NRAGE Regulates Life and Death of Neural Progenitors

Stephen E. Kendall

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NRAGE REGULATES LIFE AND DEATH OF NEURAL PROGENITORS

By

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HB.Sc. Brock University, 1999
M.Sc. University of Western Ontario, 2001

A THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy
(in Biochemistry and Molecular Biology)

The Graduate School
The University of Maine
August, 2004

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NRAGE REGULATES LIFE AND DEATH OF NEURAL PROGENITORS

By Stephen E. Kendall

Thesis Advisor: Dr. Joseph M. Verdi

An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
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The development of the central nervous system requires the orchestration of numerous instructive and permissive cues. These factors are secreted from signaling centers and function in a concentration dependent manner that effects the proliferation, survival and differentiation of neural progenitors (NP) and their differentiated progeny. The family including bone morphogenetic proteins (BMPs) are key regulators of NP expansion and survival. However, once NP become committed to a neuronal fate and begin to differentiate they compete for limited amounts of neurotrophin that facilitate further growth and survival. Here we show that the p75 neurotrophin receptor interacting protein NRAGE is expressed in NP of the developing cortex during the peak period of progenitor apoptosis. This NRAGE dependent apoptosis of NP cells is mediated through the BMP TAK1-TAB1-XIAP signaling complex leading to the activation of p38. The combined BMP activation of p38 and Smad signaling adversely effects the terminal differentiation of NPs into neurons. These results demonstrate the conserved
nature of progenitor and trophic dependent apoptosis through the utilization of NRAGE and common cell death machinery highlighting the important multifunctionality of NRAGE during neurogenesis.
DEDICATION

To my parent Barry and Heather Kendall, my sister Debra and brother Philip whom I hold with the great admiration. My unique wife Megan, what more can I say. I also wish to thank my dear friends Ian and Andre Cottee, Raechel MacDonald and Chiara Battelli whom I am forever indebted.
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CHAPTER 1

Introduction to Apoptosis and Developmental Neurobiology.

Morphological Types of Cell Death.

It has been proposed that there are three main types of cell death during normal development, on the basis of the role of the lysosomes in cell disruption. In the first type, cell death occurs without any detectable activities of endogenous lysosomes and secondary lysosome activation by tissue macrophages. This process has been referred to as heterophagocytosis. In the second type of cell death, autophagocytosis cells are eliminated after activation of their own lysosomal enzymes. In the third type, there is no obvious lysosomal intervention. The first types are by far more common and have been described by various authors.

Apoptosis.

Apoptosis was originally defined as a distinct mode of cell death on the basis of a series of characteristic ultrastructure features according to the following sequence of events: nuclear and cytoplasmic condensation, cell fragmentation and phagocytosis. However, more recent attempts to characterize apoptosis have included cell dehydration, cell membrane changes, condensation of nuclear chromatin, disintegration of the nuclear envelope, mobilization of intracellular ionized calcium, a decrease of the mitochondrial transmembrane potential, activation of caspases, and internucleosomal DNA cleavage. Therefore, a wide spectrum of methods has been developed to assess and quantify apoptosis in biological systems. Late apoptotic events such as DNA-
strand breaks can be assessed by terminal deoxynucleotidyl transferases (TdT) assays or nuclear DNA fragmentation of cells with sub G1 DNA content. More recently redistribution of the phospholipid phosphatidylserine (PS) from the inner plasma membrane leaflet to the outer leaflet has been used to determine early apoptotic events. Following apoptotic cell fragmentation into membrane-bound bodies macrophages or neighboring cells rapidly clear out and degrade apoptotic bodies within heterophagosomes.

The process of apoptosis plays an essential role in sculpting parts of the body. The formation of digits in some higher vertebrates is a well-studied example, where apoptosis eliminates the cells between developing digits. Similarly, apoptosis is involved in hollowing out solid structures to create lumina. In the early mouse embryo, for example the preamniotic cavity is formed by the death of ectodermal cells in the core of the developing embryo. Apoptosis also occurs wherever epithelial sheets invaginate and pinch off to form tubes or vesicles, as in the formation of the neural tube, lens and mammalian palate.

In the course of animal development, various structures are formed that are later removed by apoptosis. These include transient structures that are needed at one developmental stage but not at a later or structures required in one sex but not the other. However, one of the most perplexing phenomena that involve apoptosis is the overproduction of cells within a given tissue and then subsequent culling by apoptosis to adjust their numbers. In the vertebrate nervous system both neurons and oligodendrocytes are generated in excess with a large percentage being eliminated by programmed cell death. Although the
influence of cell proliferation in controlling cell numbers in animal development has received more attention the influence of apoptosis may very well be a dominant mechanism an its role during neurogenesis is an important new frontier.

**Cell Death in Early Neural Development.**

Programmed cell death is a highly phylogenetically conserved mechanism by which eukaryotic cells die following a stereotypic series of molecular and cellular events commonly referred to as apoptosis\(^9,13\). Apoptosis has been recognized to be an essential process during development where it appears to be fundamental for the control of the final numbers of neurons and glia in the central nervous system (CNS) and peripheral nervous system (PNS). Moreover, a growing body of evidence indicates that apoptosis is also responsible for the loss of neurons associated with physiological aging\(^14,15\).

Nearly all classes of neurons are produced in excess during development and it has been postulated that approximately half die before the completion of the CNS\(^16\). These large populations of neurons are then significantly reduced during the periods of naturally occurring neuronal death, primarily through activation of the apoptotic machinery. The neurotrophic theory clearly establishes the role of target-derived neurotrophic factors in the protection of projecting neurons from programmed cell death. The neurotrophic theory provides a conceptual base for the acceptance of cell death as a physiological process in
neural development, but also to generalize the significance of cell death during normal development in an organism\textsuperscript{17,18}.

Still, the general idea beyond this expanded concept is that naturally occurring neuronal death is closely related to the establishment of proper connections with their target, and under this perspective little attention has been dedicated to apoptosis of neural precursors and/or young neuroblasts at early developmental stages, which seems to be independent from synaptogenesis.

**Early Death of Proliferating Precursor Cells.**

It is generally believed that a correct balance between cell proliferation and apoptosis during development is fundamental to determining the ultimate structure, architecture, size, and shape of tissues and organs. The existence of naturally occurring cell death in the neuroepithelium at the beginning of neurulation was first described more than 50 years ago\textsuperscript{9}. Since then early neural death has been described during the formation of neural crest, and neurogenesis, with a widespread distribution throughout the CNS and in PNS ganglia\textsuperscript{9,19-31}. In these early developmental stages, differentiated neurons are rare, if not totally absent. Therefore, although a quantitative contribution of early progenitor apoptosis has been elusive, it is reasonable to assume that they must be proliferating neural precursors or newly generated neuroblasts and not functional neurons connected to their targets.

Studies utilizing knockout mice studies have provided dramatic proof of the occurrence of cell death during early neural vertebrate development in which activation of several regulatory or executor molecules in the apoptotic pathway
caused embryonic or perinatal lethality\textsuperscript{32-34}. Unexpectedly, the CNS was the most affected tissue, presenting a large excess of neurons in disorganized structures. Morphological defects were already visible well before neuronal generation, as early as embryonic day (E) 9.5 in the null mutant for the apoptotic activator Apaf-1\textsuperscript{34} and at E10.5 in the null mutants for caspase-9\textsuperscript{32} and caspase-3\textsuperscript{33}.

Since proliferation, differentiation and apoptosis are concurrent processes during early neurogenesis, determination of the relationship between proliferation and apoptosis of individual cells is an important issue to be assessed. Interestingly, a number of studies led to the conclusion that apoptotic cells enter the cell cycle shortly before death\textsuperscript{23,26,35-37}. Making the relationship between cell cycle control and apoptosis a vital mechanism in controlling cell fate.

**Neural Apoptosis at Cell Cycle Checkpoints.**

The cell cycle apparatus is composed primarily of three families of proteins: the cyclin-dependent protein kinases (CDKs), the cyclins and the cyclin-dependent kinase inhibitors (CKIs)\textsuperscript{38,39}. CDKs allow progression through the different phases of the cell cycle by phosphorylating critical serines and threonines on their target substrates. Their kinase activity is dependent on the presence of activating subunits known as cyclins, whose abundance varies substantially during the cell cycle. D-type cyclins are rapidly turned over and are synthesized in response to mitogenic stimuli. Once synthesized, they associate with CDK4 and CDK6, forming active CDK complexes in early G1 phase\textsuperscript{40,41}. In mid/late G1, cyclin E is induced and forms complexes with CDK2, whose activity appears essential for entrance into S phase\textsuperscript{42,43}. Cyclin A activity appears in
concert with the onset of DNA synthesis and it initially partners with CDK2 and later with CDC2\textsuperscript{44,45}. For mitosis to occur, cells must progress through the G2/M transition, which requires the assembly of the maturation-promoting factor (MPF) that is composed of cyclin B and CDC2. At late G2, MPF induces its own demise by activating the mitotic cyclin destruction system through the anaphase-promoting complex (APC), which acts as a ubiquitin E3 ligase for MPF.

Two major classes of CKIs have been identified. The first is the Kip family of inhibitors that consists of p21\textsuperscript{waf1}, p27\textsuperscript{Kip1} and p57\textsuperscript{Kip2}. These three CKIs can bind to most cyclin-CDKs and inhibit their activities\textsuperscript{46-48}. The Kip family of inhibitors share a common N-terminal domain for binding to and inhibiting the kinase activity of CDK-cyclin complexes. The second family of CKIs consists of the INK4 family of inhibitors, which includes p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}, p18\textsuperscript{INK4c}, and p19\textsuperscript{INK4d}. INK4 proteins are a group of ankyrin repeat-containing proteins, which specifically bind to and inactivate CDK4 and CDK6 by preventing their bindings to the cyclins\textsuperscript{49,50}.

The orderly progression of the cell cycle is driven by sequential activation of CDKs to pass key restriction or checkpoints\textsuperscript{51,52}, primarily during the G1/S and G2/M transition of the cell cycle. The checkpoints of the cell cycle ensure that critical events in a particular phase are completed before the next phase can be initiated, thereby ensuring genome integrity. Frequently, cells decide to enter or withdraw from the cell cycle at the G1 phase, and thus the G1 checkpoint at the G1/S transition is often a prime target for cell cycle regulation. Cell cycle
progression can be blocked at the G1 checkpoint in response to the status of both the intracellular and extracellular environments.

G1 phase is a period when cells make critical decisions about their fate, including the option to replicate DNA and complete cell division. The decision to enter the S phase is made at the so-called restriction point in mid-to-late G1\(^{53}\). The genes critical for G1/S transition and coordination of S-G2-M progression are regulated by the parallel retinoblastoma protein (Rb) and Myc pathways\(^{54}\).

**Retinoblastoma Protein Family.**

Among the best-characterized substrates of G1 CDKs are Rb and the Rb-related proteins p107 and p130\(^{55-57}\). Each of these Rb family members is capable of blocking progression from G1 into S\(^{58-62}\) suggesting some functional redundancy amongst Rb family members. When hypophosphorylated, Rb is active and able to bind to and repress transcription factors that promote proliferation, most notably, the E2F family of transcription factors. Phosphorylation primarily through CDK activity inactivates Rb, thereby releasing these transcription factors and driving S phase progression. Rb-deficient embryos display numerous abnormalities in the nervous system, which occur shortly after the time at which Rb would normally be expressed. In Rb\(^{−/−}\) null mice ectopic mitosis is observed in various brain regions and massive cell death occurs throughout the CNS and PNS\(^{63-66}\). A transient increase in Rb protein levels appear to be an important step in the initiation of terminal mitosis of neuronal progenitors, and is then followed by drastic reduction during terminal differentiation and maintained at low levels in postmitotic neurons\(^{67,68}\). Moreover,
an increasing body of evidence is leading to the notion that postmitotic neurons generally undergo apoptosis when the cell cycle regulators that promote Rb phosphorylation are activated. In support of this, E2F family members can trigger apoptosis, and E2F1-induced apoptosis can be specifically inhibited by Rb⁶⁹. High levels of E2F1 in a cycling cell signals division, in a quiescent cell such as a postmitotic neuron, the potent contradictory growth signals of E2F1 above a critical concentration may trigger the apoptotic cascade. The E2F1-mediated cell death of neural progenitors appears due to Rb-inactivation and was shown to be p53-dependent⁷⁰,⁷¹.

**Death of Postmitotic Neurons.**

**Neurotrophins and Neuronal Survival.**

Death of postmitotic neurons is a widely recognized phenomenon that plays a crucial role in sculpting and maintaining the architecture of the mature nervous system. A relatively large apoptotic loss of different types of neurons (and glial) occurs during vertebrate development, and the general explanation for this phenomenon is that survival of nerve cells depends on specific neurotrophic factors which are synthesized by their targets⁷²-⁷⁴. Therefore, although many neuronal types are produced in excess, only a portion of them receives sufficient proper support for survival.

Nerve growth factor (NGF) was the first in a family of small soluble molecules demonstrating survival effects on populations of mature neurons both in vitro and in vivo. The effects of these neurotrophic molecules are mediated
through two distinct classes of receptors. One class consists of members of the
Trk family of tyrosine kinase receptors while the other is made of a single
member, p75NTR. Decades of biochemical analyses have resulted in great strides
towards understanding NGF signaling potential through Trk and recently through
p75NTR. I began my studies attempting to understand the biological and signaling
potential of p75NTR by examining the expression and role of p75NTR interacting
proteins. My studies culminated with the discovery that one such interacting
protein, NRAGE is expressed in a wide variety of tissues throughout
development in a tight temporally regulated manner.

**Neurotrophins and Neurotrophin Signaling.**

The neurotrophins are a family of structurally related proteins. Four
neurotrophin genes have been identified in both mouse and humans. Each gene
codes for secretory proteins sharing four hallmark characteristics: (i) biosynthesis
as precursor proteins, (ii) characteristic spacing between their six cysteine
residues, (iii) highly basic isoelectric points, and (iv) the formation of non-
covalently bound homodimers72-77. Following the discovery of nerve growth factor
(NGF)78,79, binding studies with radiolabeled NGF suggested the presence of two
different receptors on responsive cells: one with high affinity and slow kinetics of
association/dissociation and one with relatively low affinity and fast kinetics80.
The gene encoding for the low-affinity NGF receptor was the first NGF receptor
to be cloned. Moreover, the primary sequence of its cytoplasmic domain did not
indicate any catalytic activity, nor was this receptor sufficient to account for the
physiological survival effects mediated by NGF81,82. Following the identification of
brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4/5 (NT4/5), these neurotrophins were also shown to bind to p75<sup>NTR</sup> with affinities similar to that of NGF (about 10<sup>-9</sup> M), but with relatively slower kinetics<sup>83-86</sup>. This observation lead to a nomenclature change for the low-affinity NGF receptor to reflect its wide range of physiological relevant ligands. The low-affinity NGF receptor is now commonly referred to as p75 neurotrophin receptor (p75<sup>NTR</sup>).

After the initial biochemical characterization of p75<sup>NTR</sup>, it was demonstrated that the proto-oncogene *TrkA* (tropomyosin-related kinase) had an expression pattern in the developing nervous system suggestive of a potential role in neural development. It was hypothesized that *TrkA* may mediate long-term NGF survival<sup>87</sup>. Independent studies soon demonstrated that *TrkA* bound NGF resulting in the activation of its intrinsic tyrosine kinase promoting long-term survival<sup>88,89</sup>. Kinetic and binding experiments demonstrated that this second NGF receptor binds NGF with high-affinity.<sup>75</sup> One family of highly related tyrosine kinase receptors is now recognized in mammals, each receptor selectively binding its cognate neurotrophin(s) inducing dimerization bring their cytoplasmically located tyrosine kinase activities into proximity, thereby inducing them to cross-phosphorylate. Each receptor, *TrkA*, *TrkB*, and *TrkC*, is expressed in distinct parts of the nervous system and in several isoforms<sup>90,91</sup>. NGF is the preferred ligand for *TrkA*, BDNF and NT4/5 are preferred for *TrkB*, and NT3 for *TrkC<sup>92</sup>. Ligand binding triggers receptor dimerization, autophosphorylation, and activation of well-characterized signal transduction pathways that includes
phosphatidylinositol 3 kinase, phospholipase C, and the Ras/MAP kinase cascade\textsuperscript{93,94}.

A plethora of experiments have demonstrated that the Trks mediate survival\textsuperscript{95-97}, proliferation\textsuperscript{95,98,99}, overt differentiation\textsuperscript{100-102}, synaptic plasticity\textsuperscript{103-105}, dendritic and axonal sprouting\textsuperscript{106-108}, neurotransmitter expression and long-term potentiation\textsuperscript{109-111}. However, some of the most convincing evidence comes from the tight correlation between the nature and the extent of neural losses after deletion of either a Trk receptor or its corresponding neurotrophin gene\textsuperscript{112}.

**Neurotrophin Withdrawal and Apoptosis.**

The first neurotrophin-activated signaling protein shown to mediate survival of neurons was the small GTP-binding protein Ras. Inhibition of Ras activity decreased survival many neuronal populations\textsuperscript{113,114}. Ras, which in most cases is responsible for 40-60\% of neurotrophin-dependent survival, does not act directly to promote survival. Rather, it functions by translating and directing neurotrophin-initiated signals into multiple signaling pathways. Numerous studies indicate that the two signaling pathways, PI-3K/Akt and MEK/MAPK, are the major effectors of neurotrophin and Ras-activated survival.

PI-3K was first identified as a regulator of neurotrophin mediated survival responses by Cooper and colleagues in NGF-dependent PC12 cells\textsuperscript{115}. Subsequently, many groups showed that PI-3K activity was responsible for as much as 80\% of neurotrophin regulated cell survival in cortical, cerebellar, motor, sympathetic and sensory neurons\textsuperscript{116-126}. Ras was shown to activate PI-3K survival promoting pathways through common effector proteins. Ras is not,
however, the only means by which \textit{Trk} activates PI-3K. In the Trk system, PI-3K activation is due to the combined actions of Ras and Gab-1\textsuperscript{127,128}. The primary target for active PI-3K appears to be the serine/threonine kinase Akt\textsuperscript{129,130}. Akt promotes its pro-survival activity through the phosphorylation of Bad that facilitates an association with 14-3-3, preventing it from binding and inactivating the anti-apoptotic protein Bcl-2 and Bcl-X\textsubscript{L}\textsuperscript{131,132}. Another potential target of PI-3K is the IAP (inhibitor of apoptosis) family of caspase inhibitors, which include X-linked inhibitor of apoptosis (XIAP), neuronal apoptosis-inhibitory protein (NIAP), and human inhibitor of apoptosis protein (HIAP) that function through inhibitory associations with procaspase-9\textsuperscript{133}.

Neurotrophin withdrawal reduces the activity of PI-3K permitting BH3 containing proteins, such as Bad and Bcl-2, form a mitochondrial pore increasing cytochrome c release and the activation of procaspase-9, which serves as a convergence in molecular pathways of apoptosis signaling formation of the apoptosome with subsequent cleavage of caspase-3 to the active form providing an unfettered route to apoptosis\textsuperscript{74}.

Although the role of the \textit{Trk} receptors has continued to be thoroughly investigated and the function of the other neurotrophin receptor, p75\textsuperscript{NTR}, has been more difficult to ascertain due to its lack of any obvious intrinsic catalytic activity or signaling domains. Complicating the issue, p75\textsuperscript{NTR} is often co-expressed with at least one \textit{Trk} receptor family member, making independent study of p75\textsuperscript{NTR}-mediated effects difficult. However, recent studies indicate that p75\textsuperscript{NTR} does in fact have intrinsic signaling functions as described below.
p75\textsuperscript{NTR} as a Death Receptor.

In addition to the ability of p75\textsuperscript{NTR} to modulate the binding and function of Trk receptors, this receptor has unique functions when expressed in the absence of Trk. The identification of p75\textsuperscript{NTR} as a member of the TNF receptor superfamily suggests that p75\textsuperscript{NTR} might signal cells to die, possibly using an analogous mechanism to TNF signaling. The first demonstration that p75\textsuperscript{NTR} could mediate apoptosis was by Rabizadeh and colleagues\textsuperscript{134} in two distinct neural cell lines. Since then, a wealth of in vitro evidence has accumulated to support an apoptotic role for p75\textsuperscript{NTR}. Ligand mediated Trk independent apoptosis has been shown in a variety of neural cells that include sensory neurons\textsuperscript{135,136}, sympathetic neurons\textsuperscript{137}, motoneurons\textsuperscript{138,139} and hippocampal neurons\textsuperscript{140}. p75\textsuperscript{NTR} expression is not limited to neurons; in fact oligodendrocytes and Schwann cells also express p75\textsuperscript{NTR} and have been shown to undergo an NGF-dependent p75\textsuperscript{NTR}-mediated apoptosis\textsuperscript{141,142}.

In vivo evidence for p75\textsuperscript{NTR}-mediated cell death has been found for two different central nervous system (CNS) populations. Early retinal and basal forebrain cells express p75\textsuperscript{NTR}. These cells undergo apoptosis, an effect that can be blocked by NGF antibodies\textsuperscript{143}. In cholinergic neurons of the basal forebrain there is a reduction in the number of apoptotic cells in mice lacking the p75\textsuperscript{NTR} receptor\textsuperscript{144}. Further evidence suggesting that p75\textsuperscript{NTR} independently regulates cellular apoptosis comes from transgenic mice over-expressing the intracellular domain of p75\textsuperscript{NTR}, resulting in a significant reduction in numbers of sympathetic and sensory neurons as well as cell loss in the neocortex\textsuperscript{145}. The deficit in the
peripheral nerves of these mice is primarily restricted to unmyelinated axons indicating that cell death was confined to those sensory neurons that are normally receptive to NGF and which express TrkA. Cell death occurred in neocortical cells, which do not express TrkA. This argues against a dominant negative effect; whereby, pairing of the p75NTR intracellular domain with TrkA might lead to cell death by blocking the formation of high affinity receptors for NGF. These studies suggest that the p75NTR intracellular domain independent of Trk initiated the cell death process. Moreover, in p75NTR knockout mice, the number of sympathetic neurons at two weeks of age was significantly higher than wild-type mice, indicating that apoptosis was reduced\textsuperscript{137}. p75NTR knockout mice were also reported to display reduced apoptosis, as detected by TUNEL, of spinal cord neurons at E11.5, and retinal neurons at E15.5\textsuperscript{143}.

**Isolation of Neurotrophin Receptor Interacting Factors.**

In general, it seems that neurotrophins can only induce cell death through p75NTR in cells not expressing their specific Trk receptors\textsuperscript{137,146}. By contrast, coactivation of the appropriate Trk receptors inhibits neurotrophin-induced p75NTR-mediated apoptosis, as demonstrated with cultured oligodendrocytes and neurons\textsuperscript{136}. A number of interactors and their possible functions have been described for p75NTR, many of them as a result of yeast two-hybrid screens. p75NTR interactors involved in transducing cell death include a wildly expressed zinc finger protein designated NRIF (neurotrophin receptor-interacting factor)\textsuperscript{147}. NRIF knockout mice showed reduced levels of apoptosis in the retina, and this reduction was quantitatively similar to that seen in p75NTR null
mice. These results suggest that NRIF is an essential component downstream of NGF and p75NTR in the cell death pathway. A protein designated NADE (p75NTR-associated cell death executor) associates with p75NTR after NGF binding but not after binding of BDNF, NT3 or NT4/5. It too seems to be involved in p75NTR-mediated apoptosis.

Another zinc finger protein that has been identified as an intracellular interactor of p75NTR, the Schwann cell factor-1 (SC-1), is involved in cell cycle arrest. SC-1 translocates from the cytoplasm to the nucleus after NGF, but not BDNF, stimulation of transfected COS cells. Nuclear expression of SC-1 led to loss of BrdU incorporation indicating a reduction in the rate of proliferation. The GTPase RhoA has been isolated as a p75NTR interactor in a yeast two-hybrid screen. p75NTR constitutively activates RhoA, whereas neurotrophin binding to p75NTR blocks this activation. The link to p75NTR and RhoA seems to be functionally relevant, as the modulation of neurite outgrowth in p75NTR-expressing neurons by neurotrophins mimics the results obtained following RhoA inactivation. Using coimmunoprecipitation and phosphorylation assays, a p75NTR-associated kinase, as well as ERK1 and ERK2, were shown to interact with p75NTR. The functional significance of these associations is not clear, though p75NTR is serine phosphorylated in the intracellular domain. Association of TRAF6 with p75NTR after ligand binding leads to the activation of NF-κB. Another report suggests that all members of the TRAF family interact with p75NTR. A further recently identified interactor of p75NTR, designated NRAGE (neurotrophin receptor-interacting MAGE), or MAGE-D1, mediates NGF-
dependent apoptosis in sympathetic neuron precursor cells\textsuperscript{155}. NRAGE also causes cell cycle arrest when over-expressed in cultured cells, suggesting that p75\textsuperscript{NTR} might play a role in growth control through interactions with NRAGE. Interestingly, NRAGE disrupts the interaction between p75\textsuperscript{NTR} and TrkA\textsuperscript{155}. The functional interactions between NRAGE and p75\textsuperscript{NTR} are discussed in greater detail below.

There remain many pertinent issues to address before a coherent view on naturally occurring neuronal cell death can be formed. However, evidences from transgenic and cell culture studies have delineated a picture in which a dual wave of apoptosis occurs. Early neural death of proliferating progenitors and young neuroblasts is assuming a more detailed contour, and an in depth analysis of the spatial, temporal and molecular links of proliferation, differentiation or apoptosis will surely be informative to better clarify the physiological role of this phenomena. Recently members of the bone morphogenetic family have been found as prominent regulators holding key roles during neural development including the regulation of neuronal and astrocytic determination and apoptosis in the cortex 7-10 and instructive cell fate decisions in the neural crest 11-15.

**Early Corticogenesis.**

Regionalization of the neural plate and early tube is controlled by secreted molecules produced by a grid of patterning centers\textsuperscript{156-159}. Longitudinal patterning centers are present along the ventral (axial mesoderm, and later the floor plate) and dorsal (epidermal-neuroectodermal limits and latter the roof plate) aspects of
neural plate and early neural tube. Transverse patterning centers are present at specific anterior posterior locations such as the rostral end of the neural plate (anterior neural ridge, ANR)\textsuperscript{160,161} and at the midbrain-hindbrain boundary (isthmus)\textsuperscript{162}. Each patterning center produces a distinct set of signals. Ventral centers produce sonic hedgehog (shh) and BMPs\textsuperscript{163-166}; dorsal centers produce BMPs and Wnts\textsuperscript{167,168} and transverse patterning centers produce fibroblast growth factor (FGF) and Wnts\textsuperscript{160,169,170}. Thus, the neuroepithelium adjacent to transverse and longitudinal patterning centers are exposed to a combination of signals. While some insights into the combinatorial effects of extracellular signals on cellular fate of neuroepithelial progenitors has been made, little is known how combinations of these signals regulate cell proliferation, survival and differentiation in the CNS. Furthermore, less is known how these patterning centers are established and how they affect each other.

Soon after folding of the neural plate into the neural tube, two pairs of large vesicles evaginate from its walls. First, the optic vesicles grow from a region near its rostral end, and give rise to the neural retina, pigment epithelium and optic stalks. Then, the telencephalic vesicles grow from the rostrodorsal part of the prosencephalon, and give rise primarily to the cerebral cortex and basal ganglia\textsuperscript{171}. Neural and non-neural tissues produce a number of signaling molecules including BMPs, shh and FGF8 within and adjacent to the prosencephalon\textsuperscript{157,160,168}. The expression of most BMPs is restricted to the dorsal prosencephalon, while shh expression is largely ventrally restricted and FGF8 expression is primarily in the rostrum of the ANR\textsuperscript{172-175}. Within the forebrain
BMPs repress FGF expression, and shh maintains FGF expression\textsuperscript{175,176}. The positional cue generated as a result of the combinatorial actions of these factors regulates the expression of regional specific transcription factors, which have direct effects on proliferation, survival, specification and morphogenesis.

Neuronal differentiation is promoted by both platelet-derived growth factor (PDGF) and by NT-3. The cytokines leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) are potent inducers of astrocyte production, and thyroid hormone induces oligodendrocyte differentiation\textsuperscript{177-179}. Other factors, such as BMPs can enhance both neuronal and astrocyte differentiation, depending on the age of the stimulated cortical progenitors\textsuperscript{180,181}.

**Signal Transduction of Bone Morphogenetic Proteins**

Bone morphogenetic proteins (BMPs) are members of the TGF-\(\beta\) superfamily of secreted signaling molecules\textsuperscript{182,183} with the exception of the metalloprotease BMP1. There are more than 20 members known that can be subgrouped according to the homology in their sequence\textsuperscript{182-184}. BMPs are highly conserved across the animal kingdom having been shown to participate in numerous developmental events. For example, they influence gastrulation, osteogenesis, neurogenesis, hematopoiesis and apoptosis\textsuperscript{184-197}. Despite the numerous extracellular ligands, only three Type-I and three Type-II receptors are known to mediate the signals\textsuperscript{197-203}. Following ligand binding to at least one Type-I and one Type-II receptor, the Type-II receptor phosphorylates the Type-I receptor on specific serine/threonine residues\textsuperscript{204,205}. The wide diversity of
biological effects appear to be partly due to intracellular cofactors that participate in BMP signal transduction, as well as cross talk between BMPs and other signaling pathways. Following phosphorylation of BMPR-I the R-Smads are phosphorylated and released from the receptor and recruit the common mediator Smad (Co-Smad, Smad4) into the signaling complex that is transported into the nucleus and activates the transcription of specific target gene\(^{206-212}\). In addition to Smad signaling, BMPs have recently been shown to lead to the activation of p38 through additional receptor adapter proteins TAK1/TAB1\(^{213-217}\). Furthermore, there is data showing the activation of the RAS pathway and ERK kinase pathway due to BMP stimulation\(^{218-220}\).

**Bone Morphogenetic Proteins.**

All BMPs are secreted as precursor proteins with an N-terminal hydrophobic stretch of about 50-100 amino acids. The pro-BMP is proteolytically cleaved and the mature and active form is derived from the carboxyterminal region\(^{185,221}\). A hallmark of all BMPs is the presence of a cystein knot composed of six cysteins followed by an additional cystein required for dimerization with a second monomer\(^{222-224}\).

**BMP Receptors.**

Binding of the BMP to at least one Type-I and one Type-II receptor is necessary for activation of the BMP signal\(^{203,225}\). To date three Type-I receptors have been identified [Alk2, Alk3 (BMPR-Ia), Alk6 (BMPR-Ib)] and three Type-II receptors [BMPR-II, ActRII and ActRIIB]\(^{197-200,202,203,226}\). Generally, the Type-I receptors are the high-affinity binding receptors, whereas the Type-II
receptors bind the BMPs alone with low affinity\textsuperscript{200,227,228}. Interestingly, the Type-II receptors are constitutively active serine/threonine kinase receptors. The Type-II receptor phosphorylates the Type-I receptor at a conserved GS-Box, a juxtamembrane domain enriched in glycines and serines\textsuperscript{205,229}.

The affinity for BMPs is dependent on specific Type-I and Type-II receptors\textsuperscript{200,201}, supporting the concept that receptor affinity is important for the activation of specific signal transduction pathways\textsuperscript{201,208,230,231}. The diversity of signaling is also a consequence of differential receptor expression and downstream signaling molecules that contribute to the plethora of signaling responses.

Recently, BMPR-la, BMPR-lb and BMPR-II have been shown to exist as either a preformed hetero-oligomeric complex or a homo-oligomeric complex on the cell surface\textsuperscript{232}. Moreover, the oligomerization mode of the receptors at the cell surface determines the activation of downstream signaling. Should BMP bind to the high-affinity receptor BMPR-la or BMPR-lb and then recruit BMPR-II into a hetero-oligomeric complex leads to activation of p38. The other alternative is for BMP to bind simultaneously to the preformed hetero-oligomeric complex activating Smad signaling pathway\textsuperscript{233}.

**The Smad Family and the “Canonical” Signaling Pathway.**

There are three classes of Smad proteins: the receptor-activated Smads (R-Smads), the common-mediator Smads (Co-Smads) and the inhibitory Smads (I-Smads). Structurally, R-Smads (Smad1, Smad5 and Smad8) and the Co-Smad (Smad4) are similar in that they share two highly conserved regions,
an N-terminal MH1 domain (Mad Homology 1) and a C-terminal MH2 domain separated by a less conserved linker region. However, Smad4 does not contain the C-terminal SSXS phosphorylation motif present in R-Smads, and thus not phosphorylated by the receptor. The MH1 domain of the Smad4 and all R-Smads, except for Smad2, can bind to specific DNA sequences, whereas the MH2 domain mediates protein-protein interactions with Smads promoting homo- or hetero-oligomers and additional transcriptional co-activators or co-repressors. Both R-Smads and Smad4 activate transcription primarily through their MH2 domain. This activity results, at least in part, from the ability of the MH2 domain to recruit the general transcriptional co-activators p300 and CBP. Both p300 and CBP have histone acetylase activity, enabling them to increase transcription of target genes by loosening of the chromatin structure. While Smads alone can bind to specific DNA sequences, their affinity is considered to be too weak to serve as effective and highly specific DNA binding proteins in vivo. Thus, additional and numerous DNA binding partners are required for efficient DNA binding and transcriptional regulation of target genes.

**TAK1/TAB1 and the “Non-Canonical” Signaling Pathway.**

Additional studies suggest that a mitogen-activated protein kinase (MAPK) pathway additionally mediates BMP signal transduction. These studies have shown that BMP signals can be transduced by TGF-β activated kinase (TAK1), a MAP kinase kinase kinase (MAPKKK), and TAK1 binding protein 1 (TAB1). When TAK1 alone is over-expressed in Xenopus, it induces apoptosis and this function is significantly inhibited when a kinase dead mutant is
used. However, co-injection of TAK1 together with XIAP, which belongs to the family of inhibitors of apoptosis (IAP), reduces TAK1 mediated apoptosis, but enhances ventralization of the embryo, thus mimicking BMP over-expression phenotypes. TAB1 activates TAK1 by directly binding to its catalytic domain, and over-expression of TAB1 enhances the ventralizing activity of TAK1 when co-injected. It should be noted that, in addition to BMPs other ligands such as TGF-β, interleukin-1, and tumor necrosis factor (TNF)-α have been reported to activate TAK1 in certain contexts, and that TAK1 has been implicated in the activation of JNK and p38 MAPK signaling pathways.

**Neural p38 MAPK Signaling.**

In both neural and non-neural cells the MAP kinase cascades are among such evolutionarily conserved signaling pathways that strictly control cell function and fate. Three MAP kinase cascades that converge on ERKs, JNKs and p38 MAPKs have been extensively characterized, and each consist of three classes of serine/threonine kinases, MAPK, MAPK kinase (MAPKK, also referred to as MEK) and MAPKK kinase (MAPKKK). MAPKKK phosphorylates and thereby activates MAPKK, and activated MAPKK in turn phosphorylates MAPK. Whereas the ERK cascade is primarily involved in the control of cell proliferation and differentiation by mitogens and growth factors, the JNK and p38 cascades are preferentially activated by environmental stresses such as UV radiation, X-rays, heat and osmotic shock, and by proinflammatory cytokines such as TNF-α and IL-1.
Recently, potential roles for JNKs and p38 MAPKs in neural apoptosis have received a great deal of attention. In particular, mice lacking JNKs display particular dysregulation of neural apoptosis. However, several lines of evidences have suggested that p38 MAPKs also play roles in neural apoptosis. However, these studies are primarily based upon the use of dominant negative or pharmacological inhibitors (SB203580) of p38, and thus it remains unclear whether p38 pathway is involved in neural apoptosis in vivo. Indeed, attempts to address abnormalities in neural development in mice lacking the p38α gene have met with little success as embryonic lethality results during early to mid gestation from deficits in angiogenesis. In this regard, analysis of conditional knockout mice and knockouts for other p38 isoforms will be needed to research a precise conclusion concerning a pro-apoptotic role of p38 MAPKs.

In addition to the regulation of cell death and survival as stress-activated kinases, p38 MAPKs have been shown to possess diverse biological functions including cell fate specification and control of cellular function, including neural cells. Much of the current data concerning p38 signaling in neural cell fate decision as been garnered using the rat pheochromocytoma cell line PC12. Recent studies have suggested that in addition to ERK mediated pathways, p38 signaling also participates in the nerve growth factor (NGF)-induced differentiation. BMP2 also induces neural differentiation of PC12 cells. In this process, p38 MAPKs but not ERKs are activated upon BMP treatment. Consistent with this, SB203580 and dominant negative MKK3 or MKK6 effectively inhibit BMP-induced differentiation. Thus, p38 pathway may play a
common role as a down stream target for both NGF and BMP mediated differentiation. Nevertheless, it has not been fully determined how the activity of p38 MAPK is regulated in neural differentiation, especially at the level of MAPKKK.

The p38 pathway appears to activate MEF2 transcription factors in response to calcium influx into cerebellar granule cells, and activated MEF2 regulates neural survival by stimulating MEF2 dependent gene transcription. Since MEF2 is primarily expressed in differentiating neurons but not in actively dividing neural progenitors it may act in newly formed neurons to support neural activity dependent maturation. However, concerns over p38 signaling have suggested a pro-apoptotic role for p38 in neural cells. Plausible explanations for this apparent discrepancy are that the developmental state and thus the physiological level of neural activity might control p38 pathway activation or the way in which cells respond to p38 activity might changes at neurogenesis progresses.

**NRAGE and the MAGE Family of Proteins.**

The human gene MAGE-A1 was identified by gene transfection approach involving the stimulation of cytotoxic T-lymphocytes directed against a melanoma cell line. By hybridization of cosmids with a MAGE-A1 probe, 11 closely related genes were identified. Together, these 12 genes form the MAGE-A cluster, located in the q28 region of the X chromosome. A sequencing effort directed at the Xp21 region led to the identification of the MAGE-B cluster, which is
comprised of four genes\textsuperscript{275-277}. MAGE-C, a third group, comprises two genes located in Xq26-27\textsuperscript{278}. They were identified following the analysis of cDNA libraries enriched for tumour and testis-specific sequences by representational difference analysis. These MAGE-A, -B and -C genes are not expressed in normal tissues, except in male germ-line cells, and, for some of these genes, in placental\textsuperscript{279}. Seven MAGE-A genes, two MAGE-B genes, and two MAGE-C genes are expressed in a significant proportion of tumours of various histological types. The activation of these genes appears to result from the demethylation of their promoter region\textsuperscript{280}.

Pold and colleagues\textsuperscript{281} have identified a fourth family of MAGE proteins termed MAGE-D1 located at Xp11. The full length MAGE-D1 cDNA clone was described elsewhere\textsuperscript{282} and its exon-intron structure is completely different from other MAGE genes. MAGE-D1 is expressed in a variety of normal tissues and tumours, which are in stark contrast to the expression of other MAGE proteins\textsuperscript{278}.

A notable exception to typical MAGE expression has been necdin. Necdin, which is specifically expressed in differentiated neurons, was found as a retinoic acid-inducible neural differentiated protein in the embryonal carcinoma cell line P19\textsuperscript{283}. The necdin cDNA encodes a 37Kda protein consisting of 325 amino acid residues, and the mRNA was expressed in brain, but not in non-neural tissues\textsuperscript{284}. Further immunohistochemical studies showed that necdin is a nuclear protein expressed in differentiated postmitotic neurons and believed to function as an associated growth suppressor exclusively in postmitotic neurons that are differentiated in an irreversible manner\textsuperscript{285,286}. Necdin has been shown to bind
several Rb interacting proteins. These include adenovirus E1A, the SV40 large T antigen, E2F1, and p53. Similar to Rb, necdin binds and represses E2F1 mediated transcription. Over-expression of necdin in proliferating cells induces growth arrest. Unlike necdin, Rb is expressed in neuroepithelial stem cells and at reduced levels in postmitotic neurons. While Rb is a key regulator of neural progenitor cell terminal differentiation, it may be less important in maintaining the differentiated state as p130 does. In contrast, necdin that is exclusively expressed in postmitotic neurons may have a role in the maintenance of neuronal differentiation. Thus, in neurons the existence of molecules such as necdin that are able to complement Rb/p130 function appear to be important in the maintenance of neuronal differentiation. However, it raises the question as to whether other necdin-like molecules exist in which they share a greater functional similarity to Rb. Indeed, MAGE-D1 is 32% identical and 60% similarity over the N-terminus of the protein to necdin. This high degree of structural homology leads us to the investigation of functional similarity as well.

In an attempt to elucidate novel p75NTR interacting proteins, Verdi and colleagues employed the yeast two-hybrid system to screen a rat neural crest cDNA library. A p75NTR binding partner termed NRAGE was identified. BLAST search revealed sequence homologue to human MAGE-D1. NRAGE was found to bind p75NTR in vitro and in vivo, and NRAGE associates with the plasma membrane when NGF is bound to p75NTR. NRAGE blocks the physical association of p75NTR with TrkA, and, conversely, TrkA over-expression eliminates NRAGE-mediated NGF-dependent apoptosis. Thus, indicating that
interactions between NRAGE and TrkA with p75NTR are functionally and physically exclusive. NRAGE over-expression facilitates cell cycle arrest and permits NGF-dependent apoptosis within sympathetic neuron precursor cells. Therefore, NRAGE contributes to a p75NTR-dependent cell death and suggests novel functions for MAGE family of proteins.

Since p75NTR is expressed not only in neural tissues, but many non-neural tissues as well, a description of the developmental expression of p75NTR-interacting proteins is hypothesized to reflect p75NTR receptor expression. An understanding of the developmental regulations of p75NTR-interacting proteins is important for the fundamental knowledge of when and where these proteins exert a physiologically relevant effect in vivo. Indeed, recent work has characterized p75NTR independent functions for NRAGE mediated apoptosis. NRAGE has been shown to facilitate an interleukin-3 withdrawal induced apoptosis through competitive binding to XIAP290. Moreover, additional studies suggest that NRAGE associates with members homeobox transcription factors Msx2 and Dlx5 and translocates to the nucleus291,292 and this interaction is redistributed to the plasma membrane when the tyrosine receptor kinase ROR2 is present293. Collectively these results suggest that NRAGE may function in the BMP signaling cascade regulating early neural progenitor apoptosis. In support of this the capacity for NRAGE to promote apoptosis is significantly enhanced during active proliferation of neural progenitors294. Therefore, it becomes pertinent to establish the presence of NRAGE within the neural precursor pool during peak periods of
early progenitor apoptosis and determine whether the established BMP mediated cell death in these cells is dependent on NRAGE.
CHAPTER 2
Expression Analysis of Novel p75NTR Signaling Proteins that Regulate cell Cycle Progression and Apoptosis.

Abstract

Neurotrophin Receptor-interacting MAGE (NRAGE) is the most recently identified p75NTR intracellular binding protein. Previously, NRAGE overexpression was shown to mediate cell cycle arrest and facilitate Nerve Growth Factor (NGF) dependent apoptosis of sympathetic neuroblasts in a p75NTR specific manner. Here we have examined the temporal and spatial expression patterns of NRAGE and three other p75NTR-interacting proteins over the course of murine embryogenesis to determine whether their expression is consistent with its proposed functions. We demonstrate that NRAGE mRNA and protein are expressed throughout many embryonic and adult tissues. The mRNA is constitutively expressed within each tissue across development. However, expression of NRAGE protein displays a tight temporal tissue specific regulation. During early CNS development, NRAGE protein is expressed throughout the neural tube, but by later stages of neurogenesis, NRAGE protein is restricted within the ventricular zone, subplate and cortical plate. Moreover, NRAGE protein expression is limited to proliferative neural sub-populations as we fail to detect NRAGE expression co-localized with mature/differentiation associated neural markers. Interestingly, NRAGE's expression is not restricted solely to areas of p75NTR expression suggesting that NRAGE may mediate proliferation and/or
apoptosis from other environmental signals in addition to NGF within the CNS. The distribution of NADE, NRIF and SC-1 mRNAs during murine development suggests that the action of these genes is in fact not limited to regions of p75<sup>NTR</sup> expression either. Specifically, a detailed comparison of the spatial and temporal expression domains of NADE, NRIF and SC-1 during brain development revealed regions of co-expression with p75<sup>NTR</sup> but also illustrates a distinct and discordant spatial and temporal expression. These results yield novel insights into the unique developmental characteristics of the p75<sup>NTR</sup>-interacting proteins, thus revealing their diverse signaling potential during embryonic development. Specifically, our data supports previously characterized roles for NRAGE as a mediator of precursor apoptosis and a repressor of cell cycle progression in neural development.

**Introduction**

In the course of development, the generation of neurons and glia from neural stem cells is regulated at steps that include lineage commitment, progenitor proliferation, differentiation, cellular migration and programmed cell death (apoptosis). Apoptosis is crucial for regulating not only the normal number of terminally differentiated neurons and glia, but also the number of neural progenitors<sup>16,295</sup>. In the classic neurotrophic theory, post-mitotic neurons compete for limited amounts of trophic factors, the neurotrophins, which mediate neural survival. Neurotrophins include four distinct and structurally related homodimeric
compounds. Members of this family include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4/5 (NT4/5). These molecules act on neurons by binding to distinct classes of membrane receptors. One class consists of p75 neurotrophin receptor (p75NTR), a member of the tumour necrosis factor (TNF)/Fas/CD40 receptor superfamily that binds all four neurotrophins with relatively low affinity. The other class consists of Trk receptor tyrosine kinases, including TrkA, B and C and their various isoforms. Binding of neurotrophins to Trks is specific, with NGF binding to TrkA, BDNF to TrkB and NT-3 binding to TrkC and to a lesser extent TrkA. Individual Trks are necessary and sufficient to confer a functional response to their cognate ligands in the absence of p75NTR. However, Trk and p75NTR are often co-expressed endogenously and in vitro data has shown that p75NTR increases the affinity of NGF binding TrkA and enhances TrkA autophosphorylation.

Despite the observations that Trk and p75NTR coordinate neurotrophin-dependent survival of neurons, evidence is mounting indicating that p75NTR can act independently of Trk as an inducer of cellular apoptosis. Ligand mediated p75NTR dependent apoptosis has been demonstrated within several developing neural lineages including sensory neurons, sympathetic neurons, motoneurons and within non-neural lineages such as oligodendrocytes and Schwann cells. In vivo evidence also supports a role for p75NTR in apoptosis. Endogenous NGF binding p75NTR induces apoptosis in developing chick retina neurons. Further evidence suggesting that p75NTR independently...
regulates cellular apoptosis comes from transgenic over-expression and knockout studies. Mice over-expressing the intracellular domain of the p75NTR resulted in a dramatic increase in neural death in both the central and peripheral nervous system\textsuperscript{145} and mice lacking genes for ngf and/or p75\textsuperscript{NTR} have reduced cell death in the retina and in the mantle zone of the spinal cord\textsuperscript{143,304}.

In an attempt to identify down-stream modulators of p75\textsuperscript{NTR} signaling, several groups have successfully utilized the yeast two-hybrid paradigm. Two zinc finger proteins named Schwann Cell Factor–1 (SC-1)\textsuperscript{149} and Neurotrophic Receptor Interacting Factor (NRIF)\textsuperscript{147} have been shown to interact with the juxtamembrane region of p75\textsuperscript{NTR}. A third protein, Neurotrophin Receptor Associated Cell Death Executor or NADE\textsuperscript{148} associates with the intracellular p75\textsuperscript{NTR} death domain. These p75\textsuperscript{NTR} interacting proteins functionally have been associated with either cell cycle regulation (SC-1)\textsuperscript{149} or NGF dependent apoptosis (NADE and NRIF)\textsuperscript{147,148}. SC-1 over-expression in proliferating cells induces cell cycle arrest by a currently unknown mechanism, although it may be directly involved in the down regulation of c-myc expression. NRIF knockout mice showed reduced levels of apoptosis in the retina, and this reduction was quantitatively similar to that seen in p75\textsuperscript{NTR} null mice. NADE also is involved in p75\textsuperscript{NTR}-mediated apoptosis by regulating the activation of caspase-3 upon NGF/ p75\textsuperscript{NTR} interactions\textsuperscript{146}. We have cloned and characterized an additional p75\textsuperscript{NTR} interacting protein we termed NRAGE for Neurotrophin Receptor-interacting MAGE homolog\textsuperscript{155}. NRAGE, previously reported as MAGED1, is a member of the MAGE gene family that have previously been shown to code for surface
antigens expressed by tumour cells but recently have been implicated on regulating cell cycle progression, apoptosis and neurogenic disorders. NRAGE, like SC-1 and NRIF, associates with the juxtamembrane region of p75NTR and over-expression mediates both cell cycle arrest in proliferating cells, as well as a NGF-p75NTR dependent apoptosis of sympathetic neuroblasts. Therefore, NRAGE and other p75NTR-interacting proteins may possess multiple functions in vivo as a growth arrest protein and as a pro-apoptotic protein.

Until recently, MAGE gene expression was believed to be restricted primarily to tumour cells with the exception of male germ cells and placental cells. However, one notable exception is the MAGE family member Necdin. Necdin is expressed in virtually all postmitotic neurons and is believed to function as a differentiation associated growth suppressor. Recently a sub-branch of the MAGE family tree, consisting in part with MAGED1 (NRAGE), have been reported to be expressed in a variety of normal as well as transformed cells. Therefore, since NRAGE or MAGED1 may possess multiple functions during embryogenesis, a detailed account of the spatial and temporal regulation of NRAGE, NRIF, NADE and SC-1 expression is warranted in order to decipher the functional significance of NRAGE expression during development.

Our results indicate that in general, the spatial and temporal expression of NADE, NRIF and SC-1 co-localized with known p75NTR expression in the developing nervous system. However, specific gene expression patterns exist in which these interacting proteins are expressed independent of p75NTR expression.
and each other. Also, outside the nervous system, NADE, NRIF and SC-1 mRNA expression, like that of NRAGE, was observed in a wide variety of tissues. The tissue distribution of these genes suggested that their actions are not solely limited to \( p75^{NTR} \) expression domains and may indeed have functions in regulating development independent of \( p75^{NTR} \) signaling events.

Moreover, we report the developmental expression of NRAGE during murine embryogenesis. Both the mRNA and protein are widely expressed. NRAGE message is detectable early in embryonic development and is expressed in all tissues examined into adulthood. However, the expression of NRAGE protein is strongly temporally and spatially regulated in a tissue specific manner. During cortical neurogenesis, NRAGE protein expression becomes restricted to areas of active cellular proliferation and regions where trophic dependent apoptosis predominantly occurs. In the peripheral nervous system, NRAGE protein levels fluctuate in concert with sympathetic neuroblasts exiting the cell cycle and undergoing trophic factor mediated cell death. However, this early phase of NRAGE expression is in the absence of any detectable NGF receptors strongly suggesting that NRAGE functions are independent of \( p75^{NTR} \) in mediated neuroblasts mitotic arrest or apoptosis. Taken together these results demonstrate that NRAGE protein expression yields a complex spatial and temporal regulation supporting a dual role as a growth arrest protein and a mediator of \( p75^{NTR} \) regulated apoptosis.
Materials and Methods

Embryo and Tissue Collection.

Time pregnant matings of CD1 mice were established at the Maine Medical Research Center Institute breeding facility. The observation of the vaginal plug was designated as embryonic day 0.5 (E0.5). Time pregnant Sprague-Dawley Rats were purchased from the Charles River Company (Montreal, QE, CANADA).

Pregnant mice were sacrificed on consecutive days (E7-E21). The embryos were dissected from the uterus in RNAse free phosphate-buffered saline pH 7.4 (PBS), washed in PBS and fixed in 4% paraformaldehyde (PFA) in PBS. 10μm sections of paraffin embedded tissues were generated using a progressive instrumentation microtome (Fisher).

Murine tissues (E10-adult), listed in table 1, were harvested, frozen in liquid nitrogen and stored at -80°C until use. Embryonic brain was obtained by dissecting directly caudal to the mandible and subsequently removing the skull and meninges. Neural tubes (somites 1-4, 5-12, 13-caudal) with somites removed but with adjacent sympathetic and dorsal root ganglia were maintained.

B2 Sympathetic Neuroblasts Isolations.

Sympathetic ganglia’s were dissected from embryonic Sprague Dawley rats from E13-E18 of gestation. The sympathetic chains were isolated from thoracolumbar regions and treated with a combination of 1mg/ml elastase and 1mg/ml collagenase in minimal essential medium at 37°C for 30 min. The cells
were stained with the B2 antibody and positive cells were isolated using fluorescence activated cell sorting\textsuperscript{307}. B2\textsuperscript{+} sympathetic precursors were plated in a serum-free modification of L15N2 medium as described in detail elsewhere\textsuperscript{307,308}.

**Northern Blotting.**

Mouse and rat poly-A enriched multiple tissue Northern blots were purchased from Clontech. A blot containing total RNA from normal human tissues was purchased from Invitrogen. Both human and mouse blots were probed with a 300 bp fragment of human NRAGE. This fragment was generated by polymerase chain reaction (PCR) over a common sequence (forward primer, 5' TTT AGT AGT GGC ATT CTG GG 3'; reverse primer, 5' CCC AAC TGC CAA TGA GAT GG 3'). The fragment was cloned and sequenced to verify its authenticity. The rat blot was similarly probed, with a 400 base pair (bp) cDNA containing the 5' UTR of rat NRAGE. Probes were radiolabeled with \textsuperscript{32}P-dCTP (Amersham) by random priming, and hybridizations were performed using the ExpressHyb system (Clontech) according to manufacturers recommended protocol.

**In situ hybridization.**

*In situ* hybridization of murine sections were performed according to the method of Groves\textsuperscript{309}. Negative sense control (5' to 3' orientation) and NRAGE-specific antisense (3' to 5' orientation) digoxigenin-labeled riboprobes were synthesized (Roche) from a linearized NRAGE subclone comprising a 400bp fragment of the NRAGE 5' UTR. Mouse p75\textsubscript{NTR}, NRIF, NADE and SC-1
digoxigenin-labeled riboprobes corresponding to the published GenBank sequence were also generated.

Briefly, slides were dewaxed in a series of three xylene washes, then hydrated in a graded ethanol series (100%, 90%, 70%, 50%), washed twice in RNase free PBS and incubated for 20 minutes in 0.2 N HCl-DEPC H2O. Subsequently, slides were washed twice in 2X SSC for 5 minutes and then incubated at 55°C in prehybridization buffer for 120 minutes. At this point, the prehybridization was replaced with fresh prehybridization buffer containing 1 µg/mL riboprobe and hybridization allowed to occur overnight at 55°C.

Following hybridization, slides were treated with 20 µg/mL RNAse A and washed in a series increasing stringency for 10 minutes at 45°C as described elsewhere. After washing, sections were incubated at room temperature (RT) in TBST and 10% goat serum (blocking buffer) for 1 hour at which point the blocking solution was removed and fresh blocking buffer containing a 1/200 dilution of anti-digoxigenin-Alkaline phosphatase Fab fragments (Roche) applied overnight at 4°C. Following the incubation, slides were washed in a solution containing 100mM Tris-pH 9.5, 50mM MgCl2, 100mM NaCl, 0.1% Tween-20 (AP Buffer). Visualization of bound probe was performed by incubating slides in AP buffer containing 75 mg/mL Nitro blue tetrazolium, 50 mg/mL 5-Bromo-4-Chloro-3-Indoyl-Phosphate (Roche) and 5mM Levamisole (Sigma) for up to four hours. Sections were washed extensively in PBS. NRAGE mRNA expression was visualized on an Olympus 1X70 microscope using bright field illumination (Olympus).
**Immunohistochemistry.**

In this study, two affinity purified NRAGE specific polyclonal antibodies were employed. Both α-NRAGE antibodies were generated from GST fusion constructs encoding either the N-terminal 31-396 amino acids or amino acids 53-295 of rat NRAGE. These antibodies were designed to avoid cross-reactivity with the Necdin and MAGE homology domains of NRAGE. Unless stated, all immunohistochemical procedures occurred at RT for 1 hour.

Slides were dewaxed in a series of three xylene washes for 5 minutes each. Then hydrated in a graded ethanol series [100%, 90%, 70% including 1% Sudan black B to prevent tissue autofluorescence (Sigma), 50%] and washed in PBS. Sections were incubated for 1 hour at RT in blocking buffer (10% goat serum in PBS) prior to the addition of NRAGE specific antibodies (1/100 dilution in fresh blocking buffer). Sections were then thoroughly washed in PBS before the appropriate secondary antibodies (1/200 dilution, Chemicon) were applied. Sections were then washed in PBS for 10 minutes and incubated for an additional 10 minutes in PBS containing 1 μg/mL Hoechst 33258 (Sigma). Sections were again thoroughly washed in PBS and staining visualized on an Olympus 1X70 fluorescent microscope.

For the cellular determination of NRAGE expression, adherent cortical cultures were fixed in 4% paraformaldehyde in PBS (10 minutes), rinsed in PBS and co-labeled with the α-NRAGE polyclonal antibodies and monoclonal antibodies against either the neural markers, neurofilament-68 (NF-68), neurofilament-200 (NF-200) and MAP2a/b or the glial specific marker, glial
fibrillary acidic protein (GFAP) (Sigma). Appropriate species and isotype specific secondary antibodies conjugated to FITC or Rhodamine were used as secondary detection reagents together with Hoechst 33258 (1 μg/ml).

**Semi-quantitative RT-PCR.**

Total RNA was isolated with Trizol (Invitrogen) as per manufacturer’s specifications. Total RNA was treated with DNase I (78 U/μL) (Invitrogen) to eliminate any potential genomic contamination and reprecipitated. cDNA was synthesized from 5 μg total RNA with Superscript II (Invitrogen) using an Oligo (dT)_{12-18} primer according to previously published procedures. Jack slowly began thrusting and they rocked together in synchronous rhythm with the camel’s gait. Irina dipped her tongue in and out of his mouth as she stroked and explored. They moaned at first in frustration at not being able to touch each other completely, but as they pounded together faster, they concentrated instead on the sensation of their physical connection.

All PCR reactions were performed in the linear range of amplification as determined empirically for each cDNA sample generated. An 800 bp cDNA fragment was amplified using specific primers for NRAGE that lie outside the MAGE domain. A 300 bp β-actin fragment was also amplified as a positive control and used for semi-quantitative purposes. For NRAGE the forward primer sequence was 5'-CTG CCA ATG AGA AGG CTG ATA CTG-3' and the reverse primer sequence was 5'-AAG CTG ATG GTG TCT GCC TAG CTG-3'. The β-actin forward sequence was 5'- TGT TAC CAA CTG GGA CGA CA -3' and the reverse primer sequence was 5'- CTC TCA GCT GTG GTG GTG AA -3'. The
p75 forward sequence was 5'- CAA CCA GAC CGT GTG TGA AC -3' and the reverse primer sequence was 5'- TGG CTA TGA GGT CTC GCT CT -3'. The NRIf forward sequence was 5'- GTG ATG CTA GAG ACC TTG GG -3' and the reverse primer sequence was 5'- CAC CAT GGC ATC GCT ACT GTC C -3'. The NADE forward sequence was 5'- CAT TCC CAA CAG GCA GAT G -3' and the reverse primer sequence was 5'- GGC ATA AGG CAG AAT TCA TC -3'. The SC-1 forward sequence was 5'- TGG CCA CGA AGT AGC ATT GG -3' and the reverse primer sequence was 5'- TTG ACC CGG TTT GCA ATG TG -3'. Primer sets cross an intron-exon border facilitating the detection of any potential genomic contamination in our samples. Amplification of cDNA was performed between 25-30 cycles (25 cycles for actin) [denature: 94°C, 30 seconds; anneal: 56°C, 30 seconds; extension 72°C, 40 seconds] using 1/20th of the generated cDNA. PCR products were separated on a 1% agarose gel and visualized with ethidium bromide. Resulting amplification products were analyzed using a Kodak Electrophoresis Documentation and Analysis System 120 unit. The ratio of the net intensity of actin amplification to the net intensity of the NRAGE amplification product resulted in the relative NRAGE mRNA expression level.

**Western Blotting.**

Western blots were prepared using adult and fetal CD1 mouse tissues collected from E10 through E21 solublized in Trizol (Invitrogen) and protein was obtained by exhaustive dialysis of the phenol-ethanol supernatant against 0.1% SDS, quantitated using the BCA assay, and fractionated by 8% SDS PAGE under reducing conditions. After semi-dry transfer onto Immobilon-P (Millipore),
membranes were probed with either polyclonal rabbit α-NRAGE antibodies. Blots were developed using horseradish peroxidase conjugated-goat anti-rabbit IgG (Jackson Immuno Research Laboratories Inc.) and a chemiluminescent reagent (New England Nuclear), and exposed to Fujifilm.

**Mouse Brain Cortical Cultures.**

Timed pregnant mice were sacrificed at E13 and the embryos were aseptically removed and placed in Hank’s balanced salt solution (HBSS) (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Cerebral cortex was removed, freed of meninges, dispersed with 0.05% trypsin-EDTA and filtered with 70 μm nylon cell strainers (VWR). Cells were seeded (2x10⁵ cells/mL) on poly-D-lysine coated 60 mm dishes in a medium containing 38% Neurobasal medium, 60% DMEM/F12, 2% FBS, and 0.4 μl/mL penicillin/streptomycin (Invitrogen). Once adherent (within a few hours), the cells were allow to differentiate for 5 days in serum free media comprised of 40% Neurobasal medium, 60% DMEM/F12, and N2 supplements (0.6% D-glucose, 10 mg/L insulin, 20 mg/L transferrin, 61 μM putrescine-HCl, 20 nM progesterone and 30 nM sodium selenite)³¹. Cortical astrocyte cultures were generated from E13 mouse brain by seeding cortical progenitors as above and differentiating the adherent cells in DMEM, high glucose, L-glutamine, 10% FBS and 0.4 μl/mL PS for two weeks.
RESULTS

NADE, NRIF, and SC-1 mRNAs are Extensively Expressed in Adult Mouse Tissue.

To investigate the tissue distribution of p75NTR, NADE, NRIF and SC-1 mRNAs, RT-PCR analysis was performed on total RNA isolated from selected adult mouse tissues (Fig. 1). NADE mRNA expression was ubiquitous in all

Figure 1. p75NTR-Interacting Protein mRNAs are Widely Expressed in Adult Tissues.

Adult mouse tissues were assessed for p75NTR and p75NTR-interactor mRNA expression. Reverse transcription-polymerase chain reaction products were stained with ethidium bromide on 1% agarose gels. p75NTR mRNA expression was detected in most adult tissues assessed. NADE was detected in
all tissues assessed while NRIF and SC-1 were found predominantly in $p75^{NTR}$-expressive tissues.

tissues assessed. In general, $p75^{NTR}$ interacting proteins were spatially and temporally co-expressed with $p75^{NTR}$, however the correlation was not absolute. For example, all three $p75^{NTR}$ effector transcripts were expressed in the heart even though no $p75^{NTR}$ message was identified (even after-45 cycles of amplification). Conversely, NRIF and SC-1 transcripts were not expressed in the thymus, where $p75^{NTR}$ message levels were observed. Each of the effectors also showed unique tissue distributions. SC-1 message was not observed in the lung where NRIF and NADE mRNAs were identified. Furthermore, NADE mRNA was identified in gallbladder and adrenal gland, where neither $p75^{NTR}$, NRIF or SC-1 were expressed. Collectively these results demonstrate the wide spread expression of $p75^{NTR}$-effector proteins predominantly in concurrent expression with $p75^{NTR}$, although there were specific differences between $p75^{NTR}$ and $p75^{NTR}$-effectors expression patterns implicating that $p75^{NTR}$ effector proteins have a broader and non- $p75^{NTR}$ based role in signaling events during development.

**NADE, NRIF and SC-1 mRNAs Display Distinct Temporal Regulation.**

Since all three $p75^{NTR}$ effectors' mRNAs were expressed within the brain, we chose to characterize their temporal expression throughout neurogenesis. Semi-quantitative RT-PCR analysis was performed on total RNA isolated from
E10-adult mouse brain to assess changes in mRNA levels across development (Fig. 2).

**Figure 2. Transcripts for p75\textsuperscript{NTR}-Interacting Proteins Show Discrete Temporal Expression Patterns During Brain Development.**

Semi-quantitative RT-PCR analysis of p75\textsuperscript{NTR}-interactor protein mRNAs during mouse brain development (n=4 performed in triplicate). Expression levels are equilibrated to E10 β-actin levels for each age and expressed as a ratio of the E10 p75\textsuperscript{NTR} interactor/actin levels. A ratio of 1 reflects no change in the message level compared to E10 levels.

All three p75\textsuperscript{NTR}-effector mRNA species were expressed with specific temporal trends reflecting changes in mRNA levels across development. Consistent with published reports, p75\textsuperscript{NTR} mRNA levels increase as neurogenesis progresses.
and starts to decline concurrent with the peak time of programmed cell death at E18, before leveling off to adult levels by birth (Buck et al., 1987). Whereas, NRIF gene expression gradually decreased as development progressed, SC-1 message levels increased 5-fold over the same developmental window and NADE mRNA levels remained invariant across development.

To localize p75NTR interacting protein gene expression to specific cellular regions within the brain, in situ hybridization was done on mouse serial sections of the developing brain (E7.5-P1). Sense riboprobes were used as controls with no staining detected (Fig. 3F, L, R). At E7.5, NADE and NRIF mRNA was detected in the egg cylinder, amnion, chorion, syncytiotrophoblast and uterine tissue (data not shown). Whereas, SC-1 mRNA was detected only in the embryo and syncytiotrophoblast at E7.5 (data not shown). By E11, the single proliferative neuroepithelial layer of the forebrain, midbrain and hindbrain all showed NADE, NRIF and SC-1 mRNA expression (Fig. 3A, G, M). Similar results were found for p75NTR distribution at this age (Fig. 3S). In the mouse, expansion of the cortex begins at approximately E13 with generation of the subplate. By E15 distinct cortical laminae include the marginal zone, cortical plate, subplate, intermediate zone, subventricular and ventricular zone. The cortical plate and subplate zones are transient structures with p75NTR expression specific to the subplate. In the E15 mouse, NADE, NRIF and SC-1 were identified most prominently in the subplate regions of the cortex (data not shown). Moreover, expression was observed in the subplate and cortical plate laminae from E16 through P1 for
NADE, NRIF and SC-1 (Fig. 3B, C, H, I, N, O). p75^{NTR} mRNA was identified within the cortical plate and subplate zone during mid-neurogenesis (Fig. 3T).

Figure 3. NADE, NRIF and SC-1 Expression Coincided with p75^{NTR}
Expression Areas in the Developing Cortex and Neural Tube.

However, unlike NADE, NRIF and SC-1 by late neurogenesis receptor expression was predominantly restricted to the subplate cells (Fig. 3U).

The neuroepithelial wall of the diencephalon, mesencephalon and mylenencephalon are formed from proliferative cells of the ventricular zone that migrate out into the subventricular region. NADE, NRIF and SC-1 mRNA were identified within the developing subventricular zones (data not shown).

Since p75NTR is highly expressed in the neural tube, surrounding dorsal root ganglia and sympathetic ganglia, we assessed gene expression within these regions. NADE, NRIF and SC-1 were all expressed in the mantle zone of the developing spinal cord (Fig. 3D, J, P), as was p75NTR (Fig. 3V). Also, gene expression of all three p75NTR-interactors was present in the dorsal root and sympathetic ganglia (Fig. 3E, K, Q, W).
Overall, NRIF mRNA expression phenocopies the mRNA expression of p75\textsuperscript{NTR}, supporting its role as a bona fide p75\textsuperscript{NTR} effector protein. Likewise, NADE mRNA was spatially expressed in a similar manner to p75\textsuperscript{NTR}. The mRNA expression of SC-1 coincides both spatially and temporally with that of p75\textsuperscript{NTR} mRNA. The increase in Schwann cell factor-1 mRNA over time may reflect its role in mediating the p75\textsuperscript{NTR} induced growth arrest in later development, presumably during glial development.

**NRAGE mRNA is Widely Expressed Throughout Development.**

The p75\textsuperscript{NTR} has been implicated in mediating apoptosis both \textit{in vitro} and \textit{in vivo}. The demonstration that three p75\textsuperscript{NTR} interacting proteins facilitate neurotrophin induced apoptosis in a p75\textsuperscript{NTR} dependent manner \textit{in vitro} further lends credence to the hypothesis that p75\textsuperscript{NTR} is a pro-apoptotic receptor. We wished to determine whether one such mediator of p75\textsuperscript{NTR} signaling, NRAGE, was expressed in temporal and spatial accordance to mediate such a function. To determine NRAGE gene expression across development, a mouse developmental Northern blot was probed for NRAGE using a 300 bp segment of the 5’ UTR of NRAGE to avoid the MAGE homology domain, thus insuring our results would not be confounded with other MAGE family members. A single 2.9kb band, representing the full length mRNA was detected at the earliest time point represented (E7) and at all subsequent ages examined (Fig. 4A)
Figure 4. NRAGE mRNA is Expressed Through Development and Identified in a Variety of Murine and Human Tissues.

(A) Northern analysis of a developmental time course of NRAGE. NRAGE expression is detected at E7 through E17. (B) Northern analysis of adult rat tissue expression for NRAGE. (C) Northern analysis of adult human tissue expression for NRAGE.

Examination of NRAGE mRNA expression at earlier embryonic ages by RT-PCR revealed expression at all ages tested (E3-E6) and in murine embryonic stem cells (data not shown). The presence of NRAGE gene expression at stages of development prior to the onset of neurogenesis or germ layer specification...
indicates a possible role for NRAGE other than mediating p75NTR/neurotrophin-induced apoptosis.

In general, MAGE family members are expressed only in tumour cells with the one major exception being the neuron specific MAGE member Necdin and the recently described MAGED1278,281,282,315. We then addressed whether NRAGE, like Necdin, was predominately expressed in the nervous system or was more similar to the ubiquitous expression of MAGED1278. Probing of an adult rat tissue and human tissue Northern blot using the NRAGE specific probe, revealed that unlike Necdin and most other MAGE genes, NRAGE mRNA expression was detected in most tissues. An expression profile that was more similar to MAGED278 (Fig. 4B, C). In the rat, the highest levels of expression were detected in the brain and liver with lower levels found in the remaining tissues examined. NRAGE expression pattern in human tissues was also wide spread. NRAGE mRNA was once again most prevalent in the brain, but in contrast to the tissue distribution of NRAGE mRNA in the adult rat, high levels of transcripts were also expressed in the lung and kidney with little expression in the liver. Moreover, NRAGE was detected in both skeletal muscle and spleen in adult human tissues, two tissues in rat where the levels of NRAGE mRNA were just above the threshold of detection. These differences in expression patterns and levels may reflect either differing physiological functions or tissue regulation of NRAGE between species. The significance of the expansive tissue distribution demonstrates the unique and atypical expression pattern of MAGED1 subfamily members. The diverse nature of NRAGE’s tissues distribution indicates potential
physiological relevance throughout development and into adulthood and may not be limited to signaling events transduced through the p75NTR.

The pleiotropic nature of NRAGE's physiological functions was hypothesized to be demonstrated through its gene expression distribution. Since, the highest levels of NRAGE expression were detected in rat and human brain, we asked whether this reflected a generally higher expression level of NRAGE mRNA throughout the entire brain or whether a subset of specific brain regions express varying amounts of transcripts. In addition, because each specific brain region develops at different time points during gestation, we were interested whether individual structures express NRAGE mRNA at varying levels based on their respective stages of development. To this end, we sectioned mouse embryos from E7 through adulthood and performed in situ hybridization using an NRAGE and p75NTR specific riboprobe. We employed riboprobes to increase our sensitivity and NRAGE specificity. Negative control (sense) riboprobes revealed no staining (Fig. 5F, L, P).
Figure 5. NRAGE is Expressed in Early Neural Progenitors and Differentiating Neuroblasts.
Mouse NRAGE and p75\textsuperscript{NTR} mRNA expression from E11 through Adult detected by serial section \textit{in situ} hybridization. (A, G) NRAGE expression in the neuroepithelium of the E11 cortex and the mantle zone of the spinal cord. (B, H) NRAGE mRNA in the E15 cortex detected in the ventricular zone and subplate and the mantle zone of the spinal cord. (C, I) Expression of NRAGE in the E16 cortex was detected in the ventricular zone, subplate, and cortical plate. (D) NRAGE expression in the ventricular, cortical and subplate zones in the P1 cortex. (E) Transcripts are present in the dentate gyrus, CA1, CA3 regions of the adult hippocampus (K) Within the adult cortex NRAGE mRNA is observed in layers I-VI. (M) p75\textsuperscript{NTR} was detected in the subventricular zone of the E11 cortex. (N) p75\textsuperscript{NTR} was specifically localized to the mantle of the spinal cord at E15. (O) Within the P1 cortex, p75\textsuperscript{NTR} was detected in the subplate zone. (F, L, P) No signal was detected in NRAGE and p75\textsuperscript{NTR} control cortex. LEGEND: CA1, CA3: tracks of the hippocampus, CP: cortical plate, DG: dentate gyrus, MT: mantle zone, SP: subplate, SBZ: subventricular zone, VZ: ventricular zone. Scale bars: (A-E, G-K, M, N) 100 \mu m; (O, K) 50 \mu m; (F, L, P) 200 \mu m.

Moreover, no background or signal was detected when slides were pre-treated with RNAse A and antisense probes utilized (data not shown).

Consistent with our Northern analysis, the E7 egg cylinder was found to express NRAGE (data not shown). However, the invading syncytiotrophoblast, the outer syncytial layer of the trophoblast that covers each chorionic villus of the placenta, and surrounding uterine tissue also expressed NRAGE. The presence
of NRAGE in the syncytiotrophoblast is consistent with previous studies having identified MAGE gene expression in placental tissue. From E8 through E11 NRAGE mRNA was identified in all regions of the developing neural tube (Fig. 5A). There was no apparent restriction of NRAGE expression to any particular brain segment and within each region, high levels of expression were observed throughout the neuroepithelium. Within the spinal cord, NRAGE was observed within the mantle zone (Fig. 5G). Similarly, p75\(^{NTR}\) gene expression during early neural development paralleled that of NRAGE. p75\(^{NTR}\) message was detected in the subventricular zone (Fig. 5M) and within the mantle zone of the spinal cord (data not shown).

This pattern of NRAGE tissue expression was observed until approximately E14 at which point a change in spatial distribution of message was observed. By E15, a prominent band of NRAGE gene expression was detected within the subplate and ventricular zone (Fig. 5B). The mantle zone of the spinal cord continued to express NRAGE (Fig. 5H) as well as p75\(^{NTR}\) (Fig. 5N). This segregation was complete by E16 where distinct bands of NRAGE mRNA expression are well defined within the ventricular, subplate and cortical plate zones (Fig. 5C, I). These distinct cortical regions, cortical plate, subplate and ventricular zone continued to express NRAGE mRNA into postnatal life (Fig. 5D, J), whereas p75\(^{NTR}\) was predominantly restricted to the subplate zone (Fig. 5O) at this and subsequent ages.

The adult brain was also found to express NRAGE. Within the cortex, NRAGE positive cells can be identified throughout layers I-VI (Fig. 5K). The
dentate gyrus, CA1 and CA3 regions of the hippocampus are also major sources of NRAGE mRNA in the adult brain (Fig. 5E). NRAGE mRNA was detected in numerous brain structures including, but not limited to; the neocortex, striatum, pallidum, hippocampus, thalamus, hypothalamus, tegmentum, tectum, cerebellum, pons, medulla and the spinal cord. NRAGE was also present in neural structures such as the developing pituitary or Rathke's pouch, the optic and olfactory neuroepithelium, dorsal root ganglia (DRG), superior cervical ganglia and the sympathetic chain.

NRAGE Protein Undergoes Spatial Regulation.

NRAGE mRNA undergoes a gradual spatial restriction as development progresses. We asked whether the protein follows the unique characteristics of the mRNA. To this end, we performed immunohistochemistry on serial mouse embryonic sections from E7 through adulthood. We utilized two distinct affinity purified NRAGE polyclonal antibodies: one generated to the peptide residues (53-295) of rat NRAGE and a second generated against peptides (31-396). The antibodies cross-react with rat, mouse and human protein isoforms (data not shown). There was no difference in the overall staining pattern using the two distinct antibody species. The protein expression of NRAGE revealed a similar distribution pattern to the mRNA. NRAGE protein was observable within the E7 egg cylinder and in the uterine tissue (data not shown). At E10 through E13 NRAGE, immunoreactive cells can be observed in the proliferative neural tube (Fig. 6A, B).
Mouse NRAGE protein expression from E10 through E13 detected by serial section immunohistochemistry. Panels A to F are embryos probed with α-NRAGE antibody. Panels G represents a control slide probed with pre-immunserium. **LEGEND:** DRG: Dorsal root ganglia; MT: mantle zone, VZ: ventricular zone. Scale bars: (A) 200 μm; (M-O) 100 μm.
Like the mRNA, the mantle zone of the spinal cord also expressed NRAGE protein (Fig. 6D). In addition to the CNS, neurotrophin sensitive populations in peripheral nervous namely the DRG and sympathetic ganglia also express NRAGE (Fig. 6 D, F).

Complementary to the compartmentalization of NRAGE mRNA in the neural tube, the protein also became restricted within distinct bands in the cortical, subplate and ventricular zones at E15 (Fig. 6E). Furthermore, NRAGE continued to be expressed within the mantle zone of the spinal cord (data not shown). Like the mRNA, NRAGE protein was observed in various subpopulations of cells including the neocortex, striatum, pallidum, hippocampus, thalamus, hypothalamus, tegmentum, tectum, cerebellum, pons, medulla and the spinal cord. NRAGE positive cells within the cortical and subplate zones may represent migratory neuroblasts that have recently moved from the ventricular zone. Therefore, NRAGE protein is present through the entire neuroepithelial wall within each brain region during early neural development. However, by late development cells within the proliferative ventricular zone and the transient cortical and subplate zones express NRAGE protein.

In general, cells within the ventricular zone during early neurogenesis are neural precursor cells. These cells migrate out as development progresses to form the transient subplate and cortical plate zones. These rudimentary structures act as building blocks to form the cortical laminae in the adult. The localization of NRAGE to these layers prompted an investigation as to the cellular
specificity of NRAGE expression. Immunohistochemistry was performed on developing cortical neural and glial cultures generated from E13 embryos to co-localize NRAGE protein expression with either the expression of the early neural specific marker neurofilament-68 (NF-68)<sup>316,317</sup>, or the glial specific marker glial fibrillary acidic protein (GFAP). NRAGE expression was found in both the neural and glial sub-populations. (Fig. 7A, B, D, E).
Figure 7. NRAGE Protein is Identified in Cultured Neuroblasts and not in Post-mitotic Neurons.
Immunocytochemistry for NRAGE in cortical cell cultures. (A) NF-68 was used as a marker for early neural precursor development. (B) GFAP was used as a marker for astrocytes. (C) MAP2a/b was used as a marker for maturing neurons. (D, E, F) cell stained for NRAGE. (G) Note: Early neural precursor cells are NRAGE positive, whereas maturing postmitotic neurons fail to co-localize NRAGE. Scale bar: (A-G) 100 μm

However, NRAGE protein did not co-localize with more mature neural markers such as MAP2a/b\(^{318}\) (Fig. 7C, F, G) suggesting that post-mitotic or maturing neurons attenuate or turn off the expression of NRAGE protein. Interestingly, within a subset of MAP2a/b positive neurons NRAGE was found to co-localize to nuclear compartments that displayed pyknotic nuclei characteristic of cells undergoing apoptosis (Fig. 8A).
Figure 8. NRAGE Redistributes to Nuclear and Perinuclear Compartments.

(A, B) Cortical neural progenitors were cultured in vitro for four days in differentiation media and triple stained with the postmitotic neuron marker MAP2a/b or the multipotential neural progenitor marker nestin (cyan), NRAGE (green) and DNA (red). NRAGE positive piknotic nuclei can be observed in MAP2a/b expressing neurons (white arrows), where as NRAGE is primarily cytoplasmic in nestin positive neural progenitors, which display low levels of DNA condensation (white arrows). (C-E) GFP or (F-H) NRAGE-GFP was transiently over-expressed in P19 cells and subcellular distribution was monitored over a period of 72 hours. Over the 72-hour period GFP can be observed in both the cytoplasmic and nuclear compartments. NRAGE initially occupies the cytoplasm (nuclear condensation begins) and redistributes to the membrane and
perinuclear regions after 48 hours (nuclear fragmentation begins) with almost complete co-localization to the nucleus by 72 hours (complete nuclear fragmentation) Scale bar: (A-H) 50 μm

Moreover, NRAGE expression within multipotential neural progenitors was primarily identified within the cytoplasm with little nuclear condensation observed (Fig. 8B). To further, assess NRAGE subcellular redistribution we transiently transfected NRAGE-GFP or GFP into P19 cells and observed fluorescence localization over a 72-hour period. GFP was found to occupy both cytoplasmic and nuclear compartments with no observable nuclear condensation over the 72-hour period (Fig. 8C-E). However, within the first 24 hours NRAGE-GFP expressing cells exhibited nuclear condensation with NRAGE expression displaying a punctuate cytoplasmic distribution (Fig. 8F). By 48 hours NRAGE was found primarily adjacent to the plasma membrane and a perinuclear compartment with nuclear fragmentation beginning (Fig. 8G). At 72 hours NRAGE localized to the fragmented nucleus (Fig. 8H). Therefore, these results suggest that NRAGE protein is not expressed within neural populations undergoing active neurotrophin competitive programmed cell death, but within neural progenitors undergoing terminal differentiation. Further research is required to assess NRAGE protein in more mature astrocytic or oligodendrocytes cultures.

Collectively, these data demonstrate that during embryonic neurogenesis in the CNS NRAGE spatial distribution is regulated at both the mRNA and
consequently at the protein level. NRAGE protein is expressed in all brain regions. Within the cortex, NRAGE is specifically localized to the cortical, subplate and ventricular zones. In the remainder of the brain, NRAGE is expressed within both the ventricular and subventricular zones and is restricted to neural precursors and immature neural populations.

NRAGE Protein is Temporally Regulated Throughout Development.

NRAGE potentially has multifaceted functions and thus a description of expression across development is expected to reflect endogenous physiological activities. The spatial distribution data supports the bipotential role of NRAGE during development. Therefore, a similar mechanism regulating the temporal distribution of NRAGE is expected to reflect its diverse developmental regulation. We therefore sought to characterize NRAGE mRNA and protein expression patterns across development. Utilizing semi-quantitative RT-PCR in conjunction with Western blotting we analyzed the temporal expression of NRAGE message and protein in most major embryonic and adult mouse tissues (for a complete list of tissues assayed see Table 1).
Table 1. Developmental Tissue Expression of NRAGE mRNA

<table>
<thead>
<tr>
<th>Tissue</th>
<th>E10</th>
<th>E12</th>
<th>E14</th>
<th>E16</th>
<th>E18</th>
<th>E20</th>
<th>P1</th>
<th>Adult</th>
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<td>Thoracic N.T.</td>
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Legend: NRAGE positive tissue: +, NRAGE negative tissue: -, Tissue not tested: N.A. (not applicable)

NRAGE mRNA was identified at all developmental ages for each tissue assessed with the exception of fetal and adult blood (Table 1). A common pattern of mRNA expression was observed regardless of the tissue assessed. This is demonstrated by the ratio of NRAGE message to actin message within the nervous system. The relative level of NRAGE mRNA was constitutively expressed throughout development (Fig. 9A, B).
Figure 9. **NRAGE Displays Tissue Specific Temporal Regulation.**

(A) Ethidium bromide stained agarose gels of reverse transcription-polymerase chain reaction (RT-PCR) products. First strand cDNA amplification of NRAGE resulting in a ~800 bp fragment or ~300 bp β-actin fragment and Western blots of NRAGE with an approximate molecular weight of 97 KDa. Note
NRAGE mRNA was identified at all ages, whereas the protein is regulated in a tissue specific manner. (B) The graph outlines the relative quantitation of NRAGE mRNA across development as depicted from A. NRAGE levels are equilibrated to actin levels for each age and expressed as a ratio to the E10 NRAGE/actin levels, or E12 for the sacral neural tube. An n=4 for is represented in the graph. (C) semi-quantitative RT-PCR analysis of B2+ sympathetic neuroblast NRAGE gene expression. Graph is equilibrated in the same fashion as B with an n=4. Note: NRAGE mRNA and protein levels peak concomitant in the thoracolumbar region at approximately E14.

For every tissues examined, only minimal modulation in mRNA message was observed during embryogenesis through adulthood. Intriguingly, a major exception to this typical NRAGE mRNA profile occurred within the developing thoracic neural tube. In the region that both the NGF dependent DRG and sympathetic ganglia reside, there was an elevation in both protein and mRNA message levels commencing at day E14 and again at E18 for the mRNA (Fig. 9B). Since our segments included several cell populations, we focused specifically on the expression within the developing DRG and thoracolumbar sympathetic chain over gestation (E14-E18). To this end, DRGs and B2+ sympathetic neuroblasts were isolated and RNA and protein extracted. The level of NRAGE message during gestation mimicked what was observed in our thoracic neural tube segments except the elevation of NRAGE mRNA was significantly enhanced (~3 fold) (Fig. 9C). Due to the limitation in cell number, we
were not able to quantify whether the raise in protein matched the rise in message, but since a rise in protein was also detected in the thoracic section studies we assume a significant rise in protein would follow.

Characterization of NRAGE protein expression demonstrates a distinct pattern of tissue expression regulation compared to the mRNA. Unlike the constitutive mRNA expression, the expression of NRAGE protein is under stringent temporal regulation (Fig. 9A). Within the developing mouse nervous system, NRAGE protein appears as early as E7 (first day examined) but by P1 unlike the mRNA, protein levels were under the limits of detection, even after prolonged exposure times and increasing the concentrations of the two NRAGE specific antibodies used.

Specifically, NRAGE protein levels were identified during brain development maximally at E9 through E10 and then decreasing at E12, remaining low until birth when the protein is virtually undetectable. The heaviest staining of NRAGE protein was observed between E12-E14 in the thoracic region, including dorsal root and sympathetic ganglia. Expression was detected until E20, a key chronological landmark since trophic mediated survival begins as early as E16 and continues into postnatal life\textsuperscript{319}. The presence of NRAGE protein in this segment and within these neurotrophin sensitive populations supports a role for NRAGE in this process, consistent to the observations of Salehi and colleagues\textsuperscript{155}. In the latter tissues, NRAGE appears to migrate as a doublet, possibly reflecting post-translational protein modification. In the thoracic neural tube, the upper band of this 97K doublet is particularly prominent at E16.
Possible phosphorylation and/or ubiquitination are currently being assessed as an explanation for this shift in NRAGE expression. These data indicate that despite constitutive levels of NRAGE message, the temporal expression of the NRAGE protein constitutes the greatest level of regulation.

Discussion

Developmental Expression of NADE, NRIF and SC-1 During Embryogenesis.

Our data demonstrate that primarily the \( p75^{NTR} \)-interacting proteins, NADE, NRIF and SC-1 are expressed in concert with \( p75^{NTR} \) during neurodevelopment. Temporally, the expression of \( p75^{NTR} \) with these three effector proteins also supports their predicted roles as mediators of mitotic arrest and apoptosis. What is most intriguing is the distinct expression patterns displayed by the two effectors putatively associated with apoptosis. NRIF levels decline while the levels of NADE remain invariant during neurodevelopment. Since our PCR analysis is a population assay we cannot ascertain the total message or corresponding protein levels within individual cells. However, it is interesting to speculate that the total amount of NADE and NRIF or perhaps the ratio of these two proteins in conjunction with the expression of \( p75^{NTR} \), is what makes an individual neuroblast or glioblast more or less susceptible to \( p75^{NTR} \) mediated apoptosis. In this way, \( p75^{NTR} \) mediated cell death may be an early way to limit the number of neuroblasts or glioblasts prior to overt differentiation, Trk induction and the classic target derived neurotrophin mediated apoptosis seen in later
development. However, what may be more telling regarding the roles these proteins play during development is that in addition to co-localization with p75NTR, each gene is expressed in a unique pattern independent of p75NTR and each other.

During development p75NTR is found in the transient subplate region of the cortex, cholinergic neurons of the basal forebrain, nucleus basalis, striatum and the motor columns of the spinal cord in murine and primate species\textsuperscript{138,313,320,321}. In addition to expression in the CNS, p75NTR is found in the peripheral sympathetic and sensory neurons\textsuperscript{320,322}. Within the nervous system, p75NTR has been shown to mediate a number of physiological and developmental events including; apoptosis of cholinergic neuron in the neostriatum\textsuperscript{135,323}, modulation of the cholinergic forebrain neuron physiology\textsuperscript{324}, and promotion of apoptosis in seizure-induced rats within the hippocampus, piriform cortex, and entorhinal cortex\textsuperscript{325}. Moreover, retinal and mantle zone neurons are sensitive to p75NTR-mediated apoptosis\textsuperscript{143,303}. p75NTR is also required for naturally occurring cell death within the sympathetic and sensory ganglia\textsuperscript{135,137,145}. The most recent report demonstrates that p75NTR knockout mice have severe nervous system defects, and unexpectedly show a requirement for p75NTR expression in vascular development\textsuperscript{304}. Even though expression of p75NTR in any given cell population does not predetermine whether apoptosis will occur, it is still important to address the spatial and temporal relationships of p75NTR and p75NTR-interacting proteins in order to better understand the functions of p75NTR and its interacting proteins during development.
Consistent with the diverse expression pattern of p75\textsuperscript{NTR}, all three interacting proteins examined, NADE, NRIF, and SC-1 showed a widespread tissue distribution. Within the brain, the localization of NADE, NRIF and SC-1 to the subplate and cortical plate implicates an involvement in the development of migratory maturing cells. The observation that p75\textsuperscript{NTR} is neither expressed nor required for apoptosis in these regions of the cortex does not diminish previously characterized functions of known p75\textsuperscript{NTR} interactors. NADE and NRIF expression coincide with the spatial and temporal establishment of neural circuitry, differentiation and synaptogenesis during developmental stages where high levels of trophic-dependent apoptosis are occurring.

NRIF knockout mice were previously shown to be indistinguishable from what has been observed in p75\textsuperscript{NTR}\textsuperscript{-/-} and ngf\textsuperscript{-/-} mutants\textsuperscript{326}. Our results support the widespread gene expression reported by Casademunt and colleagues\textsuperscript{326}. Similar to NADE, NRIF demonstrates p75\textsuperscript{NTR} concurrent expression in the subplate, optic vesicles, otic vesicles, mantle zone of the spinal cord and sympathetic and sensory ganglia. The strong correlation between NRIF and p75\textsuperscript{NTR} expression lends evidence to the relevance of NRIF as a bona fide p75\textsuperscript{NTR}-interacting protein mediating apoptosis \textit{in vivo}. Interestingly, other zinc finger proteins have also been shown to promote apoptosis and may work in concert with NRIF. Zac1 a tumor suppressor gene expressed in the brain was suggested as a model protein whose function may mimic NRIF's function via a non-p75 mediated pathway\textsuperscript{326,327}. A comparison of the expression domains of NRIF and Zac1
support this notion as NRIF and Zac1 have reciprocal distribution patterns within the brain\textsuperscript{327}.

The zinc finger containing protein SC-1 has the greatest stringency in its developmental expression profile. The tissue distribution is more refined than that of NADE or NRIF. Within the CNS, SC-1 was identified most prominently in the cortical and subplate regions and also in the mantle zone of the spinal cord. Likewise, expression was observed in the peripheral ganglia. The level of SC-1 mRNA increased as development progressed, with maximal levels obtained only in adulthood. SC-1 was originally identified and cloned in Schwann cells\textsuperscript{149}. Since SC-1 is expressed in both the PNS and CNS, and glial formation follows neural development, it is more likely than not that astrocytes and/or oligodendrocytes are the main expressers of SC-1. However, the precise cellular resolution of SC-1 expression in the brain remains to be determined.

We have described thus far the developmental regulation of three p75\textsuperscript{NTR} interacting proteins; NADE, NRIF and SC-1. We demonstrated that each gene has common developmental properties associated with p75\textsuperscript{NTR} expression and function. In addition, novel \textit{in vivo} roles were assessed for the unique expression characteristics relevant to each gene. The most provocative and recurring feature described to date involving any of the p75\textsuperscript{NTR}-interacting genes has been the identification of gene expression within the cortical plate. Therefore, the diverse signaling potential during development for each of these three genes strongly suggests a complex regulatory system underlying their overt characterized physiological properties.
Developmental Expression of NRAGE During Embryogenesis.

We have analyzed the expression of NRAGE, a member of the ever-growing MAGE gene family, during murine embryogenesis in order to attempt to correlate the gene expression with potential functions during development. Both the mRNA and protein are widely expressed. NRAGE mRNA was identified at the earliest embryonic day tested (E3) and the protein was found from E7 through adulthood, depending on the tissue assessed. The mRNA is constitutively expressed from embryogenesis to adulthood. However, the protein undergoes tissue specific structural and temporal regulation across development suggesting that the strongest regulation of NRAGE expression is post-transcriptional. In the developing nervous system, peak levels (E12) occur during early development when the brain and spinal cord are undergoing rapid proliferation and expansion. NRAGE mRNA and protein were apparent in all brain regions with expression detected throughout the entire neuroepithelial wall during early stages of neurogenesis. During later stages of neurogenesis, NRAGE was specifically localized to the cortical plate, subplate plate and ventricular zone of the cortex. Both the ventricular and subventricular zone of the midbrain and hindbrain express NRAGE and this expression is probably restricted to neural precursors or immature neurons since NRAGE co-localizes exclusively in within NF-68 positive cells. Localization of NRAGE to the proliferative ventricular zone is suggestive of a role in cell cycle regulation or early neural progenitors, whereas expression within the subplate and cortical
plate correlates with areas where post-mitotic neurons undergo cell death mediated by the traditional neurotrophic hypothesis.

NRAGE, like its closest family members MAGED1 is expressed in most tissues examined, supportive of a potential essential role for the cellular function of NRAGE. However, elucidation of cellular function has proven difficult to assess due to the very low levels of detectable endogenous and transfected protein expression in a variety of primary and cell lines, e.g. COS7, PC12, P19, U87, C6, MAH and 293 cells (S.K., D.G., and J.M.V. unpublished results). This narrow range of accumulation of NRAGE protein in these cell lines indicates stringent regulation for cell viability and/or normal cellular proliferation. Over-expression of NRAGE protein appears to be toxic to recipient cells, and the proportion of successfully transfected cells that express tagged NRAGE cDNAs is extremely low. Taken together that transfected cells also reduces proliferative levels it is possible that only cells in a particular stage of their cell cycle allow for the over-expression of NRAGE. These results are in agreement with previous findings from Casademunt and colleagues indicating poor cellular expression of transfected NRIF, possibly via a cell cycle dependent mechanism.

In the PNS NRAGE Correlates with Sensory and Sympathetic Neuron p75NTR Mediated Apoptosis.

For neural tube development, NRAGE is present when motoneurons of the floor plate are beginning to undergo trophic dependent programmed cell death and the lateral motor column shows high levels of p75NTR expression at
NRAGE has previously been detected in motoneurons\(^{329}\). However, the importance of \(p75^{NTR}\) mediating apoptosis within motoneuron pools \textit{in vivo}\ has been questioned since \(p75^{NTR}\) deficient mice fail to display any significant loss in motoneuron populations\(^{138,322}\).

The neural tube dissections allowed for the collection of both the sympathetic and dorsal root ganglia. The presence and timing of expression of Trk and \(p75^{NTR}\) in both these structures has been well documented\(^{30,137,329,330}\). Moreover, \(p75^{NTR}\) has been shown to be required for promoting apoptosis in these neural populations\(^{137,322,330,331}\). The timing of sensory and sympathetic neurotrophic dependent cell death commences at approximately E14 and E17 respectively\(^{135,319}\). Unlike the CNS where NRAGE protein is diminished prior to the onset of neurotrophin mediated cell death, NRAGE is expressed in the DRGs and sympathetic ganglia prior and during the period of programmed cell death.

The expression of NRAGE within the purified B2\(^+\) population of sympathetic neuroblasts reveals an interesting insight into the potential function of NRAGE in the PNS. Both NRAGE protein and message are expressed in sympathetic neuroblasts, yet \(p75^{NTR}\) is only expressed after the onset of mitotic arrest\(^{100,329}\). This means that if NRAGE mediates the cell cycle withdrawal of committed neuroblasts it is through a \(p75\) independent mechanism.

So what if any function does NRAGE and \(p75^{NTR}\) interactions have in the formation of the PNS? It is possible that NRAGE-\(p75^{NTR}\) work to cull neurons that reach their targets but have failed to reach sufficient levels of Trk on their cell surface making apoptosis an instructed extrinsic event (\(p75^{NTR}-NRAGE\) as
apposed to a default intrinsic pathway lacking NGF stimulation. This would be a twist on the classical neurotrophic theory in that neurons that do not receive sufficient neurotrophic support from lack of Trk-NGF die as a consequence, but also may be instructed to undergo apoptosis as a result of NGF- p75NTR-NRAGE activation.

In the CNS NRAGE Protein Expression Correlates with Timing of a p75NTR-Independent Neural Precursor Apoptosis.

During brain development NRAGE is present predominantly during the stages when the ventricular and subventricular region are undergoing rapid proliferation of neural precursor cells. Concomitant with differentiation and migration of neuroblasts from the ventricular region, NRAGE localizes to this proliferative layer. Although, the spatial distribution of NRAGE protein is largely independent of p75NTR receptor expression within the brain NRAGE does co-localize with p75NTR in the subplate region. p75NTR has been detected within the subplate in rat, primate and human species cerebral cortex. Furthermore, p75NTR has recently been show to promote survival of subplate neurons in vitro in a Trk independent fashion. In addition to the subplate, NRAGE co-localizes with p75NTR in basal forebrain cholinergic neurons located in the medial septum, diagonal band of Broca, nucleus basalis and striatum. The basal forebrain has been shown to be sensitive to NGF induced apoptosis. However, an involvement of NRAGE in mediating apoptosis in the striatum has yet to be determined.
Taken together these results do not preclude the possibility that NRAGE is involved in a p75<sup>NTR</sup>-mediated neural apoptosis. However, this does not seem likely since NRAGE levels are significantly reduced during the timing of trophic dependent apoptosis. However, the significant level of NRAGE in the ventricular zone certainly indicates an ancillary function to that of p75<sup>NTR</sup>. Like Necdin, NRAGE functions as a growth arrest protein and may function in mediating mitotic arrest when neuroblasts are beginning to differentiate and migrate from the ventricular zone.

The fact that NRAGE may possess roles apart from p75<sup>NTR</sup> during development does not exclude a pro-apoptotic function in a cell cycle dependent manner within the brain. Previous studies have shown that molecules acting during cell cycles progression are required for apoptosis. These include mitotic kinases<sup>338</sup>, the tumour suppressor gene p53 which is required for neural precursor cell apoptosis<sup>339</sup> and cyclin D<sub>1</sub><sup>340</sup>. There is growing support indicating the existence of two functionally distinct types of programmed cell death in the nervous system of developing mammals. This early neural cell death affects neural precursor cells and young neuroblasts involved in an active mitotic cycle, whereas trophic dependent cell death involves postmitotic neurons<sup>16</sup>. Recent estimates indicate that between 50-70% of cortical neuroblasts in the proliferative ventricular zone undergo apoptosis in murine and primate species<sup>23,29</sup>. While the precise number of neural precursor cells undergoing apoptosis is under debate<sup>26,341-343</sup> the importance of this developmental event has been inferred from the studies of caspase-3, -9 and APAF-1 deficient mice. These knockout
mice all exhibit marked ventricular zone expansion\textsuperscript{32,33,344,345}. The selective enlargement of the ventricular zone during early neurogenesis suggests that caspase-3, -9 and APAF-1 functions are essential for apoptosis during early development of the CNS and that in the absence of normal neural precursor apoptosis cell development is abnormal\textsuperscript{346}. Timing of neural precursor apoptosis within the CNS is region specific, generally cell death commences at around E10, reaching a peak around E14, and then decreasing postnatally to low levels seen in the adult. This early-programmed cell death coincides very well with both the spatial and temporal distribution of NRAGE. Taken together with the fact that NRAGE levels peak just prior to the onset of the neural precursor apoptosis and that concurrently the first cortical neurons are exiting the cell cycle at E15\textsuperscript{347} it becomes important to determine whether NRAGE may function as a molecular switch between cell proliferation and apoptosis for neural precursor cells in the developing mouse cerebral cortex.

Other studies are also consistent with the operation of extensive neuroproliferative apoptosis related to the progression of the cell cycle. Thomaidou and colleagues\textsuperscript{26} demonstrated that the commitment to die in the developing ventricular zone of the cerebral cortex is integrally related to the G1 to S transition. A primary function in linking cell cycle and cell death is exerted by proteins acting as cell cycle checkpoints. A key regulator of the G1 to S transition is E2F-1\textsuperscript{348} and mice lacking E2F-1 have a suppressed level of apoptosis\textsuperscript{349}. The p53 protein is another transcriptional regulator of the G1 to S transition and is also involved in promoting apoptosis of neural precursor cells in a caspase
dependent manner\textsuperscript{339}. Thus, it appears that cell cycle proteins are important, for at least some apoptotic mechanisms that result in the activation of caspases during early neural cell death. However, further research is required to draw definitive conclusions pertaining to NRAGE activity during neural precursor apoptosis.

NRAGE was also identified in many other non-neural tissues throughout development. Similarly the p75\textsuperscript{NTR} receptor is expressed in a variety of other non-neural tissues, both in the embryo and the adult. The gross tissue distribution of NRAGE is in agreement with the distribution of p75\textsuperscript{NTR} previously characterized in rats\textsuperscript{350,351}. However, our result also identified non-neural regions that are not known to express p75\textsuperscript{NTR}. Therefore, these results can be attributed to the proposed diverse nature of NRAGE's endogenous function as a growth arrest protein and as a mediator of a p75\textsuperscript{NTR}-independent pro-apoptotic signaling factor.

Within the majority of tissues NRAGE expression is correlated within populations of renewable cells and immature differentiated phenotypes, for example the testis (spermatozoa production), intestine (epithelial turnover), skin (dermis), spleen (lymphocyte production), liver (hepatocyte turnover), pregnant uterus, and olfactory bulb and neural precursor cells of the adult brain.

The expression of NRAGE throughout early stages of embryogenesis suggests that this protein plays an important signaling role(s) in the development of the neural tube, brain and many non-neural tissues as well. The function of NRAGE at these early times may not be mediated through neurotrophin p75\textsuperscript{NTR} interactions, as we observed in the thoracolumbar B2+ population. In this
population of purified sympathetic neuroblasts, NRAGE expression correlates with excite from the cell cycle prior to expression of Trk or p75NTR. However, under strict spatial and temporal regulation when expressed in the presence of p75NTR, NRAGE may mediate an NGF cell death-signaling cascade. This may be a novel mechanism by which sympathetic and presumable sensory neuroblasts that do not express TrkA or insufficient levels of TrkA may be culled from the population of innervating neuroblasts. When expressed in the absence of p75NTR under a different set of spatial, temporal and possibly structural regulations, NRAGE may induce cell cycle arrest potentially leading to apoptosis through a novel mechanism.
CHAPTER 3

NRAGE Regulates p38 Activation to Mediate the Transition Between BMP Induced Apoptosis and Differentiation of Neural Progenitors.

Abstract

Understanding the molecular events that govern neural progenitor lineage commitment, mitotic arrest and their differentiation into functional progeny are germane to our comprehension of neocortical development. Bone morphogenetic protein (BMP) family members play pivotal roles in regulating neural differentiation and apoptosis during neurogenesis through either Smad and/or TAK1 activation. NRAGE possesses the ability to function as a key component in the TAK1 signaling cascade stimulating p38 activation leading to the induction of apoptosis. Loss of NRAGE function in multipotential neural progenitors leads to loss of neuronal differentiation potential through perturbation of progenitor terminal mitosis. The capacity for NRAGE to modulate the cell cycle is well observed through over-expression that results in a rapid G1-S arrest with an aberrant S phase re-entry. These studies support a model in which NRAGE serves as a modulator of downstream signaling events affecting the balance between cell cycle progression, differentiation and apoptosis.

Introduction

The pseudostratified neuroepithelium of the neural tube is generated from neural progenitor cells located within the ventricular and subventricular (VZ/SVZ) zones. The fate of neural progenitors is defined by their microenvironment
including effects from extra-CNS tissues. The generation of specific cellular phenotypes, neurons, astrocytes, radial glia and oligodendrocytes occur during tightly regulated spatial and temporal windows throughout embryogenesis and early postnatal life. Regulation is complex requiring transitional changes in intrinsic cues and the availability of extrinsic growth, differentiation and survival cues. Further complicating our understanding of stem cell progression from self-renewing multipotential cell to differentiated progeny within the cortex, is the increasing recognition that programmed cell death or apoptosis is a prominent event regulating cell number throughout the entire pathway from stem cell to neural progenitor to mature differentiated phenotype. The regulation of cell numbers during all facets of neural development may be important for the correct temporal and spatial generation of cell phenotypes and consequently accurate brain morphogenesis.

The fate of neural progenitor cells is regulated in part by bone morphogenetic protein (BMP), members of the transforming growth factor-β superfamily. BMPs signal through heterotetrameric complex formations of type I (BMPR-I) and type II (BMPR-II) serine-threonine kinase receptors. The "canonical" pathway activates Smad1, Smad5 and Smad4 proteins. However, recently an alternative signaling pathway has been described in which the X-linked inhibitor of apoptosis protein (XIAP) functions as an adaptor protein bridging BMPR-Ia and TGF-β activated binding protein (TAB1), an activator of the MAPKKK TGF-β activated Kinase 1 (TAK1). TAK1 activity has been
shown to promote the activation of p38, Jun N-terminal kinases (JNKs), and Nemo-like kinase, a MAPK family member.

The multiple functions of BMPs during embryogenesis and neural development include complex actions upon the neural crest, apoptosis or differentiation of neurons and glia within the cortex and dorsal spinal cord. These apoptotic actions may be initiated through TAK1, which induces apoptosis in the neuroepithelium of Xenopus embryos and this cell death is inhibited by Bcl-2. Another prominent regulator of cell death is XIAP, which can directly bind and inhibit caspase activity. More recently, XIAP has been shown to bind TAB1, an activator of TAK1, and inhibit TAK1-induced apoptosis.

Collectively these observations suggest that BMPs regulate fundamental events during embryogenesis of the nervous system. The mechanism that governs a seemingly paradoxical switch between morphogenesis and apoptosis within the developing cortex remains unclear. However, recent work suggests that during the onset of corticogenesis in the mouse at embryonic day 13 (E13), high levels of BMPs (10-100 ng/ml) promote apoptosis and inhibit proliferation of neural progenitor cells of the ventricular zone. Moreover, by the midpoint of neurogenesis (E16), BMPs promote neural differentiation of neural progenitors. Work by McKay and colleagues describe a feed-forward mechanism whereby BMP actions are first induced through BMPR-la and then terminate through BMPR-lb. As cell surface levels of BMPR-lb exceed those of BMPR-la, BMPs cause a termination response of either cell death or cell
differentiation\textsuperscript{384}. Concurrent with the transition from BMPR-la to BMPR-1b, neural and glia progenitors are secreting BMPs thus directing both neurogenesis and gliogenesis in a concentration-dependent manner to make the critical switch to neurotrophin signals and related cytokines\textsuperscript{385,388}.

NRAGE is a recently characterized Neurotrophin Receptor interacting MAGE homologue, identified in a yeast two-hybrid screen for proteins that interact with the intracellular domain of the p75 neurotrophin receptor (p75\textsuperscript{NTR}). NRAGE was shown to mediate both cell cycle withdrawal and an NGF-specific apoptosis in sympathetic precursor cells through p75\textsuperscript{NTR}\textsuperscript{155}. The developmental characteristics of NRAGE revealed expression during early corticogenesis, specifically in neural progenitors of the ventricular zone and in differentiating neuroblast of the cortex, independent of p75\textsuperscript{NTR} expression and concomitant with the spatial and temporal occurrence of BMP-mediated apoptosis\textsuperscript{389}. This p75\textsuperscript{NTR}-independent expression suggests an alternative signaling cascade that may utilize NRAGE to regulate mitotic arrest and apoptosis of neural progenitors. Supporting this alternative for diverse cellular functions, NRAGE augments interleukin-3-withdrawal-induced apoptosis through binding endogenous XIAP\textsuperscript{290} and NRAGE is involved in UNC5-H1 induced apoptosis of neural progenitors\textsuperscript{294}.

Based on the temporal and spatial appearance of NRAGE in the ventricular zone, an active area of cell cycle withdrawal and BMP-induced apoptosis, we investigated whether NRAGE was involved in these events during cortical neurogenesis. Using a loss-of and gain-of-function approach, NRAGE expression was shown to be a prerequisite to mediate apoptosis of neural
progenitors through a BMPR-la dependent pathway. Activation of BMPRs leads to the formation of the XIAP-TAK1-TAB1 signaling complex in which NRAGE is a crucial component regulating p38 activity. The level of NRAGE protein expression is critical in maintaining a balance between cell cycle progression and apoptosis. Under low BMPR stimulation, there is a low expression of NRAGE that facilitates the formation of a TAK1-TAB1-XIAP-NRAGE complex that activates p38, driving a transient progression through the cell cycle by directly coupling growth arrest and cell cycle progression. Greater BMPR activation leads to the induction of NRAGE and at these elevated levels, NRAGE binds both members of the SMAD and TAK1 pathways triggering competitive and apposing cell cycle dynamics to initiate p53 activation and neural progenitor apoptosis. These abilities for NRAGE to regulate MAPK signaling and cell cycle progression are fundamentally important for neural differentiation and maintenance of cell number through gestation making NRAGE a key molecule during neocortical development independent of its role in neurotrophin signaling events.

**Material and Methods**

**Cell Culture.**

P19 embryonal carcinoma cells were maintained in growth medium consisting of α-MEM (Invitrogen), 7.5% heat-inactivated calf serum and 2.5% heat-inactivated fetal calf serum (HyClone) at 37°C in 5% CO₂. For apoptosis assays cells seeded at a concentration of 1 X 10⁵ cells/ml in bacteriological-grade culture dishes in growth media supplemented with 1 μM all-trans retinoic
acid (RA) and/or 10 ng/ml recombinant BMP4 (R&D Systems) or without supplementation between 1-4 days. For neural differentiation P19 cells were grown as aggregates in the presence of 1 μM RA for 4 days, trypsinized and transferred to fibronectin coated tissue culture dished in the absence of RA for an additional 4 days.

Cortical neural progenitors were isolated from timed pregnant mice sacrificed at E13 and the embryos were aseptically removed and placed in Hank’s balanced salt solution (HBSS) (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Cerebral cortex was removed, freed of meninges, dispersed with 0.05% trypsin-EDTA and filtered with 70 μm nylon cell strainers (VWR). Cells were seeded (2x10^5 cells/mL) as aggregates in a medium containing 38% Neurobasal medium, 60% DMEM/F12, 2% FBS, N2 supplemented (Invitrogen) and 0.4 μl/mL penicillin/streptomycin (Invitrogen).

**Over-expression and Loss of Function Studies.**

Over-expression studies were performed using mammalian expression vectors containing NRAGE-EGFP, NRAGE-myc, and an empty vector-EGFP and a dominant negative Smad5. Cells were transfected via Lipofectamine 2000 as per the manufactures recommended protocol (Invitrogen). Loss of function studies employed the use of antisense morpholinos directed to the start sites of the genes of interest (see table 2 for sequences, delivery and knockdown efficiency) and delivered using EPEI as recommended by the manufacture (Gene Tools).
Table 2. Morpholino Sequences and Knockdown Efficiency

<table>
<thead>
<tr>
<th>Morpholino</th>
<th>Sequence</th>
<th>Delivery Efficiency</th>
<th>Knockdown Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRAGE</td>
<td>5'-GGTTTCTGAGCCATAGCTCTCGTC-3'</td>
<td>90%</td>
<td>80%</td>
</tr>
<tr>
<td>TAK1</td>
<td>5'-AGCGCCCTTCAGCCCAGGCCC-3'</td>
<td>92%</td>
<td>85%</td>
</tr>
<tr>
<td>TAB1</td>
<td>5'-CAGGCTCCTCCTCTGCAGCCATC-3'</td>
<td>85%</td>
<td>91%</td>
</tr>
<tr>
<td>XIAP</td>
<td>5'-CATCTTCTCTGAGAAAATAGGACTTG-3'</td>
<td>88%</td>
<td>74%</td>
</tr>
<tr>
<td>Standard Control</td>
<td>5'-CCTCTTACCTCAGTTACAATTATA-3'</td>
<td>90%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Morpholinos incorporated a fluorescein tag to identify cells that have taken up the Morpholino sequence allowing purification of the population if required by fluorescence activated cell sorting. The specific p38 MAPK antagonist SB 203580 was use at a concentration of 5 μM (Calbiochem).

Over-expression of wild-type and dominant negative BMPR-la constructs was accomplished through the generation of retroviral particles. The 293 GPG retroviral packaging cell line was a generous gift from Dr. Chris Naus (UBC). 293 GPG cells were maintained in medium consisting of DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 300 μg/ml G418, 200 μg/ml puromycin, and 1.0 μg/ml tetracycline. To generate the retrovirus pSAP-expression vectors contain either wild-type or dominant negative BMPR-la were transiently transfected in our 293 GPG packaging cell line using Lipofectamine 2000. The supernatants were collected every 12~24 hours over a 4 day period. All culture supernatants were filtered through 0.45 μm syringe-mounted filters. Aliquots of 1.0 ml were storied at −80°C. Titration of retroviral supernatant was
performed using serial dilutions (1/10) of the supernatant, percent EGFP positive cells were determined using a BD FACS Calibur and taken as the ratio of viral volume per ml (CFU/ml). Cells were transfected with appropriate titers of retrovirus using 1 μg/ml polybrene.

**Immunoblotting and Immunoprecipitation.**

The following antibodies were used for biochemical analysis: α-NRAGE31 (Salehi et al., 2000), α-Caspase-3 (Pharminen), α-TAK1, α-p38, α-dp-p38, α-JNK1/2, α-β-Actin (Sigma), α-XIAP, α-BMPR-la, α-BMPR-1b, α-SMAD1, α-SMAD5, α-TAB1, α-GFP (Santa Cruz), α-NFκBp65 (BD Biosciences), α-phosphoserine, α-N-myc, α-pSMAD1, α-pRB, α-p-pRB, α-p53, α-p21waf1, α-p27kip1 (Upstate). For biochemical analysis whole cell extracts were prepared using Nonidet P-40 (NP-40) lysis buffer [137 mM NaCl, 20 mM Tris (pH8.0), 0.5 mM EDTA, 10% Glycerol, 1% NP-40] containing a protease inhibitor cocktail (Roche) supplemented with 1mM sodium orthovanadate and 1.25 mM sodium fluoride. Concentration of total soluble protein was measured using BCA Protein Assay Kit (Pierce). Lysates were immunoprecipitated using 50 μg protein, 0.1 μl (v/v) pansorbin (Calbiochem), 1 μg/mL α-N-Myc or α-GFP antibody overnight at 4°C. IPs were washed three times with lysis buffer and subjected to SDS-PAGE immunoblotting. For either immunoblotting experiments or IPs 50 μg cell lysate were subjected to 7-15% SDS-PAGE under reducing conditions. After semi-dry transfer onto Immobilon-P (Millipore), membranes were probed with appropriate primary antibody. Primary antibody detection was accomplished using an appropriate secondary horseradish peroxidase conjugate IgGs (Jackson Immuno...
Research Laboratories Inc.), developed using a chemiluminescent reagent (Amersham), and exposed to Fujifilm between 1 and 2 minutes.

**Apoptosis and Cell Survival Assays.**

The number of viable and nonviable cells was determined by trypan blue exclusion. An equal volume of 0.4% trypan blue solution (Sigma) was added to the medium and left for 1 minute. The numbers of stained and total cells were then counted within 5 minutes using a hemocytometer. Graphed data represents averages taken from four samples, denoting one replicate, and depicted as the means ± SD of four replicate experiments.

All apoptosis and fluorescence-activated cell sorting (FACS) analyses used a Becton-Dickinson FACS Vantage SE cell sorter (Enterprise IIIC laser providing simultaneous 488 nm and 350nm output, Spectra Physics Helium-Neon Laser Provides 633 nm output). Annexin-V labeling utilizes the early externalization of phosphatidylserine upon the outer leaflet of the plasma membrane by its capacity to bind annexin-V in a calcium dependent manner. Live cells were collected 24 or 48 hours post-apoptotic induction and labeled with annexin-V biotin and subsequently detected using streptavidin-PE secondary antibodies and analyzed using flow cytometry. For annexin-V assays 10 μg/mL 7-actinomycin D was used as a cell impermeable vital dye discriminating early apoptosis (annexin-V+/7-AAD−) and late apoptosis or necrotic cell death (annexin-V+/7-AAD+). Additional measures of apoptosis employed the characterization of mitochondrial membrane permeability as determined by the MitoCapture assay
kit (Calbiochem) and analyzed using flow cytometry according to manufactures specifications.

**Cell Cycle Analysis.**

For cell cycle analysis P19 cells over-expressing the genes of interest were synchronized in G0/G1 using standard serum withdrawal. Briefly cells were cultured in serum free conditions for 6 hours, transfected in serum free conditions for an additional 6 hours upon conclusion serum containing medium was replaced and cells were permitted to grow for an additional 24, 48 or 72 hours. Live cells were collected, stained using a hypotonic DNA staining solution [1 mg/ml sodium citrate, 0.3% Triton-X100, 0.1 mg/ml propidium iodide, 20 μm/mL RNAse A] and DNA content was analyzed on a Becton-Dickinson FACS Calibur using Modfit 2.0 software. NRAGE' or control' cells were gated and DNA content was measured on FL2-area. The base line location of the G0/G1 peak was set for the control' cells and determined independently for each replicate.

**Immunocytochemistry.**

Adherent cultures were grown on fibronectin coated glass coverslips, after 4 days cells were fixed in 4% paraformaldehyde in PBS (10 minutes), rinsed in PBS and labeled with the postmitotic neural markers MAP2a/b, the astrocyte specific marker glial fibrillary acidic protein (GFAP) (Sigma) and Nestin which labels multipotential progenitors (Pharmingen). Appropriate species and isotype specific secondary antibodies conjugated to Rhodamine were used together with the DNA binding dye Hoechst 33258 (1μg/ml). Percentage of cell phenotypes
were obtained for each replicate as the average of four frames and graphed data represented as the mean ± SD of four replicates for each condition (NRAGE knockdown and Control Morpholino).

**Semi-quantitative RT-PCR.**

Total RNA was isolated, treated with 1 unit of DNase I at room temperature and re-precipitated. First strand cDNA was synthesized from 5 µg total RNA with Superscript II (Invitrogen) using an Oligo (dT) 
12-18 (Invitrogen) primer according to published procedures. All PCR reactions were performed in the linear range of amplification as determined empirically using 1 µL for each cDNA sample generated. When ever possible primers were designed to cross an intron-exon boarder to distinguish cDNA amplification from any potential genomic contamination. Primer sequences and annealing temperatures are detailed in table 3.

**Table 3. RT-PCR Primer Sequences**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5'-3'</th>
<th>Reverse primer 3'-5'</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct3/4</td>
<td>ATGGGCTGGACACCTGGCTTCAGACT</td>
<td>GCCAGGGCTCTGATCAACAGCATCA</td>
<td>63°C</td>
</tr>
<tr>
<td>Hes1</td>
<td>AGAAGAGGCCAAGGGGCAAGAA</td>
<td>CAAAACACTTTGGCAGCCTC</td>
<td>54°C</td>
</tr>
<tr>
<td>Mash1</td>
<td>TCGGCACTGACTTTTGCGGC</td>
<td>GAAGCAGATCAAAAGGGG</td>
<td>56°C</td>
</tr>
<tr>
<td>Dlx5</td>
<td>CCGTCTCAGGAATCGCCAAC</td>
<td>CTGAAAGCGTGGGCTGGTG</td>
<td>56°C</td>
</tr>
</tbody>
</table>
Cycling parameters were as follows: denaturing 94°C, 30 seconds, annealing (primer specific see table 3), 30 seconds and extension at 72°C. Amplification products were separated on a 1% agarose gel and visualized with ethidium bromide. Gels depict representative experiments obtained from at least four replicates from four separate RNA pools.

**Statistics.**

All data are presented as the means ± SD of at least four independent experiments. Significance was determined by Student's t-test.

**Results**

**NRAGE is Required in the BMP Mediated RA-induced Apoptosis.**

Since, NRAGE was expressed within the developing central nervous system in the absence of p75NTR we asked whether NRAGE played a key role in the formation of the nervous system outside those roles mediated by neurotrophins. Since apoptosis is a prominent event in the regulation of cell number throughout the entire progression from stem cell to neural progenitor to mature differentiated neural cell type and NRAGE has been shown in earlier studies to mediate apoptosis in sympathetic precursor cells, we initially were interested in determining if NRAGE mediates apoptosis in the absence of p75NTR-mediate neurotrophin signaling in embryonal carcinoma (P19) cells and

<table>
<thead>
<tr>
<th>Msx2</th>
<th>ATTCGCCGCCGCCAAGACAT</th>
<th>TCTTTTCGCTTTGCCCTTC</th>
<th>56°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>TGTTACCAAATGGACGACA</td>
<td>CTCTCAGCTGTGGTGAA</td>
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</tr>
</tbody>
</table>
E13 cortical neural precursors as putative models for cortical progenitor apoptosis. Much of the detailed information regarding the effect of BMP signaling on neuroblast proliferation, differentiation and apoptosis has been garnered using these model systems. P19 cells have previously been shown to be devoid of $p75^{NTR}$ receptor expression during the first 4 days of RA and/or BMP4 induced apoptosis, but do up-regulate the receptor on post-mitotic neurons. Indeed, RT-PCR analysis demonstrates that early P19 cells treated to undergo apoptosis through RA treatment fail to express $p75^{NTR}$ receptor (Fig. 10A).
Figure 10. RA Induced Apoptosis is Mediated Through BMP Signaling.

(A) RNA was isolated from P19 cells treated with RA over a 4-day period or RNA was isolated from E13 neural progenitors following 3 or 7 days of in vitro differentiation. Note P19 cells do not express p75\textsuperscript{NTR}, but post-mitotic neurons cultured from mouse neural progenitors are p75\textsuperscript{NTR} positive. (B, C) Dose response curves were generated to determine effective dosages for the induction of apoptosis using increasing concentration of either RA or BMP4. P19 cell
survival was measured using trypan blue exclusion after apoptotic induction with 10 ng/ml BMP4, 1 μM RA or both. Presented in (D) are the means ± SD of four independent experiments demonstrating that RA enhances the number of trypan blue stained P19 cells using the BMP signaling pathway. (E, F) Presented are representative FACS histograms of P19 and E13 cortical neural progenitors (NP) treated with 10ng/ml BMP4 and 1 μM RA. Also presented is a western analysis demonstrating the extent of NRAGE suppression using NRAGE antisense morpholinos relative to standard morpholino control. Note in both cortical progenitors and P19 cells, suppression of NRAGE expression (NRAGE KD) limits the percentage of annexin-V, apoptotic, cells.

Retinoic acid (RA)-induced apoptosis of cortical progenitors and EC cells has been argued to occur via the BMP signaling pathway since BMP4 and RA appear to act synergistically to potentiate the apoptotic effect. Using two independent measures of cell survival, trypan blue exclusion and annexin-V immunoreactivity, we found that, in agreement with the previous data, cortical neural progenitors (NP) and EC cell viability was greatly compromised when treated with a combination of RA and BMP4 than when cells were treated with either molecule alone (Fig. 10D). These results do not rule out the possibility that the actions of BMP and RA are additive, perhaps due to distinct populations of BMP-sensitive and RA-sensitive cells, nor does this result formally support the hypothesis that RA leads to a BMP induced apoptosis as suggested by Rodriguez-Leon and colleagues. In an attempt to demonstrate that RA actions
were mediated by BMP, we attempted to exacerbate the effects of RA on developing neuroblasts by over-expressing BMPR-1a and treating the resulting cells with RA. Cell survival decreased precipitously (52% ± 2) to a level comparable to that of the cells treated with RA and BMP4 (48% ± 1). In contrast, cells in which BMP signaling was diminished by the expression of a dominant negative (dn) allele of BMPR-1a, that expresses only the extracellular domain and is signaling incompetent, cell viability in the presence of RA was enhanced and approached the level of untreated controls (RA treated: 72% ± 2; RA + dnBMPR-1a: 85% ± 1 p< 0.01; Untreated: 95% ± 1). Moreover, RT-PCR analysis demonstrated that BMP2 and BMP4 transcripts were greatly elevated after 8 hours of RA exposure (data not shown). These results support the hypothesis that RA-induced apoptosis of proliferating neuroblasts is mediated by the BMP signaling pathways.

We then asked if NRAGE had any role in mediating cell survival in either cell system. Cell viability was greatly enhanced in RA/BMP4 treated cells that under-expressed NRAGE using specific antisense morpholinos (Fig. 10E,F). Conversely, the vast majority of P19 cells over-expressing NRAGE underwent cell death within 24 hours, even in the absence of RA/BMP4 treatment (Fig. 10D). The addition of RA/BMP only enhanced the effect and the time course of apoptosis in NRAGE over-expressing cells. These data, taken together, demonstrate that the cell death signal, initiated by RA and BMP is relayed by the BMP signal transduction cascade and is mediated by NRAGE.
To further confirm the link between NRAGE and RA/BMP4 mediated apoptosis, we examined the expression of NRAGE protein as a function of time after RA/BMP4 treatment. NRAGE protein levels rose sharply one day after induction with RA/BMP (Fig. 11A) and in primary E13 cortical progenitors (Fig. 11B).

![Figure 11](image)

**Figure 11. NRAGE is Required for the Activation of Caspase-3 by RA/BMP.**

Lysate of P19 or NP cells harboring control morpholinos or NRAGE morpholinos treated with RA, BMP4 or both were collected daily and subjected to western analysis to determine the levels of NRAGE and active caspase-3 during apoptosis. In the absence of NRAGE expression, caspase 3 is not active and this correlates with enhanced cell survival.

Strikingly, this increase correlated with an increase in the levels of activated Caspase-3 in both systems. The activation of Caspase-3 was abrogated in cells where NRAGE levels were reduced (Fig. 11A,B) and activation was conversely enhanced in NRAGE over-expressing cells. These results suggest that NRAGE plays a pivotal role in mediating RA/BMP induction of Caspase-3 and apoptosis.
NRAGE Interacts with Members of the BMP Signaling Cascade.

NRAGE's role in the RA/BMP induced apoptosis prompted us to clarify the function of NRAGE in the BMP signaling cascade. To this end, we performed immunoprecipitation experiments using myc-tagged NRAGE over-expressed in EC cells treated with BMP4. Cell lysates were collected and Western analyses were performed using antibodies against various members of the BMP signaling cascade. NRAGE was found to weakly associate with the BMP receptors BMPR-II and BMPR-la, while it showed a strong association with BMPR-lb. Since both BMPR-I and BMPR-II are capable of existing as a heterotetramer when activated, immunoprecipitation of both type-I and type-II receptors with NRAGE was not unexpected (Fig. 12).

| NRAGE-myc | + | - | + | - | + | - | + | - | + | - | + | - | - | + | - |
| pCDNA-myc | - | + | - | + | - | + | - | + | - | + | - | + | - | + | - |

**Figure 12. NRAGE Strongly Interacts with XIAP, TAK1, TAB1 and BMPR-lb.**

Present is a western analysis of P19 cells transiently over-expressing full length NRAGE construct tagged with Myc and treated with BMP4 for 24 hours. Immunoprecipitation of the resulting lysates using the myc tag were used to precipitate potential NRAGE binding proteins. The resulting blots were probed for various members of the BMP signaling cascade.
The predominant mode of BMP signaling within the cell utilizes members of the Smad family. As a result, we also looked at NRAGE interaction with these proteins. Both Smad1 and Smad5 were found to only weakly associate with the NRAGE complex. Interestingly, NRAGE was found to strongly interact with TAK1, TAB1 and XIAP (Fig. 12). These proteins are all members of a lesser-known pathway downstream of the BMP receptors. Previous reports have shown that TAB1 (TGF-β activated binding protein) activates TAK1 (MAPKKK TGF-β activated kinase 1), which, in turn, promotes the activation of p38. XIAP (X-linked inhibitor of apoptosis) appears to be required to functionally bridge TAK1-TAB1-p38 signaling through BMPR. Interestingly, over-expression of XIAP enhances TAK1-p38 signaling, but inhibits TAK1 mediated apoptosis suggesting a complex regulatory mechanism. NRAGE interaction with these members of the BMP signaling pathway is specific since there was no interaction between NRAGE and BMP independent TAK1 binding proteins NF-κBp65 and Bcl-2 (Fig. 12, data not shown), suggesting that the main mode of BMP apoptotic activity is through this non-canonical signaling pathway of TAK1, TAB1 and XIAP.

The interaction between NRAGE, TAK1, XIAP and TAB1 appears to be a functional one since NRAGE serine phosphorylation is reduced in cells where levels of XIAP, TAK1 or TAB1 were reduced. Using immunoprecipitation followed by Western blot analysis with anti-phosphoserine antibodies, it was determined that in the presence of BMP4, serine phosphorylation of NRAGE was enhanced relative to that observed in unstimulated cells. If however, the experiment was
repeated under conditions limiting XIAP, TAK1 or TAB1 expression, phosphorylation of NRAGE was significantly reduced (Fig. 13A).

Figure 13. The TAK1, TAB1 and XIAP Complex Promotes NRAGE Phosphorylation.

(A) BMP4 treatment enhanced the serine phosphorylation of NRAGE in a XIAP-, TAK1-, TAB1-dependent manner. Protein lysates were generated from BMP4 treated P19 cells over-expressing NRAGE and harboring antisense morpholinos against TAB1, TAK1, or control (see methods and table 2). The resulting lysates were immunoprecipitated for NRAGE and immunoblotted for phosphoserine. (B) Western analysis demonstrating the effectiveness of TAB1, TAK1 and XIAP morpholinos.

A Reduction in protein levels as a result of morpholino exposure was evident as early as 24 hours after treatment (Fig. 13B). These results suggest that NRAGE, XIAP, TAK1 and TAB1 form a complex that promotes NRAGE phosphorylation and activation.
NRAGE Regulates BMP-linked Apoptosis via the BMP Target, p38 MAP Kinase.

Serine phosphorylation of NRAGE potentially leads to its activation, but the putative targets of activated NRAGE still remained to be determined. p38 MAP Kinase is a target of the BMP signaling cascade that has been shown necessary for the apoptotic response in various cell types. We hypothesized that the effect of NRAGE on the BMP mediated apoptosis might also occur via this prominent downstream regulator. To demonstrate that NRAGE is required for the activation of p38 by BMP4, we looked at p38 phosphorylation in cells expressing NRAGE at normal and reduced levels. We also monitored p38 phosphorylation as a function of TAK1 levels. This latter experiment functions as a positive control, since TAK1 is a known upstream regulator of p38. As expected, reduction of TAK1 expression via antisense morpholinos drastically perturbed activation of p38 (Fig. 14A).
Figure 14. NRAGE is Required for the Activation of p38 MAPK.

(A) Western blotting was used to detect active, doubly phosphorylated p38 in control cells and cells treated with either TAK or NRAGE morpholinos. Although all cells expressed similar levels of inactive p38, only control cells showed phosphorylation of p38. Reduced levels of NRAGE or TAK1 inhibited the appearance of active p38. In contrast, treatment with NRAGE morpholinos had
no effect on the canonical BMP pathway as indicated by the appearance of active SMAD-1. JNK was not activated as a result of BMP4 treatment. (B) NRAGE-myc was transiently over-expressed and cells were treated with or without BMP4, cell lysates were collected and immunoprecipitated. Note BMP4 treatment facilitates BMPR-la complex formation, whereas in the absence of ligand NRAGE interacts only minimal with receptor components. (C) Endogenous NRAGE was immunoprecipitated following BMP4 treatment from NP cells. Only in the presence of BMP4 does NRAGE interact with BMPR and its associated effects.

More importantly, we found a similar reduction in p38 activation in cells in which TAK1 expression was normal but NRAGE levels were diminished, suggesting that like TAK1, NRAGE is a key upstream and perhaps direct regulator of p38 activity (Fig. 14A). The down regulation of NRAGE expression appeared to have a specific effect on p38 activation, as there was no change in the activation of Smad-1 nor was JNK activated as a result of BMP4 treatment (Fig. 14A). These results demonstrate that the induction of cell death by BMP is accomplished through NRAGE's regulation of p38 activity.

We made an initial observation that when NRAGE was over-expressed, application of BMP4 significantly enhanced the capacity for NRAGE to associate with members of the BMP signaling cascade (Fig. 12). However, in the absence of ligand stimulation, prolonged NRAGE over-expression results in cellular apoptosis suggesting that accumulation of NRAGE can force activation of the
BNLP signaling complex. However, in the absence of BMP4 NRAGE over-expression was found to interact only weekly with the receptor and its signaling components (Fig. 14B). Exogenous NRAGE over-expression exceeds the level of naturally occurring NRAGE within a cell and though over-expression exceeds physiological levels it highlights the important binding capacities for NRAGE under changing cellular circumstances. We therefore, wish to assess the binding interactions of endogenous NRAGE to the BMP signaling complex in the presence and absence of BMP4 treatment. Endogenous NRAGE IP from neural progenitors was found to associate with BMPR-la and XIAP, TAK1 and TAB1 only in the presence of BMP4 (Fig. 14C). Collectively, these results suggest that in the absence of an instructive apoptotic cue NRAGE remains inactive. However, following induction of apoptotic NRAGE functions in an instructive manner signaling apoptosis through BMP. As the cellular levels of NRAGE elevate the capacity for NRAGE to associate with the BMP signaling complex is enhanced leading to p38 activation and apoptosis.

Having established a potential connection between BMP signaling and NRAGE activation of p38 leading to cellular apoptosis in cortical progenitors (Fig 10F), we performed a series of similar experiments using neural progenitor cells as they succumb to the apoptotic actions of RA and BMP in a similar manner. We have previously shown that diminishing NRAGE expression in both cell systems attenuates BMP apoptotic events perhaps NRAGE activates p38 within proliferating primary progenitors. Indeed the addition of SB203580 to neural
progenitor cells in the presence of RA/BMP resulted in attenuation of p38 activation and enhanced cell survival (Fig. 15).

**Figure 15. Neural Progenitors Require p38 MAPK to Mediate Apoptosis.**

Annexin-V labeling was used to detect neural progenitor cells undergoing apoptosis in control, RA/BMP or RA/BMP in the presence of the p38 inhibitor SB 203580 (5μm). Presented in are the X ± SD of four experiments demonstrating that inactivation of p38 rescues cells from apoptosis *p<0.01.

To further confirm a role for NRAGE in RA/BMP mediated apoptosis, we induced apoptosis in P19 cells by over-expressing NRAGE and tested the effect of treatment with the specific p38 inhibitor, SB203580. Annexin-V labeling was used to detect cells in early phases of apoptosis (NRAGE-GFP+/annexin-V+/7-AAD−), and flow cytometry was subsequently used to quantify the number of living NRAGE-GFP+ cells. In cultures over-expressing NRAGE after 48 hours, 57% ± 8 of the cells were annexin-V immunoreactive, a significantly greater number than the 1 ± 2% observed in the control cell GFP-expressing population (Fig. 16D).
Figure 16. RA/BMP4 and NRAGE Over-expression Exhibit Similar Functional Requirements to Mediate Apoptosis.

(A-C) Reducing the expression of the canonical and non-canonical BMP signaling cascade abrogates NRAGE’s ability and BMPs ability to induce apoptosis in P19 cells. (D-F) When P19 cells expressing full-length NRAGE are deprived of canonical and non-canonical BMP signaling component apoptosis is efficiently reduced. In both paradigms annexin-V immunohistochemistry was measured by FACS. Presented are representative profiles of annexin-V positive cells in each condition with the X + SD of four separate experiments.

On the other hand, the NRAGE over-expressing cell population treated with the p38 antagonist, SB203580, showed an impressive rate of survival 10% ± 9 (Fig.
In addition, using the specific caspase-3 inhibitor Z-DQMD-FMK greatly enhanced the cell survival (1% ± 1) when NRAGE was over-expressed (Fig. 16F). In other words, inhibition of p38 or the caspase cascade effectively prevented apoptosis in these NRAGE over-expressing EC cells. Interestingly, the apoptosis induced through p38 was dependent on both TAK1 and Smad5 signaling as abrogation of either molecule significantly reduced the level of cell death observed (TAK1 KD, 26% ± 4; Smad5 DN, 24% ± 7) (Fig. 16D).

As a final insurance for the involvement of NRAGE in BMP mediated apoptosis we treated our P19 cells with RA/BMP4 for a period of 48 hours and subsequently determined the requirements for BMP signaling as an instructive apoptotic cue. As was found with NRAGE over-expression, both TAK1 and Smad5 signaling are an absolute requirement for the induction of apoptosis (TAK1 KD, 3% ± 6; Smad5 DN, 8% ± 3) (Fig. 16A). Furthermore, activation of p38 is also an important regulatory event, as inhibition of p38 by SB203580 was effective in reducing the level of cell death (24% ± 7) (Fig. 16B), as was inhibition of caspase-3 (0.6% ± 3) (Fig. 16C). Collectively, these studies demarcate NRAGE as a pivotal and necessary molecule in mediating the apoptotic effects of BMP in the developing central nervous system.

**NRAGE Regulates the G1-S Cell Cycle Transition.**

Recent data suggests that uncoordinated expression of cell cycle molecules and the consequent breach of cell cycle checkpoints could be one of the primary mechanisms by which neural progenitors undergo apoptotic death during terminal exit from the cell cycle. Evidence indicates that up-regulation of
cyclin/cyclin dependent kinase activity at the G1 to S transition and deregulation of E2F transcription factors mark early stages of neuronal apoptosis. Apoptotic signals promote successive phosphorylation and dysfunction of Rb family members, resulting in sequential E2F deregulation and expression of selective E2F-responsive genes. The MAPK p38 influences cells throughout all stages of the cell cycle (G1-M). Recent work suggests that p38 activation is vital to cell cycle regulation and apoptosis of neuronal and non-neuronal cells types. Initiation of apoptosis was found to inactivate Rb by direct utilization of p38 in a cyclin independent manner resulting in unscheduled re-entry into S phase. Since NRAGE can positively regulate p38 activity and has previously been shown to promote growth arrest, we assessed NRAGE’s capacity to regulate transitions through the cell cycle. Cells over-expressing NRAGE-GFP (NRAGE-GFP) were analyzed for DNA content by flow cytometry at 24, 48 and 72 post-transfection. After 24 hours NRAGE-GFP cells exhibited an enhanced accumulation in G2-M (44 ± 3%), relative to P19 cells transfected with GFP (control-GFP) (35 ± 2%; t=7.2, p> 0.04) (Fig. 17A,B).
Figure 17. NRAGE Facilitates both Growth Arrest and Progression Through S Phase.

(A,B) NRAGE-GFP was over-expressed, cells were collected 24, 48, and 72 hours post-transfection and their cell cycle profile was analyzed using flow cytometry. After 24 hours NRAGE was driving progression through the S phase with cells accumulating at the G2-M transition. However, by 48-72 hours NRAGE was facilitating a potent G1-S arrest. (C) Disruption of p38 signaling facilitates a
rapid G1 arrest in the presence of BMP4. (D, E) Treatment with either SB203580 or BMP4 along does not significantly affect the cell cycle (F) Over-expression of both NRAGE and dominant negative Smad5 results in cells accumulating in the G2 phase. (G) Whereas, over-expression of DN Smad5 along does not significant affect the cell cycle. Collectively, these results demonstrate the capacity to functional separate NRAGE mediated cell cycle events depending on the either p38 or Smad signaling.

By 48 hours the majority (55 ± 1%) of NRAGE+GFP cells were positioned at the G1-S transition compared to just 43 ± 2% (t=9.6, p>0.02) for control cells. The percentage of cells at G1-S after 72 hours was further enhanced (64 ± 4%) compared to GFP controls (39 ± 4%; t=12.3, p>0.01). These results coincide with previous reports suggesting NRAGE acts as a growth arrest protein (Salehi et al., 2000).

However, the unique bi-functional growth arrest observed within the first 24 hours provides a provocative insight into the function of NRAGE. We have established that NRAGE is a critical component of the XIAP-TAK1-TAB1 signaling cascade leading to the activation of p38. Taken with the fact that Smad signaling is a fundamental requirement for the mitotic arrest of neural progenitors through suppressing c-myc promoter activity by dissociating p300 from E2Fs providing accumulation of p21^waf1, p27^kip1^403-405. We hypothesized that in the absence of p38 Smad signaling would dominate providing an immediate G1 arrest. In support of this we found that NRAGE over-expressing cells treated with
the p38 antagonist SB 203580 displayed a subtle G1 growth arrest (data not shown). However, with the addition of exogenously added BMP4 NRAGE^{GFP} SB 203580 treated cells rapidly exited the cell cycle displaying a prominent G1 arrest, which is maintained over the 72 hour period (Fig. 17C). Furthermore, through the use of a dominant negative Smad5, NRAGE over-expressing cells underwent a dramatic and maintained G2 arrest (Fig. 17F), a direct result of unfettered p38 activity. These findings demonstrate the capability to disassociate specific aspects of NRAGE mediated cell cycle control revealing an antagonistic bi-potential function of BMP signaling. This novel cell cycle control provides an attractive model where by altering levels of BMP stimulation a neural progenitor receives, a dynamic interplay between growth arrest and cell cycle progression is created.

**NRAGE Promotes a p53 Dependent Apoptosis.**

The observation that NRAGE mediated apoptosis requires both Smad and TAK1 signaling which possess opposing cell cycle properties suggest that the induction of apoptosis is dependent on perturbation of the cell cycle. Cell cycle and apoptosis share common participants such as Rb, E2F and p53.\(^69\) This aberrant S phase reentry stimulated by NRAGE may be the underlying cause of the pro-apoptotic action of NRAGE. Taken with the fact that there is an intimate link between cell cycle and apoptosis most often leading to induction of p53 when control is lost we wished to address the contributions of p53 to an RA/BMP and NRAGE mediated apoptosis. EC control cells or p53 knockdown cells were treated to undergo apoptosis using our standard RA/BMP technique, following a
48 hour treatment window cells were collected and stained for annexin-V and subsequently analyzed using FACS. Abrogation of p53 was effective in preserving cell viability, likewise, NRAGE mediated apoptosis was found to require p53 (Fig. 18A).

Figure 18. NRAGE Mediated Apoptosis is p53 Dependent.

(A) p53 knockdown or control morpholinos were introduced into P19 cells, which were treated with RA/BMP4 or transiently transfected with NRAGE-GFP, and the percentage apoptosis was determined using annexin-V binding. Both RA/BMP4 and NRAGE-GFP induced apoptosis require p53 as a necessary component for cell death signaling. (B) NRAGE-GFP cells were purified using FACS 24, 48 and 72 post-transfection. Cell lysates were collected and subjected the standard Western blotting techniques. NRAGE initially triggers activation of
p38. However, p38 is gradually inactivated concurrent with the activation of Rb and p53. A direct consequence of activation Rb and p53 is accumulation of p21waf1 and p27kip1.

To further elucidate changes in cell cycle machinery, cell lysates were collected from FACS purified NRAGE\(^{\text{+GFP}}\) and control\(^{\text{+GFP}}\) cells after 24, 48 and 72 hour post-transfection and then subjected to standard Western analysis. During the initial 48 hours activated p38 accumulated, consistent with the G2-M arrest, however between 48 and 72 hours active p38 was rapidly turned off, consistent with the G1 arrest (Fig. 18B). The growth arrest observed after 48 hours of NRAGE over-expression coincides with the inactivation of p38 and the losses off the inactive hyperphosphorylated form of Rb. The activation of Rb may contribute to the accumulation of the cdk inhibitors p21waf1 and p27kip1 with a sharp elevation in the phosphorylated state by 72 hours. These results are consistent with NRAGE\(^{\text{+GFP}}\) induced G1-S arrest after 72 hours. In addition, p53 levels accumulate as time progressed; supporting the p53 dependence on NRAGE\(^{\text{+GFP}}\) mediated apoptosis. These results demonstrate an antagonizing bi-functional positive feedback loop in which the accumulation of cellular NRAGE enhances progression through the cell cycle in a p38 dependent manner, concurrently stimulating Smad dependent cell cycle withdrawal. Collectively these results demonstrate that NRAGE facilitates apoptosis in a cell cycle dependent manner leading to the accumulation and activation of p53.
NRAGE is Required for the Cell Cycle Arrest and Differentiation of Multipotent Progenitors.

Terminal differentiation of post-mitotic neurons required exit from the cell cycle with increasing evidence Rb family members function as key regulators. Failure to exit the cell cycle at the appropriate time leads to apoptosis. This coupled relationship between cell cycle and differentiation is modulated at multiple stages during the development to mature cells and the aberrant deregulation of Rb during NRAGE cell cycle withdrawal suggests that NRAGE plays a key role as a regulator of terminal differentiation and apoptosis. Taking advantage of the differentiative potential of P19 cells, antisense NRAGE morpholinos or control morpholinos were used to knockdown NRAGE protein expression. Cells were then differentiated with RA to induce neurogenesis and the percentage of post-mitotic neurons was calculated using MAP2a/b expression, astrocytes were detected by GFAP expression and proliferative multipoint neural progenitors by nestin. Loss of NRAGE expression drastically perturbed the generation of neurons and this deficit appeared to be the result of a failure of neural progenitor cell cycle withdrawal (Figure 19A).
Figure 19. NRAGE Mediates the Transition of Multipotential Neural Progenitors to Post-mitotic Neuron.

(A) P19 embryonal carcinoma control or NRAGE knockdown cells were differentiated with RA for 4 days and then cultured for an additional 4 days without RA. Then subsequently stained for the post-mitotic neuron marker MAP2a/b, the glial marker GFAP and nestin for multipotential neural progenitors. In the absence of NRAGE post-mitotic neurons failed to be generated and this
deficit resulted in the accumulation of neural progenitors. Glial differentiation remained unaffected. To assess whether the inhibition was a result of perturbation in pro-neural gene expression RT-PCR was performed on RA stimulated control and NRAGE knockdown cells cultured as aggregates between 1-4 days. (B) Both Oct3/4 and Hes1 gene expression were elevated suggesting NRAGE knockdown cells remain as pluripotent neural progenitors. Interesting, loss of NRAGE did accelerate the onset of early pro-neural gene expression and BMP regulated genes. However, the expression of later pro-neural gene expression like MEF2A and MEF2C was abolished. Collectively, these results demonstrate the requirement for NRAGE to mediate the transition of neural progenitor to post-mitotic neuron.

The deficit was at the sole expense of neurogenesis as evident by an increase of 32 ± 5% in the number of multipotent progenitors with the ratio of glia remaining unaffected. Therefore, NRAGE appears to be a key component regulating the transition of multipotent progenitors to differentiated phenotype.

To determine whether this inhibition in differentiation was a direct result in failure to induce appropriate gene expression RNA was collected on days 1 through 4 of differentiation and semi-quantitative RT-PCR was performed. During RA treated cultures NRAGE knockdown did not inhibit the induction of pro-neural gene expression (Figure 19B). In fact the loss of NRAGE accelerated RA regulated gene expression by 24 hours. Furthermore, using Oct3/4 as a general measure of the state of "stemness" and Hes1 as a determinant of the frequency
of neural progenitors, NRAGE knockdown cells appeared to expand or maintain cells in a multipotent state. This enhanced neurogenic gene expression is in agreement with data obtained from neurogenesis of ES as a direct result of prolonged Oct3/4 expression\textsuperscript{406} and loss of p38 activity\textsuperscript{407}. Therefore, the apparent incapacity to generate post-mitotic neurons in NRAGE knockdown cells is not a deficit in early pro-neural gene expression per se, but may reflect an imbalance between early pro-neural genes and antagonistic bHLH factors like Hes1 and Hes5. Indeed, Hes1 levels are elevated to inappropriate levels as a result of loss of NRAGE (Fig. 19B). This imbalance may directly contribute to the loss of late neurogenic genes like MEF2A/C as NRAGE knockdown greatly perturbs the genesis of these transcription factors (Fig. 19B). Whereas, MEF2B/D transcription factors, which are not involved in neural progenitor differentiation, their gene expression remain unaffected by NRAGE knockdown (data not shown). Importantly, MEF2A/C has been shown to be an absolute requirement regulating both myogenesis and neurogenesis in a cell cycle dependent manner\textsuperscript{408-410}. The loss of MEF2C is arguably a contributing factor associated with the neurogenic deficit. Taken with the demonstration that NRAGE is a potent regulator of the cell cycle, we conclude that the neurogenic deficit is a result of failure to generate post-mitotic neuron in an NRAGE-cell-cycle dependent manner.

To further corroborate our finding we challenged sorted NRAGE\textsuperscript{GFP} or control\textsuperscript{GFP} expressing cells to differentiate in the presence of RA. As NRAGE is a potent inducer of apoptosis we maintained our cultures in the presence of the
specific caspase-3 inhibitor Z-DQMD-FMK. The overall level of neurogenesis was reduced for both our cultures, suggesting a basal level of active caspase-3 is required for the transition of stem cell to neural stem cell, however this inhibition was not absolute. In agreement with a pro-neural contribution, NRAGE over-expression significantly enhanced the number of post-mitotic neurons generated and this increase in neurogenesis cooperatively reduced the neural progenitor pool (Fig. 20).

Figure 20. NRAGE Over-expression Facilitates Neurogenesis

NRAGE-GFP and GFP EC cells were sorted by FACS and differentiated under standard RA conditions. As NRAGE over-expression is highly apoptotic cultures were maintained in the presence of a specific caspase-3 inhibitor. Note the enhanced neurogenesis as consequence of NRAGE over-expression. The increase in NRAGE drives NP out of the cell cycle early and thus reduces the overall number of NP present.

Therefore our results collectively demonstrate that NRAGE is required for the terminal differentiation of neural progenitors to post-mitotic neuron and inhibition of NRAGE activity is fatal to neurogenesis. However, NRAGE levels and activity
must be strictly regulated as failure to so quickly changes NRAGE from a pro
differentiation to pro-apoptotic molecule.

**Discussion**

The presence of apoptosis in the proliferative neuroepithelium raises the
question of how the balance between maintenance and depletion of the
progenitor pool size is appropriately controlled by the extracellular
environment. To elucidate the specific features of neural progenitor apoptosis
during neocortical development, we took advantage of primary cortical
progenitors and the P19 cell line. P19 cells differentiate into neurons and glia
when grown in the presence of RA. In addition to differentiation, RA also
induces activation of caspase-9 and 3 promoting apoptosis. Furthermore,
the apoptotic action of RA is facilitated by the addition of BMP2 or BMP4.
The actions of RA mediate apoptosis through the RA receptor α and γ, which
promote the transcription of BMP2 and BMP4.

In this study, we shown that NRAGE is a necessary and sufficient
mediator of RA/BMP induced apoptosis in both cortical progenitors and P19
cells. The RA/BMP apoptotic signal requires the activation of BMPR-1a and
subsequent formation of an NRAGE signaling complex involving XIAP, TAK1 and
TAB1. NRAGE, which has been shown in the peripheral nervous system to
mediate cell cycle withdrawal and NGF-p75 cell survival, is here shown to also
take part in BMP regulation of progenitor cell number in the forming cortex.
NRAGE functions to mediate apoptosis by forming a complex with members of
the non-canonical BMP signaling cascade TAK1, TAB1 and XIAP. This interaction leads to serine phosphorylation of NRAGE and activation of p38 and the caspase cascade leading to cell death. NRAGE plays a vital role in this pathway in that forced activation of NRAGE by over-expression leads to caspase and p38 activation and apoptosis, whereas, eliminating or diminishing NRAGE expression rescues cell viability in the presence of BMP. This places NRAGE as a mediator in determining cell death in these progenitor systems.

**XIAP as a Mediator of Cell Death**

Since NRAGE is shown to be an activator of p38 that ultimately initiates the cell death program, the question shifts to how is NRAGE regulated and activated within the cell. NRAGE binds both TAK1 and TAB1 leading to NRAGE serine phosphorylation and ultimately initiation of apoptosis. This suggests that NRAGE is a fulcrum between apoptosis and survival and that when associated with TAK1 and TAB1, NRAGE triggers death but regulated by contact with XIAP. Therefore, unlike neurotrophin survival in which cells are at the brink of death and compete for limited amounts of trophic support to quench the apoptotic signals, BMP induced cell death is an instructive signal initiating a quiescent apoptotic pathway.

**Early Progenitor Cell Death**

There appears to be two functionally distinct types of programmed cell death in the developing nervous system of mammals. An early cell death limiting the number of neural precursors and the more traditional mechanism of pruning unnecessary and miss-routed post-mitotic neurons based on trophic support.
Upper reports have suggested that up to 70% of cortical neuroblasts in the proliferative ventricular zone undergo apoptosis, though the actual percentage conceptually must be less than 50% in order to expand the neural epithelium. Whatever the exact percentage is in murine and primate species this progenitor apoptosis commences at E10, and peaking at ~E13, before decreasing postnatally to low levels seen in the adult\textsuperscript{23,26,29,342,343}. This early-programmed cell death coincides well with both the spatial and temporal distribution of NRAGE. Given the evidence presented within, it seems logical that this early cell death is mediated by the activation of NRAGE by BMPs and potentially other TAK1, TAB1 activating factors, resulting in p38 activation and initiation of the caspase. Taken together with the fact that NRAGE levels peak just prior to the onset of the apoptosis described by Blaschke et al. This further supports the important role that NRAGE plays in mediating cell survival in the early cortex.

NRAGE is a Required Element in Cell Cycle Withdrawal, Differentiation and Apoptosis of Neural Progenitors.

Our findings presented within are in agreement with previous reports demonstrating a collaborative effort between TAK1 and TAB1 induced cell death in the ventralization the \textit{Xenopus} embryo\textsuperscript{214}. The activation of TAK1 requires an association with TAB1\textsuperscript{420}. Moreover, XIAP interaction with the cytoplasmic domain of BMPR-Ia enhanced the ventralization of TAB1-TAK1, but perturbed the apoptotic affect\textsuperscript{245}. There is increasing evidence that this pro-apoptotic effect is directly regulated by p38, not JNK activity, which antagonizes the survival effects of FGF8-MAPK signaling required for neural progenitor survival\textsuperscript{421-424}. 

Abrogation of NRAGE or TAK1 expression leads to a decreased in p38 activation and reduced cell death. It is tempting to speculate that the capacity for p38 to challenge cells to undergo apoptosis is regulated in part by its ability to directly phosphorylate Rb in a cdk independent manner. In this context p38 can direct cells through the cell cycle leading to an enhanced G2-M arrest as a result of active E2F-1 cell cycle control. However, when NRAGE levels exceed a specific cell context dependent threshold NRAGE transitions from regulating required cell signaling needed to mediate cell cycle withdrawal to facilitating apoptosis of neural progenitors through cooperative actions of the apoptosis and cell cycle machinery.

In addition to the capacity for p38 to regulate Rb inactivation, p38 stimulates myocyte enhancer factor 2 (MEF2) family of transcription factors, specifically MEF2A and MEF2C, not MEF2B or MEF2D. MEFs are members of the MADS family of transcription factors originally identified as key regulators directing myogenesis. They appear to function by physically interacting with the basic helix-loop-helix (bHLH) myogenic transcription factor MyoD and myogenin to promote muscle differentiation. Furthermore, MEF2A and C are highly expressed in developing neural progenitors and cooperate with the pro-neural bHLH transcription factor Mash1 during neurogenesis, which is in agreement with our findings that NRAGE knockdown prevents the transition of progenitor to post-mitotic neuron potentially due in part to deficient MEF2A/C activation. During myogenesis the MEF2C-MyoD complex requires the hypophosphorylated or active form of Rb as an
integral component to direct muscle development\textsuperscript{434} and failure to so results in a p21\textsuperscript{waf1}-dependent apoptosis\textsuperscript{435}. Over activation of p38 leads to in excess of the hyperphosphorylated Rb incapable of functioning in the MEF2-MyoD complex promoting unscheduled re-entry into the cell cycle leading to apoptosis, whereas insufficient p38 activity fails to generate adequate levels of active MEF2C transcription factors thereby preventing the generation of the MEF2-MyoD complex inhibiting differentiation of progenitor cells. Similar effects of p38 activity are evident in our NRAGE gain and loss-of-function studies that demonstrate the capacity for NRAGE to regulate the pleiotropic functions of p38 that ultimately govern neurogenesis.

NRAGE Functions as an Important Link Between Differentiation and Apoptosis.

Though there is no direct evidence to date that Rb binds and function as a complex with MEF2-Mash1 transcription factors in neural progenitors, sufficient circumstantial evidence exists that suggests a similar mechanism that directs myogenesis may regulate neurogenesis. Neural progenitor undergoing BMP mediated apoptosis require elevated p21\textsuperscript{waf1} and p27\textsuperscript{kip1} activity\textsuperscript{392,436}. Furthermore, accumulation of non-apoptotic levels of p27\textsuperscript{kip1} is required for BMP-induced growth arrest and neural differentiation\textsuperscript{268,269,404} that is integrally linked to the activity MEF2C and Rb promoting survival under stringent p38 activity\textsuperscript{271,437,438}. 
CHAPTER 4
General Conclusions.

A requirement for neural progenitor differentiation is exit from the cell cycle in which several proteins have been implicated in controlling the G1 arrest, one of which Necdin is a close family member to NRAGE\textsuperscript{155,203,439}. Direct coupling of the cell cycle and death machinery regulating differentiation has been well documented\textsuperscript{69,440}. Unrestrained E2F activity as a result of loss of Rb function and forces unscheduled re-entry into the S phase of neural progenitors attempting to arrest in G1, thereby promoting apoptosis through p53. The importance for Rb in maintaining the G1 arrest and thus cell survival becomes very apparent in Rb\textsuperscript{1/2} null mice in which aberrant S phase re-entry results in a tremendous level of apoptosis in the progenitor pool leading to embryonic lethality by E15.5\textsuperscript{63-66}. Though this apoptosis can be circumvented in conditional Rb\textsuperscript{1/2} null mice under the control of the Foxg1 promoter\textsuperscript{441} and explained in lieu of a model proposed by Gail Martin\textsuperscript{442} in which FGF8 activates the transcription factor Foxg1 that inhibits BMP mediated apoptosis in neural progenitors. Our gain and loss-of-NRAGE studies support the pinnacle roll, which Rb plays in maintaining survival and differentiation of neural progenitors and positions p38 as a molecular switch determining life or death.

Furthermore, the results presented within demonstrate that perturbation of BMP signaling through NRAGE knockdown enhances both pro-neural and anti-neural gene expression. This imbalanced gene expression, a direct result of loss
of p38 activity leading to prolonged Oct3/4 and Hes1 expression, is sufficient to permit the transition from pluripotent stem cell to neural progenitor. However, when these progenitors are challenged to differentiate into a post-mitotic cell, in addition to cell cycle deficits, they lack sufficient levels of MEF2A/C, which is an integral component required to drive the later stages of neurogenesis. Should a progenitor be exposed to high levels of BMP stimulation, NRAGE sustains proliferation and re-entry into the cell cycle through direct yet transient coupling of Rb phosphorylation by p38 kinase activity, which is functionally antagonized via a prolonged Smad-NRAGE inhibition of c-myc and activation of p21\textsuperscript{waf1} and p27\textsuperscript{kip1}. These apposing challenges on the cell cycle, all regulated through BMPR, when in excess lead to the promotion of an NRAGE-p53 dependent apoptosis. Therefore, NRAGE has the capacity to regulate progression through the cell cycle and cell cycle arrest in an elegant cell context dependent manner ultimately affecting the overt differentiation of the progenitor pool creating a differentiation-coupled apoptosis (Fig. 21).
Figure 21. NRAGE Mediates Apoptosis of Neural Progenitors.

BMP stimulation triggers activation of NRAGE and its cooperation with the XIAP-TAK1-TAB1 complex to activate p38. Active p38 directly inactivates Rb, thus promoting progression through the cell cycle. This precocious re-entry into S phase leads to the activation of p53 that simultaneously facilitates activation of p21\textsuperscript{waf1}, promoting growth arrest and formation of the apoptosome resulting in mitochondrial leakage and caspase activation. The requirement for NRAGE
mediated growth arrest is best demonstrated through loss of function in which neural progenitors fail to differentiate, as terminal exit from the cell cycle is perturbed.

However, crosstalk of the BMP signaling pathway with other signal transduction pathways is becoming increasingly robust. Reciprocal modulation has been described between Wnt (canonical and non-canonical), TGF-β/activin, Erk-MAPK and the JAK-STAT pathway. An understanding of biological context in which these signaling pathways intersect signifies the next major frontier. It is of paramount importance to define NRAGE’s role within this greater context of cell biological signaling.

**Wnt and BMP Signaling During Neurogenesis.**

Although a role for Wnt signaling in early dorsal-ventral patterning is well established, Wnt signaling has only recently been shown to contribute to neural induction and cell survival. The Wnt signaling cascade includes the secreted Wnt ligands, Frizzled receptors, and intracellular proteins such as Dishevelled and glycogen synthase kinase 3 (GSK3). After activation of the Wnt signaling pathways, GSK3 inhibition by Dishevelled allows stabilization and nuclear localization of β-catenin, complexes with TCF DNA-binding proteins to activate transcriptional targets.

Ectodermal explants from *Xenopus* can be induced to form neuroectoderm through Wnt treatment or over-expressing the Frizzled receptor,
Dishevelled, a dominant negative form of GSK3 or a constitutively active form of β-catenin. However, the most compelling data suggesting an important in vivo role for Wnt signaling comes from mouse models over-expressing a constitutively active form of β-catenin in the ectoderm resulting in expansion of the neural progenitor pool, whereas a dominant-negative form of TCF blocks neural tissue formation and promotes apoptosis. Although it is not yet certain how Wnt signaling exerts in neurulizing activity, this process may be mediated by regulation of BMP expression. As noggin cannot down-regulate BMP4 expression during gastrulation, Wnt signaling can perform this function. In Xenopus, it has been suggested that the neural specifier (Xiro1 and Xiro2) that is activated by Wnt signaling encodes a repressor that is essential for neural development by down regulating BMP4. In zebrafish, Wnt signaling seems to play a more central role in neural patterning than in neural induction. Mutations in the Wnt signaling pathway include headless (TCF3) and bozozok, a homeodomain protein downstream of Wnt signaling. Embryos carrying mutations in either of these genes have defects in anterior-posterior neural patterning, but neural tissue is still induced. However, Wnt signaling is carefully regulated and Wnt antagonists are needed to promote telencephalic development of the ANR. One of the secreted proteins responsible for the activity of the ANR is Tcl, a member of the secreted Frizzled Related Proteins (sFRP) family. sFRPs are generally considered to antagonize Wnt activity by sequestering secreted Wnts. This suggests that the establishment of the telencephalon requires local suppression of Wnt activity. Studies in Xenopus,
chick and mouse also suggest that the telencephalon is established in a domain of low Wnt activity\textsuperscript{453-456}. The canonical Wnt pathway is used reiteratively during the development of the forebrain. Wnt signaling contributes to initial regionalization of the formation neural plate into crude AP subdivisions, then locally promotes caudalization of the forebrain anlage and later still induces proliferation and modulates patterning within individual forebrain domains. Although these roles are now reasonably well documented, the mechanisms by which Wnt and BMP signaling regulates regional fate determination within the forming CNS are still poorly understood. However, there is evidence that BMP and Wnt/β-catenin signaling directly interact in early \textit{Xenopus} embryos, TAK1 activates a distantly related MAPK family member Nemo-like kinase (NLK); this pathway culminates in the inhibitor phosphorylation of TCF-1/Lef-1 and the down-regulation of Wnt/β-catenin-inducible transcription\textsuperscript{374,457}. Therefore, it is tempting to speculate that in addition to NRAGE-TAK1-p38 activity, NRAGE may antagonize Wnt signaling through direct coupling of NRAGE and NLK. Indeed, in \textit{Drosophila} and \textit{Xenopus}, NLK is required for BMP mediated neurogenesis and apoptosis\textsuperscript{458,459}. Thus, these novel insights provide yet an additional level of BMP-NRAGE mediated regulation of neurogenesis to explore.

\textbf{FGF and BMP Signaling During Neurogenesis.}

The ability of Wnt antagonists to locally induce instructive FGF signals in the ANR\textsuperscript{451} suggests that establishment of the ANR, as a source of FGF signals is a downstream consequent of local repression of Wnt activity. Secreted FGFs
signal to target cells by binding and activating cell-surface tyrosine kinase FGF receptors (FGFRs). Once an FGF ligand is bound, the receptor dimerizes and phosphorylates intermolecular tyrosine residues, triggering initiation of FGFR signal transduction. FGFR signaling activates a number of signal transduction molecules, including those of the Ras-ERK and phospholipase C-γ pathways.

One of the most intriguing roles proposed for FGFs signals is to polarize the telencephalon, with high levels of FGF activity promoting anterior cortical fates. Specially, following neural tube closure, Fgf8 expression is localized to the ANR and removal of the ANR or treatment with inhibitors of FGF signaling causes loss of expression of telencephalic molecular markers, such as Foxg1 (Bf1). More recently, through genetically eliminating, genetically reducing or experimentally increasing Fgf8 expression in the forebrain a dose dependent effect on cell differentiation and survival was observed. In particular, either eliminating or increasing Fgf8 expression increased apoptosis, whereas reducing Fgf8 expression had the opposite effect and these result where attributed to changing BMP4 levels. In the telencephalon, FGF activity may contribute to the polarization of the cortical territories in part through repression of Emx2 transcription factor. The competence of adjacent cells to respond to FGF signals may both be regulated by Six3 and Irx proteins. Fgf8 expression in the ANR is dependent on Six3 and in a similar way to Six3, Otx2 may confer the ability of the anterior neural plate to respond to ANR signals. Indeed, the ANR of hypomorphic otx2 mutant mice is functionally
intact, but the neural plate of such embryos lacks the ability to respond to ANR signals or exogenous FGFs. The ability of FGF signaling to regulate regionalization of the telencephalon through suppression of high levels of BMP activity is an important step in the establishment of the prospective forebrain. However, with the forming neural plate, lower levels of BMP activity contribute to the initial establishment of the lateral to medial pattern. Consequently, specific thresholds of BMP activity appear to be required for the commitment of fates that derive from margins of the neural plate, such as telencephalon, dorsal eye, and epithalamus rostrally and dorsal sensory neurons and neural crest cells caudally. These observations suggest that a window of BMP activity during neurogenesis enables development of marginal and dorsal neural fates. If BMP signaling is too high, non-neural fates are promoted, whereas if it is too low, then more medial neural plate fates are promoted. Thus establishment of the ANR itself may depend upon rostral marginal neural plate cells being exposed to appropriate levels of BMP activity. Indeed, the capacity for cortical progenitors within the CNS to respond to BMPs is highly dependent on their developmental age. During early neurogenesis of the cortex, E12, BMP4 promotes apoptosis. Intriguingly, by E14 low doses of BMP4 are required to promote neurogenesis within the dorsal neocortex and as the concentration of BMP4 increases neural progenitors have a greater propensity towards apoptosis. This BMP4-mediated apoptosis of early neural progenitors is dependent on the transcription factors Msx2, ID2 and the CKI
Collectively, these studies demonstrate that BMP4 is an important switch between cell death and differentiation in a cell cycle dependent manner.

The capacity for FGFs to regulate the survival and differentiation of neural progenitors is believed to occur through inhibition of excessive BMP signaling. The ERK-MAPK pathway that mediates the effects of certain receptor tyrosine kinases (RTKs) can also modulate the BMP-Smad pathway by regulating Smad activity. Activation of RTKs by ligands such as FGF or EGF stimulates ERK mitogen-activated protein kinases. ERK in turn phosphorylates serine residues in consensus PXSP motifs located within the MH1 and MH2 domains of Smad1. This phosphorylation serves to inhibit nuclear accumulation of Smad1, although the mechanism for this inhibition remains unclear. This mechanism may underlie the observed opposing effects of mitogenic factors and BMPs during development, thereby preventing growth arrest through perturbation of nuclear translocation of NRAGE-Smad complexes. However, BMP and FGF signaling can cooperate in certain contexts, such as bone and tooth morphogenesis and erythroid differentiation.

The Smads are sometime referred to as “fast-track” molecules because they can directly transduce a signal from the plasma membrane to the gene. The JAK-STAT pathway also uses “fast-track” molecules, namely the STATS. Recently, evidence for cross talk between these two pathways has been reported. Leukemia inhibitory factor (LIF), which acts through the gp130 receptor and STAT3, can act synergistically with BMP2 in inducing astrocyte differentiation. This synergism has been shown to be due to the formation...
of a Smad1 and STAT3 complex bridged by the transcriptional co-activator p300/CBP. Moreover, the synergistic effect of BMP4 and LIF, was recently described as a mechanism for embryonic stem cell self-renewal through antagonization of ERK and inhibition of TAK1-mediated activation of p38, that consequently leads to the up-regulate of helix-loop-helix (HLH) inhibitor of differentiation (Id) transcription factors and Msx2. A phenomenon recapitulated in our NRAGE knockdown paradigm in which perturbation of NRAGE and thus p38 activity was sufficient to trap NP in an undifferentiated state. The concept for BMP facilitating stem cell self-renewal also applies to neural stem cells. In the place of LIF signaling, activation of Notch plays a key role in keeping progenitor cells from differentiating into neurons. BMP2 was shown to enhance neural progenitor cell maintenance through Notch-induced transcriptional activation of the basic-helix-loop-helix (bHLH) transcription factor Hes1 and Hes5 in which Smad1 and Notch intracellular domain complex with p300 promoting progenitor gene transcription. Collectively these results highlight the increasing awareness of the important contributions that HLH and bHLH transcription factors play in the differentiation of neural progenitor cells and the complex cross talk mediated through BMPRs in which NRAGE regulating both growth arrest and cell cycle progression ultimately affect the capacity of overt differentiation. Thus, our results yield novel insights in the dynamic interplay NRAGE regulates through BMP signaling directing stem cell self renewal, differentiation and survival.
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