that the AP-1 transcriptional activity is significantly stronger in cells expressing both receptors than in those expressing just TrkA. Moreover, AP-1 activity and neurite outgrowth are completely abolished when cells are treated with U0126 or PD098059, two specific MEK1/2 inhibitors. Our results extend those described by Leppa and colleagues who found that the NGF-mediated differentiation of PC12 cells requires the

activation of ERK1/2 for induction of c-jun mRNA synthesis, as well as for phosphorylation of the AP-1 components [75]. Specifically, the fact that JNK1/2 as well as Akt is not involved in the functional activation of the AP-1 factor suggests that ERK1/2 may activate AP-1 post-translationally. In many reports, AP-1 has been related to the p75NTR apoptotic signalling whereas other factors such as CREB and NF-kB were considered to be more specifically involved in survival and/or differentiation [83,84]. It should be noted that also in our cell model, CREB and NF-κB transcriptional activities are stimulated
in a NGF dependent manner, implying that they may participate to the differentiation process. However, our findings highlight a distinctive regulation of the AP-1 transcriptional activity specifically in determining the rate of neuronal differentiation. Such a regulation may be achieved by controlling the specific duration of ERKs activity through combinatorial expression of the two neurotrophin receptors. These findings highlight a molecular mechanism through which cells interpret differences in ERKs signal duration and give rise to distinctive biological outcomes [85]. In case of sustained ERKs phosphorylation, the activity of the AP-1 transcription factor is enhanced with consequential increased expression of the p21(CIP/WAF) and GAP-43 genes, the activity of which is respectively critical for cell growth arrest and differentiation [59–63]. This result is corroborated by experiments in which we have employed NDGA, a specific AP-1 inhibitor.
To our knowledge this is the first study in which NGF signalling has been recapitulated step-by-step within a definite cell system from the role of the membrane receptors to the expression of critical genes linked to neurite outgrowth. These findings may help understand some aspects of neuronal development in which the controlled expression of both TrkA and p75NTR may determine the fate of neurons by accelerating target innervations and differentiation within an environment endowed with limiting amounts of neurotrophins.

Material and Methods

DNA Constructs

The $p75^{NTR}$ constructs carrying point mutations were generated by Inverse Long-PCR protocol following manufacturer instructions (Herculase Enhanced DNA Polymerase, Stratagene) and using as template a pcDNA3 plasmid (Invitrogen) in which the rat $p75^{NTR}$ wild-type cDNA has been cloned ($p75^{NTR}$/pcDNA). The primer sequences used for each mutant are as follow:

Rp75NTRE377AF: 5'-TTTACCCACGCAGCCTGCCCAG-3'
Rp75NTRE377AR: 5'-GGAGTCTATATGTTCAGGCTGGTAA-3'
Rp75NTRW388AF2: 5'-GCAACGCTTGATGCCCTTTTAGCC-3'
Rp75NTRW388AR2: 5'-ACTGTCCTGGGCACCAGCGCTG-3'
Rp75NTRI411AR: 5'-TAGACTCTCCACCGCGTCAGCTC-3'
Rp75NTRI411AF: 5'-TGCAGCGAGTCCACTGCCACATC-3'

Underlined codons in either forward or reverse primer sequence indicate the A codon used to create the mutation. Introduction of the desired single point mutations was verified by plasmid clone sequencing.

The human TRADD and FADD full lenght cDNAs have been amplified by PCR from, respectively, SK-N-BE and HL-60 cells total RNA after retrotranscription. The following primers have been used:

HTRADD5BAMF: 5'-CGGGATCCGCAGCTGGGCAAAATGGGCAC-3'

HTRADD3ECOR: 5'-CGGAATTCCTAGGCCAGGCCGCCATTGG-3'

HFADDBAM1F: 5'-CGGGATCCGACCCGTTCCTGGTGC-3'

HFADDECO1R: 5'-CGGAATTCTCAGGACGCTTCGGAGG-3'.

The BamHI and EcoRI sites included in the primers and located, respectively, at the 5' and 3' ends of the RT-PCR products, were used to clone the cDNAs in the pRK7-HA expression vector to obtain in frame fusions with the HA epitope at the TRADD or FADD N-terminal. Afterward, the whole HA-TRADD cDNA has been amplified with the following forward primer:

HA5HIND1F: 5'-CCCAAGCTTACCATGGCCTACCCCTACGACG-3' and the following reverse primers:

HTRADD3NHEIR:5'-CTAGCTAGCCTAGGCCAGGCCGCCATTGG-3',

HTRADDNEG3NHEIR: 5'-CTAGCTAGCCTACGGCGGCGGCGGCTTCAC-3',

to clone, respectively, the full-length or the C-terminal truncated HATRADD (HATRADDΔDD) in the HindIII/NheI sites of the pCEP4 expression vector multicloning

site (Invitrogen). The HATRADD sequence has been verified both in the pCEP/HATRADD-FL and in the pCEP/HATRADDΔDD plasmids.

The human TNFR1 full lenght cDNA has been amplified by RT-PCR from HL-60 cells total RNA using the following primers:

TNFR1BAM1F: 5'-CGGGATCCGGCCTCTCCACCGTG-3'

TNFR1BAM1R: 5'-CGGGATCCTCATCTGAGAAGACTGGG-3'.

The amplification product has been cut at the 5' and 3' ends by BamHI and ligated into the 3xFLAG-CMV-10 expression vector (Sigma-Aldrich) after BamHI restriction reaction; the orientation of the TNFR1 cDNA respect to the plasmid vector has been assessed using a TNFR1 cDNA EcoRI asimmetric internal site.

Cell Cultures and Transfection

All cell lines were cultured at 37°C in 5% CO_2. HEK 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM), 2 mM L-glutamine, 10% fetal bovine serum (FBS) and 50 mg/ml gentamycin. The human neuroblastoma SK-N-BE cell line was grown in DMEM containing 5% FBS, 10% HS (Horse serum), 2 mM L-glutamine, 50 mg/ ml gentamycin, 250 ng/ml amphotericin B. Cells were transfected by using the polyethylene-immine method (PEI, Sigma).

SK-N-BE derived cell pools stably transfected with the expression vectors pcDNA3Neo (Invitrogen) carrying the rat wild type ($Rp75^{NTR}$) or the indicated mutant $p75^{NTR}$ cDNAs were selected in complete medium containing G418 (800mg/ml). SK-N-BE cell pools stably transfected with the $Rp75^{NTR}$/pcDNA or the pcDNA empty vector were transfected with the pCEP4Hygro (Invitrogen) expression vector carrying either the wild type or the C-term truncated human HA tagged TRADD (HA-TRADD) cDNA or the pCEP4Hygro empty vector and were selected in complete medium containing Hygromycin (800 mg/ ml), and G418 (150mg/ml). Pools of stably transfected cell clones selected with the appropriate growth inhibitor, as indicated, were characterized by checking the transfected protein/s expression level by Western-blotting and $p75^{NTR}$ cell membrane localization was checked by immunofluorescence following the procedure described in Perini et al,2002. Cell pools displaying a comparable protein expression level have been selected for further experiments.

Immunoprecipitation and Western Blot Analysis

To test the expression level of wild-type or mutant Rp75^{NTR} and HA-TRADD in the stably transfected cell pools, cell were lysed in a buffer (Lysis buffer) containing: 150 mM NaCl, 50 mM Tris pH 7,5, 0,1% NP40, 1 mM PMSF, 1mM NaPirofosfato, 1 mM NaOrtovanadato, 1x protease inhibitors cocktail, (Complete-Roche); this lysis procedure let to obtain a cell lysate enriched in membrane and cytoplasmic protein.

Protein extracts were fractioned by 4-12% SDS-PAGE (NuPage system-Invitrogen) and elettroblotted to nitrocellulose filters (Amersham Bioscinces) that were blocked for at least 30 min at room temperature in 5% nonfat dry milk in Tris-Cl pH 8.50 mM, NaCl 150 mM (blocking buffer) and incubated in blocking buffer overnight at 4°C with one of the following antibodies: anti-p75^{NTR} 9992 polyclonal antiserum raised against the rat p75^{NTR} intracellular region (Promega); anti-TRADD goat polyclonal antibody raised against C-terminal aminoacidic sequence (Santa Cruz); anti-HA monoclonal antibody (Santa Cruz) to test the HATRADD-DN expression level. Membranes were washed three times in blocking buffer with 0,1% Tween-20 and incubated in the suitable secondary HRP-conjugated antibody (Jackson-Immunoresearch Lab.) for 1 h at room temperature. Membranes were then washed as before and signals revealed using ECL (Amersham Bioscences).

The interaction between TRADD and p75^{NTR} was assessed in SKNBE transfected cell pool at 2 to 8 h after Aβ peptide stimulation or in transiently transfected HEK 293 cells, as described in the text, by Immunoprecipitation and Western blotting.

Cells were washed two times in PBS and lysed in lyses buffer. Cell lysate (1.5 mg) was precleared with protein A-agarose beads (Roche), (30 min at 4°C) and then immunoprecipitated with anti-HA Affinity Matrix overnight at 4°C. The day after, the beads with immunocomplexes were washed three times with lyses buffer and boiled in Laemmli sample buffer for 5 min at 100°. Eluted proteins were separated by SDS-PAGE and analyzed by Western blot with the indicated antibodies.

Cell death assay

SK-N-BE derived cell pools have been plated on 24-multiwell plate (3,5X10^4 cells/well); after 12-18 hours cells have been wash once with medium and exposed to the Aβ 25-35

peptide (Bachem) or to the control Aβ 35-25 peptide (Bachem) both diluted 20 μM in the culture medium. Peptide have been solved and prepared as described in Perini et al, 2002. After 48 hours cells were washed twice with PBS 1X, detached by gently pipetting and loaded in slide-carrying cell chamber to spin cells on the slide using Cytospin IV (Thermo Shandon) 4 min at 1000 rpm. Immediately after, cells have been fixed with cold Paraformaldehyde 4% in PBS 1X. After two wash with PBS 1X cells have been permeabilaized and labeled with the TUNEL technique following manufacturer instructions (TUNEL Cell Death Detection KIT-Roche). After the labeling reaction cell nuclei have been stained with Ethidium Bromide 0,7 μg/ml in PBS 1X for 7 min and then washed three time in PBS1X and mounted in antifading medium (Vectashield–Vector). The number of dead cells has been evaluated at the conventional fluorescent microscope (Zeiss-Axiophot) using selective band pass filter to detect the FITC-dTTP incorporation and filter to detect the ethidium bromide stain to check for nuclear localization of the TUNEL labeling. When indicated in the text cytotoxicity level has been evaluated also with a double Acridine orange/Ethidium Bromide in vivo staining as described (*Perini et al,2002*) To validate the antiapoptotic activity of TRADDΔDD truncated mutant, HEK 293 cells seeded on coverslips in a 6-well multiwells plate were transfected with plasmid vectors to express EGFP (to label transfected cells) and $p75^{NTR}$ or TRADDΔDD alone or together. After 12-18 hrs from the transfection cells have been twice washed, fixed in cold paraformaldehyde 4% in PBS 1X and stained for 5 min with Hoehcst 33342 diluted to 500 ng/ml in PBS1X.

Plasmid vectors

The TrkA construct was produced by cloning the corresponding cDNA into pCEP9β vector (Invitrogen). The p75NTR constructs were generated as previously reported [64,65]. Cell culture and selection of transfected cells Neuroblastoma SK-N-BE [64] cells were grown in DMEM medium containing 10% horse serum, 5% FBS and 2.5 μg/ml of gentamycin. Cells were transfected with pCEP9β empty vector or pCEP9β-TrkA or pCEP4β-p75NTR constructs by using the polyethylene-immine method (PEI, Sigma-Aldrich) [66]. After transfection, cells were split and grown in complete DMEM medium containing either 400 μg/ml of G418 (Roche) for the selection of TrkA positive pools of clones (BETrkA) or 150 μg/ml of Hygromycin (Roche) for the selection of p75NTR positive pools of clones (BEp75). Selected cell pools were collected, expanded and characterized for expression of TrkA and p75NTR proteins by Western blotting. BETrkA cells were subjected to a second round of transfection with the pCEP4β- p75NTR construct to generate BEp75/TrkA cell pools. After transfection cells were split and grown in complete DMEM medium containing both G418 (400 μg/ml) and Hygromycin (150 μg/ml). Cell pools were selected and characterized for the expression of the receptors as described above. When indicated cells were serum starved for 8 h and then treated for additional 24, 48 and 72 h with 0.1% BSA, 10 ng/ml or 100 ng/ml human recombinant NGF (Sigma) resuspended in a 0.1% BSA/PBS solution. When indicated cells were pre-incubated 1 h with0.2 μM K252a (Calbiochem), U0126 (0.5 μM, 1 μM, 5 μM; Cell Signalling), PD098059 (1 μM, 5 μM, 10 μM; Alexis Biochemicals) or NDGA-Nordihydroguaiaretic Acid (1 μM, 5 μM; Sigma-Aldrich) before adding NGF to the culture medium. Immunoprecipitation After NGF treatment, cells were washed with 1× ice-cold phosphate-buffered saline (PBS), and lysed in an IP-lysis buffer [20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% TRITON X-100, 100 mM NaF, 1 mM Na3VO4, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 2 mM phenyl-methylsulfonyl fluoride (PMSF, Sigma) and a 1× protease inhibitor cocktail (Complete, Roche)]. The lysate was incubated on ice for 10 min and clarified by centrifugation. Proteins of each sample were quantitated by the BCA protein assay (Pierce).Two milligrams of proteins from each sample was incubated O/N at 4 °C with an anti-Trk polyclonal antibody (C-14, Santa Cruz). ProteinA agarose beads (20 μl of 50% bead slurry-Pierce) were added, and samples were incubated for 3 h at 4 °C. Beads were collected, washed 3 times with 1× IP-lysis buffer and resuspended in 2× SDS sample buffer. IP-complexes were analyzed by immunoblot.

Immunoblot Cells were lysed in RIPA buffer, supplemented with phosphatase and protease inhibitors (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1mMEDTA, 1mM Na3VO4, 100 mM NaF, 1× complete protease inhibitor cocktail, 1mMPMSF). Fifty micrograms of proteins was boiled for 5min in standard 1× sample buffer, separated on a 10% SDS polyacrilamide gel and blotted onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were blocked and incubated O/N at 4 °C with the appropriate antiserum. Specifically, 9992 rabbit polyclonal antiserum raised against the intracellular region of p75NTR was a generous gift from Dr. M.V. Chao; anti-Trk(C-14), anti-ERK1/2(K-23) and anti-JNK1(C-17) were purchased from Santa Cruz; antiphospho- Tyr-100, anti-phospho-MAPK(ERK1/2), anti-phospho-SAPK/JNK, anti-phospho-Akt and anti-Akt were purchased from Cell Signaling; anti-actin was purchased from Sigma-Aldrich. Afterward, membranes were washed and incubated at room temperature for 1 h in blocking solution containing an HRP-conjugated anti-rabbit secondary antibody (Amersham Pharmacia Biotech). Signal was detected by using the ECL Western blotting detection reagents (Amersham Pharmacia Biotech). Luciferase assay 2.5×105 cells/well were plated and grown for 24 h before transfection in a 24 well plate. Cells were washed with 1× PBS then transfected with 500 ng of the appropriate responsive reporter (CREB-Luc provided by E. Ciani, NF-kB-Luc provided by K.B. Marcu, AP-1-Luc provided by M. Karin) by using the PEI method. Cells were serum starved for 8 h and then treated for additional 24 h with 0.1% BSA, 10 ng/ml or 100 ng/ml NGF (Sigma-Aldrich). When indicated, cells were pre-incubated with K252a, U0126, PD098059 or NDGA before adding NGF to the culture medium. Firefly luciferase activity was normalized to that of Renilla luciferase included as an internal control. Luciferase activity was measured by using the Luciferase Assay System (Promega) with a TD 20/20 luminometer (Turner Designs).

Real-time PCR Total RNA was extracted with TriReagent (Sigma-Aldrich). Thirty micrograms of total RNA was treated with DNASe Turbo (Ambion) to remove possible genomic DNA contamination. Two micrograms of DNA-free RNA was retrotranscribed with 5U of Thermoscript (Invitrogen) in a 20 μl volume for 1 h at 37 °C. RT reaction was brought to 100 μl final volume with DEPC water. Two microliters of the diluted RT reaction was used for QT-PCR using the IQ syber green supermix (Bio-Rad). QT-PCR was performed on an IQ5 Real-time PCR machine (Bio-Rad). Primers for QT-PCR were the following: AGCGTGGAGCAAGACAGTGG (GUSBf),

ATACAGATAGGCAGGGCGTTCG (GUSBr), GCAGGACGAGGGTAAAGAAGAGG (GAP-43f), GAGAAGAGGGTAGGGAGAGACAGG (GAP-43r), TGATTAGCAGCGGAACAAGGAG (p21f), GGAGAAACGGGAACCAGGACAC (p21r).

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