Clin. Exp. Metastasis, 1995, **13**, 67–88

The role of trophic factors and autocrine/paracrine growth factors in brain metastasis

David G. Menter, John L. Herrmann and Garth L. Nicolson

Department of Tumor Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA

(*Received 22 November 1994; accepted 22 November 1994*)

The brain is a unique microenvironment enclosed by the skull, lacking lymphatic drainage and maintaining a highly regulated vascular transport barrier. To metastasize to the brain malignant tumor cells must attach to microvessel endothelial cells, respond to brain-derived invasion factors, invade the blood-brain barrier and respond to survival and growth factors. Trophic factors are important in brain invasion because they can act to stimulate this process. In responsive malignant cells trophic factors such as neurotrophins can promote invasion by enhancing the production of basement membrane-degradative enzymes (such as type IV collagenase/gelatinase and heparanase) capable of locally destroying the basement membrane and the blood-brain barrier. We examined human melanoma cell lines that exhibit varying abilities to form brain metastases. These melanoma lines express low-affinity neurotrophin receptor $p75^{NTR}$ in relation to their brain-metastatic potentials but the variants do not express *trkA*, the gene encoding a high affinity nerve growth factor (NGF) tyrosine kinase receptor $p140^{trkA}$. Melanoma cells metastatic to brain also respond to paracrine factors made by brain cells. We have found that a paracrine form of transferrin is important in brain metastasis, and brain-metastatic cells respond to low levels of transferrin and express high levels of transferrin receptors. Brain-metastatic tumor cells can also produce autocrine factors and inhibitors that influence their growth, invasion and survival in the brain. We found that brain-metastatic melanoma cells synthesize transcripts for the following autocrine growth factors: TGFβ, bFGF, TGFα and IL-1β. Synthesis of these factors may influence the production of neurotrophins by adjacent brain cells, such as oligodendrocytes and astrocytes. Increased amounts of NGF were found in tumor-adjacent tissues at the invasion front of human melanoma tumors in brain biopsies. Trophic factors, autocrine growth factors, paracrine growth factors and other factors may determine whether metastatic cells can successfully invade, colonize and grow in the central nervous system.

Keywords: brain metastasis, growth factors, melanoma, melanotropins, nerve growth factor, neurotrophins, signal transduction, tumor progression, tyrosine receptor kinase

Introduction

The brain, because of its anatomical and physiological properties, provides a unique target for metastasis formation [1]. Due to confinement by the skull, homeostasis in the brain is highly sensitive to the slightest change in the local microenvironment. The brain is surrounded by a formidable blood-brain barrier that must be penetrated by brain-metastatic tumor cells, and lacks lymphatic drainage which could remove fluid buildup that accompanies tumor growth. Cerebral edema represents a major complication resulting from central nervous system (CNS) tumors. Brain metastases are difficult to treat, therapy is often only palliative, and brain metastases are often accompanied by additional complications. A relatively small metastasis in the brain cavity can cause severe symptoms, including paralysis, headache, seizure, and impaired cognition.

Address correspondence to: G. L. Nicolson, Department of Tumor Biology, Box 108, 1515 Holcombe Blvd., The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA. Tel: 713 792 7481; Fax: (+1) 713 794 0209.

Malignant melanoma metastasizes to the brain with one of the highest frequencies of any cancer that is capable of colonizing the CNS. Patients with disseminated malignant melanoma frequently develop metastatic lesions in the brain and spinal cord that can result in severe and debilitating neurological complications [1]. Although melanoma metastasis formation in other organs may be tolerated or remain asymptomatic, once melanoma cells colonize the brain, tumor growth often results in a rapid decline in the quality of life and death ensues. Approximately 13% of cancer patients will present with symptomatic complications related to brain metastases [2], but almost 40% of melanoma patients will be treated for complications due to brain metastases. At autopsy, 70–80% of malignant melanoma patients have CNS lesions [1].

Malignant melanomas undergo progressive changes during their pathogenesis, especially those that progress to form brain metastases. Of the phenotypic changes that occur during metastatic melanoma progression, differences in the expression of receptors for paracrine growth factors and the production of various autocrine growth factors are important [3, 4]. Progression-associated differences in growth factor production between cultured melanocytes and malignant melanoma cell lines include increased autocrine production of transforming growth factor beta (TGFβ), transforming growth factor alpha (TGFα), basic fibroblast growth factor (bFGF), keratinocyte growth factor and the A-chain of platelet-derived growth factor (PDGF) [4]. The significance of these autocrine factors in modulating the malignant properties exhibited by melanoma cells remains largely unknown but they are thought to be important in allowing malignant cells to survive in unusual environments such as the brain.

Trophism, survival, and clonal evolution within tumors

Malignant tumor growth depends on tumor cell proliferation, and this usually occurs in response to growth factors from either autocrine or paracrine sources [5]. Another mechanism leading to tumor growth is the failure to initiate programmed cell death (apoptosis) [6]. During their rapid growth phase, tumors frequently contain highly malignant cells that become clonally selected from the proliferating growth fraction and eventually become the dominant cell subpopulation [7]. When conditions within the tumor microenvironment become growth limiting, however, malignant cells may be forced to utilize other mechanisms in order to survive. Trophic factors that can support malignant cells in a state of growth stasis, suppression, or dormancy under growth limiting conditions may have a profound effect on cell survival. Consequently, tumor cells that are responsive to trophic factors may survive host selection to proliferate, further diversify and become clonally dominant. Although widely investigated in the area of developmental biology [8, 9], trophism as a support mechanism for cell survival is an area of cancer biology that remains largely unexplored.

The neurotrophins are one of the best examples of trophic substances. Neurotrophins can promote targeted tissue invasion and survival of certain neuronal cells [8], and such observations have resulted in what is known as the 'neurotrophic theory' [8]. This theory was proposed to account for the massive neuronal cell death and innervation of tissue target tissues in developing embryos [8]. The neurotrophic hypothesis states that neuronal cells that are overproduced in the embryo must compete for limited supplies of target tissue-derived neurotrophins. Hence, only small numbers of successful neurons survive and invasively innervate the target tissue and the majority of neurons then die by apoptosis. Applying the concept of trophism to rapidly growing tumors or large numbers of metastatic cells in the blood circulation or at implantation sites may account in part for tumor cell survival under growth-limiting conditions. This may be particularly true of neurotrophic effects on tumors that have neuroectodermal origins, such as malignant melanoma cells that arise from the neural crest [10].

Neurotrophins: a family of homologous trophic proteins

The neurotrophins are a family of small ($\sim$13 kDa) proteins that are highly basic (PI 9–10.5; Figure 1). The neurotrophins are synthesized as prepropeptides, whereupon they are N-terminally processed to proteins containing three interchain disulfide bonds [11]. The circulating forms of neurotrophins are nonglycosylated proteins of 26 kDa size. Each protein monomer contains an elongated central axis made of an antiparallel β-sheet structure with a flattened hydrophobic face that is involved in dimer formation [11]. At one end of each monomer there are three β-hairpin loops, and one at the opposite end comprising regions that vary between homologous neurotrophin family members [11]. In addition to nerve growth factor (NGF), all members of the

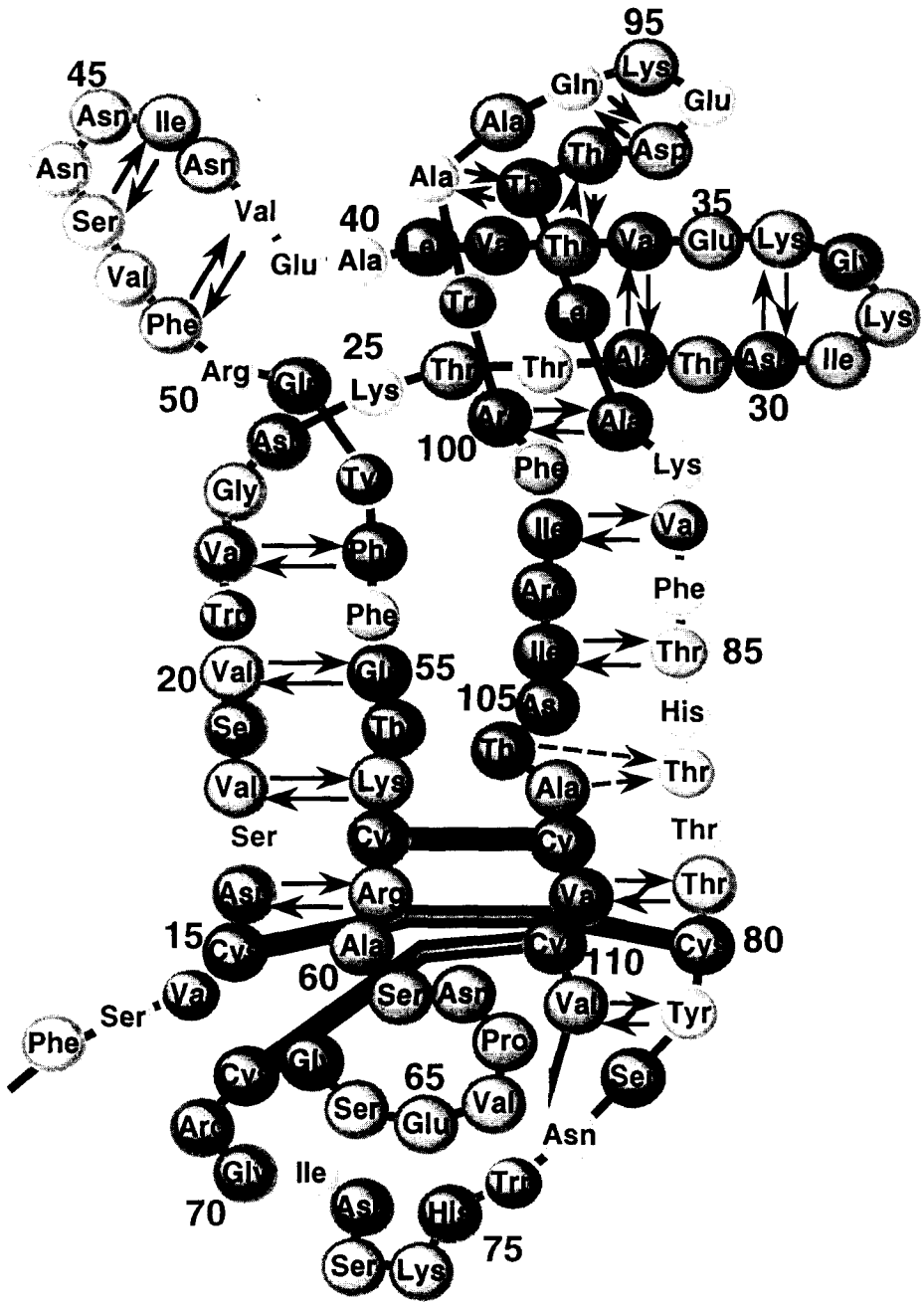

Figure 1. Diagram of a nerve growth factor (NGF) monomer and some of the important structural features. Dark shaded amino acids are the highly conserved regions tha occur along the central axis of the molecule. The light shaded amino acids make up the variable loops of the molecule. The cysteine double bonds are the heavy lines at the base of the molecule's central axis. Noncovalent interactions are represented by dashed arrows.

homologous neurotrophin protein family exhibit neurotrophic properties. Brain-derived neurotrophic factor (BDNF), isolated from brain tissue, shows significant amino acid sequence homology (50%) with NGF. Similarly, neurotrophin-3 (NT-3) isolated by various methods and neurotrophin-4 (NT-4), isolated originally from Xenopus, and its mammalian homolog neurotrophin-5 (NT-4/5) are all highly conserved in the amino acid sequence in the region of the central axis of the molecule [11]. The various neurotrophin family members derive their unique functional properties from the molecular regions that encompass much of the four variable β-hairpin loops in these molecules. These regions are involved in binding to various neurotrophin receptors. It is important to note that another neurotrophin exists, namely; ciliary neurotrophic factor (CTNF). This molecule exhibits neurotrophic properties and yet bears no homology to the other neurotrophins. The structural properties of the CTNF molecule show distinct similarities to a number of hematopoetic cytokines, including interleukin-6, leukemia inhibitory factor (LIF), granulocyte colony stimulating factor (G-CSF) and oncostatin M [12].

Neurotrophin receptors

The neurotrophin receptors can be divided into two affinity classes (Figure 2), a low-affinity receptor class ($Kd \sim 2 \times 10^{-9}$) and a high-affinity receptor class ($Kd \sim 2 \times 10^{-11}$). The gene encoding the human low-affinity nerve growth factor receptor (NGFR or $p75^{NTR}$) was cloned by Chao and coworkers [13]. This human gene encodes a 75 kDa cell surface glycoprotein made up of 399 amino acids, including a 222 amino acid extracellular domain, a 22 amino acid transmembrane domain, and a 155 amino acid cytoplasmic segment. The molecule contains four cysteine-rich extracellular domains and a G protein-binding consensus sequence in the cytoplasmic domain.

Studies originally established that the biologic effects of NGF involve a tyrosine kinase activity [14, 15]. Sequence analysis of $p75^{NTR}$, however, indicates that this molecule lacks a tyrosine kinase consensus sequence in the cytoplasmic domain [13]. Despite the absence of a tyrosine kinase domain, transfection of $p75^{NTR}$ into non-neuronal cells enhanced tyrosine kinase phosphorylation following NGF stimulation [16]. The search for a high affinity NGF receptor with tyrosine kinase activity

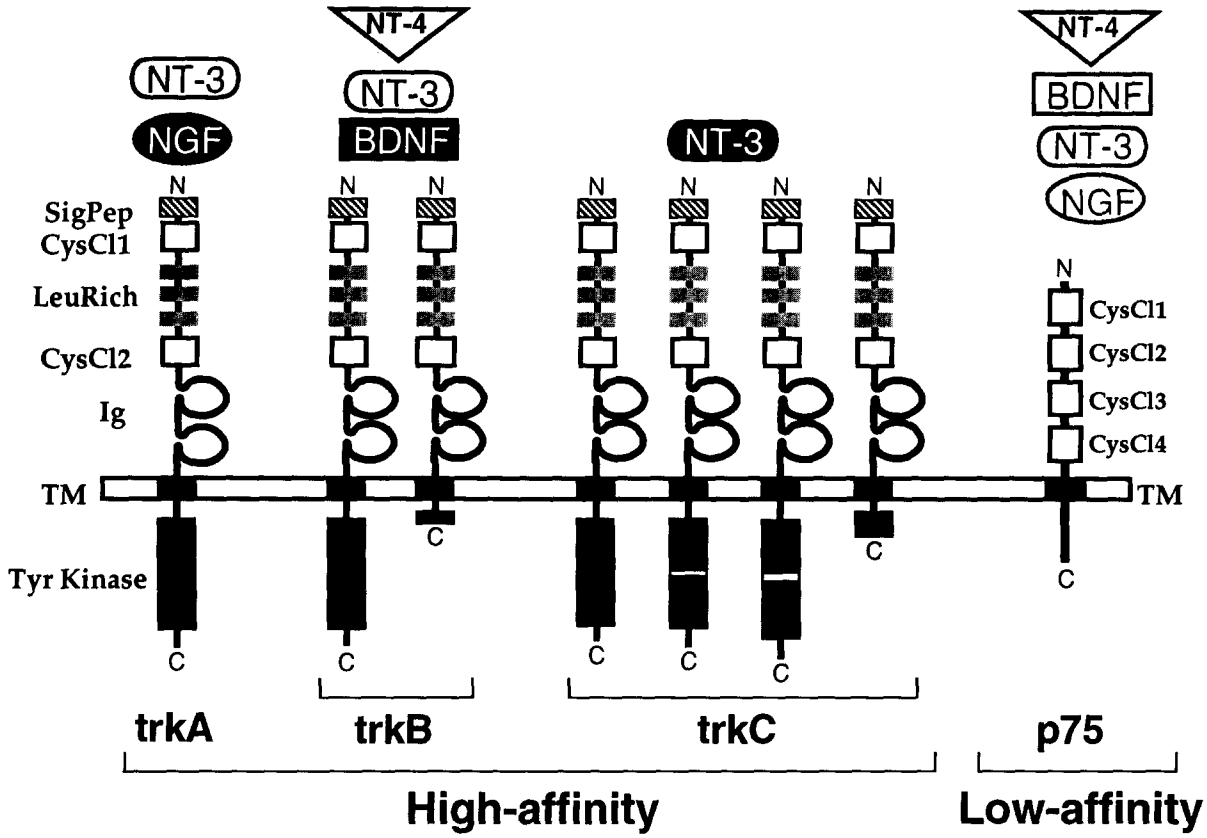

Figure 2. Schematic representation of the neurotrophins and the two different affinity class receptors that bind them. The neurotrophins are a family of small (~ 13 kDa) molecules that are highly basic (PI 9–10.5). The family consists of nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5). The high affinity class receptors ($Kd \sim 2 \times 10^{-11}$) consist of a family of tropomyosin receptor kinase (TRK) molecules. First cloned from colon carcinoma, *trkA* has been histochemically localized to nervous tissues. Hybridization cloning led to the discovery of the other family members *trkB* and *trkC*, as well as their isoforms. These isoforms contain both deletions and insertions in their cytoplasmic domains. The characteristic extracellular domain structures shared by these receptors include: a signal peptide (SigPep) and a leucine rich domain (LeuRich) flanked by two cysteine cluster regions (CysCl 1 & 2) as well as immunoglobulin C2 domains (Ig). The cytoplasmic region contains the TRK tyrosine kinase domain that is highly conserved among full length receptors. The primary ligands for each TRK receptor are NGF, BDNF and NT-3 that bind to *trkA*, *trkB* and *trkC* respectively. There is also some crossreactivity of NT-3 for 150^{trkA} as well as NT-3 and NT-4 for 145^{trkB}. The low affinity receptor class consists of only $p75^{NTR}$, that is distinguished by a series of cysteine clusters (CysCl 1–4) in the extracellular domain. The $p75^{NTR}$ cytoplasmic domain is characterized by a G protein consensus sequence. The $p75^{NTR}$ receptor binds all of the neurotrophins with low affinity ($Kd \sim 2 \times 10^{-9}$).

resulted in the discovery of the TRK family of neurotrophin receptors [17–20]. The TRK family of tyrosine receptor protein kinases consists of several receptor molecules with varying degrees of specificity for the different members of the neurotrophin family. In addition to *trkA*, hybridization cloning led to the discovery of the closely related proto-oncogenes *trkB* and *trkC*. These receptors, together with the recently discovered *trkE*, constitute the high-affinity neurotrophin family members [17–20]. Each mature 140^{trkA} kDa proto-oncogene protein contains a 375 amino acid extracellular domain, a 26 amino acid transmembrane span, and a large cytoplasmic domain of 357 amino acids. The mature *trkB* and *trkC* proto-oncogenes encode molecules of 145 kDa which are known also to exist as truncated forms or contain inserts in their tyrosine kinase domain. All of the TRK family members share distinct structural motifs in the glycosidated extracellular domain, including Ig-C2 immunoglobulin loops and a series of leucine-rich amino acid motifs that are flanked by cysteine amino acid clusters. Each mature neurotrophin receptor cytoplasmic domain contains a tyrosine kinase consensus sequence that is followed by a highly conserved 15 amino acid post-kinase domain or tail. The TRK family members are widely distributed in neuronal tissues in addition to haematopoietic cells [17–20].

Neurotrophic regulation of the death and survival of neuroectodermal derivatives

Homozygous knock-out mice lacking neurotrophins or their receptors have been very informative in elucidating the complex function of these developmental regulatory molecules [9]. There are major differences in the properties of knock-out mice depending on which neurotrophic gene has been targeted and the type of neuronal cell under observation [9]. For example, both NT-3 and its principal receptor, 145^{trkC}, regulate the proliferation and survival of neuronal precursors in addition to collateral branching of axons into target fields [21–24]. This is consistent with the lack of proprioceptor production in *trkC* (−/−) or NT-3 (−/−) mice [25, 26]. In contrast, BDNF or *trkB* gene targeting seems to affect vestibular ganglia to the greatest degree [27–29], whereas targeted disruption of NGF or *trkA* genes yields mice with defects in the superior cervical ganglia [30, 31]. Significantly, gene targeting or knock-out experiments performed with each of the neurotrophins or their receptors demonstrate profound effects on the survival of dorsal root ganglia neurons that have the same neuroectodermal origins as melanocytes. Although effects on melanocytes have not yet been described in these gene targeting studies, the effects of neurotrophins on melanocytes may be more complex. There is evidence that many neuroectodermally-derived sensory neurons switch their neurotrophin dependence from BDNF or NT-3 during early embryonic development to NGF at later stages [32]. Therefore, the targeting of more than one neurotrophin or neurotrophin receptor in homologous recombination experiments may be required to observe an effect on melanocytes in null allele mice.

Developmental changes in neurotrophin dependence may reflect the progressive increase in $p75^{NTR}$ production that occurs during the progression of melanocytes to malignant melanoma. Recently, phorbol 12-tetra decanoate 13-acetate (TPA) previously reported to induce $p75^{NTR}$ receptor production, has also been shown to induce synthesis of trk receptors [33–35]. Interestingly, primary melanocyte cultures express low levels of *trkC* that can be upregulated by TPA stimulation [35 and our unpublished observations]. Although we did not find *trkA* expression in malignant melanoma cells, we have observed the presence of *trkC* expression [36]. Since melanoma cells frequently exhibit genetic instability, they may be predisposed to switching expression of neurotrophin receptor genes to those most likely to support their survival in alternative tissue compartments.

It is now apparent that the low-affinity $p75^{NTR}$ neurotrophin receptor may have alternative functions that are dependent on the cellular context in which it is expressed. In addition to receiving differentiation or survival signals in neuronal cells, $p75^{NTR}$ may provide retrograde transport in certain neuronal cell types [37], triggering of apoptosis in certain virally transformed neuronal cells [38] or survival when expressed in neutrophils [39]. Certain properties of $p75^{NTR}$ may allow it to function in regulating survival and death of melanoma cells. In this regard, $p75^{NTR}$ may be analogous to members of the tumor necrosis factor receptor superfamily, such as Fas (Apo I), TNFRI and II, and the B cell antigen CD40, all of which regulate programmed cell death [40, 41]. Support for a $p75^{NTR}$-dependent signaling role in apoptosis was obtained by introducing $p75^{NTR}$ into a SV40 large T antigen-immortalized neuronal cell line deficient in $p75^{NTR}$ and 140^{trkA} receptors. In the absence of NGF, the $p75^{NTR}$ transfectants died. However, incubation with exogenous NGF or addition of monoclonal antibody to $p75^{NTR}$ suppressed neural cell death [38]. In the embryo, these effects are dependent on developmental stage. For example, $p75^{NTR}$ supports

sensory neuron survival from embryonic day 13 to at least postnatal day 2, but $p75^{NTR}$ can initiate apoptosis at later developmental stages [42]. In this way, $p75^{NTR}$ may play a bifunctional role as a molecular switch that signals either cell survival or cell death.

Neurotrophin receptor signaling mechanisms

The relative roles of the low- and high-affinity neurotrophin receptor classes remain unclear, but a number of studies suggest cooperativity can occur between 140^{trkA} and $p75^{NTR}$ receptors to establish functional receptor complexes for NGF. Genetic ablation of *trkA* or NGF production decreases responsiveness to painful stimuli in null allele mice. Most of these animals die by 3 weeks of age [30, 31]. In contrast, $p75^{NTR}$ $(-/-)$ receptor mice exhibit similar decreases in responsiveness to pain, but many of these animals live to adulthood [43, 44]. The differences in responsiveness to pain are explained by differential survival of various sensory neurons. The *trkA* $(-/-)$ animals show more massive losses of peripheral neurons than the $p75^{NTR}$ $(-/-)$ animals. Hempstead *et al.* [45] have suggested that the $p75^{NTR}$ gene, when transfected into NR18 (NGF unresponsive PC12) cells or COS cells, helps to establish cooperation between 140^{trkA} receptors and $p75^{NTR}$. Similarly, transfection of *trkA* into melanoma cells that express high levels of $p75^{NTR}$ results in the formation of large numbers of high-affinity NGF receptors [45], greater than expected for 140^{trkA} alone. Recent studies using immortalized MAH sympathoadrenal progenitor cells that are unresponsive to NGF support the notion that the introduction of the $p75^{NTR}$ gene can enhance catalytic activation of coexpressed 140^{trkA} receptors [37]. Coexpression of $p75^{NTR}$ and 140^{trkA} resulted in increases in downstream signaling and neurotrophin responses including mitotic arrest following neurite extension and neuronal maturation, relative to cells expressing only TrkA [19, 46, 47]. According to another model, $p75^{NTR}$ procures and presents bound neurotrophin molecules to members of the TRK receptor tyrosine kinase family to initiate signal transduction [17–20]. Little is known about $p75^{NTR}$ cooperative interactions with the other neurotrophin receptors but recent evidence based on anti-$p75^{NTR}$ antibody injections into chick embryos suggests that BDNF and NT-3 may cooperate with $p75^{NTR}$ to form functional signaling pathways [48]. Collectively, these data emphasize the importance of cooperativity between the TRK family of receptors and $p75^{NTR}$ for enhancing the neurotrophin response capabilities of cells.

The interactions between 140^{trkA} and $p75^{NTR}$ may occur at a subsequent step that does not involve physical interaction between the two receptor types. This follows from the failure to co-immunoprecipitate 140^{trkA} and $p75^{NTR}$ using various antibodies to either receptor [49]. Antibodies directed against $p75^{NTR}$ prevented high concentrations of NGF from binding to $p75^{NTR}$ on PC12 cells that contained functional 140^{trkA} receptors. This treatment was unable to prevent neurite outgrowth suggesting that distal cytosolic interactions may be involved in cooperative effects involving $p75^{NTR}$ and 140^{trkA} receptors [50]. The same effect was not observed using low concentrations of NGF while blocking NGF binding to $p75^{NTR}$ expressed in PC12 cells using BDNF or anti-$p75^{NTR}$ antibody [51]. In these studies, the Trk signaling pathway was still functional, as evidenced by c-fos activation. However, c-fos activation could be attenuated by the addition of anti-$p75^{NTR}$ antibodies competitive with NGF ligand but not by $p75^{NTR}$ saturated with BDNF ligand [51]. Although the authors concluded that the $p75^{NTR}$ increases the local concentration of NGF and stimulates 140^{trkA} activation (an argument supporting the procurement hypothesis) they could not rule out direct signaling effects by $p75^{NTR}$ as being partially responsible for their observations [51]. In addition, the crosslinking of NGF to Trk receptors yields only $p140^{trkA}$-NGF complexes but not 140^{trkA}-$p75^{NTR}$-NGF complexes [49]. Collectively, these data suggest that 140^{trkA} and $p75^{NTR}$ receptors are not in close physical proximity in the active NGF signaling complexes and may involve distal cytosolic interactions between members of the $p75^{NTR}$ and TRK signaling pathways.

Although the short cytoplasmic tail of $p75^{NTR}$ lacks a tyrosine kinase domain, some studies have implicated its involvement in signal transduction pathways. The $p75^{NTR}$ cytoplasmic tail contains a 14 amino acid mastoparan-(wasp toxin)-like domain [13, 52]. Activation of a G-stimulatory protein complex (Gs) in the presence (or absence) of NGF may lead to the production of cyclic AMP by adenylate cyclase and activation of protein kinase A (PKA) followed by transcription factor activation. This notion is supported by recent evidence indicating that the introduction of $p75^{NTR}$ into TrkA-deficient PC12 cells resulted in increased cAMP production following NGF stimulation [53]. Elevated cAMP activation of a protein kinase pathway may influence neurotrophin binding or downstream signaling via the Trk pathway. These pathways may have other as yet undiscovered functions in the presence or absence of ligand. This may explain some of the behavioral properties of cells expressing $p75^{NTR}$ in the absence of

TRK molecules [38]. Transfection studies involving sequence deletions of small segments in the cytoplasmic tail of $p75^{NTR}$ proved them to be essential for high-affinity NGF binding involving 140^{trkA} in PC12 and NIH3T3 cells [54, 55]. A 45–47 kDa serine/threonine protein kinase that is sensitive to purine analogs and known as protein kinase N (PKN) has been isolated with $p75^{NTR}$ following NGF stimulation of PC12 cells [56, 57]. The activation of this PKN in association with stimulation of ornithine decarboxylase activity may play an important role in the signaling pathways associated with $p75^{NTR}$ [58].

The cooperative interaction of downstream signals from $p75^{NTR}$/PKN in amplifying signals pre-established by 140^{trkA} may be important. In this case, when neurotrophin concentrations are high, the low-affinity activation of $p75^{NTR}$/PKN signals may amplify the 140^{trkA} response pathway. In contrast, when neurotrophin levels are low, $p75^{NTR}$ signals may be driven along an alternate pathway, allowing $p75^{NTR}$ to act as a sensitive molecular switch due to its low affinity for neurotrophins. Since the neurotrophins can cause such a wide variety of effects including cell invasion, differentiation, survival or apoptosis, it is clear that we have much to learn about the specificity of these signals and how these signaling pathways interact or become aberrant in melanoma cells.

Neurotrophin receptor signal transduction pathways

Our current knowledge of receptor tyrosine kinase (RTK) signal transduction pathways primarily involves integration of information from studies on insulin receptors, EGF receptors and neurotrophin receptors. It is generally agreed that these RTK receptors are involved in similar sequences of events that include ligand binding leading to receptor dimer formation and transactivation, resulting in tyrosine phosphorylation, with the eventual activation of serine/threonine phosphorylation cascades (Figure 3). Active signaling complexes are frequently formed by interactions between receptor phosphotyrosines and proteins containing SH2 (Src homology-2) tyrosine-binding domains. For example, the activated $pp140^{trkA}$ receptor binds to SH2-bearing phospholipase-Cγ1 (PLCγ1) [59] and a 38 kDa phosphoprotein of unknown function [16]. In contrast to other RTKs, however, this occurs in the absence of detectable associations with GTPase-activating protein (GAP). Furthermore, unlike other RTKs, phosphotidylinositol-3 kinase (PI3-K) is transiently activated [60], and accumulation of inositol phosphate-3 (IP3), calcium mobilization or an association of $pp140^{trkA}$ with p85 does not occur [60]. Activation of TRK signal pathways may involve Shc, an SH2-containing protein that induces neurite outgrowth in PC12 cells following Shc transfection [61]. In addition, a number of reports have subsequently demonstrated complex formation between the $pp140^{trkA}$ receptor and the SH2 domain of Shc [62–66]. Formation of this complex leads to tyrosine phosphorylation on Shc and the association of Shc with Grb2, another SH2-containing protein [62–66]. Binding sites for PLCγ1, p85/PI3 and Shc occur on tyrosines 785, 751 and 490, respectively [62, 63, 66]. Functional signaling complexes for PC12 cell neurite extension appear to rely on possible co-operative interactions between PLCγ1 and Shc but PI3-K does not seem to be essential [67]. The association of Shc with Grb2 can lead to further complex formation with the $p21^{ras}$ nucleotide exchange factor Son of Sevenless-1 (SOS-1). This may result in increased GTP-binding and activation of $p21^{ras}$, a GTP-binding oncoprotein originally identified in a rat sarcoma virus [68].

The downstream effectors of $p21^{ras}$ include proteins involved in serine/threonine phosphorylation cascades [69]. Studies have demonstrated that $p21^{ras}$ can coordinate the NGF-mediated, phosphorylation-dependent activation of several key growth and differentiation molecules including: (1) c-Raf-1, a cytoplasmic ser/thr kinase discovered as the oncoprotein *v-raf* in a mouse sarcoma virus; (2) mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK kinase, MEK); and (3) mitogen activated protein kinase (MAPK). The activation of MAPK can transiently induce the expression of a number of primary response genes that encode transcription factors, such as *c-fos*, *c-jun*, NGFI-B, and *krox24* [70]. The MAPK activity may also affect other ser/thr kinases and/or cytoskeletal elements [71]. Recently, MEK kinase (MEKK), a ser/thr kinase that can activate MEKs independently of c-Raf-1, has been observed to phosphorylate MEK in PC12 cells as they respond to NGF [72].

The recent connection between Ras and Raf pathways has prompted a great deal of research interest [69]. The association of Ras with Raf may help to explain the coupling of signal transduction pathways initiated by the tyrosine kinases to the ser/thr phosphorylation cascades that result in cell differentiation or division. Following a series of postranslational modifications (farnesylation, proteolysis and carboxymethylation), Ras is known to be transported to and becomes situated on the inner leaflet of the plasma membrane. The c-Raf-1 protein is characterized by three highly conserved regions,

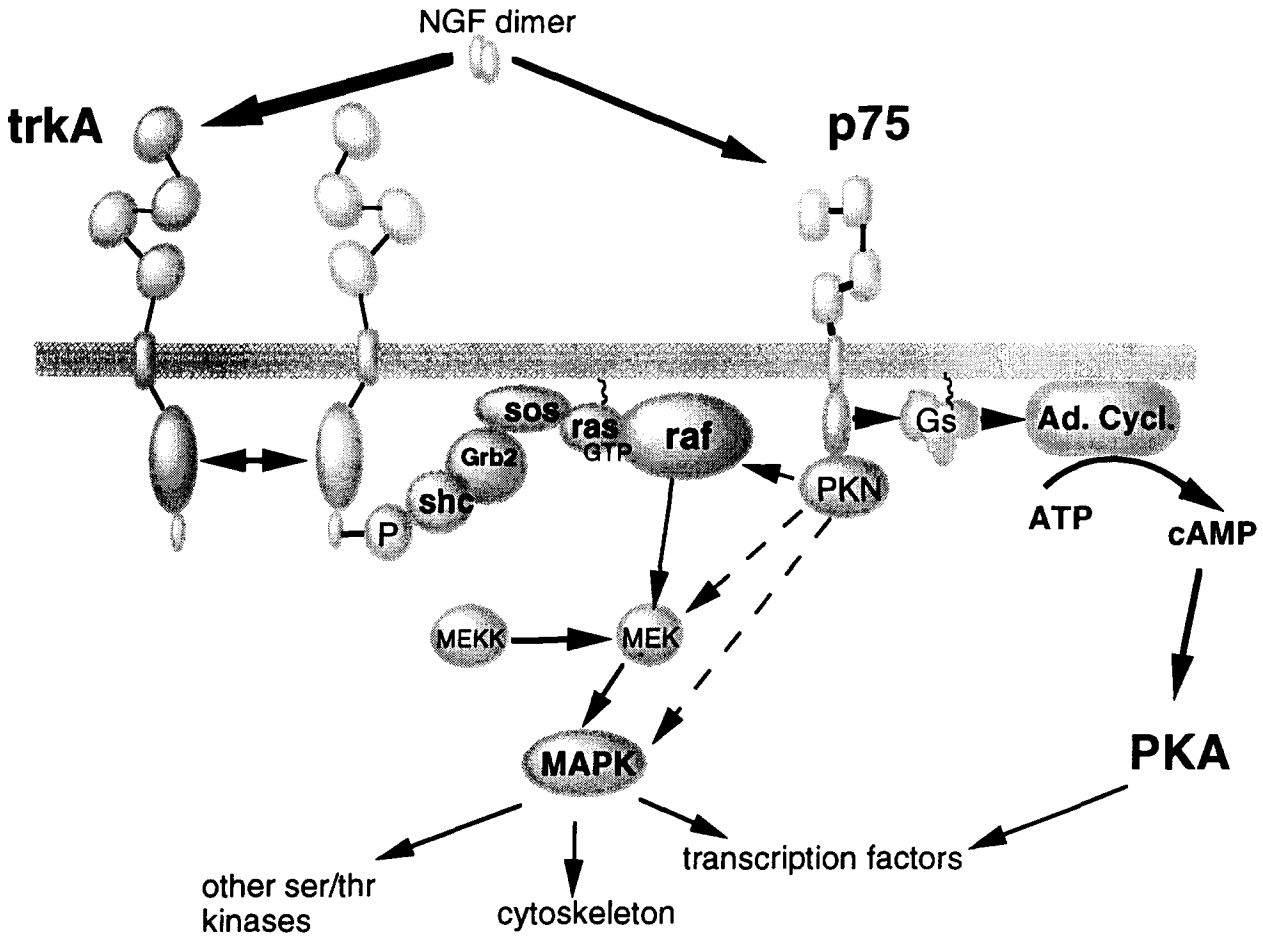

Figure 3. A diagram of the possible mechanisms involved in neurotrophic signal transduction. The dimerized form of the NGF molecule, as an example, binds to either 140^{trkA} or $p75^{NTR}$. Homodimerization followed by phosphorylation of 140^{trkA} and possible recruitment of $p75^{NTR}$ leads to the cytosolic binding of src homology 2 (SH2) phosphotyrosine binding proteins. The SH2 domain of Shc binds to the phosphotyrosine at position 490 on the Trk cytoplasmic domain. Phosphorylation of Shc promotes the SH2 driven binding and activation of Grb2 that binds to and activates Son of Sevenless 1 (SOS 1) a nucleotide exchange factor. The action of SOS 1 elicits the stabilization of GTP association with p21ras. The activated form of p21ras-GTP is stably associated with the plasma membrane during it's recruitment to the signaling complex leading to Raf-1 binding to p21ras-GTP. Raf-1 activation occurs by an unknown mechanism that may utilize as yet unidentified factors and causes the ser/thr phosphorylation of MEK (MAPK/ERK) that in turn phosphorylates mitogen activated protein kinase (MAPK) on ser/thr. MAPK activation leads to further activation of other ser/thr kinases, cytoskeletal elements and transcription factors (excluding fos/jun). The binding of NGF to $p75^{NTR}$ can initiate G-stimulatory protein complexes (Gs) to activate cyclic adenosine monophosphate (cAMP) production by adenylate cyclase and activation of protein kinase A (PKA) followed by transcriptional factor activation. The $p75^{NTR}$ receptor is associated with the ser/thr phosphorylate protein kinase N (PKN) which may interact directly with Raf 1, MEK or MAPK to amplify the NGF response. Other MEK kinases (MEKK) may act on the ser/thr kinase cascade independently of Raf-1.

CR-1 containing a zinc finger, CR-2 with no distinctive motif and CR-3 which inscribes a catalytic domain [69]. Recent studies have demonstrated that Raf-1 associates with the plasma membrane and becomes constitutively activated if the Ras-derived CAAX farnesylation motif is added to the carboxyl terminal of Raf-1 [73, 74]. Furthermore, starvation of cells stimulates the formation of a membrane-bound, activated Raf-1 complex containing two molecular chaparones, heat shock protein 90 (hsp90) and an unspecified 50 kDa protein [75]. Activated Raf-1 phosphorylates MEK but the mechanism of Raf activation remains uncertain. It has been speculated that other ligands, phosphorylation reactions by downstream ERKs, or other kinases or zinc finger interactions may be essential for Raf-1 activation [69]. It is interesting that there may be convergent pathways between the Trk signaling pathway and the

cooperative interaction of $p75^{NTR}$ by virtue of activating Raf-1 by $p75^{NTR}$/PKN or direct activation of MEK or MAPK by $p75^{NTR}$/PKN (Figure 3). Recently, MEK (ERK1) and MAPK (ERK2) were found to co-immunoprecipitate with $p75^{NTR}$ lending support for this hypothesis [76]. CHO cells transfected with $p75^{NTR}$ alone, however, did not result in activation of downstream effector molecules such as Raf-1 or MAPK, indicating that $p75^{NTR}$ alone is probably insufficient to activate this pathway [77].

Activation of the sphyngomyelin cycle may be an alternate signaling pathway for $p75^{NTR}$. Recent studies using $p75^{NTR}$-expressing T9 glioma cells and addition of cell-permeable ceramide analogs resulted in growth inhibition and the formation of dendritic cell processes [78]. The sphyngomyelin pathway also seems to be important during signaling by TNFα receptors, and this pathway appears to involve a ceramide-activated protein phosphatase [79]. This alternate form of signal transduction by $p75^{NTR}$ may be important to cells invading the brain. Brain tissue injured by tumor cell invasion may provide a ready source of ceramide that might also influence invading cells.

Neurotrophin receptors and progression of malignant melanoma cells

Malignant melanoma cells are transformed melanocytes derived from the neural crest and their expression of certain cell surface receptors is indicative of this origin. During malignant progression melanoma cells show progression-associated increases in the expression of $p75^{NTR}$ for neurotrophins [3, 36]. In situ examination of $p75^{NTR}$ expression revealed increased synthesis in advanced stages of malignant melanoma [80]. Human melanoma cells established in short term tissue culture from brain metastases exhibit characteristic chromosomal alterations [81]. There is a high frequency of translocation or deletion breakpoints at the 23q locus on chromosome 11 [81]. There is also a high frequency of terminal translocations at the q25 locus on chromosome 17 or an isochromosome for the long arm of chromosome 17 [81]. It is significant that, although $p75^{NTR}$ expression was not examined in these cells, [81] the gene is located at 17q21–22 and may be amplified in tumor cells containing the isochromosome. The neural cell adhesion molecule (NCAM) locus is at 11q23 and may also be important in establishing melanoma brain metastases.

We have examined the role of neurotrophin receptors in brain invasion and colonization of malignant melanomas. Using a human melanoma variant cell subline (70W) that has the capacity to form colonies in the brains of nude mice, we have studied the effects of neurotrophins and growth factors on the malignant properties of these cells. The 70W subline was derived as one of a series of human MeWo melanoma cell variants selected by treatment with wheat germ agglutinin [82]. Parental MeWo cells exhibit intermediate metastatic potential compared with other cell lines, such as the nonmetastatic subline 3S5 and the brain-metastatic 70W subline. Using the MeWo melanoma cell lines, we have shown that overexpression of $p75^{NTR}$ is associated with brain colonization and enhancement of extracellular matrix invasion [36].

Neurotrophins enhance matrix invasion and degradative enzyme activities of brain-metastatic melanoma cells

To evaluate the effect of NGF on brain-metastatic melanoma cells we used Matrigel™-coated membranes, and as a chemoattractant we placed brain microvessel endothelial cell-conditioned medium in the lower chamber of a Transwell™ apparatus. The presence of brain endothelial cell motility factors in the invasion assay was essential; endothelial cell motility factors from lung or liver did not substitute for brain endothelial cell motility factors. Melanoma cells that migrate into the lower chamber of the Transwell™ apparatus must first degrade the matrix barrier.

Following NGF treatment, brain-invasive 70W melanoma cells showed a 7.9-fold increase in the extent of matrix invasion over a 72 h period (Figure 4). In NGF treated 70W cells, there were corresponding increases in MMP-2 type IV collagenase/gelatinase A and heparanase activities, but there was a slight decrease in NGF-treated 70W cells in the production of MMP-9 type IV collagenase/gelatinase B (Figure 5). In comparison, NGF treatment induced a 2.1-fold increase in matrix invasion by MeWo parental cells and no increase in invasion of nonmetastatic 3S5 cells (Figure 4). NGF treatment of MeWo cells also increased their production of MMP-2 gelatinolytic activity and increased their heparanase activity. The noninvasive, nonmetastatic 3S5 cells exhibited a slight decrease in gelatinase A and heparanase activities (Figure 5). The ability to invade a reconstituted basement membrane was only apparent if the cells were grown on extracellular matrix and placed on an invasion substrate in the presence of brain microvessel endothelial cell-conditioned medium, suggesting that in addition to their response to neurotrophic factors, melanoma cells must receive the appropriate matrix and paracrine motility signals to be highly invasive

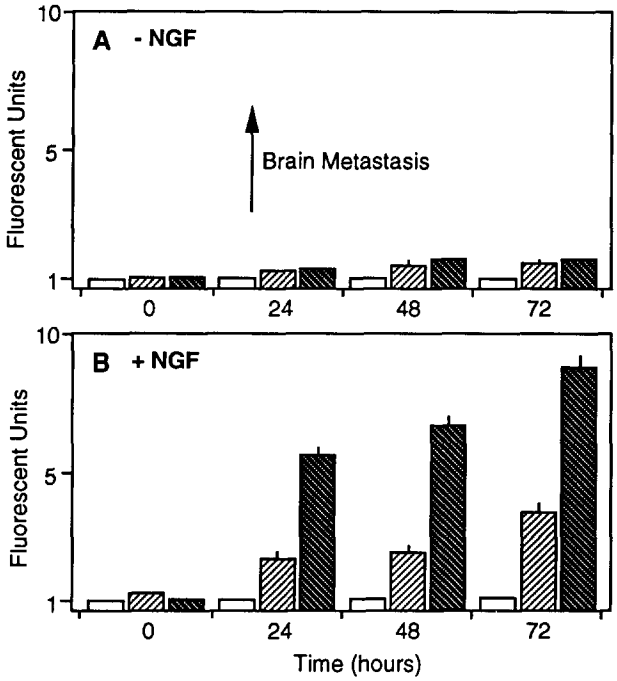

Figure 4. Treatment of human melanoma cells with 2.5S NGF enhances invasion of ECM as shown in an invasion assay using a Transwell™ apparatus [5]. Briefly, reconstituted basement membrane Matrigel™ (Collaborative Research, Bedford, MA) was applied to the upper surface of a polycarbonate filter insert in a Transwell™ chamber (Costar, Cambridge, MA). Cells were seeded into the upper compartment in serum-free medium with or without the addition of 50 ng/ml 2.5S NGF. The lower compartment contained human brain microvessel endothelial cell-conditioned medium as a chemoattractant. The cells were incubated for 72 h on Matrigel™ and the number of cells that penetrated the Matrigel and filter were counted after fixation and staining [5]. ■, 70W; ▨, MeWo; □, 3S5.

[36, 83]. In fact, adhesive contact with RGD-containing substratum may be essential for the proper expression and function of $p75^{NTR}$ [36].

Elevated $p75^{NTR}$ expression in brain-metastatic 70W melanoma cells

We examined the expression of $p75^{NTR}$ on brain-metastatic 70W cells by $p75^{NTR}$ immunoprecipitation analysis of radioiodinated cell surface proteins. The anti-$p75^{NTR}$ monoclonal antibody Me20.4 specifically precipitated more of an appropriately sized, radio-iodinated component in lysates of the 70W and A875 cells (Figure 6, lanes 3 and 4) than of the MeWo or 3S5 cell lysates (Figure 6, lanes 1 and 2). The expression of $p75^{NTR}$ on the surface of the MeWo parental cell line was low but detectable, and we could not detect $p75^{NTR}$ on nonmetastatic 3S5 cells. This indicates that the amount of $p75^{NTR}$ expressed on the cell surface by the nonmetastatic melanoma cells was below the sensitivity of the immunoprecipitation assay, or the $p75^{NTR}$ exists as a cytoplasmic form of the mature cell surface $p75^{NTR}$. The observed Mr for the protein immunoprecipitated by the anti-$p75^{NTR}$ antibody was ~90 000 for MeWo cells and ~97 000 for 70W cells, consistent with other reports on $p75^{NTR}$ [13, 84]. The observed difference in Mr between the MeWo and 70W $p75^{NTR}$ receptors probably reflects differences in glycosylation resulting from lectin selection of the 70W

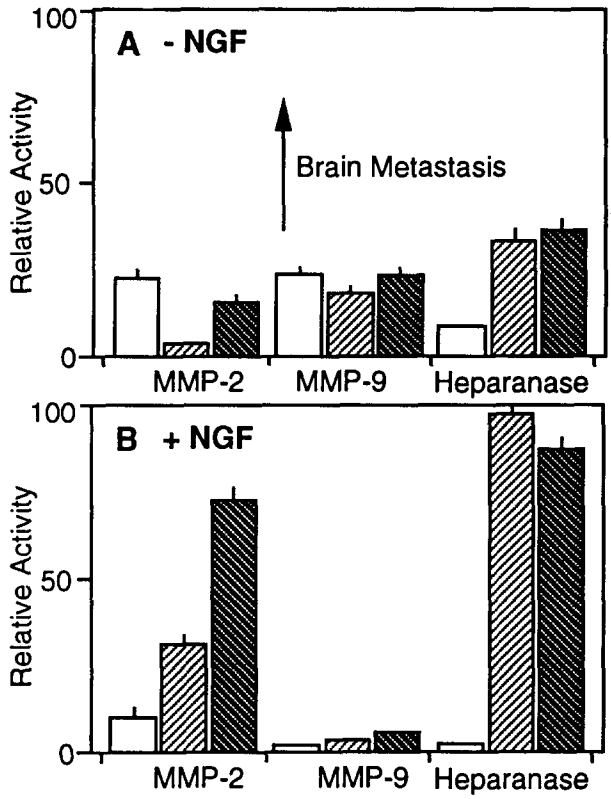

Figure 5. NGF-induced production of degradative enzymes in human MeWo melanoma sublines. Degradative enzymes were determined by assaying the melanoma cell-conditioned medium (72 h) in the upper chamber of a Transwell™ apparatus that contained endothelial cell-conditioned medium in the lower chamber. Gelatinase A and gelatinase B activities are represented as the level of increase following NGF treatment after digitizing and integrating the total gelatinolytic area per cm^2 on a zymogram using computer analysis [5]. Heparanase activity is represented as the level of increase following NGF treatment and separation by HPLC [14]. The increases in degradative enzymes were found in the following order: 70W > MeWo > 3S5. Standard deviations did not exceed 10% in the primary data used for these comparisons. ■, 70W; ▨, MeWo; □, 3S5.

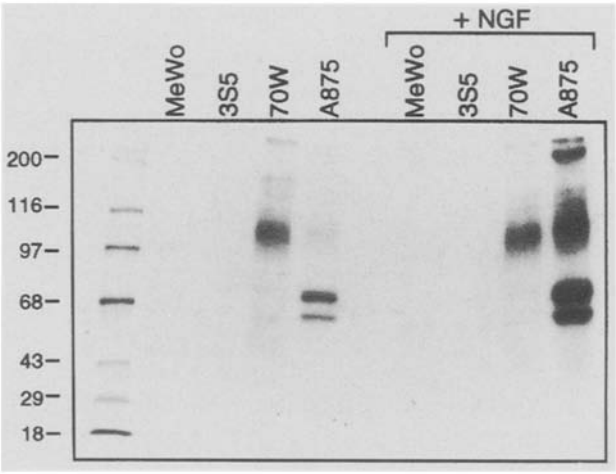

Figure 6. NGF receptor $p75^{NTR}$ expression correlates with invasive behavior and the ability to form brain metastases. Immunoaffinity isolation of cell surface radioiodinated proteins was performed using anti-$p75^{NTR}$ monoclonal antibody and resolved by SDS–PAGE. We used a vectorial cell surface-labeling procedure that retains biological activity in whole cells [141]. Briefly, subconfluent cell cultures were washed and removed with buffer containing 2 mM EDTA. After resuspension, 2×10^7 tumor cells were radioiodinated with ^{125}I-sulfosuccinimidyl-3(4-hydroxyphenyl)proprionate (Pierce, Rockford, IL). Radiolabeled cells were solubilized in CHAPS solubilization buffer at five times the packed cell volume. CHAPS detergent cell lysates were precleared with affinity purified rabbit anti-mouse IgG(H + L) and recombinant protein A/G (Oncogene Science, Manhassat, NY). After centrifugation, the supernatants were immunoprecipitated with the appropriate antibody: 10 μg of anti-$p75^{NTR}$ ME20.4 monoclonal antibody (Amersham, Arlington Heights, IL). After washing, agarose beads were treated with SDS sample buffer and electrophoretically resolved on a 6–16% moving boundary velocity gradient-SDS-polyacrylamide gradient gel electrophoresis. Gels were fixed, vacuum dried and exposed to Kodak XAR 5 film for 72 h with an intensification screen at −70°C and then photographically processed. Weakly immunoreactive products were observed in samples from MeWo parental cells (lanes 1 and 6) and nonmetastatic 3S5 cells (lanes 2 and 7). Strongly immunoreactive products were observed in samples from brain-metastatic 70W cells (lanes 3 and 8) and A875 cells established from brain metastasis (lanes 4 and 9). NGF (10 mg) increased the amounts of immunoreactive products (lanes 7–10).

cells. We also observed in the A875 melanoma cell lysates the presence of lower Mr forms of $p75^{NTR}$ that were previously described to be the result of proteolytic processing of the low-affinity NGF receptor (Figure 6, lane 4) [85].

To evaluate if NGF binding involves the formation of NGF receptor complexes, immunoprecipitation was performed in the presence of 10 μg of exogenous NGF. Addition of excess NGF caused a significant increase in the amount of immunoprecipitate formed and an increased organization of proteins into high Mr immunocomplexes (Figure 6, lanes 7 and 8). A complex of Mr ~200 000 was increased in amount by prior treatment with NGF or the presence of excess exogenous NGF. Similar ~200 kDa complexes with high affinity for NGF have been reported to be formed on A875 cells after NGF treatment [86]. In addition we observed receptor complexes by immunofluorescence using anti-$p75^{NTR}$ that are rapidly turned over following treatment (unpublished observations). These data suggest that once neurotrophins such as NGF bind to melanoma cells, multimeric complexes containing $p75^{NTR}$ form by lateral aggregation of receptors.

Brain-metastatic 70W melanoma cells spontaneously produce growth factors

The production of autocrine and paracrine growth factors by MeWo melanoma cells could influence their invasion and growth in the brain. To determine those that could be important in brain metastasis, we examined 70W melanoma cells for the synthesis of various growth factor transcripts by reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR revealed the production of TGF-β1 and bFGF (Figure 7a, lanes 1 and 3) as well as TGFα and interleukin IL-1β (Figure 7b, lanes 1 and 3). There was no transcript observed using PDGF primers. All primers were intron-spanning to ensure that the PCR products were RNA-derived and not derived from genomic DNA contamination. The TGF-β1, bFGF, TGFα and IL-1β products migrated at the expected product sizes (161bp, 177bp, 441bp, 802bp, respectively). Thus we concluded that brain metastatic 70W cells expressed certain cytokines that might be important in conditioning the brain microenvironment or alternatively, these factors could operate to stimulate autocrine growth.

Brain tissue neurotrophin production at the melanoma tumor invasion front

After establishing that MeWo 70W melanoma cells could produce growth factors, including TGFβ, TGFα, bFGF, and IL-1β, we reasoned that these might act as paracrine factors influencing neurotrophin production in the brain. Many of these factors can stimulate brain astrocytes or oligodendrocytes to produce neurotrophins. Therefore, we examined

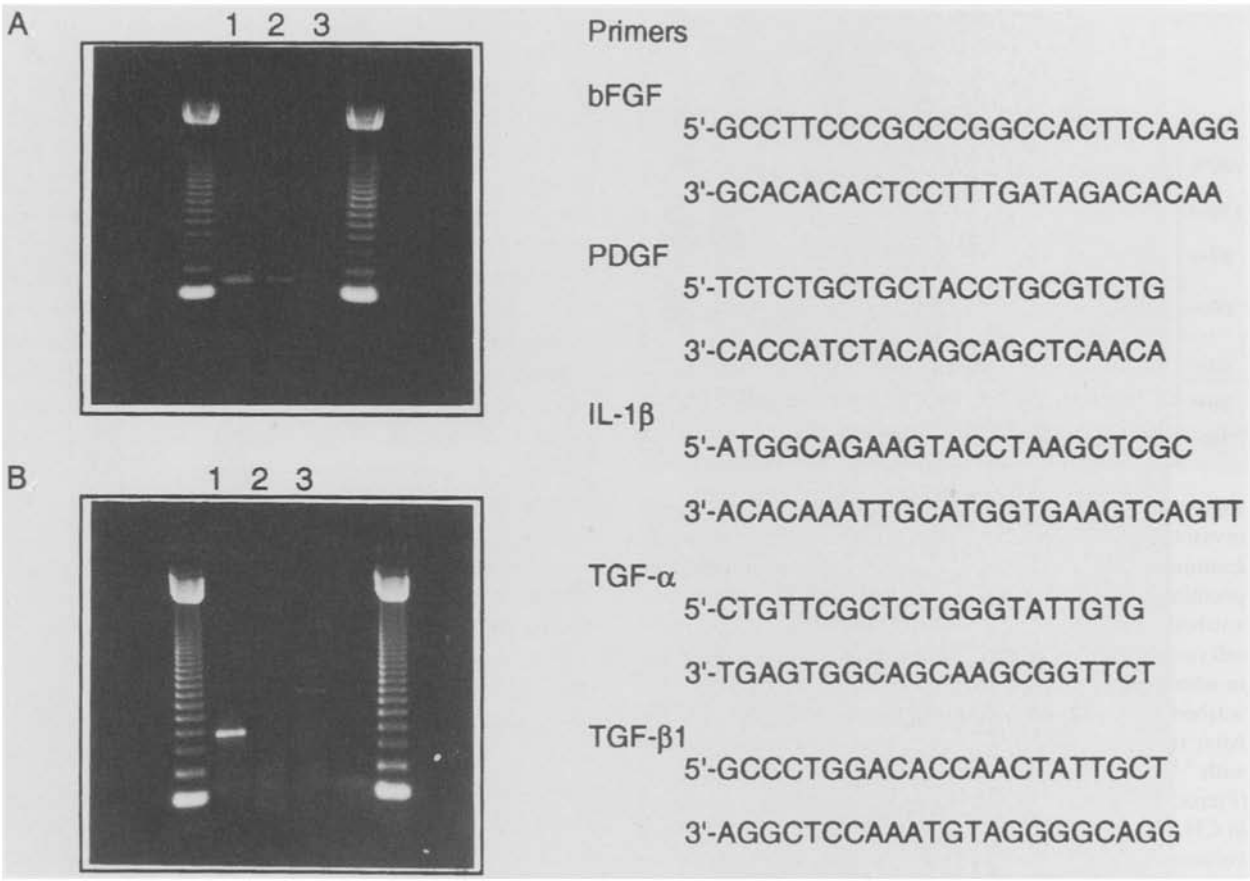

Figure 7. Detection of growth factor transcripts produced by brain-colonizing 70W cells. Total RNA was isolated from subconfluent cell cultures by extraction with guanidinium thiocyanate-phenol-chloroform [142]. Total RNA was used as a template for first strand cDNA synthesis using Superscript AMV reverse transcriptase (Gibco/BRL, Gaithersburg, MD) and oligo-d(T) as an extension primer. Oligonucleotide primers were prepared by GenoSys Biotechnologies (The Woodlands, TX) for PCR analysis based on their specificity using GCG Sequence Analysis Software (Genetics Computer Group, Madison, WI) and the OLIGO-4 Primer/Probe Analysis Software Package (National Biosciences, Plymouth, MN). Primer, template, salt concentration and annealing temperatures were provided by OLIGO-4. The amplification profile consisted of an initial template denaturation step at 94°C for 2 min followed by 30 cycles of 94°C, 1 min; 60°C, 1 min; 72°C, 1 min. Amplification products were analyzed on a 1% agarose/TBE gel using a 123 bp ladder fragment size standard (Gibco/BRL). All primer sequences were based on published cDNA sequences for the various cytokines. RT-PCR demonstrated the production of TGF-β1 and bFGF, (a1 and a3) in addition to TGFα and IL-β1 (β1 and β3). The expected product sizes of 161 bp, 177 bp, 441 bp, and 802 bp, correspond to TGF-β1, bFGF, TGFα and IL-β1, respectively. The use of intron-spanning primers assured that all products were derived from cDNA, not genomic DNA.

whether brain-invading melanoma cells can induce changes in NGF concentration or distribution at the invading edge of melanoma tumors in vivo. Brain-tissue samples from human melanoma metastases and uninvolved brain tissues in adjacent sections were examined immunohistochemically for the presence of NGF. Hematoxylin-eosin staining confirmed the presence of brain invasive melanoma and adjoining brain tissue with extensive gliosis [10]. Staining of serial sections with anti-NGF monoclonal antibody revealed increased concentrations of NGF in the tumor-adjacent tissue at the invasive front. Staining was highest at the interface between melanoma and adjacent normal brain tissue and gradually decreased in concentration until NGF was undetectable at more distant sites [10]. Controls without primary antibody [10] or uninvolved brain tissue distant from the melanoma lesion [10] possessed very low or undetectable concentrations of NGF.

Paracrine growth factors and melanoma brain metastasis

Tumor cells metastatic to brain must respond to local growth factors in the brain environment. A major

tissue-derived paracrine growth factor for metastatic melanoma cells has been purified to homogeneity [87] and found to be a transferrin (Tf) [88]. Tf-like factors (TfLFs) are probably used as paracrine growth stimulators at organ sites such as lung, bone and brain. TfLFs and Tf may utilize the same receptor on melanoma cells, the ~180 kDa dimeric Tf receptor. We examined the ^{125}I-labeled Tf-binding properties and growth response to Tf of tumor cell sublines of different metastatic properties. In the murine B16 melanoma system, we found that brain-colonizing sublines exhibited the greatest growth response to Tf, followed in order by the ovary-colonizing, the highly lung-colonizing, and finally the poorly lung-colonizing sublines. The rank order of binding of ^{125}I-labeled Tf to the B16 cell sublines paralleled their growth responses to Tf [89]. We also found a close relationship between the binding of ^{125}I-labeled Tf, growth responses to Tf, and spontaneous metastatic potential in a rat mammary adenocarcinoma model. The results indicated that Tf receptor numbers increased as spontaneous metastatic properties increased in the following order: high brain-metastasizing ability > high lung-metastasizing ability > poor metastatic capability [90]. Examination of the responses of human melanoma cell lines to Tf in the absence of FBS indicated that the brain-metastatic sublines responded best to Tf (Figure 8A) and expressed the highest numbers of Tf receptors (Figure 8B), suggesting that Tf response may be an important property of brain-metastatic melanoma cells.

Overexpression of particular growth factor receptors may be important in metastatic cell growth response at certain sites, and also in stimulating the growth responses of normal cells at sites of wounding or inflammation. Tumor cells that express high numbers of Tf receptors should be able to respond to low, limiting concentrations of Tf that exist in some tissue compartments, such as the brain. In the brain, Tfs, or more likely Tf-like growth factors (TfLFs), are probably used as paracrine growth factors during fetal development [91]. With the possible exception of the choroid plexus, uninjured adult brain does not synthesize large amounts of Tf, and Tf is normally present in limited quantities in the brain, probably due to its poor penetration through the blood-brain barrier. When malignant cells metastasize to brain, it may be advantageous for them to express high numbers of Tf receptors and to respond to low concentrations of Tf. Alternatively, brain synthesizes TfLFs that may not possess the same efficiency in binding to the Tf receptor. We have recently found that fetal brain synthesizes relatively large amounts of a TfLF that we have called TfLF-3 [92]. It is likely

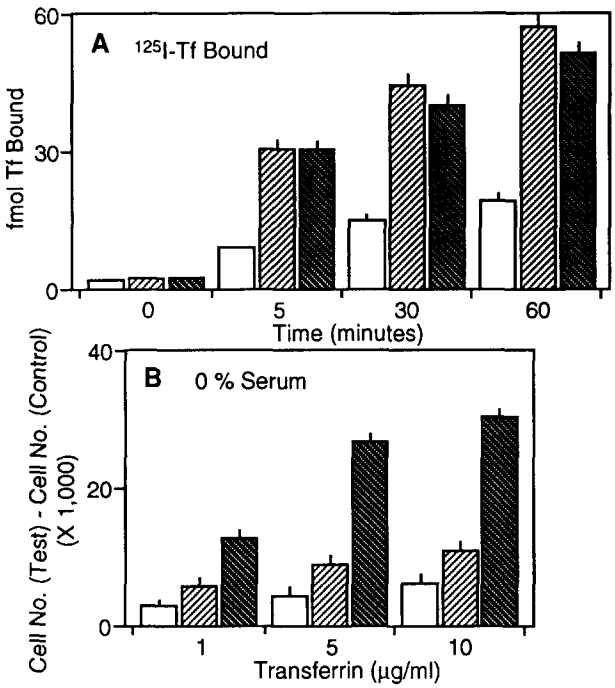

Figure 8. Binding of ^{125}I-labeled Tf to human MeWo melanoma variants and their growth responses to Tf. **A**; time course of binding of ^{125}I-labeled Tf. Murine or human Tf (5 mg/ml) in phosphate-buffered saline (PBS) was saturated with iron by incubation with ferric ammonium citrate (0.1 mg/ml) in 0.01 M NaHCO3 for 4 h at room temperature. Unbound iron ions were removed by dialysis against PBS overnight. Iron-saturated Tf was labeled with ^{125}I according to Inoue *et al.* [90]. The specific activities of ^{125}I-labeled Tf ranged from 60 to 65 cpm/fmol. Confluent cell monolayers were incubated with ^{125}I-labeled Tf for various times at 4°C washed with PBS, and cell-associated radioactivity was determined. Nonspecific binding of ^{125}I-labeled Tf was estimated by the amount of labeling in the presence of a 200–1000-times excess concentration of unlabeled Tf. ■, 70W; ▨, MeWo; □, 3S5. **B**; growth response to Tf in the absence of fetal bovine serum at 72 h. Cells were plated at 1000 cells/well in 96-well plates or at 5000 cells/well in 24-well plates in medium containing 1% fetal bovine serum. After 24 h, the medium was changed to that containing 0.3% or 0% serum and increasing amounts of human-holo-Tf was added. After 3–6 days, the cells were enumerated using a crystal violet cell quantitation assay.

that TfLF-3 is only one of several growth and inhibitory factors important in the organ preference of metastatic cells to the brain [93]. TfLF-3 is synthesized only in low amounts in uninjured brain, but after wounding (and probably after brain injury by invading malignant cells) TfLF-3 synthesis is dramatically increased. The expression of TfLF-3 then appears to return to normal low levels once the brain injury is repaired. In the normal brain Tfs/TfLFs

are produced primarily by oligodendrocytes and astrocytes in the choroid plexus, cerebral plexus, amygdala, hippocampus, brain stem and cerebellar Purkinje cells [94–96]. The production of Tfs (more likely TfLFs) by brain astrocytes can be induced by both IL-1 and TNFα [97]. Injury to the brain leads to a significant increase in both Tfs/TfLFs and TfR expression [98]. Similarly, during brain injury astrocytes can also respond to bFGF, EGF, IL-1α and interleukin 2 (IL-2) [99].

Some cell types in the brain, such as oligodendrocytes and type 2 astrocyte (O-2A) progenitor cells, can respond to trauma-associated mitogenic cues from bFGF and PDGF [100]. Microglial cells also synthesize cytokines in response to trauma, such as bFGF, IL-1 and TNFα [101–103]. Brain trauma that occurs during the pathogenesis of glioma brain tumors often leads to the production of both bFGF and vascular endothelial cell growth factor (VEGF). The levels of bFGF and VEGF are the highest in the anaplastic astrocytes that surround abnormal blood vessels, usually areas of endothelial cell proliferation [104]. Brain microvascular endothelial cells are also important sources of cytokines [105]. Furthermore, since endothelial cells respond to angiogenesis factors released by tumor cells, a reciprocal relationship probably exists between tumor cells and specific organ-derived endothelial cells at the secondary site (Figure 9). Coculture and conditioned medium experiments have provided important information about the reciprocal cytokine relationships between tumor cells and their parenchymal counterparts (reviewed in [89]). This reciprocal cytokine regulation of growth probably also extends to parenchymal cell types as well as to extracellular matrix (Figure 9). The observation of metastatic growth explosion at certain

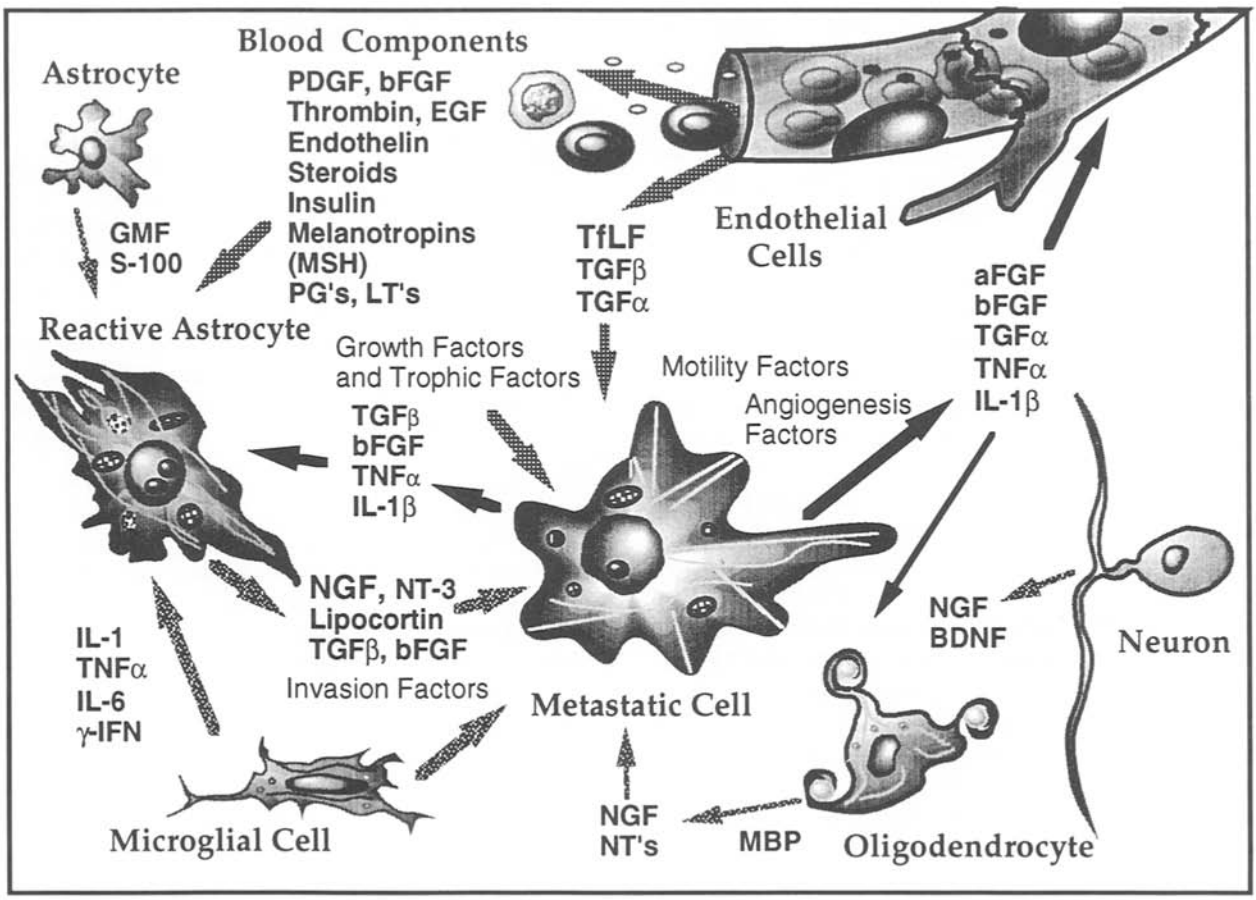

Figure 9. Reciprocal interactions between brain-invading melanoma cells and normal cells in the brain microenvironment. Tumor cells release cytokines that can affect host cells such as parenchymal cells, endothelial cells, glial cells, oligodendrocytes, astrocytes and host tissue extracellular matrix. Reactive astrocytes can arise from stimulation by blood derived vasogenic factors, factors released by brain-invading melanoma cells and factors released from other brain cells. In turn, the host cells release factors that stimulate or inhibit tumor cell motility and proliferation. Astrocytes, oligodendrocytes and neurons can release neurotrophins in response to brain-invading melanoma cells.

organ sites can easily be explained by the notion that reciprocal release of cytokines and other factors by tumor and host cells stimulate the motility, invasion, and growth of both tumor and host cells.

In the confined environment of the brain, inhibitory cytokines help to moderate the growth stimulatory effects of many other growth factors. Following brain injury there is an increase in TGFβ production coincident with an inhibition of astrocyte proliferation. It is thought that TGFβ suppresses the mitotic effects of bFGF and EGF on astrocytes and microglial cells [99, 106, 107]. Interestingly, inhibition of astrocyte cell division is accompanied by transient increases in NGF production, whereas BDNF levels remain unchanged [99, 106, 107]. Certain melanoma cells that colonize the meninges and ventricles are growth stimulated by TGF-β while others that colonize the brain parenchyma are growth inhibited by TGF-β [108]. Other growth factors, such as EGF, IL-1β, TNFα acidic fibroblast growth factor (aFGF) and bFGF, can also stimulate NGF synthesis, a response that can be potentiated by treatment with dibutyryl-cAMP [109–113]. Another cell type that can increase the synthesis of NGF in response to bFGF treatment are the fibroblasts found in the meninges [110]. An important inhibitory cytokine for melanoma cells produced by astrocytes after TNF treatment was identified as interleukin 6 (IL-6) [114, 115]. Neurotrophin BDNF and NGF production is regulated in hippocampal neurons and astrocytes by glutamate and the gamma-aminobutyrate (GABA) system in response to neuronal activities and cytokines [116]. Another neurotrophin, ciliary neurotrophic factor (CTNF) can be produced by astrocytes [117]. The normal role of these neurotrophins is to promote neuronal cell survival, but they also can promote the survival of brain metastatic cells [118].

The brain as a unique compartment for the invasion and growth of malignant melanoma cells

Homeostasis and the control of material flow into the brain is strictly regulated by the blood-brain barrier. Anatomically, the barrier is defined by specialized endothelial cells that are jointed by an extensive network of tight junctions. The endothelial barrier is supported by a thick basement membrane and underlying astrocytes that control the traffic of ions, nutrients and cells into the brain. This is a formidable barrier that metastatic cells must breach to invade into and colonize the brain parenchyma. As discussed above, invasion into brain requires that metastatic cells increase their expression of certain cell surface receptors, degradative enzymes, growth factors and possibly cytokines, and they must respond to invasion-stimulating cytokines such as neurotrophins and paracrine growth factors.

Brain-metastasizing melanoma cells express relatively high levels of basement membrane hydrolytic enzymes, such as type IV collagenases, cathepsins, plasminogen activators and heparanase [89]. For example, we found that murine and human melanoma cells that possess high brain-colonization properties secreted the highest amounts of various basement membrane-degrading enzymes [89]. Although highly metastatic cells generally expressed higher amounts of degradative enzymes than nonmetastatic cells, as discussed above, some of these enzymes may be induced to even higher levels by the microenvironment (paracrine invasion factors), or they can be provided by certain normal cells (microvessel endothelial cells, among others) [119]. If the appropriate paracrine signals are received by malignant cells, they can be stimulated to increase their synthesis and release of blood-brain barrier-degrading enzymes. For example, as discussed in previous sections, we found that brain-metastatic human and murine melanoma cells are sensitive to exogenous NGF [36, 83] and treatment of brain metastatic cells with NGF increases their expression of MMP-2 type IV collagenase, gelatinase A and heparanase (Figure 5).

Cellular responses to brain tissue injury as a paradigm for brain metastasis

Astroglial cells constitute the primary cellular response following brain injury [120]. Astrocytes are the predominant cell type in the brain, outnumbering neurons by a factor of ten to one. These numbers mean that astrocytes make up one-third of the cerebral cortex; however, as a population of cells they are widely heterogeneous [121]. Astrocytes are organized into a well developed syncytium containing gap junctions that mediate homeostasis and intercellular communication [122]. Astrocytes are influenced by neuronal interactions and regulate brain function including: the blood brain barrier; water, ion and basal metabolism; immune responses neuronal cell migration; neurite outgrowth, and functional synapse formation [120]. The integrated organization of the astrocytic cellular compartment of the brain provides tremendous potential for large scale recruitment of astrocytes in response to tumor cell invasion.

One of the earliest pathological responses to brain trauma involves astrocyte swelling occurring

predominantly in the perivascular astrocytic endings [123, 124]. In experimental brain tumors, cerebral edema has been associated with significant alterations in vascular permeability [125]. If the blood-brain barrier is compromised, astrocyte swelling may involve vasogenic edema. In this case, the astrocytes swell as they take up proteins and water that may induce cytotoxicity due to increases in potassium ion and glutamate uptake [126]. This may also include the production of arachidonic acid metabolites [prostaglandins (PG's) and leukotrienes (LT's)] and diffusion of cytokines into the astrocytic cell compartment [120, 124], (Figure 9). It is generally believed that astrocyte swelling is caused by increases in intracellular osmolarity followed by water influx. This may occur without loss of blood-brain barrier integrity and may simply represent a redistribution of water from the neuronal cell compartment to the astrocytic cell compartment. This mild form of astrocyte swelling is generally not as severe as the astrocyte swelling that can result from vasogenic edema associated with trauma caused by tumor cell invasion. If astrocyte swelling becomes too severe it can cause astroglial cells to depolarize, leading to the loss of homeostatic ion gradients and membrane rupture resulting in cell death. These dynamic astrocyte changes in response to tumor cell invasion can lead to increased intracranial pressure and further complications. The massive tumor-induced response by astrocytes is a possible reason for small metastases causing severe symptoms, such as paralysis, headache, seizures, and impaired cognition.

In addition to astrocyte swelling, the most profound cellular response of brain tissue to invasive injury is the production of reactive astrocytes (fibrous astrocytes). This is a condition known as reactive astrocytosis or reactive gliosis [120]. Histologically reactive astrocytes exhibit cytoplasmic hypertrophy in the form of dense, elongated cellular processes or a fibrous-appearing glial scar. These fibrous processes stain positively for glial fibrillary acidic protein (GFAP) and vimentin intermediate filaments [120]. Unlike fibrosis in other scar tissues, gliosis consists predominantly of cellular processes or glial fibers and lacks collagen or equivalent fibrous extracellular matrix proteins. Reactive astrocytes often contain enlarged nuclei having multiple nucleoli (Figure 9). This is accompanied by increases in the numbers of organelles such as mitochondria, golgi, endoplasmic reticulum, lysosomes, microtubules and dense bodies. Membrane alterations include increases in hemi-desmosomes and gap junctions [120]. Receptor up-regulation includes p185^{neu}, p145^{kit} and class II histocompatibility antigens [127, 128]. The induction of reactive astrocyte formation involves a number of cellular products from different brain cells including: glial maturation factor (GMF), S100 protein from astrocytes; IL-1, TNFα, IL-6 and γ-IFN from microglial cells; myelin basic protein (MBP) from oligodendrocytes; K^+, ATP and bFGF from neurons. Vasogenic edema leads to the influx of thrombin platelet derived growth factor steroids, insulin and various cytokines from the blood and lymphocytes as well as endothelin, ATP and bFGF from endothelial cells. The induction of reactive astrocytes, when associated with tumor cell invasion, is probably initiated by endogenous factors in the brain in addition to those provided by the invading tumor cells. The reactive astrocytes, for example, in addition to NGF and NT-3, can synthesize S100 protein, lipocortin (a precursor to β-melanocyte stimulating hormone, β-MSH), TGFβ and bFGF which can affect the reciprocal cytokine loops described previously (Figure 9). We have observed extensive reactive astrocytosis or gliosis in brain tissue associated with melanoma invasion front illustrating the cellular response of the adjacent brain tissue [10]. As previously mentioned, the adjoining brain produces high levels of NGF in comparison with uninvolved brain tissue in addition to the morphological changes observed [10]. Thus, brain-metastatic melanoma cells may induce the production of brain cytokines such as NGF that aid in the invasion and survival of melanoma cells in the CNS.

Melanotropic peptides may modulate astroglial cell and melanoma cell behavior during brain metastasis formation

The melanotropins are small stress-released brain neuropeptides that are derived from pro-opiomelanocortin (POMC) precursor molecules [129]. Pro-opiomelanocortin is proteolytically cleaved by a subtilisin-like serine protease, proprotein convertase (PC) [130] to form an N-terminal fragment containing γ-melanocyte-stimulating-hormone (γ-MSH); adrenocorticotropic hormone (ACTH) containing the sequence for α-melanocyte-stimulating-hormone (α-MSH) and β-lipocortin that contains both β-melanocyte-stimulating-hormone (β-MSH) and β-endorphin sequences (Figure 10A). Melanotropins are derived from the pituitary and hypothalamic neurons [131] and are capable of bidirectional passage across the blood barrier [132]. Traditionally, the melanotropins are known to control adrenal steroidogenesis (ACTH) and melanogenesis (MSH). It is now apparent that melanotropins have a

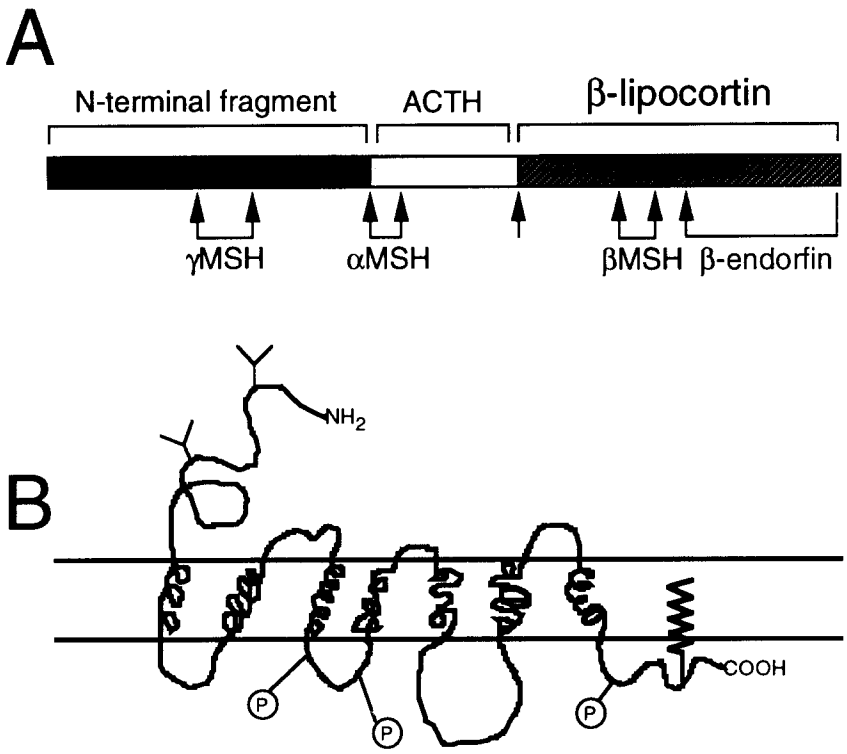

Figure 10. **A**: The structure and proteolytic processing of pro-opiomelanocortin (POMC). The arrows indicate the proteolytic cleavage sites contained within POMC. A large N-terminal fragment is the segment from which is derived γ-melanocyte-stimulating-hormone (γ-MSH). The internal fragment contains adrenocorticotrophic hormone (ACTH) that includes (α-MSH). The C-terminal fragment contains the sequences for β-lipocortin, β-melanocyte-stimulating-hormone (β-MSH) and β-endorphin. **B**: The MSH receptor contains seven trans-membrane domains with two extracellular N-glycosidation sites (Y symbols). The second intracellular domain contains two serine-phosphorylation sites and the consensus sequence for cAMP-dependent protein kinase recognition. The C-terminal segment contains a tyrosine-phosphorylation site and a site for the covalent attachment of palmitic acid (zig-zag line).

wide range of additional mitogenic and trophic functions, including mediating nerve regeneration and functional recovery from CNS trauma; modulating electrophysiological activity of nerve cells, regulating neuromuscular synapse formation; promoting growth stimulation and stimulating morphological differentiation of brain astrocytes [133]. The melanocortin receptors belong to a family of G-protein coupled receptors that rely on extracellular calcium for activity [134]. The melanoma MSH receptor consists of a 317 amino acid 34.8 kDa protein that has 7 trans-membrane domains (Figure 10B). The N-terminus has two potential N-glycosylation sites at positions 15 and 29, and the C-terminus has one phosphorylation site and a palmitoylation site. The second intracellular loop has two phosphorylation sites and contains consensus sequences for cAMP-dependent protein kinase recognition [133]. Stimulation of melanoma cells results in elevation of cAMP leading to melanogenesis, differentiation and growth inhibition of melanoma cells [135–137]. Surprisingly, treatment of murine melanoma cells with MSH can either enhance or suppress metastasis formation depending on the melanoma cells that are analyzed [136, 138]. Melanotropins can affect not only invading melanoma cells but also the morphology and behavior of brain astroglial cells (Figure 9). Notably, α-MSH can act within the brain to mediate a neurogenic anti-inflammatory response to a wide range of cytokines [139, 140]. If melanotropins alter the responsiveness of astrocytes to swelling or gliosis for example, this could significantly modulate their effectiveness in responding to invading melanoma cells. The pleiotropic nature of melanotropin effects may have important effects systemically following stress-induced release. A greater understanding of the signaling mechanisms used by MSH receptors and their role in melanoma metastasis to brain may provide useful targets for diagnosis and therapy in patients with melanoma.

Concluding comments

Clearly, we have much more to learn about the mechanisms utilized by melanoma cells in colonizing the brain. In this review we have attempted to identify certain topics that require further examination: 1) The response by brain tissues to invading melanoma cells requires further research emphasis. In particular, we need a better understanding of what triggers the reactive astrocytosis and the interactions between brain cells and invading melanoma cells. 2) We need a greater understanding of the brain microenvironment and the reciprocal cytokine signaling circuits that enable melanoma cells to invade and colonize the

brain. Since many of the cytokines involved in brain metastasis are effective at stimulating both the brain tissue and the invading melanoma cells, this will be one of the most difficult problems to decipher. It will be important to establish clearly, if possible, those growth factors that are essential in maintaining brain homeostasis versus those that are needed by invading melanoma cells. 3) The precise role of neurotrophins and melanotropins in promoting melanoma cell invasion, triggering differentiation and maintaining the survival or initiating apoptosis of both melanoma cells and brain tissues. 4) Understanding the trophic responses of brain-metastatic tumor cells may be important to understand not only how melanoma cells invade and colonize the brain but also how these factors promote tumor cell survival. The implications of trophic support as a tumor cell survival mechanism are certainly far reaching. Although neurotrophins and melanotropins are some of the best examples of trophic substances, certain growth factors not thought of as typical trophic factors may actually support survival of certain tumor cells. The implications of these phenomena may be particularly profound for the tumor biologist, clinician and most importantly the cancer patient.

Acknowledgements

These studies were supported by a grant from the US National Institute of Health and the National Foundation for Cancer Research, Inc. to G. L. Nicolson.

References

1. Steck PA and Nicolson GL, 1993, Metastasis to the central nervous system, In: Levine A and Schmidek H, eds. *Molecular Genetics of Nervous System Tumors*, New York: Wiley and Sons, pp 371–9.
2. Wright DC and Delaney TF, 1989, Treatment of metastatic cancer to the brain, In: DeVita VT, Hellman S and Rosenburg SA, eds. *Cancer: principals and practice of oncology*, New York: J. B. Lippincott Co, pp 2245–61.
3. Herlyn M, Thurin J, Balaban G *et al.* 1985, Characteristics of cultured human melanocytes isolated from different stages of tumor progression, *Cancer Res*, **45,** 5670–6.
4. Albino AP, Davis BM and Nanus DM, 1991, Induction of growth factor RNA expression in human malignant melanoma: markers of transformation, *Cancer Res*, **51,** 4815–20.
5. Nicolson GL, 1993, Paracrine and autocrine growth mechanisms in tumor metastasis to specific sites with particular emphasis on brain and lung metastasis, *Cancer Metastasis Rev*, **12,** 325–43.
6. Raff CM, 1992, Social controls on cell survival and cell death, *Nature*, **356,** 397–400.
7. Nowell PC, 1976, The clonal evolution of tumor cells, *Science*, **194,** 23–8.
8. Raff MC, Barres BA, Burne JF *et al.* 1993, Programmed cell death and the control of cell survival: lessons from the nervous system, *Science*, **262,** 695–700.
9. Snider WD, 1994, Functions of the neurotrophins during nervous system development: what knockouts are teaching us, *Cell*, **77,** 627–38.
10. Menter DG, Herrmann JL, Marchetti D and Nicolson GL, 1995, in press, Involvement of neurotrophins and growth factors in brain metastasis formation, *Invasion Metastasis*, in press.
11. Bradshaw RA, Blundell TL, Lapatto R, McDonald NQ and Murray RJ, 1993, Nerve growth factor revisited, *Trends Biochem Sci*, **18,** 48–52.
12. Ip NY and Yancopoulos GD, 1992, Ciliary neurotrophic factor and its receptor complex, *Prog Growth Factor Res*, **4,** 139–55.
13. Johnson D, Lanahan A, Buck CR *et al*, 1986, Expression and structure of the human NGF receptor, *Cell*, **47,** 545–54.
14. Maher PA, 1988, Nerve growth factor induces protein-tyrosine phosphorylation, *Proc Natl Acad Sci USA*, **85,** 6788–91.
15. Miyasaka T, Chao MV, Sherline P and Saltiel AR, 1990, Nerve growth factor stimulates a protein kinase in PC-12 cells that phosphorylates microtubule-associated protein-2, *J Biol Chem*, **265,** 4730–5.
16. Ohmichi M, Decker SJ and Saltiel AR, 1992, Nerve growth factor stimulates the tyrosine phosphorylation of a 38-kDa protein that specifically associates with the src homology domain of phospholipase C-gamma 1, *J Biol Chem*, **267,** 21601–6.
17. Chao MV, 1992, Neurotrophin receptors: a window into neuronal differentiation, *Neuron*, **9,** 583–93.
18. Meakin SO and Shooter EM, 1992, The nerve growth factor family of receptors, *Trends Neurosci*, **15,** 323–31.
19. Barbacid M, 1993, Nerve growth factor: a tale of two receptors, *Oncogene*, **8,** 2033–42.
20. Saltiel AR and Decker SJ, 1994, Cellular mechanisms of signal transduction for neurotrophins, *BioEssays*, **16,** 405–11.
21. Kalcheim C, Carmeli C and Rosenthal A, 1992, Neurotrophin 3 is a mitogen for cultured neural crest cells, *Proc Natl Acad Sci USA*, **89,** 1661–5.
22. Birren SJ, Lo L and Anderson DJ, 1993, Sympathetic neuroblasts undergo a developmental switch in trophic dependence, *Development*, **119,** 597–610.
23. DiCicco BE, Friedman WJ and Black IB, 1993, NT-3 stimulates sympathetic neuroblast proliferation by promoting precursor survival, *Neuron*, **11,** 1101–11.
24. Schnell L, Schneider R, Kolbeck R, Barde YA and Schwab ME, 1994, Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion, *Nature*, **367,** 170–3.
25. Ernfors P, Lee KF, Kucera J and Jaenisch R, 1994, Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents, *Cell*, **77,** 503–12.
26. Klein R, Silos SI, Smeyne RJ *et al.* 1994, Disruption of the neurotrophin-3 receptor gene trkC eliminates la

muscle afferents and results in abnormal movements, *Nature*, **368,** 249–51.
27. Klein R, Smeyne RJ, Wurst W *et al.* 1993, Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death, *Cell*, **75,** 113–22.
28. Ernfors P, Lee KF and Jaenisch R, 1994, Mice lacking brain-derived neurotrophic factor develop with sensory deficits, *Nature*, **368,** 147–50.
29. Jones KR, Farinas I, Backus C and Reichardt LF, 1994, Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development, *Cell*, **76,** 989–99.
30. Crowley C, Spencer SD, Nishimura MC *et al.* 1994, Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons, *Cell*, **76,** 1001–11.
31. Smeyne RJ, Klein R, Schnapp A *et al*, 1994, Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene, *Nature*, **368,** 246–9.
32. Buchman VL and Davies AM, 1993, Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons, *Development*, **118,** 989–1001.
33. Peacocke M, Yaar M, Mansur CP, Chao MV and Gilchrest BA, 1988, Induction of nerve growth factor receptors in cultured human melanocytes, *Proc Natl Acad Sci USA*, **85,** 5282–6.
34. Yaar M and Gilchrest BA, 1991, Human melanocyte growth and differentiation: a decade of new data. *J Invest Dermatol*, **97,** 611–7.
35. Yaar M, Eller MS, DiBenedetto P *et al.* 1994, The trk family of receptors mediates nerve growth factor and neurotrophin-3 effects in melanocytes, **94,** 1550–62.
36. Herrmann JL, Menter DG, Hamada J *et al.* 1993, Mediation of NGF-stimulated extracellular matrix invasion by the human melanoma low-affinity p75 neurotrophin receptor: melanoma p75 functions independently of trkA, *Mol Biol Cell*, **4,** 1205–16.
37. Verdi JM, Birren SJ, Ibanez CF *et al.* 1994, p75LNGFR regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells, *Neuron*, **12,** 733–45.
38. Rabizadeh S, Oh J, Zhong LT *et al.* 1993, Induction of apoptosis by the low-affinity NGF receptor, *Science*, **261,** 345–8.
39. Kannan Y, Usami K, Okada M, Shimizu S and Matsuda H, 1992, Nerve growth factor suppresses apoptosis of murine neutrophils, *Biochem Biophys Res Commun*, **186,** 1050–6.
40. Beutler B and van Huffel C, 1994, Unraveling function in the TNF ligand and receptor families, *Science*, **264,** 667–8.
41. Smith CA, Farrah T and Goodwin RG, 1994, The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death, *Cell*, **76,** 959–62.
42. Barrett GL and Bartlett PF, 1994, The p75 nerve growth factor receptor mediates survival or death depending on stage of sensory neuron development, *Proc Natl Acad Sci USA*, **91,** 6501–5.
43. Lee KF, Li E, Huber LJ *et al.* 1992, Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system, *Cell*, **69,** 737–49.
44. Lee KF, Bachman K, Landis S and Jaenisch R, 1994, Dependence on p75 for innervation of some sympathetic targets, *Science*, **263,** 1447–9.
45. Hempstead BL, Martin ZD, Kaplan DR, Parada LF and Chao MV, 1991, High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor, *Nature*, **350,** 678–83.
46. Hempstead BL, Schleifer LS and Chao MV, 1989, Expression of functional nerve growth factor receptors after gene transfer, *Science*, **243,** 373–5.
47. Berg MM, Sternberg DW, Hempstead BL and Chao MV, 1991, The low-affinity p75 nerve growth factor (NGF) receptor mediates NGF-induced tyrosine phosphorylation, *Proc Natl Acad Sci USA*, **88,** 7106–10.
48. von Bartheld CS, Kinoshita Y, Prevette D *et al.* 1994, Positive and negative effects of neurotrophins on the isthmo-optic nucleus in chick embryos, *Neuron*, **12,** 639–54.
49. Meakin SO and Shooter EM, 1991, Molecular investigations on the high-affinity nerve growth factor receptor, *Neuron*, **6,** 153–63.
50. Weksamp G and Reichardt LF, 1991, Evidence that biological activity of NGF is mediated through a novel subclass of high affinity receptors, *Neuron*, **6,** 649–63.
51. Barker PA and Shooter EM, 1994, Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells, *Neuron*, **13,** 203–15.
52. Feinstein DL and Larhammar D, 1990, Identification of a conserved protein motif in a group of growth factor receptors, *FEBS Lett*, **272,** 7–11.
53. Knipper M, Beck A, Rylett J and Breer H, 1993, Neurotrophin induced cAMP and IP3 responses in PC12 cells. Different pathways, *Febs Lett*, **324,** 147–52.
54. Hempstead BL, Patil N, Thiel B and Chao MV, 1990, Deletion of cytoplasmic sequences of the nerve growth factor receptor leads to loss of high affinity ligand binding, *J Biol Chem*, **265,** 9595–8.
55. Hantzopoulos PA, Chitra S, Glass DJ, Goldfarb MP and Yancopoulos GD, 1994, The low affinity NGF receptor, p75, can collaborate with each of the trks to potentiate functional responses to the neurotrophins, *Neuron*, **13,** 187–201.
56. Volonte C and Greene LA, 1992, Nerve growth factor-activated protein kinase N. Characterization and rapid near homogeneity purification by nucleotide affinity–exchange chromatography, *J Biol Chem*, **267,** 21663–70.
57. Volonte C, Ross AH and Greene LA, 1993, Association of a purine-analogue-sensitive protein kinase activity with p75 nerve growth factor receptors, *Mol Biol Cell*, **4,** 71–8.
58. Volonte C and Greene LA, 1990, Induction of ornithine decarboxylase by nerve growth factor in PC12 cells: dissection by purine analogues, *J Biol Chem*, **265,** 11050–5.
59. Ohmichi M, Decker SJ, Pang L and Saltiel AR, 1991, Phospholipase C-gamma 1 directly associates with the p70 trk oncogene product through its src homology domains, *J Biol Chem*, **266,** 14858–61.
60. Ohmichi M, Decker SJ and Saltiel AR, 1992, Activation of phosphatidylinositol-3 kinase by nerve growth factor involves indirect coupling of the trk proto-oncogene with src homology 2 domains, *Neuron*, **9,** 769–77.

61. Rozakis-Adcock M, McGlade J, Mbamalu G *et al.* 1992, Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases, *Nature*, **360**, 689–92.
62. Obermeier A, Halfter H, Wiesmuller KH *et al.* 1993, Tyrosine 785 is a major determinant of Trk-substrate interaction, *EMBO J*, **12**, 933–41.
63. Obermeier A, Lammers R, Wiesmuller KH *et al.* 1993, Identification of Trk binding sites for SHC and phosphatidylinositol 3'-kinase and formation of a multimeric signaling complex, *J Biol Chem*, **268**, 22963–6.
64. Borrello MG, Pelicci G, Arighi E *et al.* 1994, The oncogenic versions of the Ret and Trk tyrosine kinases bind Shc and Grb2 adaptor proteins, *Oncogene*, **9**, 1661–8.
65. Ohmichi M, Matuoka K, Takenawa T and Saltiel AR, 1994, Growth factors differentially stimulate the phosphorylation of Shc proteins and their association with Grb2 in PC-12 pheochromocytoma cells, *J Biol Chem*, **269**, 1143–8.
66. Stephens RM, Loeb DM, Copeland TD *et al.* 1994, Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses, *Neuron*, **12**, 691–705.
67. Obermeier A, Bradshaw RA, Seedorf K *et al.* 1994, Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLC gamma, *EMBO J*, **13**, 1585–90.
68. Satoh T, Nakafuku M and Kaziro Y, 1992, Function of Ras as a molecular switch in signal transduction, *J Biol Chem*, **267**, 24149–52.
69. Avruch J, Zhang X and Kyriakis JM, 1994, Raf meets Ras: completing the signal transduction pathway, *Trends Biochem Sci*, **19**, 279–83.
70. Batistatou A, Volonte C and Greene LA, 1992, Nerve growth factor employs multiple pathways to induce primary response genes in PC12 cells, *Mol Biol Cell*, **3**, 363–71.
71. Taylor LK, Swanson KD, Kerigan J, Mobley W and Landreth GE, 1994, Isolation and characterization of a nerve growth factor-regulated Fos kinase from PC12 cells, *J Biol Chem*, **269**, 308–18.
72. Lange-Carter CA and Johnson GL, 1994, Ras-dependent growth factor regulation of MEK kinase in PC12 cells, *Science*, **265**, 1458–61.
73. Leevers SJ, Paterson HF and Marshall CJ, 1994, Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane, *Nature*, **369**, 411–4.
74. Stokoe D, Macdonald SG, Cadwallader K, Symons M and Hancock JF, 1994, Activation of Raf as a result of recruitment to the plasma membrane, *Science*, **264**, 1463–7.
75. Wartmann M and Davis RJ, 1994, The native structure of the activated Raf protein kinase is a membrane-bound multi-subunit complex, *J Biol Chem*, **269**, 6695–701.
76. Volente C, Angelastro JM and Greene LA, 1993, Association of protein kinases ERK1 and ERK2 with p75 nerve growth factor receptors, *J Biol Chem*, **268**, 21410–5.
77. Ohmichi M, Pang L, Decker SJ and Saltiel AR, 1992, Nerve growth factor stimulates the activities of the raf-1 and the mitogen-activated protein kinases via the trk protooncogene, *J Biol Chem*, **267**, 14604–10.
78. Dobrowsky RT, Werner MH, Castellino AM, Chao MV and Hannun YA, 1994, Activation of the sphyngomyelin cycle through the low-affinity neurotrophin receptor, *Science*, **265**, 1596–9.
79. Wolff RA, Dobrowsky RT, Bielawaska A, Obeid LM and Hannun YA, 1994, Role of ceramide-activated protein phosphatase in ceramide-mediated signal transduction, *J Biol Chem*, **269**, 19605–9.
80. Ross AH, Grob P, Bothwell M *et al.* 1984, Characterization of nerve growth factor receptor in neural crest tumors using monoclonal antibodies, *Proc Natl Acad Sci USA*, **81**, 6681–5.
81. Morse HG, Gonzalez R, Moore GE and Robinson WA, 1992, Preferential chromosome 11q and/or 17q aberrations in short-term cultures of metastatic melanoma resections from the brain, *Cancer Genet Cytogenet*, **64**, 118–26.
82. Ishikawa M, Dennis JW, Man S and Kerbel RS, 1988, Isolation and characterization of spontaneous wheat germ agglutinin-resistant human melanoma mutants displaying remarkably different metastatic profiles in nude mice, *Cancer Res*, **48**, 665–70.
83. Marchetti D, Menter D, Jin L, Nakajima M and Nicolson GL, 1993, Nerve growth factor effects on human and mouse melanoma cell invasion and heparanase production, *Int J Cancer*, **55**, 692–9.
84. Kaplan DR, Hempstead BL, Martin ZD, Chao MV and Parada LF, 1991, The trk proto-oncogene product: a signal transducing receptor for nerve growth factor, *Science*, **252**, 554–8.
85. Zupan AA, Osborne PA, Smith CE *et al.* 1989, Identification, purification and characterization of truncated forms of the human nerve growth factor receptor, *J Biol Chem*, **264**, 11714–20.
86. Buxser S, Puma P and Johnson GL, 1985, Properties of the nerve growth factor receptor. Relationship between receptor structure and affinity, *J Biol Chem*, **260**, 1917–26.
87. Cavanaugh PG and Nicolson GL, 1989, Purification and some properties of lung-derived growth factor that differentially stimulates the growth of tumor cells metastatic to the lung, *Cancer Res*, **89**, 3928–33.
88. Cavanaugh PG and Nicolson GL, 1991, Lung-derived growth factor for lung-metastasizing tumor cells: identification as a transferrin, *J Cell Biochem*, **47**, 261–7.
89. Nicolson GL, Menter DG, Herrmann JL *et al*, 1994, Tumor metastasis to brain: role of endothelial cells, neurotrophins and paracrine growth factors, *Crit Rev Oncogenesis*, in press.
90. Inoue T, Cavanaugh PG, Steck PA, Brunner N and Nicolson GL, 1993, Differences in transferrin response and numbers of transferrin receptors in rat and human mammary carcinoma lines of different metastatic potentials, *J Cell Physiol*, **156**, 212–7.
91. Mescher AL and Muniam SI, 1988, Transferrin and the growth-promoting effect of nerves, *Int Rev Cytol*, **110**, 1–26.
92. Jia LB, Cavanaugh PG and Nicolson GL, 1994, Paracrine growth factors for metastatic breast cancer cells: cloning of three new transferrin-like growth factor cDNAs that may be involved in the growth stimulation of breast cancer cells at secondary sites, *Proc Am Assoc Cancer Res*, **35**, 44.

93. Nicolson GL, 1993, Cancer progression and growth: relationship of paracrine and autocrine growth mechanisms to organ preference of metastasis, *Exp Cell Res,* **204,** 171–80.
94. Connor JR, Menzies SL, StMartin SM and Mufson EJ, 1990, Cellular distribution of transferrin, ferritin and iron in normal and aged human brains, *J Neurosci Res,* **27,** 595–611.
95. Constam DB, Philipp JP, Malipiero UV *et al.* Differential expression of transforming growth factor-beta 1, -beta 2 and -beta 3 by glioblastoma cells, astrocytes and microglia, *J Immunol,* **148,** 1404–10.
96. Morris CM, Candy JM, Bloxham CA and Edwardson JA, 1992, Immunocytochemical localisation of transferrin in the human brain, *Acta Anat (Basel),* **143,** 14–8.
97. Oh YJ, Markelonis GJ and Oh TH, 1993, Effects of interleukin-1 beta and tumor necrosis factor-alpha on the expression of glial fibrillary acidic protein and transferrin in cultured astrocytes, *Glia,* **8,** 77–86.
98. Orita T, Akimura T, Nishizaki T *et al,* 1990, Transferrin receptors in injured brain, *Acta Neuropathol (Berlin),* **79,** 686–8.
99. Hunter KE, Sporn MB and Davies AM, 1993, Transforming growth factor-betas inhibit mitogen-stimulated proliferation of astrocytes, *Glia,* **7,** 203–11.
100. Fressinaud C, Laeng P, Labourdette G, Durand J and Vallat JM, 1993, The proliferation of mature oligodendrocytes in vitro is stimulated by basic fibroblast growth factor and inhibited by oligodendrocyte-type 2 astrocyte precursors, *Dev Biol,* **158,** 317–29.
101. Merrill JE, 1992, Tumor necrosis factor alpha, interleukin 1 and related cytokines in brain development: normal and pathological, *Dev Neurosci,* **14,** 1–10.
102. Fischer HG, Nitzgen B, Germann T *et al,* 1993, Differentiation driven by granulocyte-macrophage colony-stimulating factor endows microglia with interferon-gamma-independent antigen presentation function, *J Neuroimmunol,* **42,** 87–95.
103. Matsuda S, Fujita H, Imaoka S, Okumura N and Sakanaka M, 1993, Immunoelectron microscopic localization of basic FGF in neuroglias and neurons of the trigeminal mesencephalic and motor nuclei, *Okajimas Folia Anat Jpn,* **69,** 335–43.
104. Alvarez JA, Baird A, Tatum A *et al.* 1992, Localization of basic fibroblast growth factor and vascular endothelial growth factor in human glial neoplasms, *Mod Pathol,* **5,** 303–7.
105. Fabry Z, Fitzsimmons KM, Herlein JA *et al.* 1993, Production of the cytokines interleukin 1 and 6 by murine brain microvessel endothelium and smooth muscle pericytes, *J Neuroimmunol,* **47,** 23–34.
106. Lindholm D, Hengerer B, Zafra F and Theonen H 1990, Transforming growth factor-beta 1 stimulates expression of nerve growth factor in the rat CNS, *Neuroreport,* **1,** 9–12.
107. Lindholm D, Castren E, Kiefer R, Zafra F and Thoenen H, 1992, Transforming growth factor-beta 1 in the rat brain: increase after injury and inhibition of astrocyte proliferation, *J Cell Biol,* **117,** 395–400.
108. Takamitsu F, Fan D, Staroselsky AH *et al.* 1993, Critical factors regulating site-specific brain metastasis of murine melanomas, *Int J Oncol,* **3,** 789–99.
109. Ono T, Saito H, Kishimoto T, Okumoto T and Miyamoto K, 1991, Stimulation of biosynthesis of nerve growth factor by acidic fibroblast growth factor in cultured mouse astrocytes, *Neurosci Lett,* **126,** 18–20.
110. Yoshida K and Gage FH, 1991, Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes, *Brain Res,* **538,** 118–26.
111. Yoshida K and Gage FH, 1992, Cooperative regulation of nerve growth factor synthesis and secretion in fibroblasts and astrocytes by fibroblast growth factor and other cytokines. *Brain Res,* **569,** 14–25.
112. Yoshida K, Kakihana M, Chen LS *et al.* 1992, Cytokine regulation of nerve growth factor-mediated cholinergic neurotrophic activity synthesized by astrocytes and fibroblasts, *J Neurochem,* **59,** 919–31.
113. Yoshida T and Takeuchi M, 1993, Establishment of an astrocyte progenitor cell line: induction of glial fibrillary acidic protein and fibronectin by transforming growth factor-beta 1, *J Neurosci Res,* **35,** 129–37.
114. Lu C, Vickers MF and Kerbel RS 1992, Interleukin 6: a fibroblast-derived growth inhibitor of human melanoma cells from early but not advanced stages of tumor progression, *Proc Natl Acad Sci USA,* **89,** 9215–9.
115. Sawada M, Suzumura A and Marunouchi T, 1992, TNF alpha induces IL-6 production by astrocytes but not by microglia, *Brain Res,* **583,** 296–9.
116. Zafra F, Lindholm D, Castren E, Hartikka J and Thoenen H, 1992, Regulation of brain-derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes, *J Neurosci,* **12,** 4793–9.
117. Sendtner M, Arakawa Y, Stockli KA, Kreutzberg GW and Thoenen H, 1991, Effect of ciliary neurotrophic factor (CNTF) on motoneuron survival, *J Cell Sci Suppl,* **15,** 103–9.
118. Menter DG, Herrmann JL and Nicolson GL, 1994, The metastatic melanoma neurotrophin receptor (p75) is a cell survival (menocytosis) receptor, *Clin Exp Metastasis,* **12,** 82a.
119. Levi MR, 1987, The nerve growth factor 35 years later, *Science,* **237,** 1154–62.
120. Norenberg MD, 1994, Astrocyte responses to CNS injury, *J Neuropath Exp Neurol,* **53,** 213–20.
121. Wilken GP, Marriot DR and Chlolewinski AJ, 1990, Astrocyte heterogeneity, *Trends Neurosci,* **13,** 43–6.
122. Kettenmann H, Orkand RK and Schachner M, 1983, Coupling among identified cells in mammalian nervous system cultures, *J Neurosci,* **3,** 506–16.
123. Kimelberg HK and Ransom BR, 1986, Physiological aspects of astrocyte swelling, In: Fedoroff S and Verandakis A eds. *Astrocytes,* Orlando: Academic Press, pp 129–66.
124. Hirano A, Kawanami T and Llena JF, 1994, Electron microscopy of the blood brain barrier, *Microsc Res Tech,* **27,** 543–56.
125. Lantos PL, Luthert PJ and Deane BR, 1984, Vascular permeability and cerebral oedema in experimental brain tumors. In: Inaba Y, Klatzo and Spatz M eds. *Brain Edema,* New York: Springer-Verlag, pp 40–7
126. Klatzo I, Chui E, Fujiwara K and Spatz M, 1980, Resolution of vasogenic brain edema (VBE), *Adv Neurol,* **28,** 359–73.
127. Frank E, Pulver M and DeTribolet N, 1986, Expression of class II major histocompatibility antigens on reactive astrocytes and endothelial cells within

gliosis surrounding metastases and abscesses, *J Neuroimmunol,* **12,** 29–36.
128. Kristt DA, Reedy E and Yarden Y, 1993, Receptor tyrosine kinase expression in astrocytic lesions: similar features in gliosis and glioma, *Neurosurg,* **33,** 106–15.
129. Strand FL, Zuccarelli LA, Williams KA *et al,* 1993, Melanotropins as growth factors, In: Vaudry H and Eberle AN eds. *The melanotropic peptides, Ann N Y Acad Sci,* **680**, 29–50.
130. Mbikay M, Seidah and NG and Chretien M, 1993, From pro-opiomelanocortin to cancer, possible role of convertases in neoplasia, In: Vaudry H and Eberle AN eds. *The melanotropic peptides, Ann N Y Acad Sci,* **680**, 13–9.
131. Jegou S, Blasquez C, Delbende C, Bunel DT and Vaudry H, 1993, Regulation of a-melanocyte-stimulating hormone release from hypothalamic neurons, In: Vaudry H and Eberle AN eds. *The melanotropic peptides, Ann N Y Acad Sci,* **680**, 260–78.
132. Banks WA and Kastin AJ, 1992, Bidirectional passage of peptides across the blood brain barrier, *Prog Brain Res,* **91,** 139–48.
133. Eberle AN, Siegrist W, Bagutti C *et al.* 1993, Receptors for melanocyte stimulating hormone on melanoma cells, In: Vaudry H and Eberle AN eds. *The melanotropic peptides, Ann N Y Acad Sci,* **680**, 320–41.
134. Mountjoy KG, Robbins LS, Mortrund MT and Cone RD, 1992, The cloning of a family of genes that encode the melanocortin receptors, *Science,* **257,** 1248–51.
135. Salomon Y, Zohar M, Dejordy JO *et al.* 1993, Signaling mechanisms controlled by melanocortins in melanoma, lacrimal and brain astroglial cells, In: Vaudry H and Eberle AN (eds.), *The melanotropic peptides, Ann N Y Acad Sci,* **680**, 364–79.
136. Bennett DC, Holmes A, Devlin L and Hart IR, 1994, Experimental metastasis and differentiation of murine melanoma cells: actions and interactions of factors affecting different intracellular signaling pathways, *Clin Exp Metastasis,* **12,** 385–97.
137. Siegrist W, Stutz S and Eberle AN, 1994, Homologous and heterologous regulation of a-melanocyte stimulating hormone receptors in human and mouse melanoma cell lines, *Cancer Res,* **54,** 2604–10.
138. Kameyama K, Vieira WD, Tsukamoto K, Law LW and Hearing VJ, 1990, Differentiation and the tumorigenic and metastatic phenotype of murine melanoma cells, *Int J Cancer,* **45,** 1151–8.
139. Hiltz ME, Catania A and Lipton JM, 1992, Alpha-MSH peptides inhibit acute inflammation induced in mice by rIL-1 beta, rIL-6, rTNF-alpha and endogenous pyrogen but not that caused by LTB-4, PAF and rIL-8, *Cytokine,* **4,** 320–8.
140. Ceriani G, Macaluso A, Catania A and Lipton JM, 1994, Central neurogenic anti-inflammatory action of alpha-MSH: modulation of peripheral inflammation induced by cytokines and other mediators of inflammation, *Neuroendocrinol,* **59,** 138–43.