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Neurotrophin dependence mediated by p75^{NTR}: contrast between rescue by BDNF and NGF

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Abstract

During development, neurons pass through a critical phase in which survival is dependent on neurotrophin support. In order to dissect the potential role of p75^{NTR}, the common neurotrophin receptor, in neurotrophin dependence, we expressed wild-type and mutant p75^{NTR} in cells that do not express endogenous p75^{NTR} or Trk family members (NIH3T3). Expression of wild-type p75^{NTR} created a state of neurotrophin dependence: cells could be rescued by nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), or neurotrophin-3 (NT-3), but not by a mutant NGF that binds well to Trk A but poorly to p75^{NTR}. Similarly, expression of p75^{NTR} in human prostate cancer cells in culture rendered a metastatic tumor cell line (PC-3) sensitive to the availability of neurotrophins for survival. Moreover, expression of mutant p75^{NTR}'s in another neurotrophin-insensitive cell line (HEK293T) showed that a domain within the intracellular domain governs alternate responses to neurotrophins: the carboxy terminus of the intracellular domain of p75^{NTR} including the sixth alpha helix domain is necessary for rescue by BDNF, but not NGF. These results, when considered with previous studies of the timing of p75^{NTR} expression, support the hypothesis that p75^{NTR} is a mediator of neurotrophin dependence during the critical phase of developmental cell death and during the progression of carcinogenesis in prostate cancer.

Keywords: p75^{NTR}; apoptosis; neurotrophin; dependence; prostate cancer

Abbreviations: p75^{NTR}, the common neurotrophin receptor; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3

Introduction

Developing neurons, and at least some non-neuronal cells,[1] pass through a critical phase during development.[2,3] During this phase, trophic factor support, which may be derived from targets of innervation, is required for survival. However, the withdrawal of trophic factor support prior to, or following, the critical period, has substantially less effect on cell survival.

Although this critical period was described nearly 50 years ago,[2] the molecular basis for this phenomenon is unknown. We found previously that the overexpression of p75^{NTR}, the common neurotrophin receptor, enhanced apoptosis in neural cell lines following serum withdrawal.[4] Complementary results were obtained by Barrett and Bartlett,[5] who found that the down-regulation of p75^{NTR} expression by antisense oligonucleotides at E19-P4 prevents apoptosis in response to nerve growth factor withdrawal.

It was therefore proposed that the expression of p75^{NTR} in developing cells may force the cells into a choice: in the absence of trophic support, apoptosis is induced, whereas in the presence of trophic support, survival is enhanced.[4,6,7] This profile supports p75^{NTR} as a candidate for mediation of the phenomenon of neurotrophin dependence. This idea is compatible with the temporal pattern of p75^{NTR} expression: for example, developing chick motor neurons undergo their naturally occurring cell death from E5 to E12, concurrently with their expression of p75^{NTR}.[8] The idea is also compatible with the known inducers of p75^{NTR} expression, such as axotomy and β-amyloid peptide, which are both pro-apoptotic.[9,10]

Evaluation of cells coexpressing p75^{NTR} and Trk A, B, or C, is complicated by putative effects of each on the other: for example, Taglialatela *et al*[11] showed that BDNF could act through p75^{NTR} to rescue PC12 cells, but this effect was blocked by Trk A expression. Therefore, in order to evaluate the effect of p75^{NTR} in the absence of the expression of Trk A, B, or C, we utilized the extensively studied, Trk-negative cell line, NIH3T3.[12–14] These cells survive fairly well in serum-free medium, but the expression of p75^{NTR} induced a state of neurotrophin dependence. Although this finding is inconclusive when considered alone, it nonetheless provides further support for the hypothesis that neurotrophin dependence is mediated at least in part by p75^{NTR}. Moreover, it is compatible with recent findings that p75^{NTR}-null mice demonstrate a reduction in developmental neuronal death in the basal forebrain cholinergic neurons,[15,16] and that the neuronal loss characteristic of hemizygous NGF mice is rescued by crossing with the p75^{NTR}-null mice.[17] The latter experiment exemplifies the role of p75^{NTR} in mediating neurotrophin dependence: in the absence of NGF, basal forebrain cholinergic neurons which were once dependent on NGF for survival, remain viable since they no longer express the mediator of neurotrophin dependence, p75^{NTR}.

Neurotrophin dependence is not limited to neurons. Prostate epithelial cells, for example, express $p75^{NTR}$ and depend on paracrine interaction with and secretion of NGF or an NGF-like protein by prostate stromal cells for development and growth.[18] Interestingly, all four metastatic prostate carcinoma cell lines evaluated for $p75^{NTR}$ expression were shown to lack $p75^{NTR}$ expression.[19] Here we show that returning $p75^{NTR}$ to one of these prostate tumor cell lines, PC-3, renders these cells dependent on neurotrophins for survival.

The mechanisms by which a survival signal is transduced by NGF through $p75^{NTR}$ may include the activation and nuclear translocation of the transcription factor NF-κB.[20,21] This pathway is elicited in response to NGF, but not BDNF, though both bind with similar affinity to $p75^{NTR}$.[13,22] This differential response suggests that there may exist separate domains within $p75^{NTR}$ that are responsible for signalling survival in response to activation by each of the three neurotrophins. Here we have deleted regions within the cytoplasmic domain of $p75^{NTR}$ to determine the domain necessary for BDNF to signal survival, and show that it is not required for NGF-mediated apoptosis inhibition.

Results

NIH3T3 cells do not express Trk A, B, or C.[12–14] These fibroblastic cells are relatively resistant to apoptosis induction by serum withdrawal (Figure 1). This property was unaffected by transfection with the control vector, pBabepuro. In contrast, expression of $p75^{NTR}$ altered the response of the cells to serum-free medium markedly: approximately 80% of the cells underwent apoptosis within 3 days (Figure 1).

Apoptosis was prevented by binding of neurotrophins to $p75^{NTR}$, returning the survival to the rate seen in the absence of $p75^{NTR}$ expression (Figure 1). Unlike with neural cells, enhancement of survival beyond that recorded with vector alone was not observed.[4]

Addition of NGF (200 ng/ml), BDNF (200 ng/ml), or NT-3 (200 ng/ml), returned survival to the level observed with the vector control. The effect of each of the three neurotrophins on the cells expressing $p75^{NTR}$ was statistically significant ($P<0.006$; two-tailed *t*-test, $n=4$), but no significant effect ($P<0.5$) of NGF on the control transfectants was observed (Figure 1).

A triple mutant of NGF[23] was used, in order to ensure that the effects observed were secondary to the binding of NGF, BDNF, or NT-3 to $p75^{NTR}$. The triple mutant (K32A+K34A+E35A) was derived by Ibañez *et al*[23] and shown to bind to Trk A with an affinity that is unaltered from that of the wild-type NGF; in contrast, the affinity for $p75^{NTR}$ was shown to be approximately 100-fold lower. Unlike for NGF, BDNF, and NT-3, addition of the triple mutant NGF (200 ng/ml) did not have a statistically significant effect ($P<0.1$) on cell survival, although a trend toward improved survival did occur (Figure 1).

PC-3 prostate carcinoma cells do not express $p75^{NTR}$.[19] However, when $p75^{NTR}$ expression is returned, these cells become dependent upon the availability of neurotrophins for survival (Figure 2). Upon transfection with the expression vector pBabepuro-$p75^{NTR}$, there was a marked reduction in the number of colonies that were able to survive upon selection with puromycin relative to cells transfected with the control vector, pBabepuro. However, the number of colonies increased sharply with the addition of 50 ng/ml of exogenous NGF or BDNF to cells that were selected for the expression of $p75^{NTR}$ (Figure 2). The neurotrophins had minimal effects on cells expressing the control vector (Figure 2). Similar phenomena were also

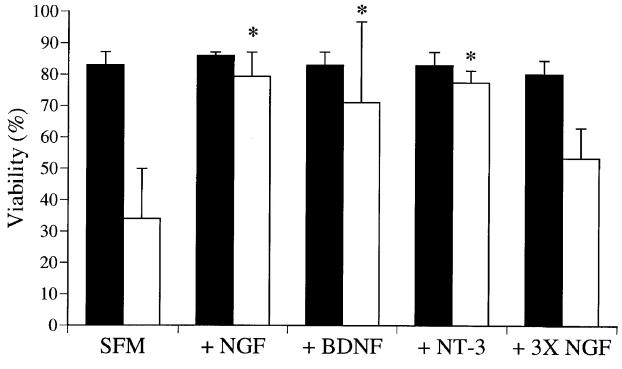

Figure 1 $p75^{NTR}$-induced apoptosis in NIH3T3 cells is inhibited by NGF, BDNF, and NT-3, and not by the Trk A-favored triple mutant NGF (3X NGF). NIH3T3 cells expressing pBabepuro-$p75^{NTR}$ (black columns) or pBabepuro control vector (white columns) were placed in serum-free media (SFM) or serum-free media supplemented with 200 ng/ml NGF, BDNF, NT-3, or 3X NGF. NGF, BDNF, and NT-3 significantly inhibited apoptosis in cells expressing $p75^{NTR}$ (*$P<0.006$; two-tailed *t*-test). 3X-NGF, however, did not significantly inhibit $p75^{NTR}$-induced apoptosis ($P<0.1$; two-tailed *t*-test). These data represent one of four separate transfections, all of which exhibited similar results. The error bars represent standard deviations

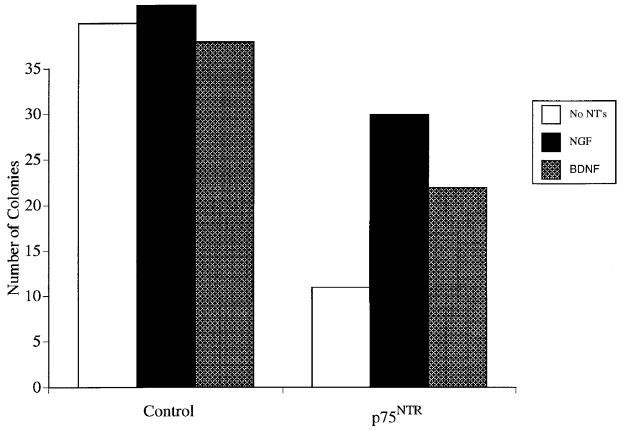

Figure 2 PC-3 metastatic prostate carcinoma transfected with $p75^{NTR}$ exhibit reduced growth in the absence of neurotrophins. Upon transfection and at the start of puromycin selection, PC-3 cells transfected with pBabepuro-$p75^{NTR}$ or pBabepuro control vector (Control) were grown in the absence (white columns) or presence of 200 ng/ml NGF (black columns) or 200 ng/ml BDNF (stippled gray columns). After 18 days of selection with puromycin, throughout which the neurotrophins were replaced every 3 days, total number of viable colonies of PC-3 cells were tabulated by microscopy. These data represent one of three separate transfections. Similar results were observed in all three experiments

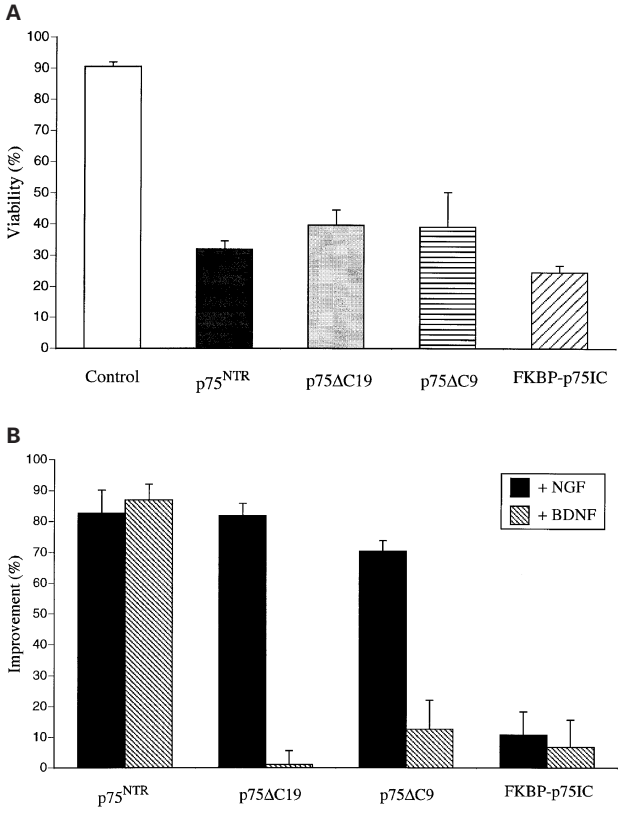

Figure 3 BDNF rescue of HEK293T cells expressing $p75^{NTR}$ requires the carboxy 19 amino acid tail of the receptor. (**A**) HEK293T cells transiently transfected with pcDNA3-$p75^{NTR}$, pcDNA3-p75ΔC19, pcDNA3-p75ΔC9, or FKBP-p75IC underwent significantly ($P<0.000001$; two-tailed *t*-test) more apoptosis than HEK293T transfected with pcDNA3 control vector (Control). (**B**) Mutant $p75^{NTR}$-transfected HEK293T cells show a different per cent improvement in response to NGF or BDNF over their respective untreated, transfected cells in (**A**). NGF (100 ng/ml, black columns) rescued HEK293T cells expressing $p75^{NTR}$, p75ΔC19, or p75ΔC9, but not those expressing FKBP-p75IC, which is lacking the extracellular domain of the receptor. BDNF (100 ng/ml, striped columns), however, rescued HEK293T cells expressing $p75^{NTR}$, but not those expressing p75ΔC19 or p75ΔC9. The domain deleted in these mutants is thus required for the anti-apoptotic effect of BDNF, but not NGF. As with NGF, BDNF failed to rescue FKBP-p75IC. These data represent eight separate transfections. Error bars represent standard error

observed in transfected R2 cerebellar neural cells[4] and PC12 NRA5 cells[4] (data not shown).

Not surprisingly, the expression of a mutant $p75^{NTR}$ that lacks the extracellular and transmembrane domains, FKBP-p75IC (Rabizadeh *et al*, submitted), induces apoptosis in HEK293T cells that is not inhibited by either of these neurotrophins (Figure 3). This result demonstrates that in the absence of $p75^{NTR}$, HEK293T cells are not responsive to NGF or BDNF. Deleting the carboxy terminal 19 amino acid residues, which includes the sixth alpha helix,[24] from $p75^{NTR}$ produces a receptor that is as potent an inducer of apoptosis as the wild-type receptor (Figure 3; Rabizadeh *et al*, submitted). However, expression of this truncated receptor, p75ΔC19, in HEK293T cells induces apoptosis that is inhibited only by NGF and not by BDNF (Figures 3 and 4). As with the PC-3 and NIH3T3 cells, expression of the wild-type $p75^{NTR}$ induced

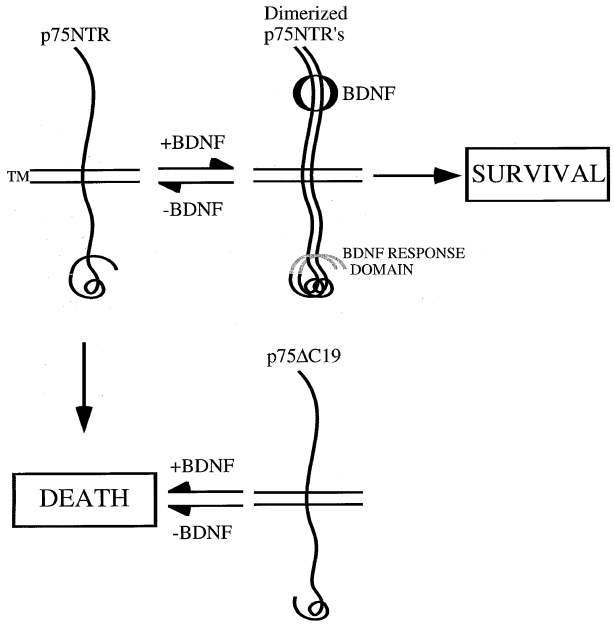

Figure 4 Summary diagram of BDNF rescue of $p75^{NTR}$ through the BDNF response domain. Hypothesis: BDNF effects the dimerization of $p75^{NTR}$ by a mechanism that requires the carboxy terminal 19 amino acids of the receptor. Because of the absence of the BDNF response domain, addition of BDNF does not rescue p75ΔC19-induced death. TM: transmembrane domain

HEK293T cells to undergo apoptosis that was inhibited by NGF and BDNF (Figure 3).

Though BDNF was unable to rescue p75ΔC19-induced apoptosis, it had a slight effect on cells expressing another truncated $p75^{NTR}$ receptor, p75ΔC9 (Figure 3B). This latter receptor expresses the $p75^{NTR}$ protein truncated at the carboxy terminus of the sixth alpha helical domain.[24] Like the wild-type receptor and p75ΔC19, p75ΔC9 induced apoptosis which could be inhibited by 100 ng/ml of NGF (Figure 3B).

Discussion

These results demonstrate that in NIH3T3, a cell line that does not express Trk, the expression of $p75^{NTR}$ leads to a state of neurotrophin dependence, which is absent in the control transfectants. This state is revealed in the absence of serum, since serum was used to allow both control and $p75^{NTR}$-expressing cells to proliferate; nonetheless, even in the presence of serum, $p75^{NTR}$ expression leads to a higher rate of apoptosis.[6] Thus, $p75^{NTR}$ expression is lost rapidly with passage of the cells.

It is noteworthy that $p75^{NTR}$ has been shown to modulate apoptosis both in the absence of ligand binding[4,5] and in the presence of ligand binding.[25,26] These results may not be mutually exclusive, since these results appear to depend on cell type, state of differentiation, and possibly on Trk coexpression.[7]

The neurotrophin theory states that neurons compete for a limiting amount of growth factor,[27] but the mechanism by which a cell would transduce a signal for cell death based

on lack of trophic factors is unknown. It has been suggested that a default cell death program exists, such that cells that are not stimulated by growth factors to prevent apoptosis will by default undergo apoptosis.[27] However, the molecular basis of the posited default program has not been defined. The demonstration that the antisense inhibition of $p75^{NTR}$ blocks dorsal root ganglion neuron apoptosis induced by NGF withdrawal during the critical phase[5] argues that $p75^{NTR}$ mediates a part (perhaps the initial part) of the postulated default program of cell death. Moreover, the notion that the default death pathway may originate from a receptor that is intimately involved with the trophic response (along with Trk), which interrupts the death pathway, is appealing.

The current results do not suggest a mechanism by which $p75^{NTR}$ initiates the apoptotic program. However, they demonstrate that the ability of $p75^{NTR}$ to induce apoptosis does not depend on an inhibition of Trk phosphorylation, since apoptosis may be induced in the absence of Trk expression. We have previously shown that $p75^{NTR}$ induces apoptosis in the presence of Trk expression as well.[4]

Metastatic prostate carcinomas have been shown to express Trk A and lack $p75^{NTR}$, though normal prostate epithelia express both receptors.[28] In fact, Pflug *et al*[19] have shown an inverse relationship between the expression of $p75^{NTR}$ and the progression of prostate carcinoma. Though normally expressed in epithelial cells, $p75^{NTR}$ expression is reduced in benign and malignant prostate tissue, and completely absent in cell lines derived from metastatic prostate tumor cells. In the latter group, the possibility exists that these cells have been able to survive outside of the prostate microenvironment (i.e. away from the NGF-secreting prostate stromal cells) because of their lack of dependence on a neurotrophin-mediated survival signal. Moreover, our results indicate that returning $p75^{NTR}$ expression to these cells renders them dependent on neurotrophins for survival again. Whereas prostate cells fail to show any Trk phosphorylation with BDNF,[28] both BDNF and NGF promote the survival of PC-3 cells expressing $p75^{NTR}$.

Although NGF, BDNF, and NT-3 inhibit $p75^{NTR}$-induced cell death with similar efficiency, it is unlikely that all three signal through the receptor in the same manner. For example, only NGF can activate NF-κB through $p75^{NTR}$.[20] On the other hand, the carboxy 19 amino acid tail of the receptor is necessary for BDNF, but unnecessary for NGF, to signal survival. This domain has therefore been dubbed the BDNF response domain. A deleted $p75^{NTR}$ mutant lacking the carboxy terminal 9 or 19 amino acid residues induces apoptosis at least as well as the wild-type receptor. However, apoptosis induced by these mutations is rescued only by NGF and not by BDNF. Further deletions beyond the amino terminus of the BDNF response domain inhibits the receptor from inducing apoptosis in the absence or presence of neurotrophins (Rabizadeh *et al*, submitted). The experiments determining the BDNF response domain correspond well with data from studies demonstrating that dimerization of these mutant receptors does not inhibit their ability to induce apoptosis, whereas it does inhibit the wild-type receptor (Wang *et al*, submitted). Combining these results, it is hypothesized that BDNF inhibits $p75^{NTR}$-induced apoptosis by a mechanism requiring the carboxy terminal 19 amino acids of $p75^{NTR}$, and the dimerization of the receptor (Figure 4). Our results, however, do not preclude NGF from activating a survival signal through the carboxy terminus of $p75^{NTR}$ as well. It is apparent, however, that there exists another domain(s) through which NGF prevents $p75^{NTR}$-induced apoptosis.

In the carboxy tail of $p75^{NTR}$, the only known interacting protein is the phosphatase FAP-1, which inhibits $p75^{NTR}$-induced apoptosis (Yano *et al*, submitted). A single point mutation at the carboxy terminal residue of $p75^{NTR}$ (valine at residue 396) renders the receptor inaccessible to FAP-1 (Yano *et al*, submitted). Yet this mutant $p75^{NTR}$ is still rescued by BDNF (Wang *et al*, submitted), thereby suggesting that BDNF does not inhibit $p75^{NTR}$-induced apoptosis by activating FAP-1. Hence, the signal pathway mechanism of rescue by BNDF through the carboxy terminus of $p75^{NTR}$ remains to be deciphered.

The application of the concept of neurotrophin dependence states mediated by receptors such as $p75^{NTR}$, the androgen receptor,[29] and DCC[30] to the studies of developmental neurobiology, neurodegenerative diseases, and cancer may lead to new therapeutics for their associated diseases.

Materials and Methods

Plasmid construction

The expression constructs pBabepuro, pBabepuro-$p75^{NTR}$, pcDNA3, pcDNA3-$p75^{NTR}$, pcDNA3-p75ΔC19, and FKBP-p75IC were derived as described earlier,[4] (Rabizadeh *et al*, submitted). The construct pcDNA3-p75ΔC9 was was derived by deleting the final nine amino acid codons from the wild-type pcDNA3-$p75^{NTR}$ via the PCR-based site-directed mutagenesis techniques described earlier.[30] The sequences of the oligonucleotide primers used in this process were as follows: 5′ GAGAGTCTATGCTGAGTCGAGCATGCATCT was the forward primer and 5′ AGATGCATGCTCGACTCAGCATAGACTCTC was the reverse primer.

Cell culture and transfection

NIH3T3 fibroblast and HEK293T cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. PC-3 prostate carcinoma were grown and maintained in DMEM/F12 supplemented with 5% FBS at 37°C and 5% CO_2. For transfection, PC-3 and NIH3T3 cells were seeded at 70% density in 10 cm plates and transfected with the vectors pBabepuro-$p75^{NTR}$ and pBabepuro (Control) using the lipid-transfection agent DOTAP (Boehringer-Mannheim Biochemicals) as described earlier.[4] Upon selection with 5 μg/ml puromycin, the expression of $p75^{NTR}$ in NIH3T3 cells was assessed via flow cytometry as before.[31]

PC-3 cells were either untreated or treated with 50 ng/ml NGF or 50 ng/ml BDNF upon selection with 5 μg/ml puromycin. The media, neurotrophins, and puromycin were replaced every 3 days. After 18 days of selection with puromycin, transfected PC-3 cells were washed with PBS, stained with the protein dye Coomassie Blue,[32] and the total number of viable colonies of PC-3 cells was tabulated by microscopy.

HEK293T cells were transiently transfected with the vectors pcDNA3-p75^{NTR}, pcDNA3-p75ΔC19, pcDNA3-p75ΔC9, FKBP-p751C, or pcDNA3 (control) via the calcium/phosphate method described previously.[29] 100 ng/ml of either NGF or BDNF were added to the cells at 6 h after transfection. Expression of p75^{NTR}, p75ΔC19, p75ΔC9, and FKBP-p75IC was assessed by flow cytometry or Western blot analysis as before.[30,31]

Induction of apoptosis and assessment of viability

Upon transfection and selection, transfected NIH3T3 cells were plated onto 96-well plates at a density of approximately 7500 cells per well. Serum was then withdrawn from these cells and 50 ng/ml of either NGF or BDNF was added to the media. Apoptosis was then quantitated every day be detection of cellular uptake of propidium iodide by a Cytofluor as described previously.[31]

Upon transfecting and incubating HEK293T cells in the calcium/phosphate/DNA/neurotrophin mixture for 20–24 h, 40–50 μM of the apoptosis inducing agent tamoxifen[33] was added to enhance the apostat of transfected cells.[34] Dying floating cells were collected and centrifuged for 10 min at 1500 r.p.m. The pellets were then resuspended in 150 μl of DMEM. An equal volume of Trypan Blue was added to the cells in DMEM and the dead cells were quantitated in a hemacytometer as previously described.[4]

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