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NGF and ProNGF: Regulation of Neuronal and Neoplastic Responses through Receptor Signaling

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Abstract

Nerve growth factor (NGF) and its precursor (proNGF) are primarily considered as regulators of neuronal function that induce their responses via the tyrosine kinase receptor TrkA and the pan-neurotrophin receptor p75NTR. It has been generally held that NGF exerts its effects primarily through TrkA, inducing a cascade of tyrosine kinase-initiated responses, while proNGF binds more strongly to p75NTR. When this latter entity interacts with a third receptor, sortilin, apoptotic responses are induced in contrast to the survival/differentiation associated with the other two. Recent studies have outlined portions of the downstream phosphoproteome of TrkA in the neuronal PC12 cells and have clarified the contribution of individual docking sites in the TrkA endodomain. The patterns observed showed a similarity with the profile induced by the epidermal growth factor receptor, which is extensively associated with oncogenesis. Indeed, as with other neurotrophic factors, the distribution of TrkA and p75NTR is not limited to neuronal tissue, thus providing an array of targets outside the nervous systems. One such source is breast cancer cells, in which NGF and proNGF stimulate breast cancer cell survival/growth and enhance cell invasion, respectively. This latter activity is exerted via TrkA (as opposed to p75NTR) in conjunction with sortilin. Another tissue overexpressing proNGF is prostate cancer and here the ability of cancer cells to induce neuritogenesis has been implicated in cancer progression. These studies show that the non-neuronal functions of proNGF/NGF are likely integrated with their neuronal activities and point to the clinical utility of these growth factors and their receptors as biomarkers and therapeutic targets for metastasis and cancer pain.

Keywords

NGF; Growth Factor; Receptor Tyrosine Kinase; Signaling; Phosphorylation

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Introduction

Protein phosphorylation as a means to regulate and perpetrate cellular signaling mechanisms has been one of the dominant themes of biological research for several decades (Biarc *et al*., 2010). In humans alone, there are over 600 enzymes devoted to adding or removing this modification (approximately divided 5 to 1 between kinases and phosphatases) (Blume-Jensen and Hunter, 2001). These alterations can directly affect biological activity, as exemplified by the regulation of glycogen phosphorylase (which was the pioneering discovery that launched this field) (Krebs and Fischer, 1964), or, more often, exert their effects by altering protein-protein interactions. Moreover there has evolved an elaborate system, as manifested in specific recognition domains (Pawson, 2002), for recognizing key phosphorylation sites that lead to the formation of molecular complexes and that are required for the flux of information in dynamic signaling pathways. These domains, such as SH2 and PTB, are usually found in proteins that also have other domains that recognize different structural elements or contain effectors that generate new modifications or associations. The extent to which protein phosphorylations occur, even in resting (unstimulated) cells, in terms of both range and variety of sites, is sufficiently vast (thousands of loci) that it is unlikely that they are all of equal physiological significance (Gnad *et al*., 2011). Indeed many may be spurious, resulting from the substantial number of protein kinases that are active in any given cell at any given moment and the lack of tight substrate specificity for many of them. For the most part, these probably form a 'background' that may be of some general advantage to cells, since the accumulated negative charge from these sites may tend to keep cytoplasmic proteins away from membrane structures, which must be able to recruit, i.e. be available for binding of, certain signaling entities following stimulation in order to transmit their signals. Ascertaining which phosphorylations are essential and which are not (and which are introduced by which kinase) remains a singularly important challenge.

Protein phosphorylations in mammalian species occur primarily on serine, threonine and tyrosine residues and in that relative order of abundance (serine phosphorylations being the most prevalent). Protein kinases with specificity for tyrosine make up about 20% of the family and are found both as cytoplasmic and integral membrane bound entities. This latter group constitute the so-called receptor tyrosine kinases (RTKs), which are subdivided into 20 families; some of these contain only a single member, such as MUSK or RET while the ephrins have more than a dozen members. Likewise the ligand families that activate these entities also can be singular in nature or spread among multiple homologous members.

Although the overall organization of the RTKs is generally the same, with each containing an extracellular (or exo-) domain, a transmembrane domain and an intracellular (or endo-) domain, there are notable distinguishing differences in the intra-domain organization of the exo- and endo-moieties. The exodomains, whose function is basically to provide the recognition and subsequent binding of the activating ligands, are composed of many different folding motifs (sometimes in tandem arrays) and these show considerable variability, although domains of the same basic motif are found in different families. On the other hand, the endodomains, which all contain the eponymous tyrosine kinase, actually share little similarity in the non-kinase regions that are found between the transmembrane

and kinase domains (juxtamembrane domain) and the kinase domain and the C-terminus. These segments vary considerably in length and in function. Moreover, the distribution of tyrosine residues, a subset of which are phosphorylated in each case and generally provide docking sites for adaptor/scaffold/effector moieties, are also significantly different. This provides, in turn, a number of distinct means for propagating the signal from that receptor (Bradshaw *et al*., 2013). In the light of this diversity, it is somewhat surprising that there is considerable uniformity in the downstream pathways that are activated by different RTK families. In the main, RTKs stimulate three main pathways: the activation of ERKs via Ras, GTP binding proteins and several other kinases; the activation of phospholipase Cγ with the resulting production of diacylglycerol and inositol triphosphate from the cleavage of phosphoinositides; and the activation of the several Akt pathways via the agency of phosphoinositide-3-kinase (PI3K) (Schlessinger, 2000; Choudhary and Mann, 2010). These events are accompanied by a broad stimulation of protein kinases, producing extensive modifications (primarily on serine and threonine residues) as well as other reversible modifications, such as N^{ε}-acetylation, ubiquitination and O-glycosylation with GlcNAc(Zeidan and Hart, 2010). The extent of these alterations and the full description of their impact on cellular activities and responses in any system remain to be elucidated.

Neurotrophins and their receptors

Neurotrophic factors are a broad group of growth factors and cytokines whose principal targets are neurons of the peripheral and central nervous systems. They can stimulate neurite growth, maintain viability and induce differentiation, among other activities. The first such substance to be defined, and the prototype of the class, was NGF (Levi-Montalcini, 1987). It was originally observed in two mouse tumor cell lines by its ability to induce fiber outgrowth of sympathetic and sensory neurons, but its discovery in the male mouse submandibular gland opened the way for the detailed molecular characterization (Shooter, 2001), including sequence analysis (Angeletti and Bradshaw, 1971), of the mature protein. Cloning experiments established that it was, not surprisingly, elaborated as a prepro protein, with a signal sequence of 19 residues and a pro segment of 120 residues (Scott *et al*., 1983; Ullrich *et al*., 1983). Several years later, a homolog of NGF that was primarily found in the brain, was isolated, characterized and designated brain-derived neurotrophic factor (BDNF) (Barde *et al*., 1982). Molecular cloning experiments defined two more members of this family, neurotrophins 3 and 4 (NT3 and −4) (Maisonpierre *et al*., 1991; Ip *et al*., 1992). Although early observations suggested that NGF behaved like a hormone (Frazier *et al*., 1972) and iodinated tracer-binding experiments supported the presence of a cell surface receptor, its identification and characterization proceeded relatively slowly. This was due in part to a variety of measurements that yielded conflicting results with regard to both binding properties and molecular mass (Raffioni *et al*., 1993). On the one hand, there was compelling evidence that biological activity (generally defined as neurite outgrowth from PC12 cells) was associated with a molecule of about ~130 kDa (Kouchalakos and Bradshaw, 1986); on the other, there was strong evidence for the existence of a receptor protein of half that mass and this was basically confirmed by cloning experiments (Chao *et al*., 1986; Radeke *et al*., 1987). This latter entity eventually became known as p75NTR or the pan- neurotrophin receptor because it bound all four members of the neurotrophin family

with about the same affinity. However, it did not contain a kinase or other known effectors as part of its endodomain. The enigma was finally resolved in 1991 when TrkA, an RTK (of the molecular mass previously predicted) was identified and cloned (Kaplan *et al*., 1991; Klein *et al*., 1991). Two additional members of this RTK family, TrkB and C with specificities for the other neurotrophins were eventually identified. The final participant in this group is sortilin (also known as NTSR3 for neurotensin receptor 3 or GP110 for glycoprotein 110). It has multiple functions and binds several different types of ligands including proNGF and proBDNF. This interaction is involved in apoptosis and appears to function in concert with p75NTR (Hempstead, 2014). In this regard, it has been shown that the pan-neurotrophin receptor also binds more avidly to the proneurotrophins than it does to the corresponding mature forms. A portrait of this group of ligands and receptors is shown in Fig 1.

The neurotrophins are expressed in a broad array of tissues, consistent with the view that they mainly function as target-derived survival factors (Kaplan and Miller, 2000; Reichardt, 2006; Hempstead, 2014). NGF was initially envisioned as a peripheral nervous system agent but it is clear that it has some central nervous system functions as well. In contrast, BDNF is primarily important in the brain, and as such has received considerable attention as a target for common CNS maladies, such as Parkinson's disease. As a result, its receptors are commonly found on responsive neurons, although they are also found on other non-neuronal tissues too. The potential importance of NGF (and proNGF) in the responses of both normal and neoplastic non-neuronal tissues will be elaborated on in subsequent sections below.

TrkA induced signaling

As with other RTKs, the activation of TrkA by ligand binding results in the formation of a number of phosphorylated tyrosines on its endodomain, most notably the three found in the activation loop and those at position 490 in the juxtamembrane domain and 785 in the C-terminal domain. It is generally held that these result from autocatalysis but the involvement of another tyrosine kinase (activated by the receptor kinase) has not been ruled out. Although these modifications also lead to other tyrosine phosphorylations, a much more striking outcome is the plethora of downstream phosphorylations that occur on a host of intracellular proteins. Of course there is a high level of 'baseline' (unstimulated) modifications and those that might relate to growth factor stimulated responses should be reflected in significant change, i.e. be either up- or down-regulated, from the control. In situations of acute stimulation (stimulus added as a signal bolus addition) the total amount of phosphorylation peaks about 20 minutes after addition of the ligand. In order to better define the TrkA phosphoproteome at this time point, PC12 cells, a well-studied paradigm with many neuronal characteristics (including their response to NGF) were engineered to express hybrid TrkA receptors that were designed to specifically avoid endogenous signaling and to allow dissection of the participating tyrosines. Basically, the extracellular domain of the human Platelet-Derived Growth Factor (PDGF) receptor was fused to the transmembrane and intracellular domain of rat TrkA (termed PTR) and stably transfected into PC12 cells. Derivatives in which Y490 and Y490/Y785 were mutated to phenylalanine were also constructed and expressed. The chimeric receptors were appropriately responsive to PDGF (but not untransfected cells which have no PDGF receptors) in all cases. The

phosphoproteome changes induced, compared to unstimulated cells, were quantified by growing each transfected cell line in media with isotopically labeled amino acids (SILAC) and measuring the released tryptic peptides by MS/MS following TiO_2 enrichment (Biarc *et al*., 2012). As shown in Fig 2, there were 988 peptides with greater than a 2-fold change that were identified in all four samples (unstimulated PC12 cells and stimulated samples of the wild type receptor and the two mutants). Further analyses of these samples underscored the central role of Y490 in activating the Erks and effecting changes in transcription while Y785 (which is known to activate PLCγ) is more involved in cell cycle/mitotic control. Interestingly these studies also established that there was still signaling by the double mutant, indicating at least one additional docking site (perhaps involving the activation loop tyrosines) that was strongly manifested in CK2 regulation (Biarc *et al*., 2013).

One analysis that was particularly interesting was a comparison of these findings with a similar set of phosphopeptide identifications from the stimulation of HeLa cells by EGF at the same time point (Olsen *et al*., 2006). Plotted using the catalytic specificity motifs of 16 groups of kinases, the data revealed a high degree of similarity, suggesting that the pathways (kinases?) stimulated were substantially overlapping, despite the fact that the two cell types were different and from different species (rat vs. human) (Fig 3). In view of the heavy involvement of EGF in many cancers, as well as some other RTK members (Drake *et al*., 2014), it raises questions of whether there might not be a similar involvement of NGF and the other neurotrophins in cancer as well. Obviously, since their normal targets (neurons) are basically post-mitotic, they would not, at first pass, appear to be good candidates for such a role. However, this would ignore the fact that there are well established roles for NGF outside both the central and peripheral nervous systems and this provides potential opportunities for both ligands and receptors to be of oncologic significance (Kruttgen *et al*., 2006). Indeed, essentially all of the so-called neurotrophic factors appear to function with this kind of dual functionality, which may be an important consideration in their deployment as potential biomarkers or therapeutic targets.

NGF and breast cancer

The first indication of NGF involvement in breast cancer was the discovery of a stimulatory effect on the proliferation of several mammary tumor-derived epithelial cell lines (Descamps *et al*., 1998). These cells expressed both TrkA and the p75NTR receptor and the effects were clearly demonstrated to require the activation of the MAP kinases via the TrkA receptor. Subsequently, it was shown that the activation of p75NTR (and the transcription factor NF-κB) lead to an anti-apoptotic effect that was dependent on TRADD (Descamps *et al*., 2001; El Yazidi-Belkoura *et al*., 2003). Thus in breast cancer cells, this dual activation of TrkA and p75 leads to the stimulation of cell proliferation and survival, respectively, a situation in which the two receptors initiate separate signaling pathways that ultimately lead to different biological effects, albeit that there are interconnections between them. The direct demonstration that breast cancer cells produce NGF thus provides all the elements of an autocrine loop involving NGF and its receptors (Dolle *et al*., 2003) and as its inhibition results in a diminished tumor growth in a preclinical animal model (Adriaenssens *et al*., 2008), it underscores the potential value of NGF as a therapeutic target.

The role of TrkA in the signaling processes of breast cancer cells appears to be in part different than in their neuronal counterparts. Com et al. (Com *et al*., 2007) used proteomics to determine a number of TrkA signaling partners in MCF-7 breast cancer cells, in particular Ku70; a protein involved in DNA repair that has also been found to be associated with EGF receptor signaling (Bandyopadhyay *et al*., 1998). Interestingly it is not involved in TrkA signaling in PC12 cells but clearly plays a role in the prevention of breast cancer cell apoptosis. Indeed, in the absence of this regulator, TrkA can act as a pro-apoptotic agent. Therefore it is not only p75NTR, but also TrkA that can participate in the resistance to apoptosis induced by NGF. In a separate study, Lagadec et al. (Lagadec *et al*., 2010) identified a second DNA repair protein, Ku86, in tumor cells over expressing TrkA and showed that PI3K-Akt and ERK/p38 MAP kinases were activated and required for the maintenance of a more aggressive cellular phenotype. In addition to the stimulating effect of NGF on breast cancer cell survival and proliferation, altered expression of TrkA is also associated with tumor progression to effusion and clearly enhances growth and metastasis of breast cancer cells (Davidson *et al*., 2004; Lagadec *et al*., 2010).

In neuronal cells, there is considerable debate about the role of p75NTR as a regulator of TrkA activity and whether there is a direct interaction between the two receptors (Reichardt, 2006). While they certainly exert an effect on each other with respect to function and response, there is no compelling basis to assume that physical complexes actually form. This is also true in breast cancer cells but there siRNA or pharmacological inhibitors have also established that there is no particular effect of one receptor on the functionality of the other receptor. Therefore, it appears that in breast cancer cells, TrkA and p75 are working rather independently from one another (Fig. 4B).

ProNGF as an active growth factor

Although it is not surprising that the neurotrophins are synthesized as precursors that contain pro-domains in addition to their mature sequences, it is unusual that these entities are important ligands in their own right. A precursor of NGF was first detected in 1977 by immunoprecipitation of radiolabeled protein synthesized in tissue samples of mouse submaxillary gland (Berger and Shooter, 1977) and was subsequently confirmed by cloning experiments, which provided molecular details (Scott *et al*., 1983; Ullrich *et al*., 1983). It has since been detected in a number of tissues (Hempstead, 2014) and was reported to be the sole detectable form (by Western blot) of the protein in the brain (Fahnestock *et al*., 2001). Because there are two alternative spliced forms along with various glycosylated intermediates, proNGF can be observed in multiple forms. The demonstration that proNGF had a higher affinity for the p75NTR receptor than TrkA, and further is bound to sortilin via its pro peptide to promote pro-apoptotic activities in concert with p75NTR, provided a clear rationale for its prevalence. It is still unclear what regulates the processing events (or lack thereof), which are thought to be performed by furins and proconvertases intracellularly (Seidah *et al*., 1996) and by plasmin and MMPs after secretion (Teng *et al*., 2010) and determine the amounts of proNGF vs. the mature form in any given situation. The end result in neuronal cells is that proNGF, in the absence of processing, is an active product that promotes apoptosis via p75NTR/sortilin complexes and counters the effect of NGF, acting via TrkA or p75NTR, to stimulate survival and differentiation (Fig 4A).

While most attention has been focused on the role of proneurotrophins in the nervous system, proNGF has been associated with other types of tissues as well. Both dermal and cardiac responses have been described (Hempstead, 2014). However, a more compelling involvement in the behavior of several tumor types suggests that, like several other members of the greater RTK family including both ligands and receptors, it may be of much more significance in the management of oncological pathologies[66]. For example, proNGF can stimulate invasion of melanoma cells through a mechanism involving p75NTR and sortilin (Truzzi *et al*., 2008). These cells are also of neuroectodermal origin and express all the members of the neurotrophin family and its three distinct receptors and utilize both TrkA and p75NTR in promoting proliferation. In this case the p75NTR-sortilin complex is implicated in promoting migration.

As described above, breast cancers express and respond to NGF, and therefore the discovery that proNGF is secreted by tumor cells was not overly surprising (Demont *et al*., 2012). However the determination that it stimulates their migration/invasion through an autocrine loop mediated by TrkA and sortilin was unexpected. This somewhat controversial observation is the first indication of a biologically significant TrkA-sortilin partnership. The signaling pathway requires the phosphorylation of TrkA as well as the activation of Src and Akt, but not the MAP-kinases. Moreover, in contrast to melanoma cells, p75NTR is not involved. In addition, a comparison between proNGF levels and clinicopathological parameters revealed a correlation with lymph node invasion. In invasive ductal carcinomas, which represent the majority of breast cancers, there was no correlation with histological grade, tumor value, axillary lymph node status, age and presence of estrogen receptors, although a statistically significant association was obtained between the quantity of proNGF and lymph node invasion, suggesting a link to metastasis (Demont *et al*., 2012). Indeed, proNGF may serve as a biomarker of metastasis and possibly as a therapeutic target in breast cancer. As described below, prostate tumors also express proNGF.

Neurotrophin-induced neurogenesis in tumor tissues

The tumor microenvironment represents an additional area of great importance in understanding the factors controlling neoplastic tissue growth and progression, particularly as they relate to metastases and all factors and elements involved in these processes impact it (Swartz *et al*., 2012). In breast cancer, tumor neovascularization and macrophage invasion are generally held to be the most important elements of the microenvironment influencing tumor development, and NGF/proNGF contribute to both of these (Hondermarck, 2012). Angiogenesis requires the activation and proliferation of endothelial cells (usually recruited from the pre-existing vascular bed) and vascular endothelial cell growth factor (VEGF) and the fibroblast growth factors (FGFs), that stimulate other members of the RTK family, are key components of this activity. Indeed, inhibiting angiogenesis has been an important target for cancer therapeutics (Gimbrone *et al*., 1972). NGF has also been reported to promote angiogenesis and/or induce the expression of proangiogenic molecules in several tissues (Cantarella *et al*., 2002), including breast cancer (Romon *et al*., 2010). Related to tumor angiogenesis is the facilitation of the infiltration of immune cells. The link between inflammation and cancer involves a variety of cytokines and chemokines and NGF is produced by various immune cells (Leon *et al*., 1994; Nilsson *et al*., 1997). It has recently

been shown that breast cancer NGF can stimulate TrkA signaling in tumor-associated macrophages, increasing IL-10 production (Ley *et al*., 2013).

A third potential contributor to the tumor microenvironment is from nerve fibers induced to infiltrate the tumor. The reverse situation, perineural invasion, whereby tumors infiltrate and follow nerve fibers occurs frequently and has been well documented in pancreatic, prostatic and breast cancer (Villers *et al*., 1989; Karak *et al*., 2010). The occurrence of perineural invasion does not generally lead to a good prognosis. Nerve fibers are commonly found in the microenvironment, but there is a paucity of information about what they might contribute to the growth and expansion of tumors. Ayala et al (Ayala *et al*., 2008; Magnon *et al*., 2013) were among the first to suggest tumors promote neurogenesis in prostate cancer and suggested the overexpression of semaphorin 4F might be mechanistically responsible. Recently, Magnon et al (Magnon *et al*., 2013) reported a study of autonomic nerve formation in prostate cancer, establishing that fibers from both the sympathetic (adrenergic) and parasympathetic (cholinergic) systems were present, with the former dominating the early stages. The density of these fibers was directly correlated to the Gleason prostate cancer score, and in an animal model, denervation resulted in a decrease in tumor engraftment and metastasis. Thus, these new autonomic nerve projections affected both cancer initiation and progression.

The mechanisms responsible for stimulating the growth of these peripheral neurons into the prostate tumors were not addressed. Entschladen et al. (Entschladen *et al*., 2006) put forth the idea that neurogenesis (they termed it neoneurogenesis) could be induced by tumors through the production of neurotrophic factors. It had already been reported (Delsite and Djakiew, 1999) that a proNGF molecule of 22 kDa is expressed by human prostatic stromal cells, as detected immunologically, but mature NGF, which would be expected to be the agent capable of attracting sympathetic and/or sensory neurites was not detected in these studies. To address whether NGF or proNGF may be involved in prostate tumor-directed neurogenesis, a cohort of 120 human prostate samples was examined by immunohistochemistry (Pundavela *et al*., 2014). ProNGF was readily detected in the cytoplasm of the cancer cells but much less so in the stromal cells. Importantly quantification of these observations indicated that the levels detected correlated with the Gleason scores of these samples (n=104, coefficient of correlation $\tau B = 0.51$) and this pattern matched the neurite invasion data of Magnon et al. (Magnon *et al*., 2013). In keeping with previous observations (Delsite and Djakiew, 1999), mature NGF was not detected in prostate cancer cells. Western blot analysis of three prostate cancer-derived cell lines compared with normal prostate epithelial cells, transformed non-tumorigenic prostate epithelial cells and benign prostate hyperplasia (BPH) cells indicated that the tumor and BPH cells showed a prominent band at 60 kDa that was largely absent in the normal cells. This form of proNGF was previously described in uterine samples (Lobos *et al*., 2005); indeed several high molecular mass forms have been observed that are, at least in part, derived from glycosylation and alternative splicing. However, the detailed molecular characterization of the proNGF produced by prostate tumor cells has not been determined and it could contain other modifications as well. Determining the nature of the alterations

that lead to the higher molecular mass forms will be an important step in evaluating the usefulness of proNGF as prostate cancer biomarker.

To ascertain whether the proNGF identified immunologically was capable of inducing the peripheral neuron infiltration of the tumors (Magnon *et al*., 2013), the prostate cancer cell line PC-3 was incubated with two NGF-responsive cell lines, PC-12 and 50B11, in Transwell Boyden chambers (Pundavela *et al*., 2014). Both of these paradigms extend neurites when exposed to germane neurotrophic agents, such as NGF. PC-3 cells were able to induce neurite outgrowth with both test cell lines whereas control normal cells did not. Moreover the responses were inhibited by anti-proNGF sera but were not affected by an isotype anti-sera. Clearly the proNGF observed to be present in prostate tumor cells is exported in a manner sufficient to induce the nerve infiltration observed (Magnon *et al*., 2013). However, it is not known if proNGF is acting on its own to stimulate neurite outgrowth in prostate tumors or if it requires processing to mature NGF.

Given the responses to prostate tumors, it is reasonable to assume that other tumors might also induce neurogenesis from peripheral neurons that could impact tumor growth and progression. Albo et al (Albo *et al*., 2011) and Tomita et al (Tomita, 2012) have reported neoneurogenesis in colon cancer and Zhao et al (Zhao *et al*., 2014) have very recently made similar observations for breast cancer where they observed PGP 9.5 positive fibers in over 60% of a cohort of 144 cases of invasive ductal carcinoma. In an independent study[1], nerve fibers were imaged in a cohort of primary invasive breast cancers by immunohistochemistry with the same neuronal marker. Neurites were detected in 20% of tumors and there was an association with NGF expression and lymph node invasion, suggesting a relationship with the metastatic potential. Although broader studies will be required to confirm and extend these observations, it already seems clear that many types of tumors have the potential to express NGF (and/or proNGF) and that these factors, in turn, may induce peripheral nerve infiltration into the tumor microenvironment, resulting in further stimulation of tumor growth and metastases. Such effects may not be limited to the neurotrophins but may be stimulated by other neurotrophic factors as well.

ProNGF/NGF stimulated nerve infiltration in solid tumors may also participate in cancer pain. Indeed NGF is also a mediator of pain that acts though the activation of TrkA in endings of sensory neurons (Pezet and McMahon, 2006). Blocking antibodies against NGF, and pharmacological inhibitors against TrkA, have been developed and some are already in clinical trials for their potent analgesic effect in rheumatoid and back pain (Longo and Massa, 2013). Interestingly, in the mouse it has been shown that anti-NGF antibodies can decrease the pain caused by bone metastasis and to attenuate bone destruction (Jimenez-Andrade *et al*., 2011; McCaffrey *et al*., 2014). Therefore targeting NGF/proNGF in cancer could also have an additional impact by reducing cancer pain.

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Conclusions

NGF and its precursor, proNGF, clearly have multiple roles in both neuronal and non-neuronal targets as exerted through three receptor types. Importantly these seem to manifest themselves in different ways and with different phenotypic responses. These differences are most acute when comparing normal and neoplastic tissues. Thus, as shown in Fig 5, NGF and proNGF (and presumably other neurotrophic factors) can directly affect tumor cells or they can influence the composition and responses of the cells that are an important part of the microenvironment, stimulating such tumor sensitive processes as angiogenesis, immune responses and pain. This places NGF/proNGF in a central role for the diagnosis and management of many breast and prostate cancers and its detection and inhibition may become important clinically.

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Abbreviations

BDNF	Brain-Derived Neurotrophic Factor
NGF	Nerve Growth Factor
PDGF	Platelet-Derived Growth Factor
RTK	Receptor Tyrosine Kinase
SILAC	Stable Isotope Labeling of Amino Acids in Culture

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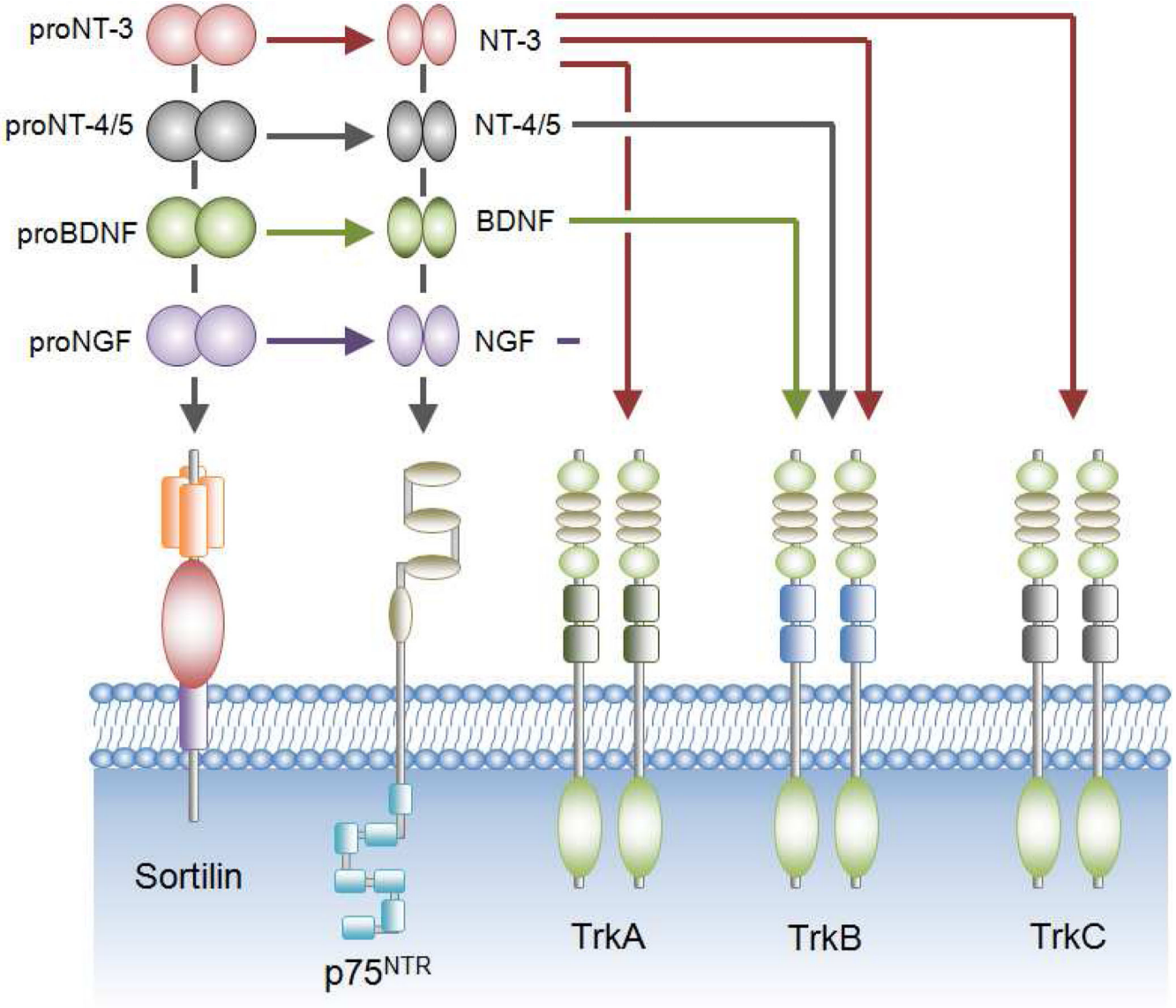

Figure 1. Binding of neurotrophins and proneurotrophins to Trk receptors and p75NTR

NGF, BDNF, NT-3, NT-4/5 as well as their respective precursors (proNGF, proBDNF, proNT, proNT-4/5) all bind to the pan-neurotrophin receptor $p75^{NTR}$ while Trk receptors bind neurotrophins with different specificities. Sortilin binds only the precursor forms.

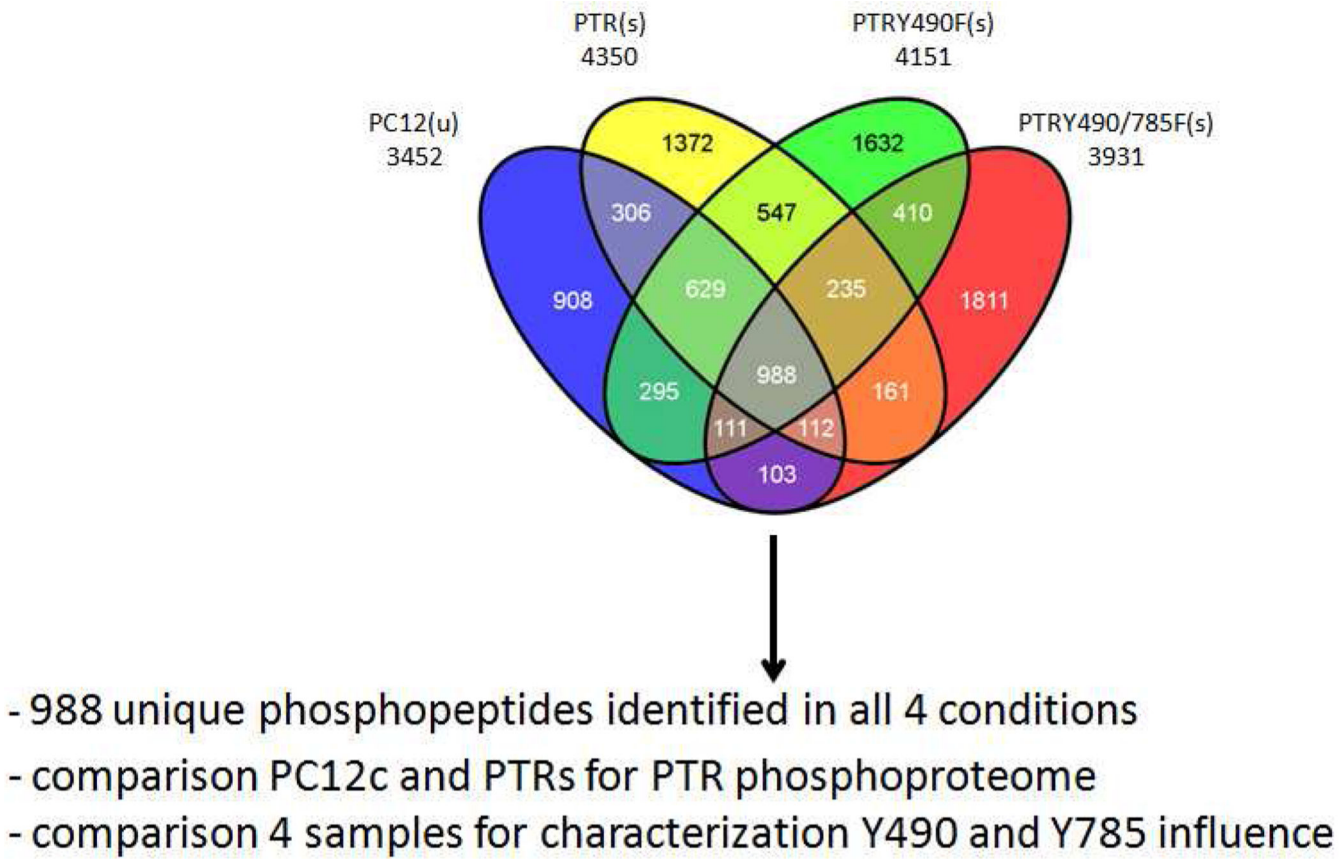

Figure 2. Overlap of phosphopeptides identified upon receptor stimulation

Venn diagram describing the phosphopeptides identified in PC12 cells (PC12c), PC12 cells stably transfected with chimeric receptor PTR stimulated for 20 min with PDGF-BB (PTRs), PTR Y490F stimulated (PTR Y490F(s)) or PTR Y490F/Y785F stimulated with a peptide false-positive rate of 0.5%. 988 phosphopeptides (gray part) were identified in all four conditions. Adapted from (Biarc *et al*., 2013).

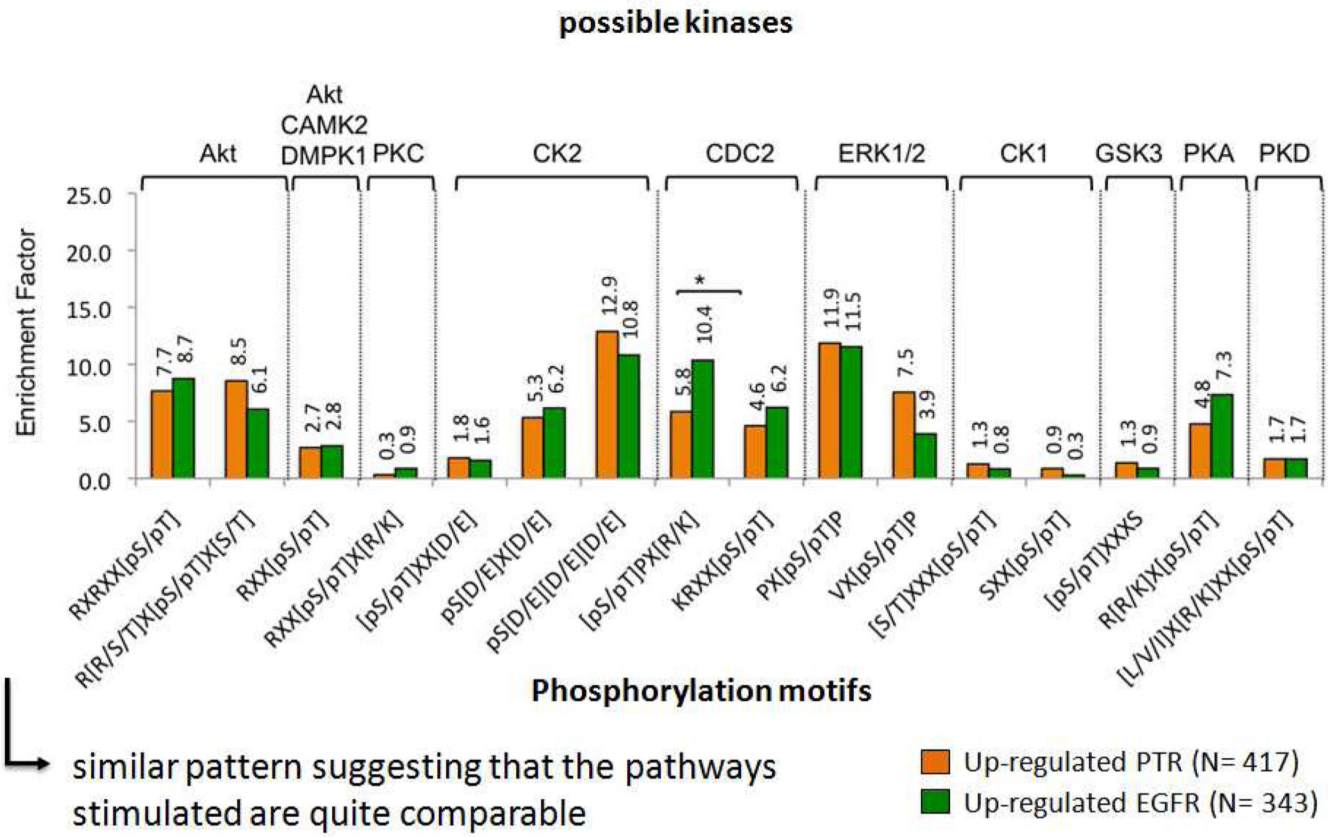

Figure 3. Phosphorylation motifs

Sixteen phosphorylation motifs modified by different kinases that are represented at the top of the figure were analyzed for the regulated phosphopeptides by determining their enrichment in each population. Plotted is the enrichment factor (how frequently phosphorylation in a particular motif was observed in comparison to the motif's frequency in all rat proteins) in up-regulated phosphopeptides upon stimulation of the TrkA chimera in PC12 cells (orange bars) and EGFR in Hela cells (Olsen *et al*., 2006) (green bars). Adapted from (Biarc *et al*., 2013).

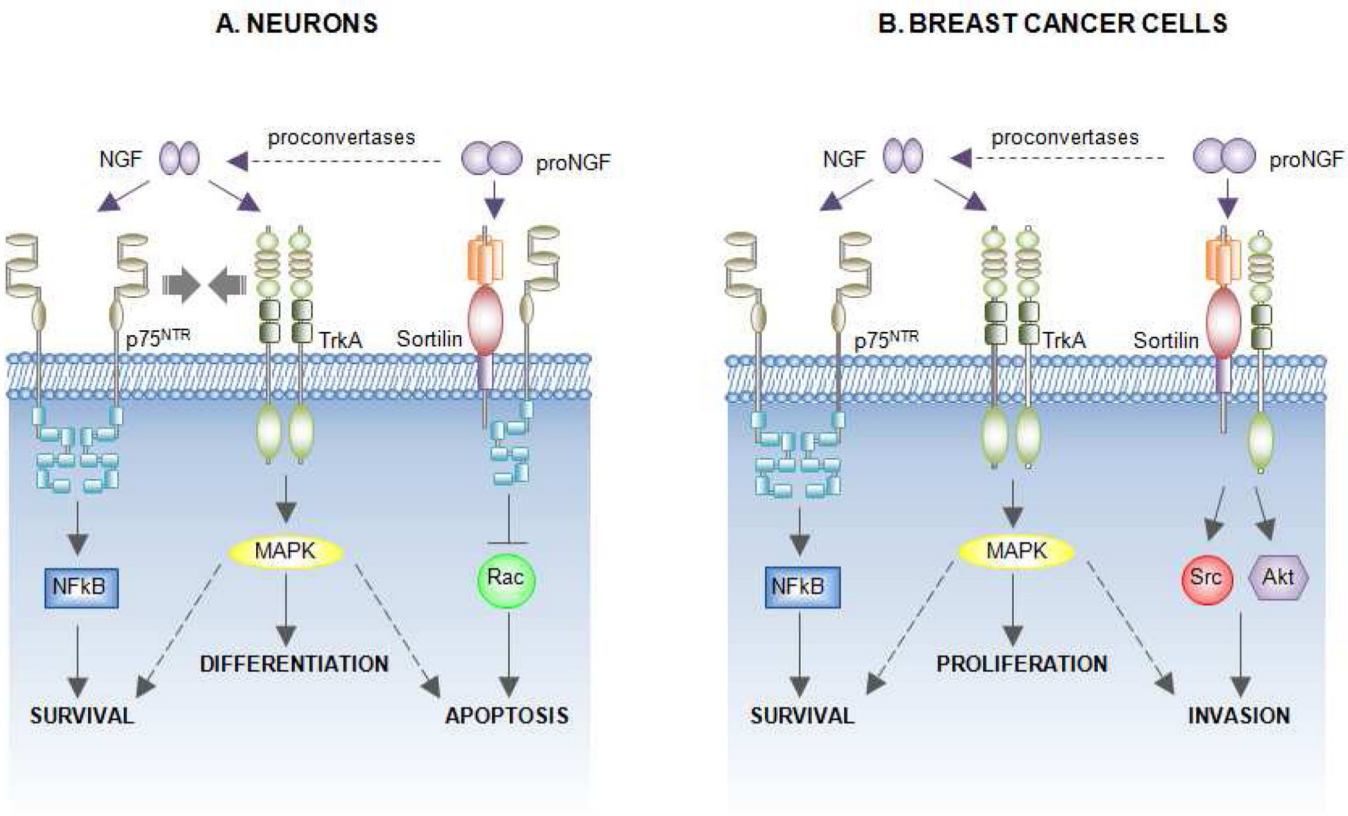

Figure 4. ProNGF/NGF signaling in neurons and breast cancer cells

A) In neurons NGF stimulates survival and differentiation through TrkA and p75NTR and via a signaling involving the MAP kinases and NFkB. ProNGF stimulate a complex between p75NTR and sortilin that leads to the inhibition of RAC (Rho GTPase). B) In breast cancer cells, NGF stimulates TrkA and p75NTR leading to the activation of cell proliferation and survival, respectively. ProNGF binds to a complex TrkA/sortilin to stimulate cancer cell migration and invasion via the activation of Src and Akt.

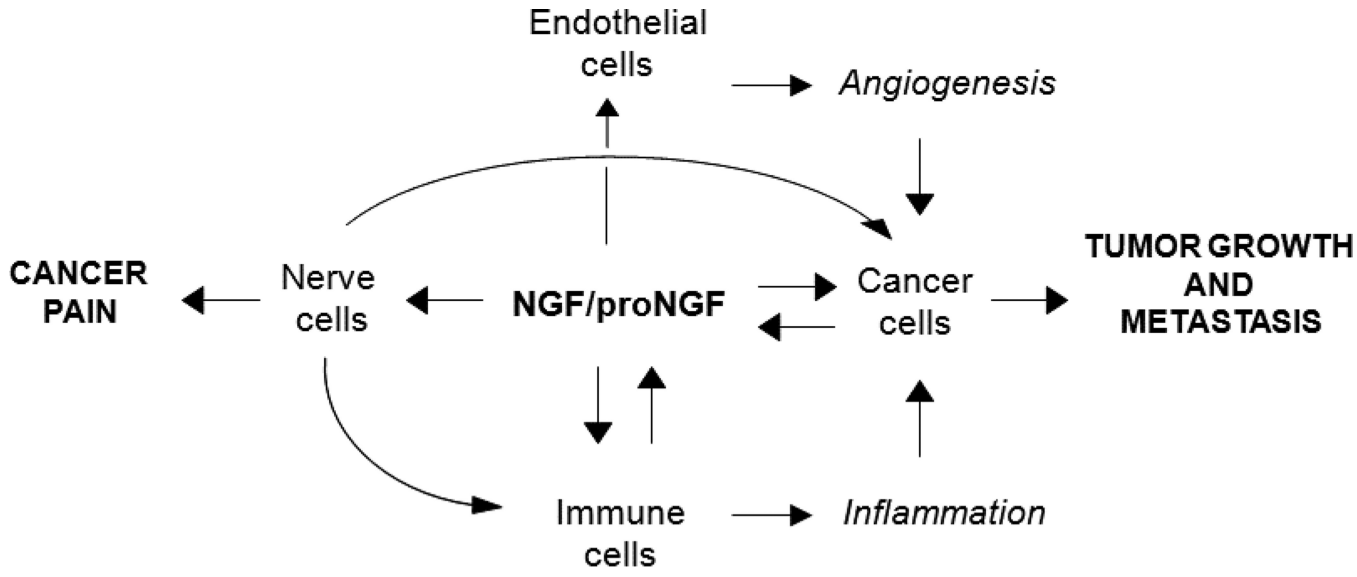

Figure 5. ProNGF/NGF impact on cancer progression

ProNGF/NGF produced by cancer cells activates cancer cells growth and dissemination via an autocrine loop of stimulation, and stimulate various cell types in the tumor microenvironment. Immune cells, endothelial cells and nerves in the tumor microenvironment are activated, leading to the stimulation of inflammation, neoangiogenesis and nerve infiltration. The presence of nerve fibers in the tumor microenvironment could contribute to the feeling of pain in and around the tumor.