Nerve growth factor (NGF) exerts its pro-apoptotic effect via the P75^{NTR} receptor in a cell cycle-dependent manner

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Abstract Nerve growth factor (NGF), the prototypic member of the neurotrophin family of growth factors, exerts its action via two receptors, P75^{NTR} and TrkA, the expression of which varies at the cell surface of neuroblastoma cells (SH-SY5Y cells) in a cycle phase-specific manner. NGF was pro-apoptotic on growing cells expressing preferentially P75^{NTR} and exhibited a potent anti-apoptotic effect on quiescent cells, when TrkA was prevalent at the cell surface, showing that NGF can have a dual action on SH-SY5Y cells depending on the relative cell surface expression of TrkA and P75^{NTR}. The pro-apoptotic activity of NGF but not its anti-apoptotic activity was abrogated by an antibody against the extracellular domain of P75^{NTR} and in cells isolated from P75^{NTR} knock-out mice indicating that NGF exhibits a pro-apoptotic activity via P75^{NTR} exclusively. On the other hand, we showed that the anti-apoptotic activity of NGF was specifically mediated by an interaction with TrkA with no contribution of P75^{NTR}, as demonstrated on SK-N-BE cells transfected with TrkA in which NGF was a potent anti-apoptotic compound but did not exhibit any pro-apoptotic activity. These results support the hypothesis that the survival response to NGF depends on its binding to TrkA without any involvement of P75^{NTR} which in turn selectively mediates the pro-apoptotic activity of NGF with no contribution of TrkA and show that, depending on the growth state of the cells, NGF exhibits dual pro- or anti-apoptotic properties via P75^{NTR} and TrkA, respectively.

Key words: Neuroblastoma cell; Apoptosis; P75^{NTR}; TrkA; Nerve growth factor

1. Introduction

Nerve growth factor (NGF) is a member of a structurally and functionally related family of proteins (termed neurotrophins) with pleiotropic roles [1], including critical functions in the development, survival and differentiation of neurons [2–9]. The action of NGF is mediated by two classes of cell surface receptors: TrkA [10,11], a member of the Trk family of tyrosine kinase receptors, and P75^{NTR}, a low affinity receptor that also binds other neurotrophins such as BDNF or NT-3 [12,13]. The role of P75^{NTR} in the functional response to NGF is still controversial [14,15]. Some reports indicate that P75^{NTR} collaborates with TrkA in the formation of high-affinity binding sites and in NGF signal transduction [16–19], but others suggest that TrkA alone may be sufficient to support the activity of NGF on cell differentiation [20]. P75^{NTR} also possesses unique, Trk-independent signalling properties which involve ceramide production [21] and activation of the transcription factor NFκB [22]. Moreover, recent evidence indicates that NGF uses a two-receptor system to dictate signalling pathways leading to either cell survival or suicide, but this topic has been a continuing source of controversy [23–25]. Recently, Urdiales et al. [26] showed that TrkA was expressed preferentially at the cell surface in the early G1 and M phases of the cell cycle whereas P75^{NTR} was only detectable during the late G1, S and G2 phases.

These observations led us to examine the effect of NGF on the apoptotic response of SH-SY5Y, a neuroblastoma cell line, and to evaluate the respective roles of TrkA and P75^{NTR} in the pro- and anti-apoptotic activities of NGF.

2. Materials and methods

2.1. Materials

The human neuroblastoma cell line SH-SY5Y was kindly provided by Dr D. Caput (Sanofi recherche, Labège, France). Dulbecco's modified Eagle medium (DMEM), RPMI 1640 medium, geneticin, gentamicin, pyruvate, anti-PPLO and phosphate-buffered saline (PBS) were from Gibco BRL (Cergy-pontoise, France). Foetal calf serum (FCS), penicillin, streptomycin sulfate and glutamine were from Boehringer Mannheim (Meylan, Claix, France). Human recombinant NGF and hygromycin B were obtained from Sigma Chemical (L'Isle d'Abeau, France). 35 mm dishes biocoat collagen I, culture slides biocoat collagen I and T-75 flasks biocoat collagen I were from Becton Dickinson (Le Pont de Claix, France). The antibody against the extracellular domain of P75^{NTR} (CHEX) was kindly given by Dr L.F. Reichardt (San Francisco, CA, USA).

2.2. Cell cultures

Human neuroblastoma SH-SY5Y cells were routinely cultured in DMEM culture medium supplemented with 5% FCS, pyruvate Na 1 mM, anti-PPLO 5 ml, gentamicin 0.1 mg/ml and 4 mM glutamine on pre-coated collagen T-75 flasks. Human neuroblastoma SK-N-BE cells were cultured in RPMI 1640 culture medium supplemented with 15% FCS, 2 mM glutamine and 50 mg/ml gentamicin. SK-N-BE cells expressing TrkA were maintained in the same medium supplemented with 500 μg/ml geneticin. Murine aortic smooth muscle cells (ASMCs) were isolated from P75^{NTR} knock-out (KO) mice or the corresponding wild-type mice (C129-Ngfrt^{m1Jae}) (Jackson Labs, ME, USA) [27] and routinely cultured in DMEM culture medium supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin sulfate and 4 mM glutamine.

2.3. Generation of neuroblastoma cells expressing TrkA

Subconfluent human neuroblastoma SK-N-BE cells were transfected with a plasmid vector carrying the cDNA of the human TrkA gene under the control of the cytomegalovirus promoter and the bacterial neo gene. G418 (800 μg/ml)-resistant colonies were isolated, grown and analysed for TrkA expression by a Western blot.

2.4. Measurement of apoptosis

SH-SY5Y cells were seeded in 35 mm Petri dishes biocoat collagen

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(10^5 cells/well) in DMEM+5% FCS and grown for 24 h. Culture medium was then aspirated, cells were rinsed with PBS and fresh medium+5% FCS or 0.2% FCS was added in the presence of saline or NGF at the indicated concentrations. SK-N-BE cells were seeded in 35 mm Petri dishes (10^5 cells/well) in RPMI 1640 culture medium+15% FCS and grown for 24 h. Culture medium was then aspirated, cells were rinsed and fresh medium+15% FCS or 0.2% FCS was added in the presence of saline or NGF (100 ng/ml). ASMCs were seeded in 35 mm Petri dishes in DMEM+10% FCS (5×10^4 cells) for 3 days in a humidified 5% CO_2 incubator at 37°C. Culture medium of non-confluent ASMCs was then replaced with fresh medium+10% or 0.2% FCS in the presence of saline or NGF at the indicated concentrations. 48 h later, apoptosis was measured with a photometric enzyme immunoassay (cell death detection ELISA, Boehringer Mannheim, Germany) for the quantitative detection of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes). Apoptosis was expressed as the mean number of oligonucleosomes/10^5 cells ± S.D. Results were from three different experiments performed in triplicate.

2.5. Terminal-deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling (TUNEL) assay

SH-SY5Y cells were seeded on biocoat collagen eight well culture slides (5×10^4 cells/well) in DMEM+5% FCS and grown for 24 h. Culture medium was then aspirated, cells were rinsed with PBS and fresh medium+5% FCS or 0.2% FCS was added in the presence of saline or NGF (100 ng/ml). 48 h later, the cells were rinsed two times with PBS, fixed for 30 min at room temperature in a 4% paraformaldehyde solution, washed with PBS and incubated for 30 min at room temperature in 0.3% hydrogen peroxide in methanol. Cells were then rinsed twice with PBS and measurement of DNA fragmentation by the TUNEL assay was carried out using the in situ cell death detection kit POD (Boehringer Mannheim, Germany) according to the manufacturer's instructions.

2.6. Immunofluorescence staining of $P75^{NTR}$

SH-SY5Y neuroblastoma cells were seeded on coverslips pre-coated with collagen and incubated for 48 h in DMEM+5% or 0.2% FCS. The cells were then fixed as described above, washed with PBS and incubated for 30 min at room temperature in 0.3% hydrogen peroxide in methanol. The cells were then incubated for 2 min with PBS containing 0.1% triton X-100 and 0.1% sodium citrate at 4°C, washed three times with PBS and incubated at room temperature for 30 min with an anti-$P75^{NTR}$ antibody (Promega, Charbonnières, France) (dilution 1/200) in PBS. The cells were then washed three times and incubated for 30 min in PBS with a biotinylated secondary antibody (rabbit anti-goat IgG) (Vector Laboratories, Burlingam, USA). Cells were then rinsed, incubated for 30 min in avidin-HRP complex (Vectastain ABC reagent, Vector Laboratories, Burlingam, USA) and developed for 5 min with the DAB substrate kit peroxidase (Vector Laboratories, Burlingam, USA).

2.7. Flow cytometry analysis

Cells were harvested by trypsin (0.05%) EDTA (0.02%), treated with PBS containing 2% BSA and exposed for 20 min at 0°C to an anti-$P75^{NTR}$ antibody (4 μg/ml) (monoclonal antibody 192-IgG, Boehringer Mannheim, Germany). After three washes in PBS, the cells were exposed for 20 min at 0°C to a phycoerythrin-labelled antibody (goat anti-mouse IgG) (dilution 1/50) (Immunotech, Marseille, France) in PBS+2% BSA. The analysis was performed on a FacsStar Plus flow cytometer (Becton Dickinson, Paris, France). Emission fluorescence was measured with a DF575/26 filter. Data acquisition and analysis were performed with CellQuest software.

3. Results and discussion

3.1. Cell cycle phase-specific expression of $P75^{NTR}$

Although the effect of NGF on cell survival has been acknowledged for a long time, the exact role(s) plaid by $P75^{NTR}$ in the functional response to NGF is still highly controversial and contradictory results have been obtained. For example, in neonatal dorsal root ganglion neurons, $P75^{NTR}$ appeared to

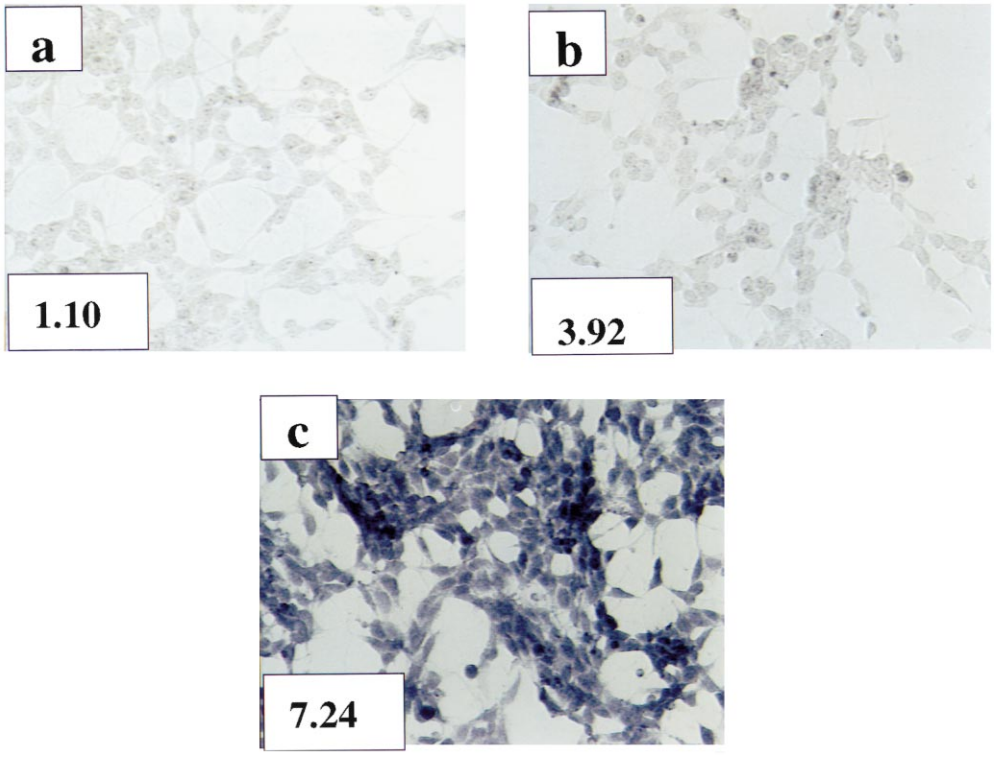
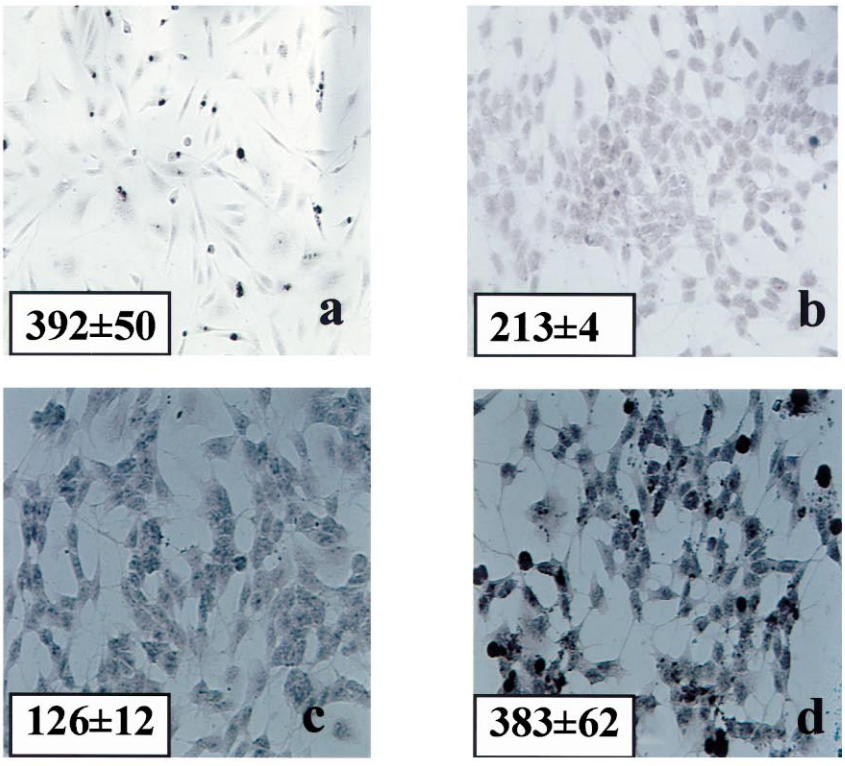


Fig. 1. Immunohistochemical detection of $P75^{NTR}$ on SH-SY5Y cells. SH-SY5Y cells were incubated for 48 h in culture medium containing 0.2% FCS (b) or 5% FCS (c). A blank is shown in (a). Immunohistochemical detection of $P75^{NTR}$ was performed as described under Section 2. Magnification: ×500. On the figures, the quantitative immunodetection of $P75^{NTR}$ is shown, determined at the cell surface by flow cytometry. Units are arbitrary.



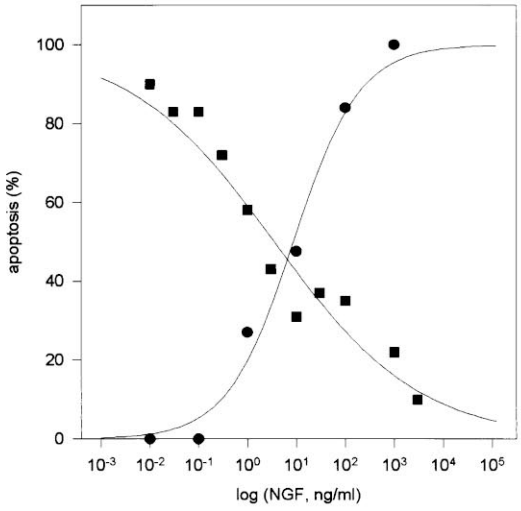


Fig. 2. Effect of NGF on the apoptosis of SH-SY5Y cells. Subconfluent monolayers of SH-SY5Y cells were incubated for 48 h in culture medium containing 5% FCS (a and b) or 0.2% FCS (c and d) in the absence (a and c) or presence (b and d) of NGF (100 ng/ml). Apoptotic cells were detected by TUNEL. Magnification: ×500. On the figures, the quantitative determination of mono/oligonucleosomes is shown, measured with an ELISA kit as described under Section 2 and expressed as mean number of oligonucleosomes/10^5 cells ± S.D. ($n=9$). (e) SH-SY5Y cells were pre-incubated for 48 h in medium containing 5% FCS (●) or 0.2% FCS (■) in the presence of increasing concentrations of NGF. Apoptosis was measured with an ELISA kit. Data are reported as % apoptosis compared to cells in 5% FCS ($n=9$).

mediate a pro-apoptotic signal [28], whereas in other cell systems, NGF behaved as a pro-apoptotic factor [29,30]. In order to determine if these contradictory results could be explained by a differential cell cycle-dependent expression of $P75^{NTR}$ at the cell surface, we have examined the effect of NGF on the apoptotic response of SH-SY5Y, a human neuroblastoma cell line, and evaluated the role plaid by $P75^{NTR}$ in the pro-apoptotic activity of NGF. On subconfluent SH-SY5Y cells, under experimental conditions where the cells were exponentially growing (in 5% FCS), the expression of $P75^{NTR}$ strongly increased at the cell surface (Fig. 1c), whereas when the cells were cultured in 0.2% FCS (quiescent cells), a decreased expression of $P75^{NTR}$ could be observed (Fig. 1b). These results are in agreement with those recently reported by Urdiales et al. [26] on PC-12 cells.

3.2. Dual effect of NGF on the apoptosis of SH-SY5Y cells

Under experimental conditions which allowed the cells to

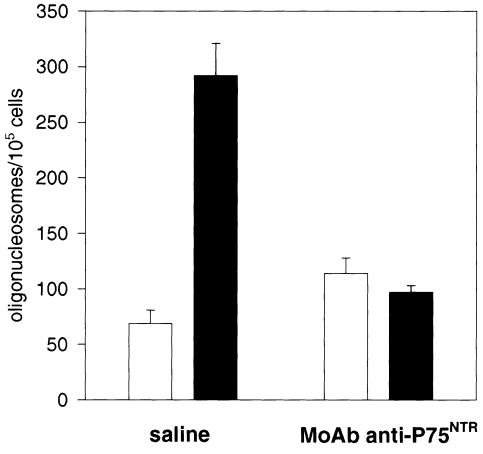


Fig. 3. Effect of an antibody against the extracellular domain of $P75^{NTR}$ on the pro-apoptotic effect of NGF on SH-SY5Y cells. Subconfluent monolayers of SH-SY5Y cells were incubated for 48 h in medium containing 5% FCS with saline or the antibody against the extracellular domain of $P75^{NTR}$, in the presence (full bars) or absence (empty bars) of NGF (100 ng/ml). Apoptosis was measured with an ELISA kit and expressed as mean number of oligonucleosomes/10^5 cells ± S.D. ($n = 9$).

proliferate and to express preferentially $P75^{NTR}$, NGF at the concentration of 100 ng/ml induced apoptosis of SH-SY5Y cells (Fig. 2b). This pro-apoptotic effect of NGF was demonstrated by TUNEL labelling and confirmed by the dosage of mono/oligonucleosomes (Fig. 2b and e). NGF induced a dose-dependent pro-apoptotic effect which was detected from the concentration of 1 ng/ml and reached a maximum at 1 μg/ml (Fig. 2e). The concentration that induced a half-maximum effect (ED_{50}) was 11.4 ng/ml. This pro-apoptotic effect of NGF was time-dependent and was significant after 18 h of incubation (not shown). In contrast, under experimental conditions where the level of $P75^{NTR}$ expression at the cell surface was low (cells cultured in 0.2% FCS for 24 h), NGF at the concentration of 10 ng/ml inhibited the apoptosis of SH-SY5Y cells induced by FCS deprivation (Fig. 2d and e). The dose-dependent anti-apoptotic activity of NGF was detected from the concentration of 0.01 ng/ml and reached a maximum at 10 ng/ml (Fig. 2e). The ED_{50} value was 0.26 ng/ml, showing that NGF was anti-apoptotic at lower doses compared to those exhibiting a pro-apoptotic effect. This difference in efficacy might be due to a higher affinity of NGF for TrkA than for $P75^{NTR}$ as demonstrated several times on other cell types [10–13]. These results therefore show that NGF exhibits dual pro- or anti-apoptotic properties via $P75^{NTR}$ and TrkA, respectively, and show that NGF exerts a pro-apoptotic effect on exponentially growing cells over-expressing $P75^{NTR}$, whereas it exerts a potent anti-apoptotic activity on quiescent cells when $P75^{NTR}$ expression is decreased but when TrkA expression is prevalent at the cell surface. These results support our hypothesis that the survival response to NGF depends on the growth state of the cells and further extend the observations made by Urdiales et al. [26].

To study in further detail how NGF exerted a pro-apoptotic effect, we determined the effect of an antibody against the extracellular domain of $P75^{NTR}$ [25]. Under culture conditions where $P75^{NTR}$ was preferentially expressed at the cell surface (growing cells), this antibody abrogated the pro-apoptotic effect of NGF (Fig. 3). In contrast, under experimental conditions which allowed the cells to express preferentially TrkA (0.2% FCS), it did not block the anti-apoptotic effect of NGF (not shown). This observation therefore shows that NGF exerts its anti-apoptotic effect in a $P75^{NTR}$-independent manner and provides compelling evidence that apoptosis is a consequence of the NGF/$P75^{NTR}$ interaction and that the anti-apoptotic activity of NGF is selectively mediated by a NGF/TrkA signalling pathway with no contribution of $P75^{NTR}$.

The ongoing dissection of the roles of $P75^{NTR}$ and TrkA in NGF signalling has generated a number of apparent paradoxes. Recent studies have suggested that TrkA alters the conformation of $P75^{NTR}$ in the absence of ligand [31] and that TrkA and $P75^{NTR}$ co-localise on the cell surface [32]. The synergy of $P75^{NTR}$ and TrkA in inducing optimal trophic signalling has been demonstrated [33]. On the other hand, it has been shown that $P75^{NTR}$-mediated apoptosis of developing forebrain neurons was restricted to cells expressing $P75^{NTR}$ but not TrkA [22]. In addition, TrkA expression has been shown to block ligand-mediated ceramide production induced by $P75^{NTR}$ [21]. These observations, consistent with a model of mutual receptor modulation [15], led us to examine the effect of NGF on cells lacking one of the two receptors.

In order to confirm that $P75^{NTR}$ is only involved in the pro-apoptotic NGF signalling, we have prepared primary cultures of ASMCs from $P75^{NTR}$ KO mice and from the corresponding wild-types which have been shown to express both $P75^{NTR}$ and TrkA at their surfaces [34]. Like in SH-SY5Y and PC-12 cells [26], this expression varied in a cell cycle phase-specific manner (not shown). Under experimental conditions which allowed the cells to preferentially express $P75^{NTR}$ (cultured in 10% FCS), NGF (100 ng/ml) exhibited a pro-apoptotic effect on ASMCs from wild-type mice but did not show any effect on ASMCs isolated from $P75^{NTR}$ KO mice (Table 1).

Table 1
Effect of NGF on the apoptosis of cells which do not express $P75^{NTR}$

		Mouse ASMCs		SK-N-BE	
		Wild-type	$P75^{-/-}$	Wild-type	TrkA-transfected
Quiescence (0.2% FCS)	Control	ND	ND	683 ± 30	690 ± 25
	+NGF	ND	ND	700 ± 45	162 ± 39
Growth (10% FCS)	Control	25 ± 2	39 ± 3	ND	ND
	+NGF	190 ± 20	50 ± 5	ND	ND

Subconfluent monolayers of ASMCs from $P75^{NTR}$ KO mice and from the corresponding wild-types or human neuroblastoma cells SK-N-BE and SK-N-BE cells transfected with TrkA were pre-incubated for 48 h in culture medium containing 0.2 or 10% FCS in the absence or presence of NGF (100 ng/ml). Apoptosis was measured with an ELISA kit and expressed as mean number of oligonucleosomes/10^5 cells ± S.D. ($n = 9$). ND = not determined.

These results further confirm that NGF exerts its pro-apoptotic effect in a $P75^{NTR}$ manner exclusively. On the other hand, the neuroblastoma cell line SK-N-BE was chosen to study the TrkA-mediated survival effect of NGF. On this cell line which does not express $P75^{NTR}$ nor TrkA [35], NGF did not show any pro- or anti-apoptotic activity (Table 1), but when this cell line was transfected with TrkA [35], NGF at the concentration of 10 ng/ml presented a potent anti-apoptotic activity suggesting that the survival response to NGF depends on its interaction with TrkA without any involvement of $P75^{NTR}$.

These results support the hypothesis that the survival response to NGF depends on its binding to TrkA without any involvement of $P75^{NTR}$, which in turn selectively mediates the pro-apoptotic activity of NGF with no contribution of TrkA and show that, depending on the growth state of the cells, NGF exhibits dual pro- or anti-apoptotic properties via $P75^{NTR}$ and TrkA, respectively.

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