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NRAGE Regulates Life and Death of Neural Progenitors

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NRAGE REGULATES LIFE AND DEATH OF NEURAL PROGENITORS

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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 mulitfunctionality of NRAGE during neurogenesis.

DEDICATION

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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[1]. 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[2].

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[3]. 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[4]. 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[5-7]. Similarly, apoptosis is involved in hollowing out sold 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[8]. 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[9].

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[10]. 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[11,12]. 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[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[9]. Since then early neural death has been described during the formation of neural crest, and neurogenesis, with a widespread distribution through out the CNS and in PNS ganglia[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[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[34] and at E10.5 in the null mutants for caspase-9[32] and caspase-3[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[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)[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[40,41]. In mid/late G1, cyclin E is induced and forms complexes with CDK2, whose activity appears essential for entrance into S phase[42,43]. Cyclin A activity appears in

concert with the onset of DNA synthesis and it initially partners with CDK2 and later with CDC2[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 $p21^{waf1}$, $p27^{Kip1}$ and $p57^{Kip2}$. These three CKIs can bind to most cyclin-CDKs and inhibit their activities[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 $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$. 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[49,50].

The orderly progression of the cell cycle is driven by sequential activation of CDKs to pass key restriction or checkpoints[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 Rb[69]. 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-dependent[70,71].

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 targets[72-74]. 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, $p75^{NTR}$. Decades of biochemical analyses have resulted in great strides towards understanding NGF signaling potential through *Trk* and recently through $p75^{NTR}$. I began my studies attempting to understand the biological and signaling potential of $p75^{NTR}$ by examining the expression and role of $p75^{NTR}$ 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 homodimers[72-77]. 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 kinetics[80]. 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 NGF[81,82]. 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 $p75^{NTR}$ with affinities similar to that of NGF (about 10^{-9} M), but with relatively slower kinetics[83-86]. 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 ($p75^{NTR}$).

After the initial biochemical characterization of $p75^{NTR}$, it was demonstrated that the proto-oncogene *Trk*A (tropomyosin-related kinase) had an expression pattern in the developing nervous system suggestive of a potential role in neural development. It was hypothesized that *Trk*A may mediate long-term NGF survival[87]. Independent studies soon demonstrated that *Trk*A bound NGF resulting in the activation of its intrinsic tyrosine kinase promoting long-term survival[88,89]. Kinetic and binding experiments demonstrated that this second NGF receptor binds NGF with high-affinity[75]. 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, *Trk*A, *Trk*B, and *Trk*C, is expressed in distinct parts of the nervous system and in several isoforms[90,91]. NGF is the preferred ligand for *Trk*A, BDNF and NT4/5 are preferred for *Trk*B, and NT3 for *Trk*C[92]. 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[93,94].

A plethora of experiments have demonstrated that the *Trks* mediate survival[95-97], proliferation[95,98,99], overt differentiation[100-102], synaptic plasticity[103-105], dendritic and axonal sprouting[106-108], neurotransmitter expression and long-term potentiation[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[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 of survival many neuronal populations[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[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[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 *Trk* activates PI-3K. In the *Trk* system, PI-3K activation is due to the combined actions of Ras and Gab-1[127,128]. The primary target for active PI-3K appears to be the serine/threonine kinase Akt[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-XL[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[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[74].

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

p75^{NTR} as a Death Receptor.

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

In vivo evidence for p75^{NTR}-mediated cell death has been found for two different central nervous system (CNS) populations. Early retinal and basal forebrain cells express p75^{NTR}. These cells undergo apoptosis, an effect that can be blocked by NGF antibodies[143]. In cholinergic neurons of the basal forebrain there is a reduction in the number of apoptotic cells in mice lacking the p75^{NTR} receptor[144]. Further evidence suggesting that p75^{NTR} independently regulates cellular apoptosis comes from transgenic mice over-expressing the intracellular domain of p75^{NTR}, resulting in a significant reduction in numbers of sympathetic and sensory neurons as well as cell loss in the neocortex[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 *Trk*A. Cell death occurred in neocortical cells, which do not express *Trk*A. This argues against a dominant negative effect; whereby, pairing of the $p75^{NTR}$ intracellular domain with *Trk*A might lead to cell death by blocking the formation of high affinity receptors for NGF. These studies suggest that the $p75^{NTR}$ intracellular domain independent of *Trk* initiated the cell death process. Moreover, in $p75^{NTR}$ knockout mice, the number of sympathetic neurons at two weeks of age was significantly higher than wild-type mice, indicating that apoptosis was reduced[137]. $p75^{NTR}$ 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[143].

Isolation of Neurotrophin Receptor Interacting Factors.

In general, it seems that neurotrophins can only induce cell death through $p75^{NTR}$ in cells not expressing their specific *Trk* receptors[137,146]. By contrast, coactivation of the appropriate *Trk* receptors inhibits neurotrophin-induced $p75^{NTR}$-mediated apoptosis, as demonstrated with cultured oligodendrocytes and neurons[136]. A number of interactors and their possible functions have been described for $p75^{NTR}$, many of them as a result of yeast two-hybrid screens. $p75^{NTR}$ interactors involved in transducing cell death include a wildly expressed zinc finger protein designated NRIF (neurotrophin receptor-interacting factor)[147]. NRIF knockout mice showed reduced levels of apoptosis in the retina, and this reduction was quantitatively similar to that seen in $p75^{NTR}$ null

mice. These results suggest that NRIF is an essential component downstream of NGF and $p75^{NTR}$ in the cell death pathway. A protein designated NADE ($p75^{NTR}$-associated cell death executer) associates with $p75^{NTR}$ after NGF binding but not after binding of BDNF, NT3 or NT4/5[148]. It too seems to be involved in $p75^{NTR}$-mediated apoptosis.

Another zinc finger protein that has been identified as an intracellular interactor of $p75^{NTR}$, the Schwann cell factor-1 (SC-1), is involved in cell cycle arrest[149]. 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 $p75^{NTR}$ interactor in a yeast two-hybrid screen[150]. $p75^{NTR}$ constitutively activates RhoA, whereas neurotrophin binding to $p75^{NTR}$ blocks this activation. The link to $p75^{NTR}$ and RhoA seems to be functionally relevant, as the modulation of neurite outgrowth in $p75^{NTR}$-expressing neurons by neurotrophins mimics the results obtained following RhoA inactivation. Using coimmunoprecipitation and phosphorylation assays, a $p75^{NTR}$-associated kinase[151], as well as ERK1 and ERK2[152], were shown to interact with $p75^{NTR}$. The functional significance of these associations is not clear, though $p75^{NTR}$ is serine phosphorylated in the intracellular domain. Association of TRAF6 with $p75^{NTR}$ after ligand binding leads to the activation of NF-κB[153]. Another report suggests that all members of the TRAF family interact with $p75^{NTR}$[154]. A further recently identified interactor of $p75^{NTR}$, designated NRAGE (neurotrophin receptor-interacting MAGE), or MAGE-D1, mediates NGF-

dependent apoptosis in sympathetic neuron precursor cells[155]. NRAGE also causes cell cycle arrest when over-expressed in cultured cells, suggesting that $p75^{NTR}$ might play a role in growth control through interactions with NRAGE. Interestingly, NRAGE disrupts the interaction between $p75^{NTR}$ and *Trk*A[155]. The functional interactions between NRAGE and $p75^{NTR}$ 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[156-159]. Longitudinal pattering 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)[160,161] and at the midbrain-hindbrain boundary (isthmus)[162]. Each patterning center produces a distinct set of signals. Ventral centers produce sonic hedgehog (shh) and BMPs[163-166]; dorsal centers produce BMPs and Wnts[167,168] and transverse patterning centers produce fibroblast growth factor (FGF) and Wnts[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[171]. Neural and non-neural tissues produce a number of signaling molecules including BMPs, shh and FGF8 within and adjacent to the prosencephalon[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[172-175]. Within the forebrain

BMPs repress FGF expression, and shh maintains FGF expression[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[177-179]. Other factors, such as BMPs can enhance both neuronal and astrocyte differentiation, depending on the age of the stimulated cortical progenitors[180,181].

Signal Transduction of Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily of secreted signaling molecules[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[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[184-197]. Despite the numerous extracellular ligands, only three Type-I and three Type-II receptors are known to mediate the signals[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[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 carboxylterminal 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[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[205,229].

The affinity for BMPs is dependent on specific Type-I and Type-II receptors[200,201], supporting the concept that receptor affinity is important for the activation of specific signal transduction pathways[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-Ia, BMPR-Ib and BMPR-II have been shown to exist as either a preformed hetero-oligomeric complex or a homo-oligomeric complex on the cell surface[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-Ia or BMPR-Ib 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[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[195,234]. 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[235-238]. Both R-Smads and Smad4 activate transcription primarily through their MH2 domain[239]. This activity results, at least in part, from the ability of the MH2 domain to recruit the general transcriptional co-activators p300 and CBP[240-244]. 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*[237]. 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)[213,214]. 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[245]. 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[213,214,246,247], and that TAK1 has been implicated in the activation of JNK and p38 MAPK signaling pathways[215,216,247-249].

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[250-252]. 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[253].

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[254,255]. However, several lines of evidences have suggested that p38 MAPKs also play roles in neural apoptosis[256-259]. 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[260-263]. 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[264-266]. 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[267]. BMP2 also induces neural differentiation of PC12 cells[268,269]. 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[270]. 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[271]. However, concerns over p38 signaling have suggested a pro-apoptotic role for p38 in neural cells[256-259]. 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[272]. By hybridization of cosmids with a MAGE-A1 probe, 11 closely related genes were identified[273]. Together, these 12 genes form the MAGE-A cluster, located in the q28 region of the X chromosome[274]. A sequencing effort directed at the Xp21 region led to the identification of the MAGE-B cluster, which is

comprised of four genes[275-277]. MAGE-C, a third group, comprises two genes located in Xq26-27[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[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[280].

Pold and colleagues[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[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[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[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[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[285,286]. Necdin has been shown to bind

several Rb interacting proteins. These include adenovirus E1A, the SV40 large T antigen, E2F1, and p53[287,288]. Similar to Rb, necdin binds and represses E2F1 mediated transcription[287]. Over-expression of necdin in proliferating cells induces growth arrest[285]. Unlike necdin, Rb is expressed in neuroepithelial stem cells and at reduced levels in postmitotic neurons[68,289]. 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 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 $p75^{NTR}$ interacting proteins, Verdi and colleagues[155] employed the yeast two-hybrid system to screen a rat neural crest cDNA library. A $p75^{NTR}$ binding partner termed NRAGE was identified. BLAST search revealed sequence homologue to human MAGE-D1. NRAGE was found to bind $p75^{NTR}$ *in vitro* and *in vivo*, and NRAGE associates with the plasma membrane when NGF is bound to $p75^{NTR}$. NRAGE blocks the physical association of $p75^{NTR}$ with *Trk*A, and, conversely, *Trk*A over-expression eliminates NRAGE-mediated NGF-dependent apoptosis. Thus, indicating that

interactions between NRAGE and *Trk*A with $p75^{NTR}$ 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 $p75^{NTR}$-dependent cell death and suggests novel functions for MAGE family of proteins.

Since $p75^{NTR}$ is expressed not only in neural tissues, but many non-neural tissues as well, a description of the developmental expression of $p75^{NTR}$-interacting proteins is hypothesized to reflect $p75^{NTR}$ receptor expression. An understanding of the developmental regulations of $p75^{NTR}$-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 $p75^{NTR}$ independent functions for NRAGE mediated apoptosis. NRAGE has been shown to facilitate an interleukin-3 withdrawal induced apoptosis through competitive binding to XIAP[290]. Moreover, additional studies suggest that NRAGE associates with members homeobox transcription factors Msx2 and Dlx5 and translocates to the nucleus[291,292] and this interaction is redistributed to the plasma membrane when the tyrosine receptor kinase ROR2 is present[293]. 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 progenitors[294]. Therefore, it becomes pertinent to establish the presence of NRAGE within the neural precursor pool during peek periods of

early progenitor apoptosis and determine whether the established BMP mediated cell death in these cell is dependent on NRAGE.

CHAPTER 2

Expression Analysis of Novel $p75^{NTR}$ Signaling Proteins that Regulate cell Cycle Progression and Apoptosis.

Abstract

Neurotrophin Receptor-interacting MAGE (NRAGE) is the most recently identified $p75^{NTR}$ intracellular binding protein. Previously, NRAGE over-expression was shown to mediate cell cycle arrest and facilitate Nerve Growth Factor (NGF) dependent apoptosis of sympathetic neuroblasts in a $p75^{NTR}$ specific manner. Here we have examined the temporal and spatial expression patterns of NRAGE and three other $p75^{NTR}$-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 $p75^{NTR}$ 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 $p75^{NTR}$ 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 $p75^{NTR}$ but also illustrates a distinct and discordant spatial and temporal expression. These results yield novel insights into the unique developmental characteristics of the $p75^{NTR}$-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[16,295].

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)[296,297]. These molecules act on neurons by binding to distinct classes of membrane receptors[76]. One class consists of p75 neurotrophin receptor ($p75^{NTR}$), a member of the tumour necrosis factor (TNF)/Fas/CD40 receptor superfamily that binds all four neurotrophins with relatively low affinity[298,299]. 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[94,112]. Individual Trks are necessary and sufficient to confer a functional response to their cognate ligands in the absence of $p75^{NTR}$[300]. However, Trk and $p75^{NTR}$ are often co-expressed endogenously and in vitro data has shown that $p75^{NTR}$ increases the affinity of NGF binding TrkA[301] and enhances TrkA autophosphorylation[100,302].

Despite the observations that Trk and $p75^{NTR}$ coordinate neurotrophin-dependent survival of neurons, evidence is mounting indicating that $p75^{NTR}$ can act independently of Trk as an inducer of cellular apoptosis[299]. Ligand mediated $p75^{NTR}$ dependent apoptosis has been demonstrated within several developing neural lineages including sensory neurons[135,136], sympathetic neurons[137], motoneurons[138,139] and within non-neural lineages such as oligodendrocytes[141] and Schwann cells[142]. *In vivo* evidence also supports a role for $p75^{NTR}$ in apoptosis. Endogenous NGF binding $p75^{NTR}$ induces apoptosis in developing chick retina neurons[303]. Further evidence suggesting that $p75^{NTR}$ independently

regulates cellular apoptosis comes from transgenic over-expression and knockout studies. Mice over-expressing the intracellular domain of the $p75^{NTR}$ resulted in a dramatic increase in neural death in both the central and peripheral nervous system[145] and mice lacking genes for *ngf* and/or $p75^{NTR}$ have reduced cell death in the retina and in the mantle zone of the spinal cord[143,304].

In an attempt to identify down-stream modulators of $p75^{NTR}$ signaling, several groups have successfully utilized the yeast two-hybrid paradigm. Two zinc finger proteins named Schwann Cell Factor–1 (SC-1)[149] and Neurotrophic Receptor Interacting Factor (NRIF)[147] have been shown to interact with the juxtamembrane region of $p75^{NTR}$. A third protein, Neurotrophin Receptor Associated Cell Death Executor or NADE[148] associates with the intracellular $p75^{NTR}$ death domain. These $p75^{NTR}$ interacting proteins functionally have been associated with either cell cycle regulation (SC-1)[149] or NGF dependent apoptosis (NADE and NRIF)[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 $p75^{NTR}$ null mice. NADE also is involved in $p75^{NTR}$-mediated apoptosis by regulating the activation of caspase-3 upon NGF/ $p75^{NTR}$ interactions[148]. We have cloned and characterized an additional $p75^{NTR}$ interacting protein we termed NRAGE for Neurotrophin Receptor-interacting MAGE homolog[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[155,278,282,305,306]. NRAGE, like SC-1 and NRIF, associates with the juxtamembrane region of $p75^{NTR}$ and over-expression mediates both cell cycle arrest in proliferating cells, as well as a NGF-$p75^{NTR}$ dependent apoptosis of sympathetic neuroblasts. Therefore, NRAGE and other $p75^{NTR}$-interacting proteins may posses 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[279]. 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[285,286]. 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[155,278,282,305,306]. 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 $p75^{NTR}$ expression in the developing nervous system. However, specific gene expression patterns exist in which these interacting proteins are expressed independent of $p75^{NTR}$ 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 caudle to the mandible and subsequently removing the skull and meninges. Neural tubes (somites 1-4, 5-12, 13-caudle) 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 37^0C for 30 min. The cells

were stained with the B2 antibody and positive cells were isolated using fluorescence activated cell sorting[307]. $B2^+$ sympathetic precursors were plated in a serum-free modification of L15N2 medium as described in detail elsewhere[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 ^{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[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 $p75^{NTR}$, 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 H_2O. Subsequently, slides were washed twice in 2X SSC for 5 minutes and then incubated at 55°C in prehybridization buffer[309] 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[309]. 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 $MgCl_2$, 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[155]. 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[100]. 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 boarder 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[310], 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 ($2x10^5$ 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)[311]. 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 $p75^{NTR}$, 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

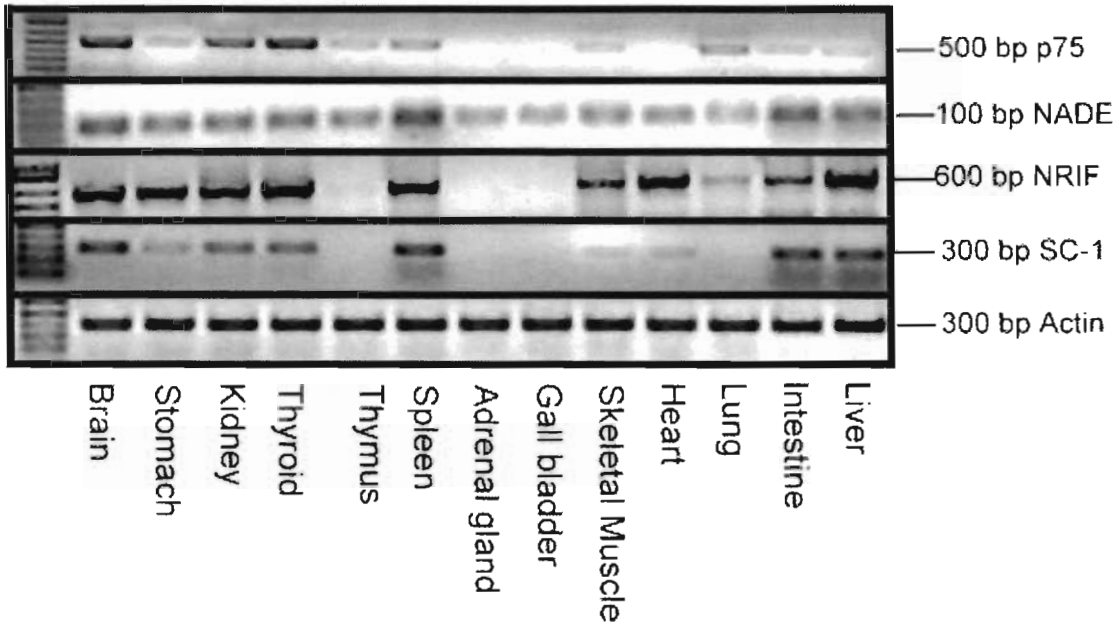

Figure 1. $p75^{NTR}$-Interacting Protein mRNAs are Widely Expressed in Adult Tissues.

Adult mouse tissues were assessed for $p75^{NTR}$ and $p75^{NTR}$-interactor mRNA expression. Reverse transcription-polymerase chain reaction products were stained with ethidium bromide on 1% agarose gels. $p75^{NTR}$ 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).

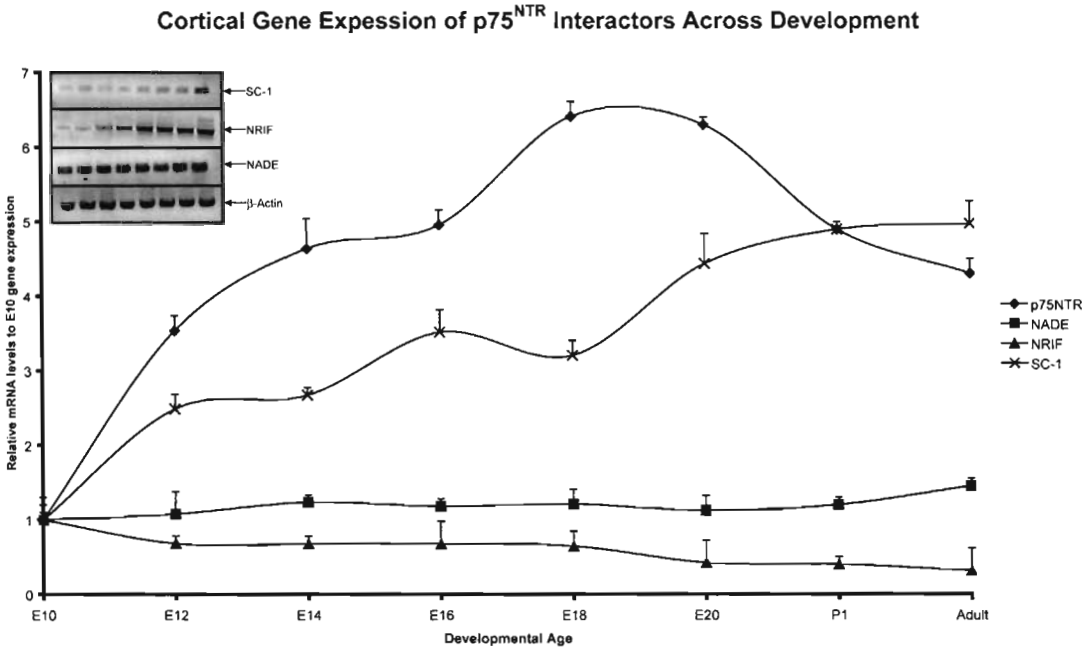

Figure 2. Transcripts for p75^{NTR}-Interacting Proteins Show Discrete Temporal Expression Patterns During Brain Development.

Semi-quantitative RT-PCR analysis of p75^{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^{NTR} interactor/actin levels. A ratio of 1 reflects no change in the message level compared to E10 levels.

All three p75^{NTR}-effector mRNA species were expressed with specific temporal trends reflecting changes in mRNA levels across development. Consistent with published reports, p75^{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 $p75^{NTR}$ 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 $p75^{NTR}$ 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 $p75^{NTR}$ expression specific to the subplate[312-314]. 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).

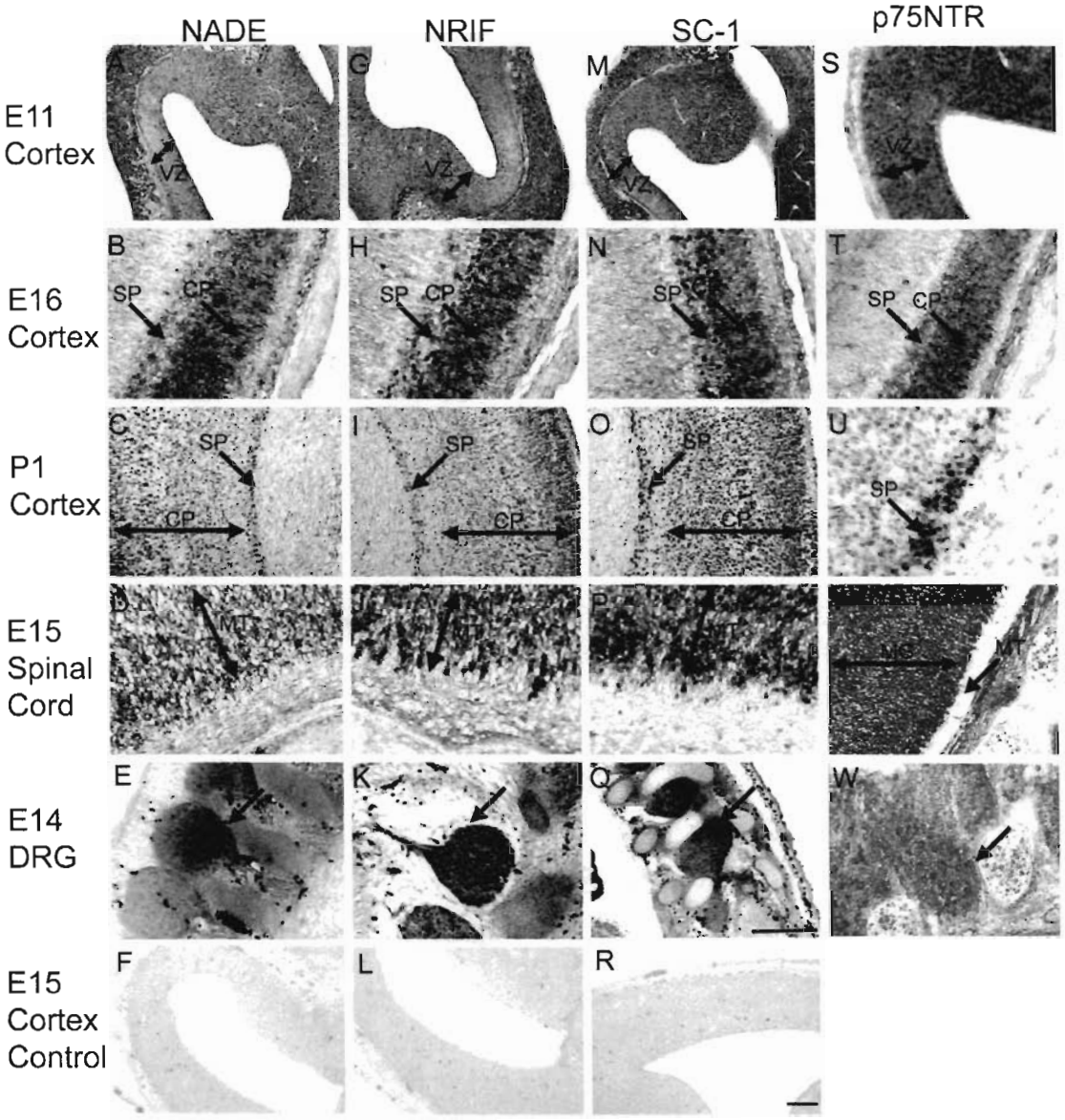

Figure 3. NADE, NRIF and SC-1 Expression Coincided with $p75^{NTR}$ Expression Areas in the Developing Cortex and Neural Tube.

Shown are *in situ* hybridizations for NADE, NRIF, SC-1 and $p75^{NTR}$ mRNA using serial sections of mouse embryonic tissue. Black staining and arrows denote positive gene expression. Panels **(A, G, M, S)**: E11 cortex, **(B, H, N, T)**: 16 cortex, **(C, I, O, U)**: P1 cortex, **(D, J, P, V)**: E15 thoracic spinal cord, **(E, K, Q, W)**: E14 DRGs, **(F, L, R)** E15 control cortex. LEGEND: CP: cortical plate, MT: mantle zone, SP: subplate, VZ: ventricular zone. Scale bar for **(A, F, G, L, M, Q-S)** = 100 μm. Scale bar for **(B-E, H-K, N-P, T-V)** = 100 μm.

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 mylenecephalon 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 $p75^{NTR}$ 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 $p75^{NTR}$ (Fig. 3V). Also, gene expression of all three $p75^{NTR}$-interactors was present in the dorsal root and sympathetic ganglia (Fig. 3E, K, Q, W).

Overall, NRIF mRNA expression phenocopies the mRNA expression of $p75^{NTR}$, supporting its role as a bona fide $p75^{NTR}$ effector protein. Likewise, NADE mRNA was spatially expressed in a similar manner to $p75^{NTR}$. The mRNA expression of SC-1 coincides both spatially and temporally with that of $p75^{NTR}$ mRNA. The increase in Schwann cell factor-1 mRNA over time may reflect its role in mediating the $p75^{NTR}$ induced growth arrest in later development, presumably during glial development.

NRAGE mRNA is Widely Expressed Throughout Development.

The $p75^{NTR}$ has been implicated in mediating apoptosis both *in vitro* and *in vivo*. The demonstration that three $p75^{NTR}$ interacting proteins facilitate neurotrophin induced apoptosis in a $p75^{NTR}$ dependent manner *in vitro* further lends credence to the hypothesis that $p75^{NTR}$ is a pro-apoptotic receptor. We wished to determine whether one such mediator of $p75^{NTR}$ 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)

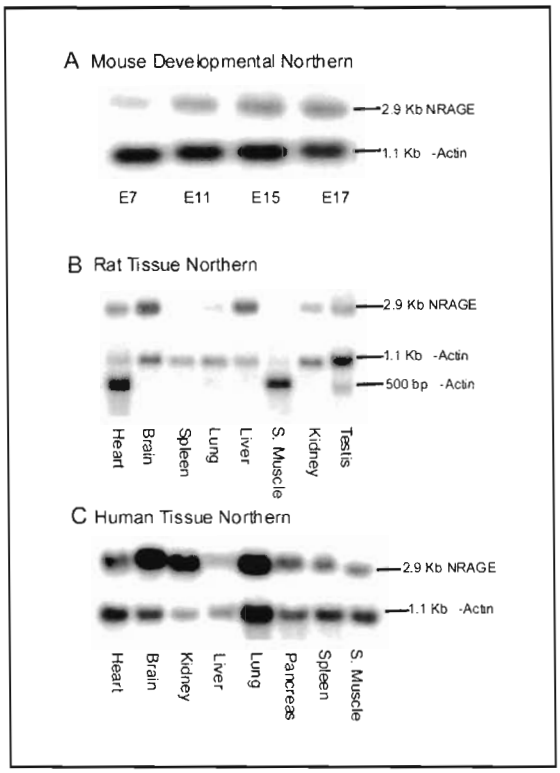

Figure 4. NRAGE mRNA is Expressed Through Development and Identified in a Variety of Murine and Human Tissues.

(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 $p75^{NTR}$ /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 MAGED1[278,281,282,315]. We then addressed whether NRAGE, like Necdin, was predominately expressed in the nervous system or was more similar to the ubiquitous expression of MAGED1[278]. 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 MAGED[278] (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 $p75^{NTR}$.

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 $p75^{NTR}$ specific riboprobe. We employed riboprobes to increase our sensitivity and NRAGE specificity. Negative control (sense) riboprobes revealed no staining (Fig. 5F, L, P).

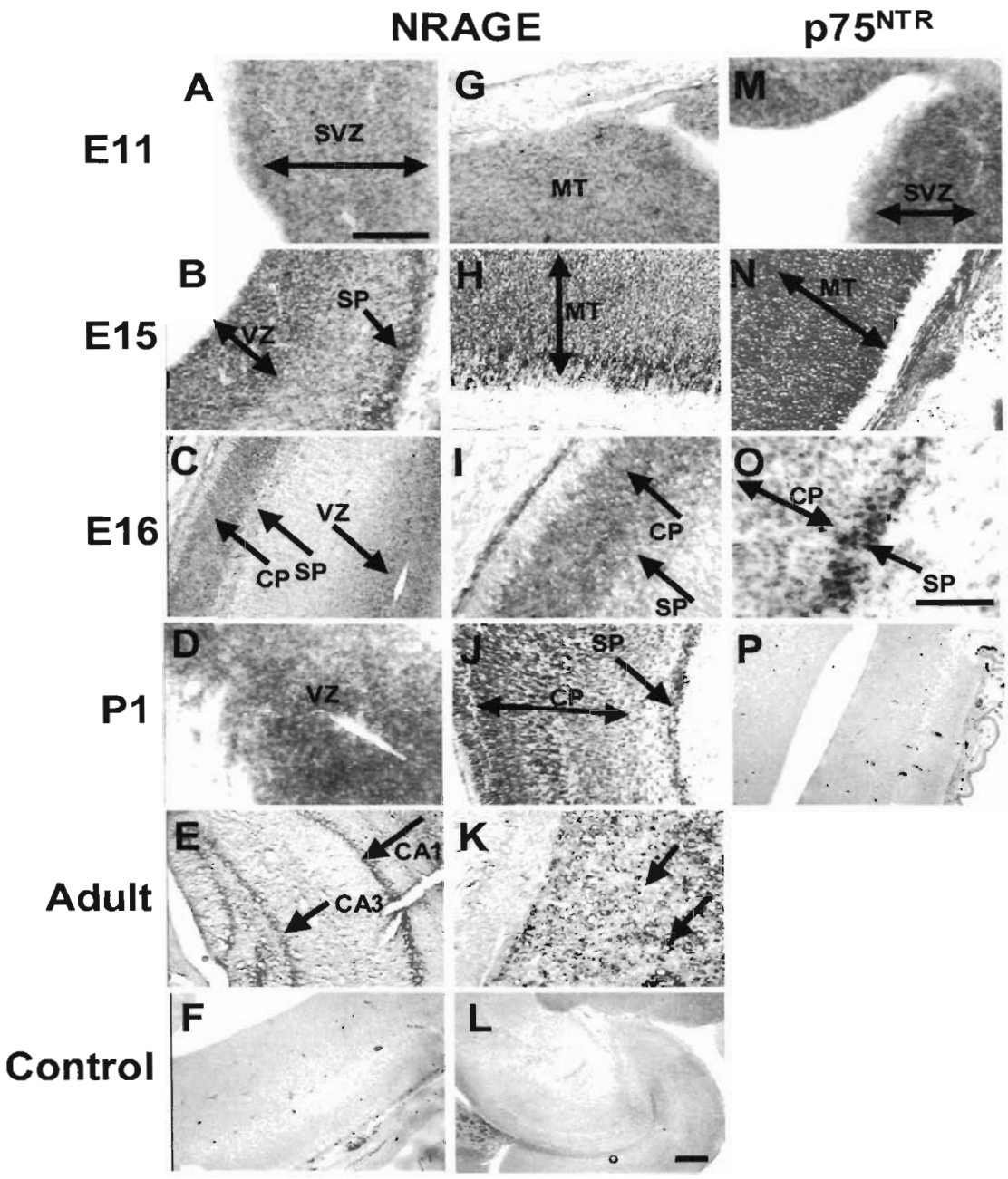

Figure 5. NRAGE is Expressed in Early Neural Progenitors and Differentiating Neuroblasts.

Mouse NRAGE and $p75^{NTR}$ mRNA expression from E11 through Adult detected by serial section *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)** $p75^{NTR}$ was detected in the subventricular zone of the E11 cortex. **(N)** $p75^{NTR}$ was specifically localized to the mantle of the spinal cord at E15. **(O)** Within the P1 cortex, $p75^{NTR}$ was detected in the subplate zone. **(F, L, P)** No signal was detected in NRAGE and $p75^{NTR}$ 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 μm: **(O, K)** 50 μm: **(F, L, P)** 200 μm.

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[279]. 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).

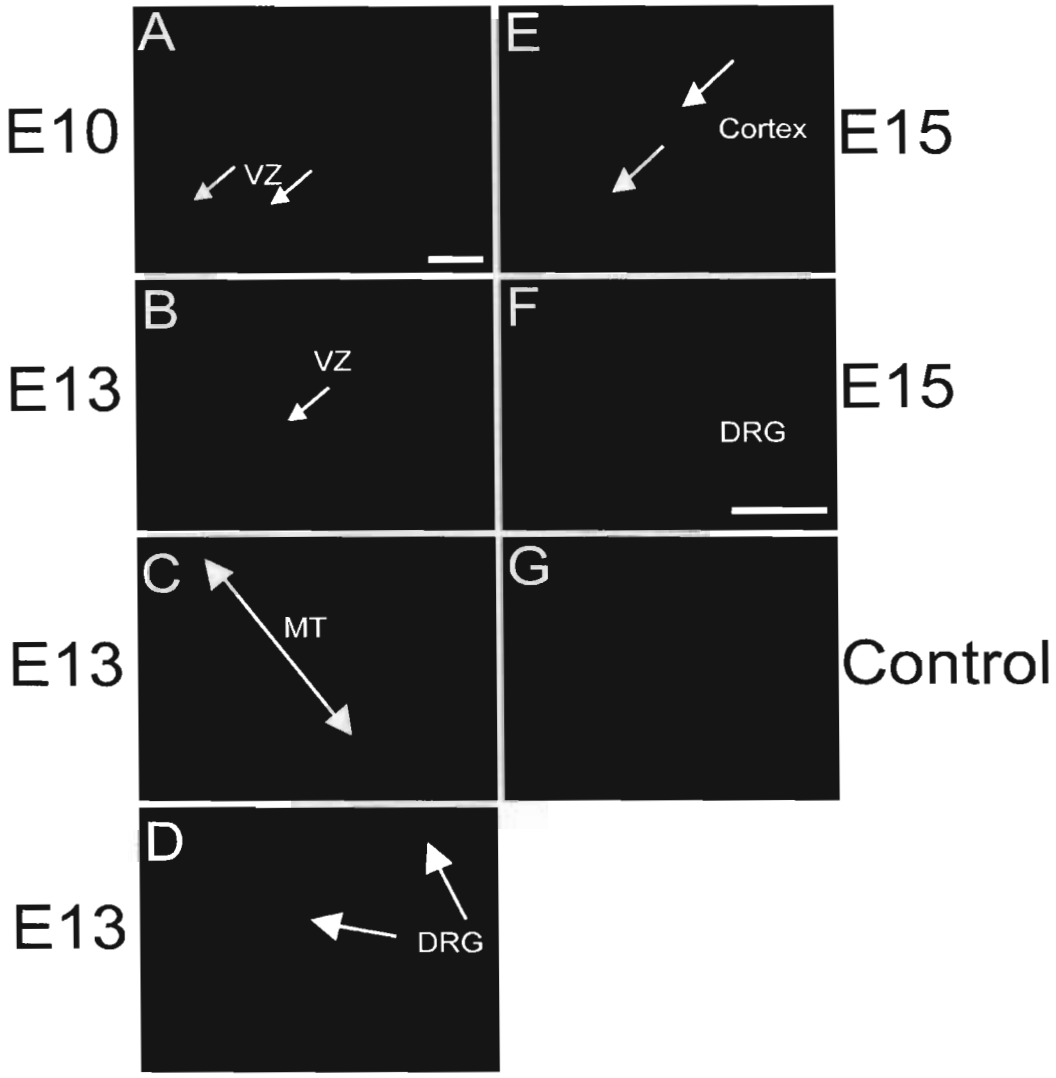

Figure 6. NRAGE Protein Distribution Recapitulates the mRNA Expression.

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)[316,317], 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).

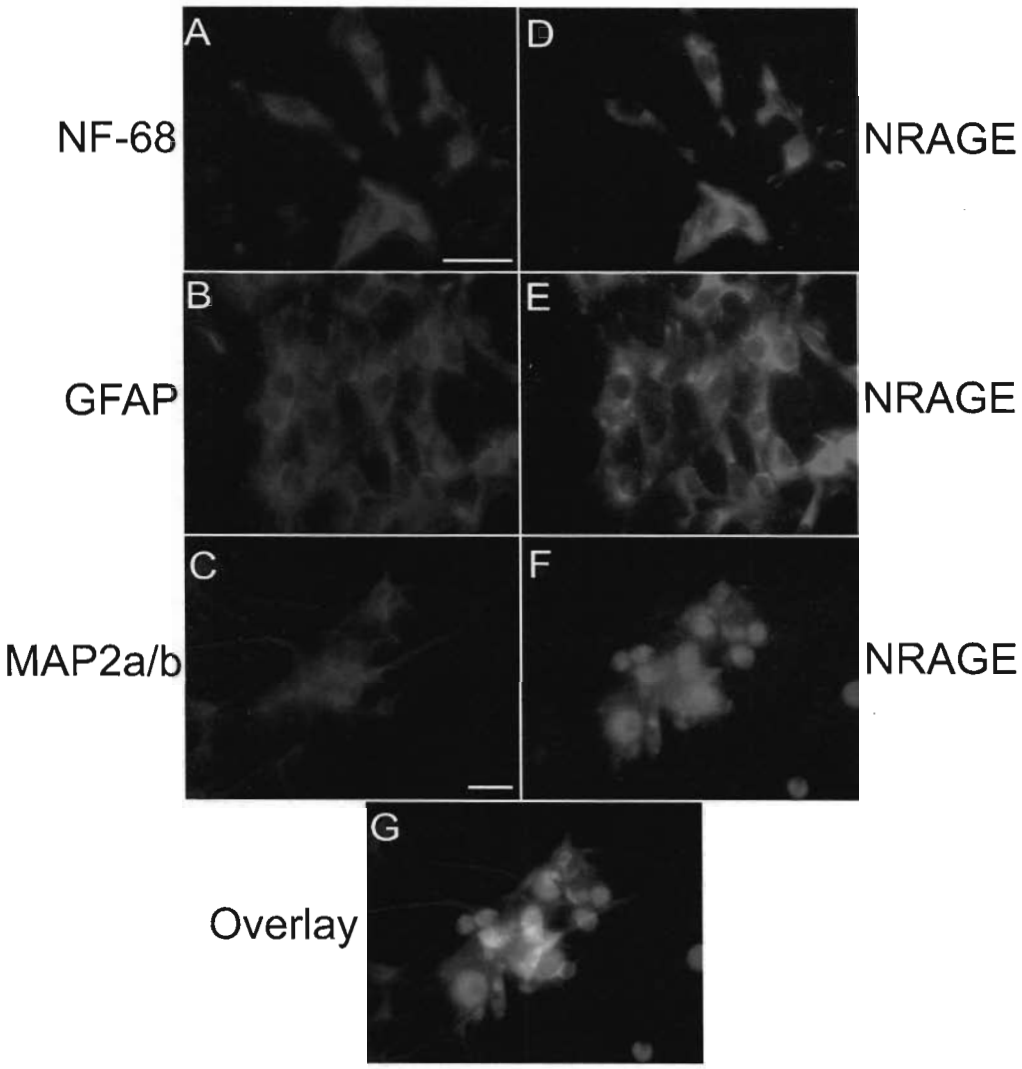

Figure 7. NRAGE Protein is Identified in Cultured Neuroblasts and not in Post-mitotic Neurons.

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).

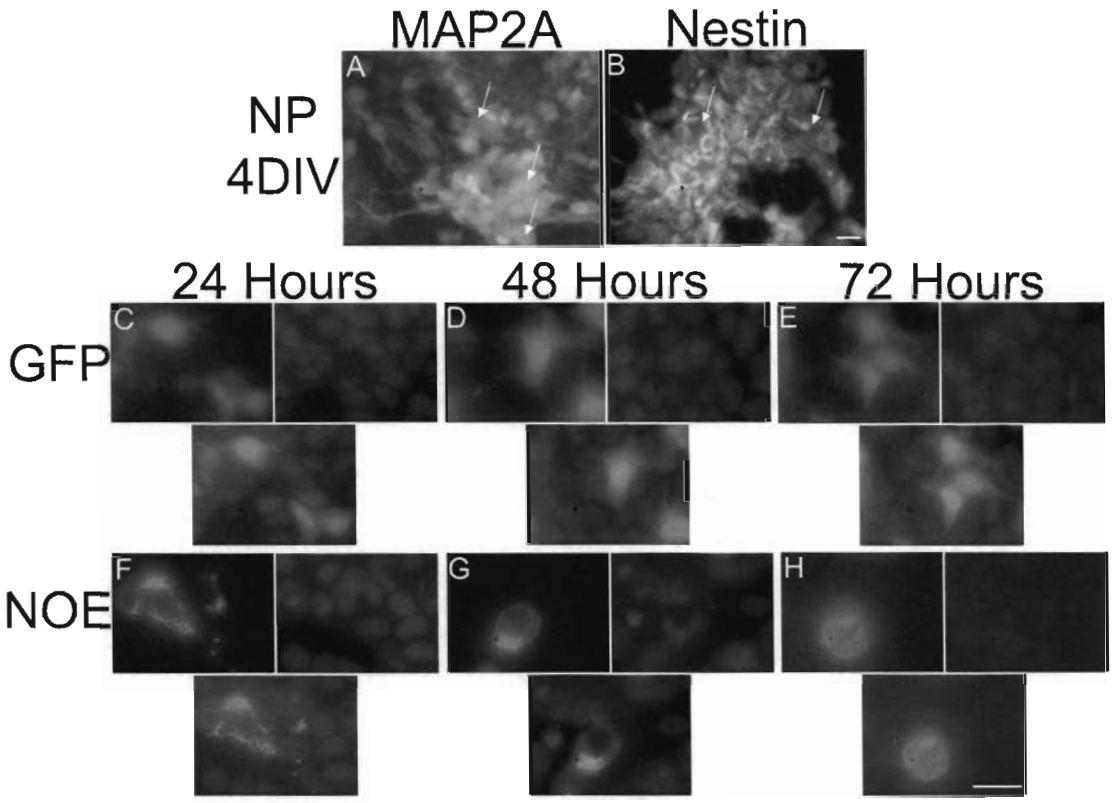

Figure 8. NRAGE Redistributes to Nuclear and Perinuclear Compartments.

(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

Tissue	*E10*	*E12*	*E14*	*E16*	*E18*	*E20*	*P1*	*Adult*
Brain	+	+	+	+	+	+	+	+
Cervical N.T.	+	+	+	+	+	+	+	+
Thoracic N.T.	+	+	+	+	+	+	+	+
Sacral N.T.	N.A.	+	+	+	+	+	+	+
Heart	+	+	+	+	+	+	+	+
Liver	+	+	+	+	+	+	+	+
Lung	N.A.	N.A.	N.A.	+	+	+	+	+
Kidney	N.A.	N.A.	N.A.	+	+	+	+	+
Muscle	N.A.	N.A.	N.A.	+	+	+	+	+
Intestine	N.A.	N.A.	N.A.	+	+	+	+	+
Thyroid	N.A.	N.A.	N.A.	+	+	+	+	+
Thymus	N.A.	N.A.	N.A.	+	+	+	+	+
Spleen	N.A.	N.A.	N.A.	+	+	+	+	+
Blood	N.A.	N.A.	N.A.	N.A.	N.A.	-	-	-

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).

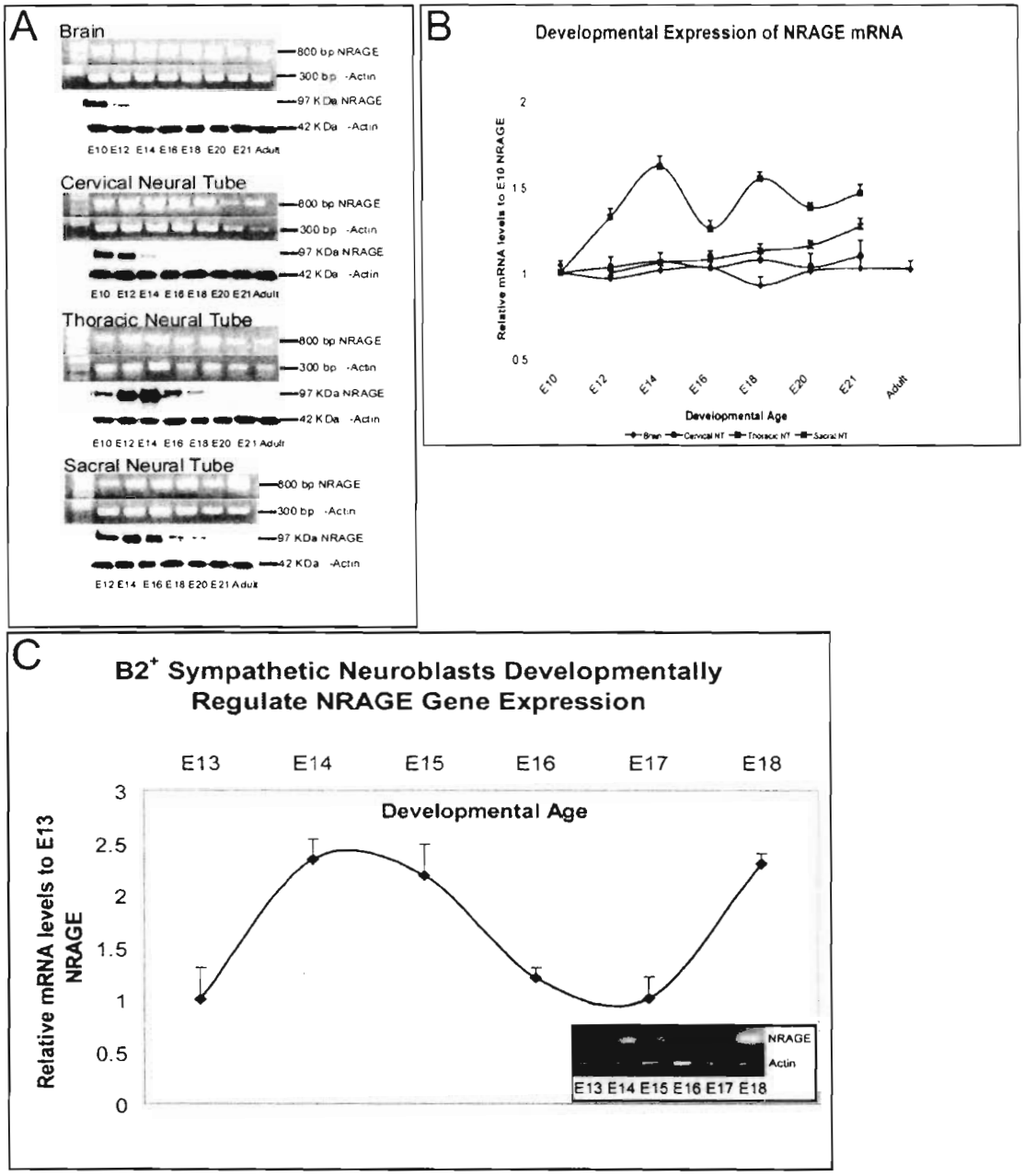

Figure 9. NRAGE Displays Tissue Specific Temporal Regulation.

(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[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[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 patters 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 $p75^{NTR}$, each gene is expressed in a unique pattern independent of $p75^{NTR}$ and each other.

During development $p75^{NTR}$ 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[138,313,320,321]. In addition to expression in the CNS, $p75^{NTR}$ is found in the peripheral sympathetic and sensory neurons[320,322]. Within the nervous system, $p75^{NTR}$ has been shown to mediate a number of physiological and developmental events including; apoptosis of cholinergic neuron in the neostriatum[135,323], modulation of the cholinergic forebrain neuron physiology[324], and promotion of apoptosis in seizure-induced rats within the hippocampus, piriform cortex, and entorhinal cortex[325]. Moreover, retinal and mantle zone neurons are sensitive to $p75^{NTR}$-mediated apoptosis[143,303]. $p75^{NTR}$ is also required for naturally occurring cell death within the sympathetic and sensory ganglia[135,137,145]. The most recent report demonstrates that $p75^{NTR}$ knockout mice have severe nervous system defects, and unexpectedly show a requirement for $p75^{NTR}$ expression in vascular development[304]. Even though expression of $p75^{NTR}$ in any given cell population does not predetermine whether apoptosis will occur, it is still important to address the spatial and temporal relationships of $p75^{NTR}$ and $p75^{NTR}$ -interacting proteins in order to better understand the functions of $p75^{NTR}$ and its interacting proteins during development.

Consistent with the diverse expression pattern of $p75^{NTR}$, 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 $p75^{NTR}$ is neither expressed nor required for apoptosis in these regions of the cortex does not diminish previously characterized functions of known $p75^{NTR}$ 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 $p75^{NTR-/-}$ and $ngf^{-/-}$ mutants[326]. Our results support the widespread gene expression reported by Casademunt and colleagues[326]. Similar to NADE, NRIF demonstrates $p75^{NTR}$ 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 $p75^{NTR}$ expression lends evidence to the relevance of NRIF as a bona fide $p75^{NTR}$-interacting protein mediating apoptosis *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[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[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[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 $p75^{NTR}$ interacting proteins; NADE, NRIF and SC-1. We demonstrated that each gene has common developmental properties associated with $p75^{NTR}$ expression and function. In addition, novel *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 $p75^{NTR}$-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[155] 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 collogues[147] indicating poor cellular expression of transfected NRIF, possibly via a cell cycle dependent mechanism.

In the PNS NRAGE Correlates with Sensory and Sympathetic Neuron p75^{NTR} Mediated Apoptosis.

For neural tube development, NRAGE is present when motoneurons of the floor plate are beginning to undergo trophic dependent programmed cell death[328] and the lateral motor column shows high levels of p75^{NTR} expression at

this time[329]. NRAGE has previously been detected in motoneurons[155]. However, the importance of $p75^{NTR}$ mediating apoptosis within motoneuron pools *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- $p75^{NTR}$-NRAGE activation.

In the CNS NRAGE Protein Expression Correlates with Timing of a $p75^{NTR}$-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 $p75^{NTR}$ receptor expression within the brain NRAGE does co-localize with $p75^{NTR}$ in the subplate region. $p75^{NTR}$ has been detected within the subplate in rat, primate and human species cerebral cortex[314,332-334]. Furthermore, $p75^{NTR}$ has recently been show to promote survival of subplate neurons *in vitro* in a Trk independent fashion[335]. In addition to the subplate, NRAGE co-localizes with $p75^{NTR}$ in basal forebrain cholinergic neurons located in the medial septum, diagonal band of Broca, nucleus basalis and striatum[313,333,336,337]. The basal forebrain has been shown to be sensitive to NGF induced apoptosis[143]. 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 $p75^{NTR}$-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 $p75^{NTR}$. 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 $p75^{NTR}$ 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[338], the tumour suppressor gene p53 which is required for neural precursor cell apoptosis[339] and cyclin D_1[340]. 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[16]. Recent estimates indicate that between 50-70% of cortical neuroblasts in the proliferative ventricular zone undergo apoptosis in murine and primate species[23,29]. While the precise number of neural precursor cells undergoing apoptosis is under debate[26,341-343] 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[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[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[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[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[348] and mice lacking E2F-1 have a suppressed level of apoptosis[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[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 concludes pertaining to NRAGE activity during neural precursor apoptosis.

NRAGE was also identified in many other non-neural tissues throughout development. Similarly the $p75^{NTR}$ 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 $p75^{NTR}$ previously characterized in rats[350,351]. However, our result also identified non-neural regions that are not known to express $p75^{NTR}$. 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 $p75^{NTR}$-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 $p75^{NTR}$ 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 $p75^{NTR}$. However, under strict spatial and temporal regulation when expressed in the presence of $p75^{NTR}$, 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 $p75^{NTR}$ 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 posses 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 multipotenital 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 server 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[352]. The fate of neural progenitors is defined by their microenvironment

including effects from extra-CNS tissues[178,353-363]. 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[19,23,29,173,343,364-368]. 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[225,369]. The "canonical" pathway activates Smad1, Smad5 and Smad4 proteins[229,370,371]. 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)[213,245,372]. TAK1 activity has been

shown to promote the activation of p38[215,217,373] Jun N-terminal kinases (JNKs)[216,247], and Nemo-like kinase[374] a MAPK family member.

The multiple functions of BMPs during embryogenesis and neural development include complex actions upon the neural crest[21,241,375-382], apoptosis or differentiation of neurons and glia within the cortex[180,181,383-385] and dorsal spinal cord[386]. 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[214]. Another prominent regulator of cell death is XIAP, which can directly bind and inhibit caspase activity[387]. More recently, XIAP has been shown to bind TAB1, an activator of TAK1, and inhibit TAK1-induced apoptosis[245].

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[173,181,383]. Moreover, by the midpoint of neurogenesis (E16), BMPs promote neural differentiation of neural progenitors[383]. Work by McKay and colleagues[384] describe a feed-forward mechanism whereby BMP actions are first induced through BMPR-Ia and then terminate through BMPR-Ib. As cell surface levels of BMPR-Ib exceed those of BMPR-Ia, BMPs cause a termination response of either cell death or cell

differentiation[384]. Concurrent with the transition from BMPR-Ia 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[385,388].

NRAGE is a recently characterized **N**eurotrophin **R**eceptor interacting M**AGE** homologue, identified in a yeast two-hybrid screen for proteins that interact with the intracellular domain of the p75 neurotrophin receptor ($p75^{NTR}$). NRAGE was shown to mediate both cell cycle withdrawal and an NGF-specific apoptosis in sympathetic precursor cells through $p75^{NTR155}$. 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 $p75^{NTR}$ expression and concomitant with the spatial and temporal occurrence of BMP-mediated apoptosis[389]. This $p75^{NTR}$-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[290] and NRAGE is involved in UNC5-H1 induced apoptosis of neural progenitors[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-Ia 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% CO_2. For apoptosis assays cells seeded at a concentration of 1 X 10^5 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).

Table2. Morpholino Sequences and Knockdown Efficiency

Morpholino	Sequence	Delivery Efficiency	Knockdown Efficiency
NRAGE	5'-GGTTTCTGAGCCATAGCTCTCGTC-3'	90%	80%
TAK1	5'-AGCGCCCTTCAGCCCGGAGCCC-3'	92%	85%
TAB1	5'-CAGGCTCCTCCTCTGCGCCGCCATC-3'	85%	91%
XIAP	5'-CATCTTCTCTGGAAAATAGGACTTG-3'	88%	74%
Standard Control	5'-CCTCTTACCTCAGTTACAATTTATA -3'	90%	0%

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-Ia 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 puromycine, and 1.0 μg/ml tetracycline. To generate the retrovirus pSAP-expression vectors contain either wild-type or dominant negative BMPR-Ia 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-$NRAGE_{31-396}$ (Salehi et al., 2000), α-Caspase-3 (Pharmingen), α-TAK1, α-p38, α-dp-p38, α-JNK1/2, α-β-Actin (Sigma), α-XIAP, α-BMPR-Ia, 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-$p21^{waf1}$, α-$p27^{kip1}$ (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 $\pm$ SD of four replicate experiments.

All apoptosis and fluorescence-activated cell sorting (FACS) analyses used a Becton-Dickinson FACS Vantage SE cell sorter (Enterprise IIC 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][390] and DNA content was analyzed on a Becton-Dickinson FACS Calibur using Modfit 2.0 software. NRAGE^{+GFP} or control^{+GFP} 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^{+GFP} 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[389]. 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

Gene	Forward primer 5′-3′	Reverse primer 3′-5′	Annealing Temperature
Oct3/4	ATGGCTGGACACCTGGCTTC AGACT	GCCAGGCTCCTGATCAACAG CATCA	63°C
Hes1	AGAAGAGGCGAAGGGCAAG AA	CAAAAAACCTTGGCAGCCTC T	54°C
Mash1	TCGGCACTGACTTTTGCGGC TGCTTT	GAAGCACGATCAAAGGGGG ACGAA	56°C
Wnt-1	ACGTTGCTACTGGCACTGAC	CCATTTGCACTATCGCACAG	58°C
Neuro D1	TCAGCATCAATGGCAACT	TGACTCGCTCATGATGCGA	56°C
MEF2A	AGAAATGCCGACAGCCTACA A	TCACCCATGTGTCCATCCTC A	54°C
MEF2C	AGCAAGAATACGATGCCATC	GAAGGGGTGGTGGTACGGT C	56°C
Dlx5	CCGTCTCAGGAATCGCCAAC	CTGAAAGCTGGCTGGCTGGT	56°C

Msx2	ATTCGCCGCCGCCAAGACAT A	TCTTTTCGCCTTGGCCCTTC G	56°C
β-Actin	TGTTACCAACTGGGACGACA	CTCTCAGCTGTGGTGGTGAA	56°C

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 $p75^{NTR389}$ 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[23,29] 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 $p75^{NTR}$-mediate neurotrophin signaling in embryonal carcinoma (P19) cells and

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[383,391-394]. P19 cells have previously been shown to be devoid to $p75^{NTR}$ receptor expression during the first 4 days of RA and/or BMP4 induced apoptosis, but do up-regulate the receptor on post-mitotic neurons[76,395]. Indeed, RT-PCR analysis demonstrates that early P19 cells treated to undergo apoptosis through RA treatment fail to express $p75^{NTR}$ receptor (Fig. 10A).

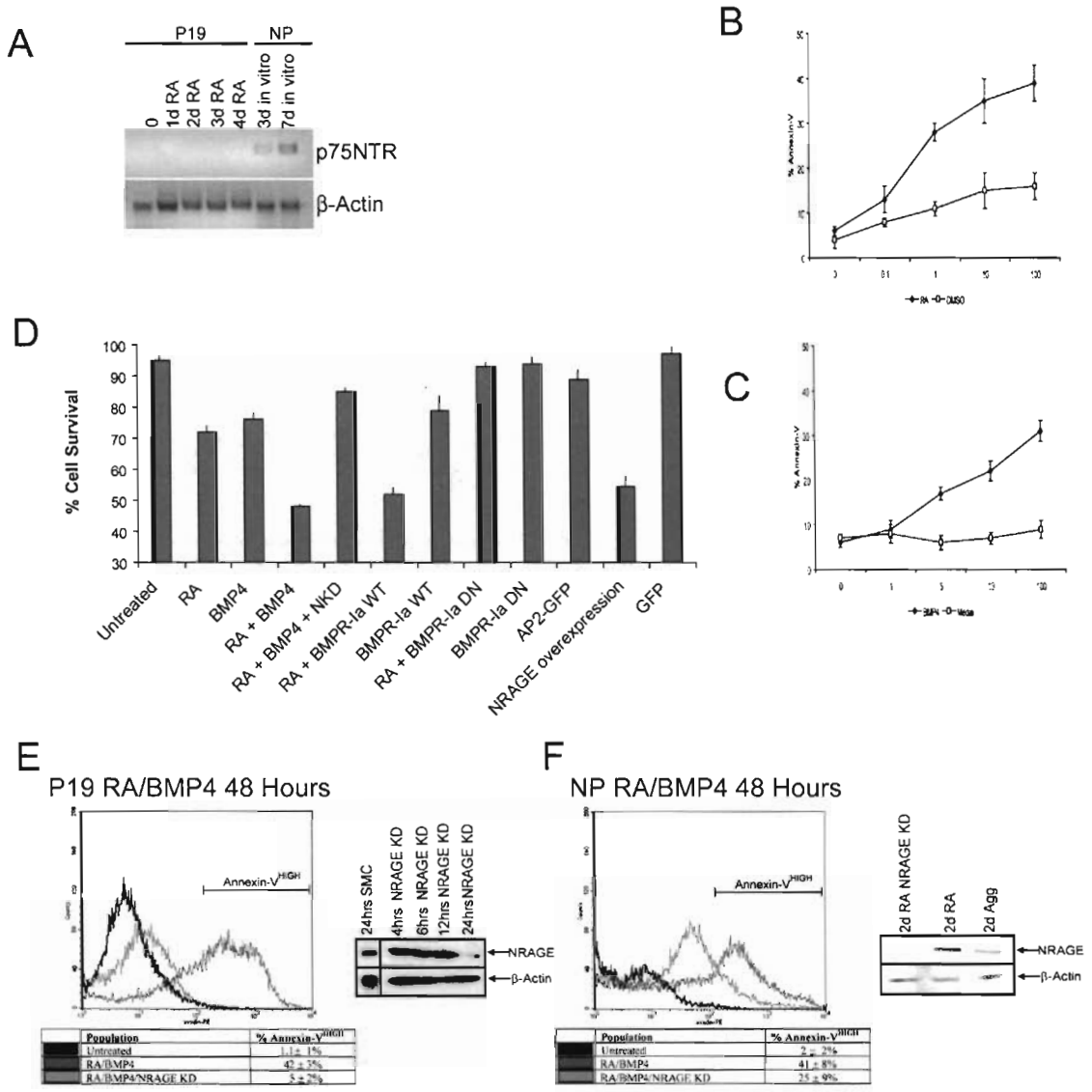

Figure 10. RA Induced Apoptosis is Mediated Through BMP Signaling.

(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 $p75^{NTR}$, but post-mitotic neurons cultured from mouse neural progenitors are $p75^{NTR}$ 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[396]. Using two independent measures of cell survival, trypan blue exclusion and annexin-V immunoreactivity, we found that, in agreement with the previous data[396], 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 Rodriquez-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% $\pm$ 2) to a level comparable to that of the cells treated with RA and BMP4 (48% $\pm$ 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% $\pm$ 2; RA + dnBMPR-1a: 85% $\pm$ 1 $p \leq 0.01$; Untreated: 95% $\pm$ 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).

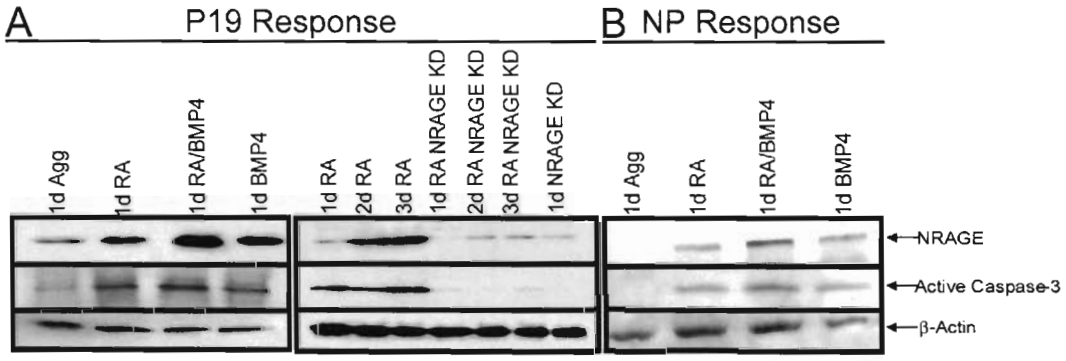

Figure 11. NRAGE is Required for the Activation of Caspase-3 by RA/BMP.

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-Ia, while it showed a strong association with BMPR-Ib. 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).

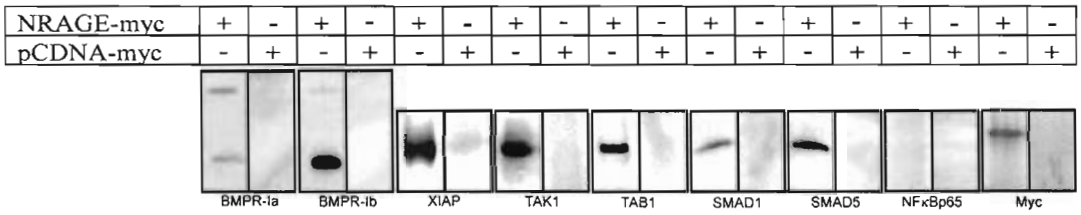

Figure 12. NRAGE Strongly Interacts with XIAP, TAK1, TAB1 and BMPR-Ib.

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[245]. 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-kβp65 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).

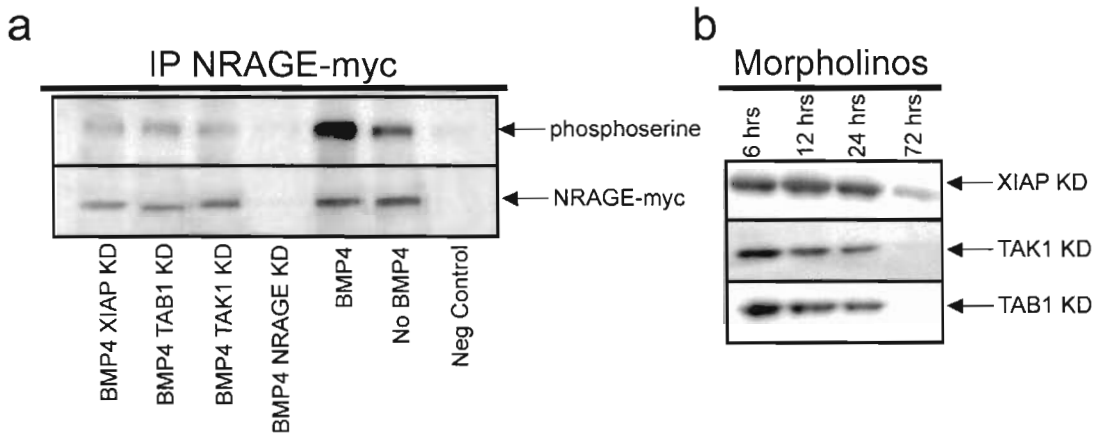

Figure 13. The TAK1, TAB1 and XIAP Complex Promotes NRAGE Phosphorylation.

(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 immunoprecipated 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).

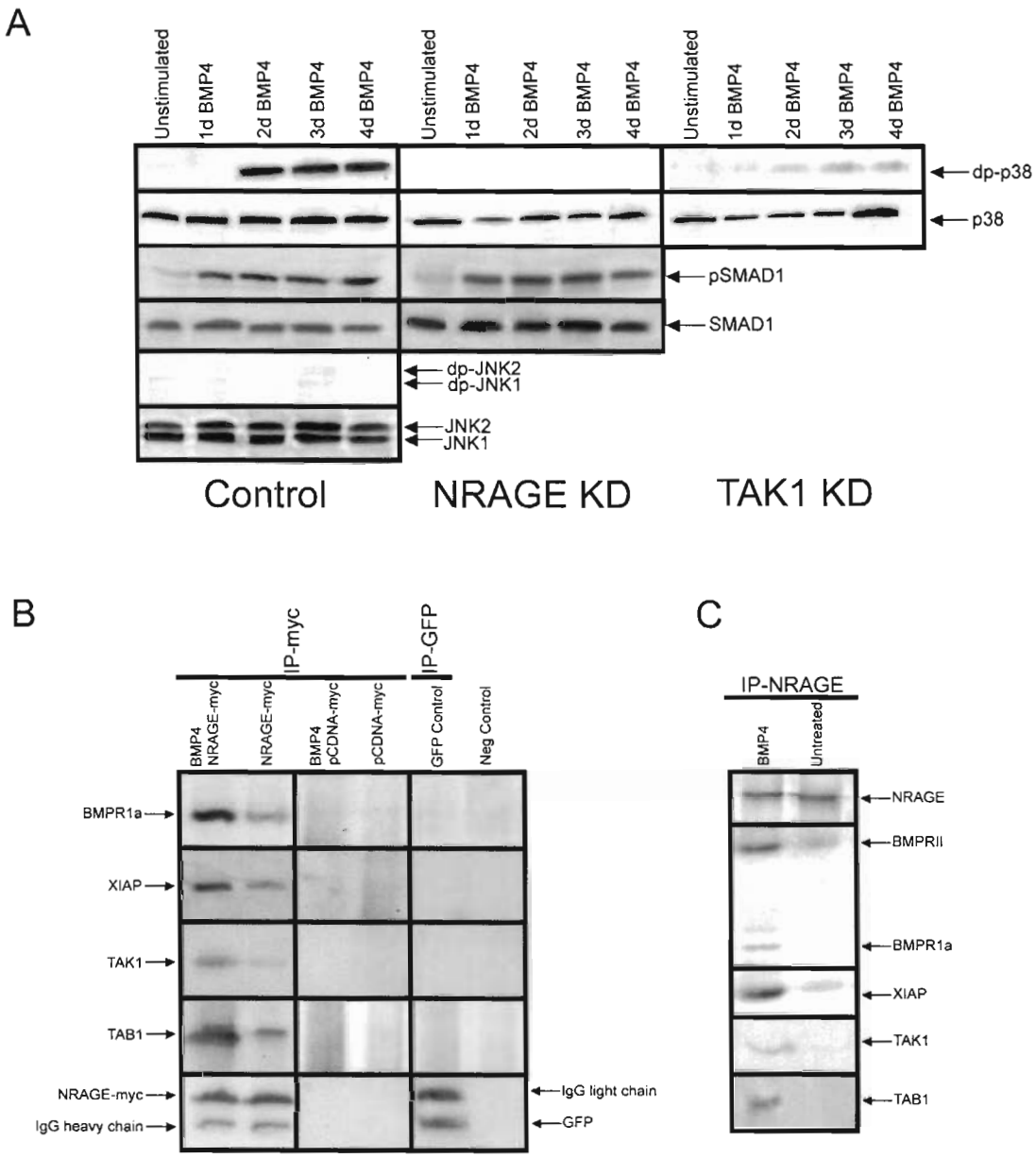

Figure 14. NRAGE is Required for the Activation of p38 MAPK.

(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-Ia 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

BMP 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-Ia 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 apoptosis 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).

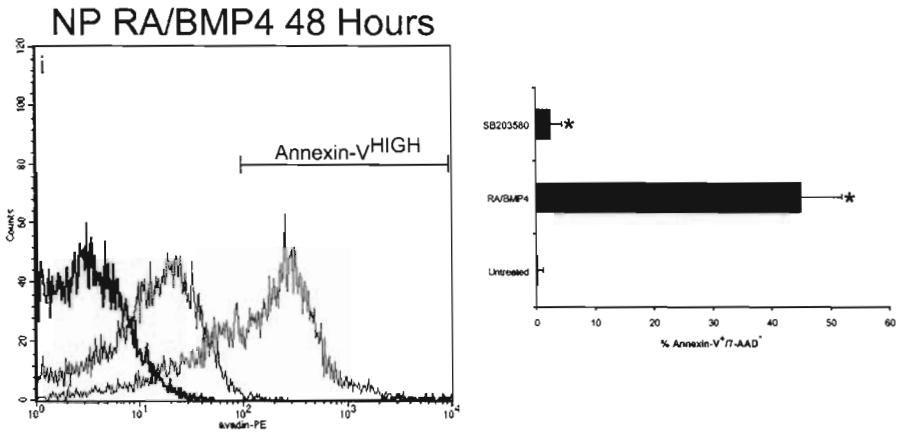

Figure 15. Neural Progenitors Require p38 MAPK to Mediate Apoptosis.

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).

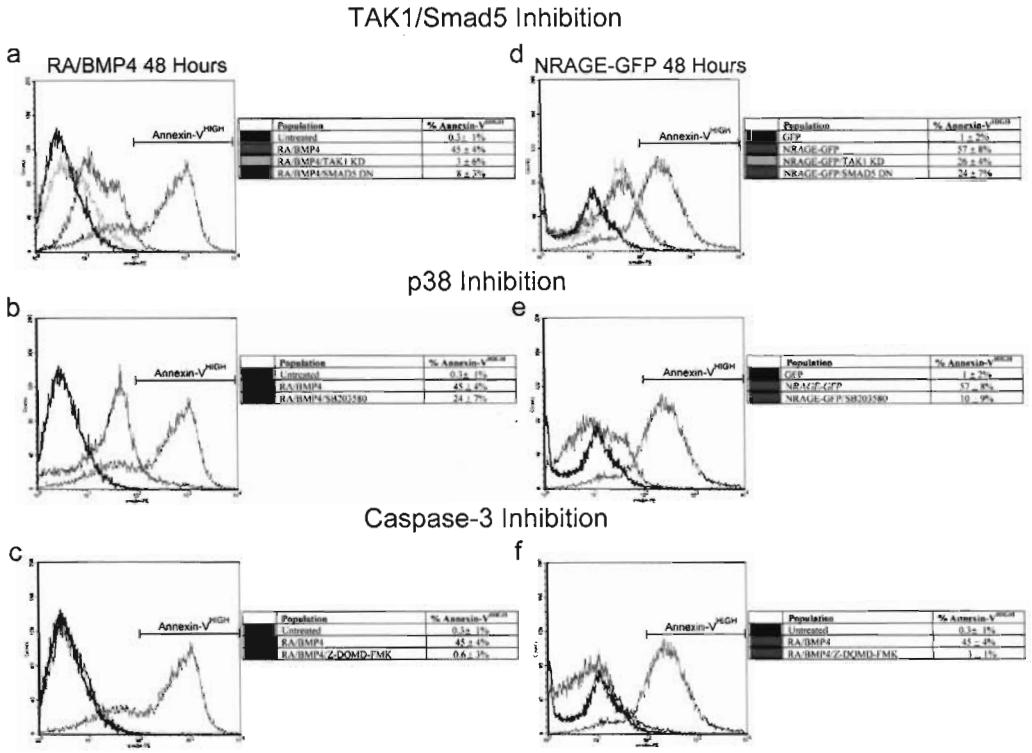

Figure 16. RA/BMP4 and NRAGE Over-expression Exhibit Similar Functional Requirements to Mediate Apoptosis.

(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% $\pm$ 9 (Fig.

16E). 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. Interesting, 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[69]. 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 [397-400]. 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[401,402]. 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 $\pm$ 3%), relative to P19 cells transfected with GFP ($control^{+GFP}$) (35 $\pm$ 2%; $t=7.2$, $p> 0.04$) (Fig. 17A,B).

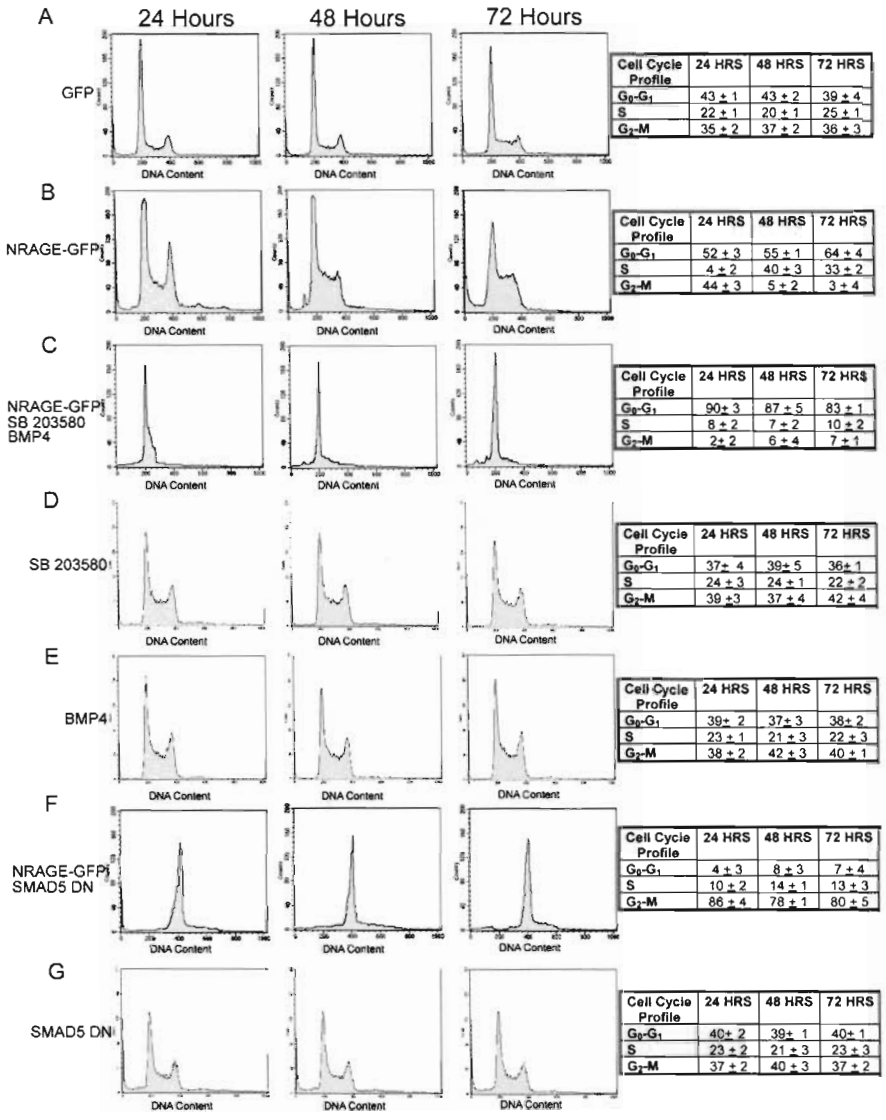

Figure 17. NRAGE Facilitates both Growth Arrest and Progression Through S Phase.

(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 $\pm$ 1%) of NRAGE^{+GFP} cells were positioned at the G1-S transition compared to just 43 $\pm$ 2% ($t=9.6$, $p>0.02$) for control cells. The percentage of cells at G1-S after 72 hours was further enhanced (64 $\pm$ 4%) compared to GFP controls (39 $\pm$ 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$^{kip1403-405}$. 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 underling 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).

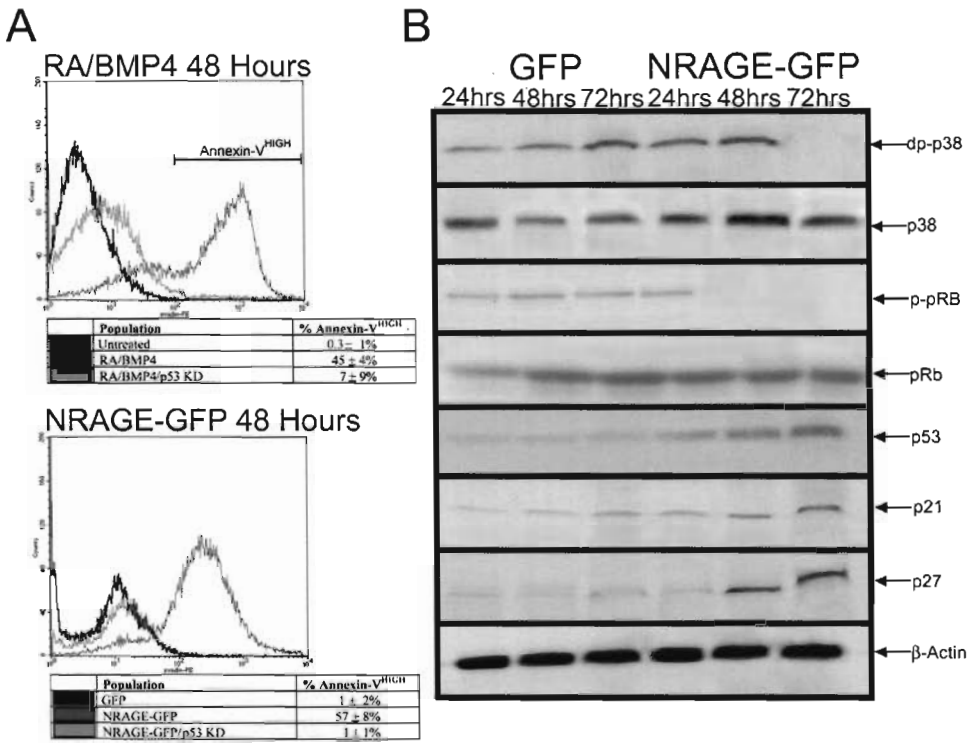

Figure 18. NRAGE Mediated Apoptosis is p53 Dependent.

(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 $p21^{waf1}$ and $p27^{kip1}$.

To further elucidate changes in cell cycle machinery, cell lysates were collected from FACS purified $NRAGE^{+GFP}$ and $control^{+GFP}$ 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 $p21^{waf1}$ and $p27^{kip1}$ with a sharp elevation in the phosphorylated state by 72 hours. These results are consistent with $NRAGE^{+GFP}$ induced G1-S arrest after 72 hours. In addition, p53 levels accumulate as time progressed; supporting the p53 dependence on $NRAGE^{+GFP}$ mediated apoptosis. These results demonstrate an antagonizing bi-functional positive feedback loop in which the accumulation of cellular NRAGE enhance 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).

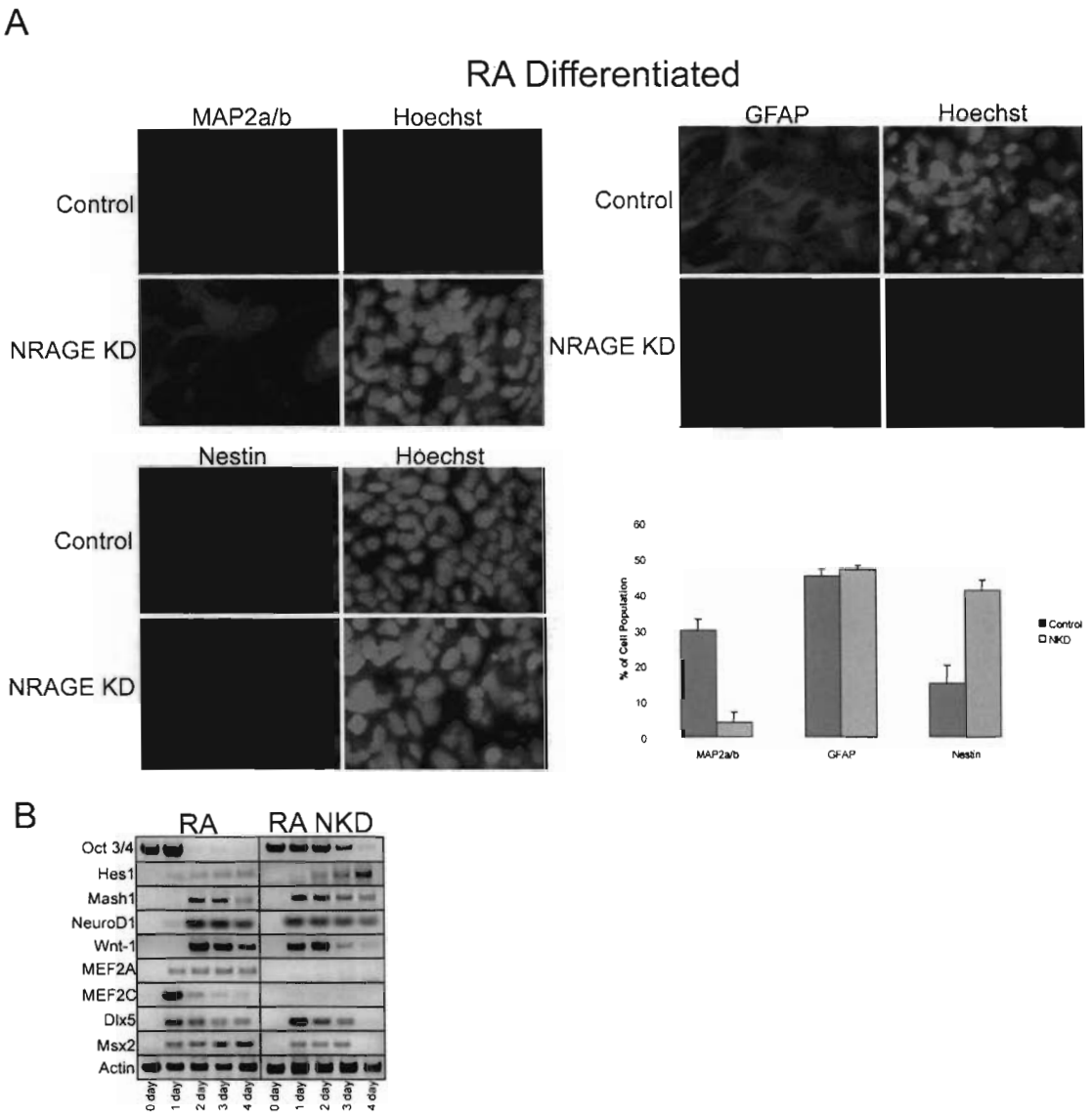

Figure 19. NRAGE Mediates the Transition of Multipotential Neural Progenitors to Post-mitotic Neuron.

(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 resulted 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 accelerates 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 $\pm$ 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[406] and loss of p38 activity[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[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^{+GFP} or control^{+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).

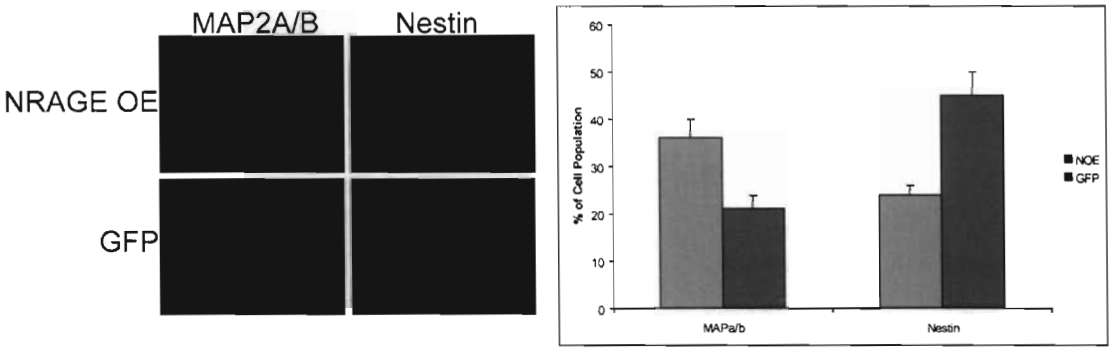

Figure 20. NRAGE Over-expression Facilitates Neurogenesis

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[346]. 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[411]. In addition to differentiation, RA also induces activation of caspase-9 and 3 promoting apoptosis[412-414]. Furthermore, the apoptotic action of RA is facilitated by the addition of BMP2 or BMP4[415,416]. The actions of RA mediate apoptosis through the RA receptor α and γ[417], which promote the transcription of BMP2 and BMP4[418,419].

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-Ia 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-$p75^{NTR}$ 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[16].

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[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 *Xenopus* embryo[214]. The activation of TAK1 requires an association with TAB1[420]. Moreover, XIAP interaction with the cytoplasmic domain of BMPR-Ia enhanced the ventralization of TAB1-TAK1, but perturbed the apoptotic affect[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[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[399,401]. 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[425,426]. 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[427-430]. 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[408,409]. Furthermore, MEF2A and C are highly expressed in developing neural progenitors[410] and cooperate with the pro-neural bHLH transcription factor Mash1 during neurogenesis[431-433], 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[434] and failure to so results in a p21^{waf1}-dependent apoptosis[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^{waf1} and p27^{kip1} activity[392,436]. Furthermore, accumulation of non-apoptotic levels of p27^{kip1} is required for BMP-induced growth arrest and neural differentiation[268,269,404] that is integrally linked to the activity MEF2C and Rb promoting survival under stringent p38 activity[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[155,283,439]. Direct coupling of the cell cycle and death machinery regulating differentiation has been well documented[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 $Rb^{-/-}$ 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[63-66]. Though this apoptosis can be circumvented in conditional $Rb^{-/-}$ null mice under the control of the Foxg1 promoter[441] and explained in lieu of a model proposed by Gail Martin[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 $p21^{waf1}$ and $p27^{kip1}$ [403-405]. 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).

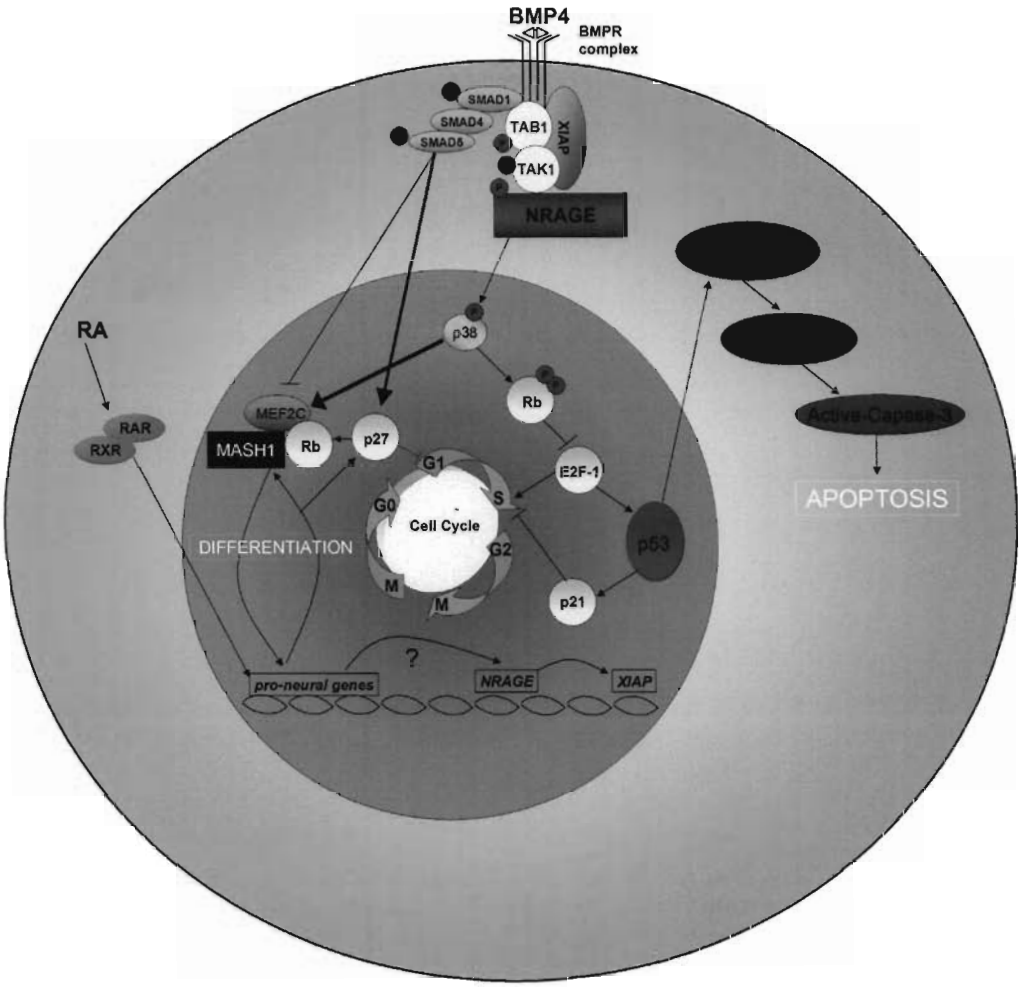

Figure 21. NRAGE Mediates Apoptosis of Neural Progenitors.

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 $p21^{waf1}$, 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 increasing more robust. Reciprocal modulation has been described between Wnt (canonical and non-canonical), TGF-b/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[443,444]. 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[445].

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[443]. 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[446]. 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[443]. 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[447]. 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[448-450]. 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 Tlc[451], a member of the secreted Frizzled Related Proteins (sFRP) family. sFRPs are generally considered to antagonize Wnt activity by sequestering secreted Wnts[452]. 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[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 *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[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 *Drosophila* and *Xenopus*, NLK is required for BMP mediated neurogenesis and apoptosis[458,459]. Thus, these novel insights provide yet an additional level of BMP-NRAGE mediated regulation of neurogenesis to explore.

FGF and BMP Signaling During Neurogenesis.

The ability of Wnt antagonists to locally induce instructive FGF signals in the ANR[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)[460-463]. Once an FGF ligand is bound, the receptor dimerizes and phosphorylates intermolecular tyrosine residues, triggering initiation of FGFR signal transduction[464-466]. FGFR signaling activates a number of signal transduction molecules, including those of the Ras-ERK and phospholipase C-γ pathways[464,466-468].

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[469,470]. Specially, following neural tube closure, *Fgf8* expression is localized to the ANR[172,175] and removal of the ANR or treatment with inhibitors of FGF signaling causes loss of expression of telencephalic molecular markers, such as Foxg1 (Bf1)[160,471]. 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[442]. 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[175,442,472]. 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 activity[456,473] 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[474].

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[475]. 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 rosstrally[476,477] and dorsal sensory neurons and neural crest cells caudally[478,479]. 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[451,480]. 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[383,481,482]. 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[181,383]. This BMP4-mediated apoptosis of early neural progenitors is dependent on the transcription factors Msx2, ID2 and the CKI

p21[waf1436]. 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[483]. 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[484,485], 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[486-489].

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[490]. 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[491,492]. 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[407]. 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[493]. 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.

REFERENCES

1. Schweichel JU, Merker HJ. The morphology of various types of cell death in prenatal tissues. Teratology 1973;7(3):253-66.

2. Clarke PG. Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol (Berl) 1990;181(3):195-213.

3. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26(4):239-57.

4. Clodi K, Kliche KO, Zhao S, Weidner D, Schenk T, Consoli U, Jiang S, Snell V, Andreeff M. Cell-surface exposure of phosphatidylserine correlates with the stage of fludarabine-induced apoptosis in chronic lymphocytic leukemia and expression of apoptosis-regulating genes. Cytometry 2000;40(1):19-25.

5. Mori C, Nakamura N, Kimura S, Irie H, Takigawa T, Shiota K. Programmed cell death in the interdigital tissue of the fetal mouse limb is apoptosis with DNA fragmentation. Anat Rec 1995;242(1):103-10.

6. Jacobsen MD, Weil M, Raff MC. Role of Ced-3/ICE-family proteases in staurosporine-induced programmed cell death. J Cell Biol 1996;133(5):1041-51.

7. Tang MK, Leung AK, Kwong WH, Chow PH, Chan JY, Ngo-Muller V, Li M, Lee KK. Bmp-4 requires the presence of the digits to initiate programmed cell death in limb interdigital tissues. Dev Biol 2000;218(1):89-98.

8. Coucouvanis E, Martin GR. Signals for death and survival: a two-step mechanism for cavitation in the vertebrate embryo. Cell 1995;83(2):279-87.

9. Glucksmann A. Local factors in the histogenesis of hypertrophic scars. Br J Plast Surg 1951;4(2):88-103.

10. Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. Cell 1997;88(3):347-54.

11. Barde YA. Trophic factors and neuronal survival. Neuron 1989;2(6):1525-34.

12. Oppenheim RW. Cell death during development of the nervous system. Annu Rev Neurosci 1991;14:453-501.

13. Saunders JW, Jr. Death in embryonic systems. Science 1966;154(749):604-12.

14. Taglialatela G, Caprioli A, Giuliani A, Ghirardi O. Spatial memory and NGF levels in aged rats: natural variability and effects of acetyl-L-carnitine treatment. Exp Gerontol 1996;31(5):577-87.

15. Kaufmann JA, Bickford PC, Taglialatela G. Oxidative-stress-dependent up-regulation of Bcl-2 expression in the central nervous system of aged Fisher-344 rats. J Neurochem 2001;76(4):1099-108.

16. de la Rosa EJ, de Pablo F. Cell death in early neural development: beyond the neurotrophic theory. Trends Neurosci 2000;23(10):454-8.

17. Raff MC. Social controls on cell survival and cell death. Nature 1992;356(6368):397-400.

18. Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y, Jacobson MD. Programmed cell death and the control of cell survival: lessons from the nervous system. Science 1993;262(5134):695-700.

19. Ferrer I, Soriano E, del Rio JA, Alcantara S, Auladell C. Cell death and removal in the cerebral cortex during development. Prog Neurobiol 1992;39(1):1-43.

20. Jeffs P, Jaques K, Osmond M. Cell death in cranial neural crest development. Anat Embryol (Berl) 1992;185(6):583-8.

21. Graham A, Francis-West P, Brickell P, Lumsden A. The signalling molecule BMP4 mediates apoptosis in the rhombencephalic neural crest. Nature 1994;372(6507):684-6.

22. Homma S, Yaginuma H, Oppenheim RW. Programmed cell death during the earliest stages of spinal cord development in the chick embryo: a possible means of early phenotypic selection. J Comp Neurol 1994;345(3):377-95.

23. Blaschke AJ, Staley K, Chun J. Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. Development 1996;122(4):1165-74.

24. Yaginuma H, Tomita M, Takashita N, McKay SE, Cardwell C, Yin QW, Oppenheim RW. A novel type of programmed neuronal death in the cervical spinal cord of the chick embryo. J Neurosci 1996;16(11):3685-703.

25. Morales AV, Serna J, Alarcon C, de la Rosa EJ, de Pablo F. Role of prepancreatic (pro)insulin and the insulin receptor in prevention of embryonic apoptosis. Endocrinology 1997;138(9):3967-75.

26. Thomaidou D, Mione MC, Cavanagh JF, Parnavelas JG. Apoptosis and its relation to the cell cycle in the developing cerebral cortex. J Neurosci 1997;17(3):1075-85.

27. Weil M, Jacobson MD, Raff MC. Is programmed cell death required for neural tube closure? Curr Biol 1997;7(4):281-4.

28. Hensey C, Gautier J. Programmed cell death during Xenopus development: a spatio-temporal analysis. Dev Biol 1998;203(1):36-48.

29. Blaschke AJ, Weiner JA, Chun J. Programmed cell death is a universal feature of embryonic and postnatal neuroproliferative regions throughout the central nervous system. J Comp Neurol 1998;396(1):39-50.

30. Verdi JM, Groves AK, Farinas I, Jones K, Marchionni MA, Reichardt LF, Anderson DJ. A reciprocal cell-cell interaction mediated by NT-3 and neuregulins controls the early survival and development of sympathetic neuroblasts. Neuron 1996;16(3):515-27.

31. Gould TW, Burek MJ, Sosnowski JM, Prevette D, Oppenheim RW. The spatial-temporal gradient of naturally occurring motoneuron death reflects

the time of prior exit from the cell cycle and position within the lateral motor column. Dev Biol 1999;216(2):611-21.

32. Kuida K, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MS, Rakic P, Flavell RA. Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell 1998;94(3):325-37.

33. Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic P, Flavell RA. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 1996;384(6607):368-72.

34. Yoshida H, Kong YY, Yoshida R, Elia AJ, Hakem A, Hakem R, Penninger JM, Mak TW. Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell 1998;94(6):739-50.

35. Galli-Resta L, Ensini M. An intrinsic time limit between genesis and death of individual neurons in the developing retinal ganglion cell layer. J Neurosci 1996;16(7):2318-24.

36. Ninomiya Y, Adams R, Morriss-Kay GM, Eto K. Apoptotic cell death in neuronal differentiation of P19 EC cells: cell death follows reentry into S phase. J Cell Physiol 1997;172(1):25-35.

37. Azuma-Hara M, Taniura H, Uetsuki T, Niinobe M, Yoshikawa K. Regulation and deregulation of E2F1 in postmitotic neurons differentiated from embryonal carcinoma P19 cells. Exp Cell Res 1999;251(2):442-51.

38. Hunter T, Pines J. Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. Cell 1994;79(4):573-82.

39. Sherr CJ. G1 phase progression: cycling on cue. Cell 1994;79(4):551-5.

40. Lukas J, Bartkova J, Bartek J. Convergence of mitogenic signalling cascades from diverse classes of receptors at the cyclin D-cyclin-dependent kinase-pRb-controlled G1 checkpoint. Mol Cell Biol 1996;16(12):6917-25.

41. Matsushime H, Roussel MF, Ashmun RA, Sherr CJ. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell 1991;65(4):701-13.

42. Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM, Pagano M. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Mol Cell Biol 1995;15(5):2612-24.

43. Resnitzky D, Gossen M, Bujard H, Reed SI. Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. Mol Cell Biol 1994;14(3):1669-79.

44. Pagano M, Pepperkok R, Verde F, Ansorge W, Draetta G. Cyclin A is required at two points in the human cell cycle. Embo J 1992;11(3):961-71.

45. Resnitzky D, Hengst L, Reed SI. Cyclin A-associated kinase activity is rate limiting for entrance into S phase and is negatively regulated in G1 by p27Kip1. Mol Cell Biol 1995;15(8):4347-52.

46. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 1993;75(4):805-16.

47. Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev 1994;8(1):9-22.

48. Lee MH, Reynisdottir I, Massague J. Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes Dev 1995;9(6):639-49.

49. Carnero A, Hannon GJ. The INK4 family of CDK inhibitors. Curr Top Microbiol Immunol 1998;227:43-55.

50. Chellappan SP, Giordano A, Fisher PB. Role of cyclin-dependent kinases and their inhibitors in cellular differentiation and development. Curr Top Microbiol Immunol 1998;227:57-103.

51. Pardee AB. G1 events and regulation of cell proliferation. Science 1989;246(4930):603-8.

52. Murray A. Cell cycle checkpoints. Curr Opin Cell Biol 1994;6(6):872-6.

53. Bartek J, Bartkova J, Lukas J. The retinoblastoma protein pathway and the restriction point. Curr Opin Cell Biol 1996;8(6):805-14.

54. Walworth NC. Cell-cycle checkpoint kinases: checking in on the cell cycle. Curr Opin Cell Biol 2000;12(6):697-704.

55. Weinberg RA. The retinoblastoma protein and cell cycle control. Cell 1995;81(3):323-30.

56. Beijersbergen RL, Bernards R. Cell cycle regulation by the retinoblastoma family of growth inhibitory proteins. Biochim Biophys Acta 1996;1287(2-3):103-20.

57. Mulligan G, Jacks T. The retinoblastoma gene family: cousins with overlapping interests. Trends Genet 1998;14(6):223-9.

58. Goodrich DW, Wang NP, Qian YW, Lee EY, Lee WH. The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. Cell 1991;67(2):293-302.

59. Hinds PW, Mittnacht S, Dulic V, Arnold A, Reed SI, Weinberg RA. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell 1992;70(6):993-1006.

60. Zhu L, van den Heuvel S, Helin K, Fattaey A, Ewen M, Livingston D, Dyson N, Harlow E. Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. Genes Dev 1993;7(7A):1111-25.

61. Claudio PP, Howard CM, Baldi A, De Luca A, Fu Y, Condorelli G, Sun Y, Colburn N, Calabretta B, Giordano A. p130/pRb2 has growth suppressive properties similar to yet distinctive from those of retinoblastoma family members pRb and p107. Cancer Res 1994;54(21):5556-60.

62. Vairo G, Livingston DM, Ginsberg D. Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. Genes Dev 1995;9(7):869-81.

63. Clarke AR, Maandag ER, van Roon M, van der Lugt NM, van der Valk M, Hooper ML, Berns A, te Riele H. Requirement for a functional Rb-1 gene in murine development. Nature 1992;359(6393):328-30.

64. Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA, Weinberg RA. Effects of an Rb mutation in the mouse. Nature 1992;359(6393):295-300.

65. Lee EY, Chang CY, Hu N, Wang YC, Lai CC, Herrup K, Lee WH, Bradley A. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature 1992;359(6393):288-94.

66. Lee EY, Hu N, Yuan SS, Cox LA, Bradley A, Lee WH, Herrup K. Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. Genes Dev 1994;8(17):2008-21.

67. Slack RS, El-Bizri H, Wong J, Belliveau DJ, Miller FD. A critical temporal requirement for the retinoblastoma protein family during neuronal determination. J Cell Biol 1998;140(6):1497-509.

68. Callaghan DA, Dong L, Callaghan SM, Hou YX, Dagnino L, Slack RS. Neural precursor cells differentiating in the absence of Rb exhibit delayed terminal mitosis and deregulated E2F 1 and 3 activity. Dev Biol 1999;207(2):257-70.

69. Liu DX, Greene LA. Neuronal apoptosis at the G1/S cell cycle checkpoint. Cell Tissue Res 2001;305(2):217-28.

70. Macleod KF, Hu Y, Jacks T. Loss of Rb activates both p53-dependent and independent cell death pathways in the developing mouse nervous system. Embo J 1996;15(22):6178-88.

71. Tsai KY, Hu Y, Macleod KF, Crowley D, Yamasaki L, Jacks T. Mutation of E2f-1 suppresses apoptosis and inappropriate S phase entry and extends survival of Rb-deficient mouse embryos. Mol Cell 1998;2(3):293-304.

72. Oppenheim RW. Cyclic GMP and neurone death. Nature 1985;313(5999):248.

73. Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y, Jacobson MD. Programmed cell death and the control of cell survival. Philos Trans R Soc Lond B Biol Sci 1994;345(1313):265-8.

74. Miller FD, Kaplan DR. Neurotrophin signalling pathways regulating neuronal apoptosis. Cell Mol Life Sci 2001;58(8):1045-53.

75. Barbacid M. Structural and functional properties of the TRK family of neurotrophin receptors. Ann N Y Acad Sci 1995;766:442-58.

76. Bothwell M. Functional interactions of neurotrophins and neurotrophin receptors. Annu Rev Neurosci 1995;18:223-53.

77. Ibanez CF. Emerging themes in structural biology of neurotrophic factors. Trends Neurosci 1998;21(10):438-44.

78. Levi-Montalcini R, Hamburger V. Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. J Exp Zool 1951;116(2):321-61.

79. Cohen S, Levi-Montalcini R. Purification and properties of a nerve growth-promoting factor isolated from mouse sarcoma 180. Cancer Res 1957;17(1):15-20.

80. Sutter A, Riopelle RJ, Harris-Warrick RM, Shooter EM. The heterogeneity of nerve growth factor receptors. Prog Clin Biol Res 1979;31:659-67.

81. Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao M. Expression and structure of the human NGF receptor. Cell 1986;47(4):545-54.

82. Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM. Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 1987;325(6105):593-7.

83. Rodriguez-Tebar A, Dechant G, Barde YA. Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron 1990;4(4):487-92.

84. Ernfors P, Wetmore C, Olson L, Persson H. Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. Neuron 1990;5(4):511-26.

85. Squinto SP, Stitt TN, Aldrich TH, Davis S, Bianco SM, Radziejewski C, Glass DJ, Masiakowski P, Furth ME, Valenzuela DM and others. trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell 1991;65(5):885-93.

86. Hallbook F, Ibanez CF, Persson H. Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron 1991;6(5):845-58.

87. Martin-Zanca D, Barbacid M, Parada LF. Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. Genes Dev 1990;4(5):683-94.

88. Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF. The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 1991;252(5005):554-8.

89. Klein R, Jing SQ, Nanduri V, O'Rourke E, Barbacid M. The trk proto-oncogene encodes a receptor for nerve growth factor. Cell 1991;65(1):189-97.

90. Bothwell M. Keeping track of neurotrophin receptors. Cell 1991;65(6):915-8.

91. Kaplan DR, Miller FD. Signal transduction by the neurotrophin receptors. Curr Opin Cell Biol 1997;9(2):213-21.

92. Barbacid M. The Trk family of neurotrophin receptors. J Neurobiol 1994;25(11):1386-403.

93. Klesse LJ, Meyers KA, Marshall CJ, Parada LF. Nerve growth factor induces survival and differentiation through two distinct signaling cascades in PC12 cells. Oncogene 1999;18(12):2055-68.

94. Kaplan DR, Miller FD. Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 2000;10(3):381-91.

95. Glass DJ, Nye SH, Hantzopoulos P, Macchi MJ, Squinto SP, Goldfarb M, Yancopoulos GD. TrkB mediates BDNF/NT-3-dependent survival and proliferation in fibroblasts lacking the low affinity NGF receptor. Cell 1991;66(2):405-13.

96. Ip NY, Li Y, Yancopoulos GD, Lindsay RM. Cultured hippocampal neurons show responses to BDNF, NT-3, and NT-4, but not NGF. J Neurosci 1993;13(8):3394-405.

97. Kawamoto K, Okada T, Kannan Y, Ushio H, Matsumoto M, Matsuda H. Nerve growth factor prevents apoptosis of rat peritoneal mast cells through the trk proto-oncogene receptor. Blood 1995;86(12):4638-44.

98. Di Marco E, Mathor M, Bondanza S, Cutuli N, Marchisio PC, Cancedda R, De Luca M. Nerve growth factor binds to normal human keratinocytes through high and low affinity receptors and stimulates their growth by a novel autocrine loop. J Biol Chem 1993;268(30):22838-46.

99. Janet T, Ludecke G, Otten U, Unsicker K. Heterogeneity of human neuroblastoma cell lines in their proliferative responses to basic FGF, NGF, and EGF: correlation with expression of growth factors and growth factor receptors. J Neurosci Res 1995;40(6):707-15.

100. Verdi JM, Birren SJ, Ibanez CF, Persson H, Kaplan DR, Benedetti M, Chao MV, Anderson DJ. p75LNGFR regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells. Neuron 1994;12(4):733-45.

101. Rabin SJ, Cleghon V, Kaplan DR. SNT, a differentiation-specific target of neurotrophic factor-induced tyrosine kinase activity in neurons and PC12 cells. Mol Cell Biol 1993;13(4):2203-13.

102. Gao WQ, Zheng JL, Karihaloo M. Neurotrophin-4/5 (NT-4/5) and brain-derived neurotrophic factor (BDNF) act at later stages of cerebellar granule cell differentiation. J Neurosci 1995;15(4):2656-67.

103. Wang T, Xie K, Lu B. Neurotrophins promote maturation of developing neuromuscular synapses. J Neurosci 1995;15(7 Pt 1):4796-805.

104. Thoenen H. Neurotrophins and neuronal plasticity. Science 1995;270(5236):593-8.

105. Kang H, Jia LZ, Suh KY, Tang L, Schuman EM. Determinants of BDNF-induced hippocampal synaptic plasticity: role of the Trk B receptor and the kinetics of neurotrophin delivery. Learn Mem 1996;3(2-3):188-96.

106. Morfini G, DiTella MC, Feiguin F, Carri N, Caceres A. Neurotrophin-3 enhances neurite outgrowth in cultured hippocampal pyramidal neurons. J Neurosci Res 1994;39(2):219-32.

107. Mamounas LA, Altar CA, Blue ME, Kaplan DR, Tessarollo L, Lyons WE. BDNF promotes the regenerative sprouting, but not survival, of injured serotonergic axons in the adult rat brain. J Neurosci 2000;20(2):771-82.

108. Fryer RH, Kaplan DR, Kromer LF. Truncated trkB receptors on nonneuronal cells inhibit BDNF-induced neurite outgrowth in vitro. Exp Neurol 1997;148(2):616-27.

109. Kang H, Schuman EM. Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. Science 1995;267(5204):1658-62.

110. Lessmann V. Neurotrophin-dependent modulation of glutamatergic synaptic transmission in the mammalian CNS. Gen Pharmacol 1998;31(5):667-74.

111. Gooney M, Lynch MA. Long-term potentiation in the dentate gyrus of the rat hippocampus is accompanied by brain-derived neurotrophic factor-induced activation of TrkB. J Neurochem 2001;77(5):1198-207.

112. Bibel M, Barde YA. Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. Genes Dev 2000;14(23):2919-37.

113. Borasio GD, Markus A, Wittinghofer A, Barde YA, Heumann R. Involvement of ras p21 in neurotrophin-induced response of sensory, but not sympathetic neurons. J Cell Biol 1993;121(3):665-72.

114. Nobes CD, Tolkovsky AM. Neutralizing anti-p21ras Fabs suppress rat sympathetic neuron survival induced by NGF, LIF, CNTF and cAMP. Eur J Neurosci 1995;7(2):344-50.

115. Yao R, Cooper GM. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science 1995;267(5206):2003-6.

116. Crowder RJ, Freeman RS. Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J Neurosci 1998;18(8):2933-43.

117. Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME. Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 1997;275(5300):661-5.

118. D'Mello SR, Borodezt K, Soltoff SP. Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: possible involvement of PI 3-kinase in IGF-1 signaling. J Neurosci 1997;17(5):1548-60.

119. Bartlett SE, Reynolds AJ, Weible M, Heydon K, Hendry IA. In sympathetic but not sensory neurones, phosphoinositide-3 kinase is important for NGF-dependent survival and the retrograde transport of 125I-betaNGF. Brain Res 1997;761(2):257-62.

120. Mazzoni IE, Said FA, Aloyz R, Miller FD, Kaplan D. Ras regulates sympathetic neuron survival by suppressing the p53-mediated cell death pathway. J Neurosci 1999;19(22):9716-27.

121. Vaillant AR, Mazzoni I, Tudan C, Boudreau M, Kaplan DR, Miller FD. Depolarization and neurotrophins converge on the phosphatidylinositol 3-

kinase-Akt pathway to synergistically regulate neuronal survival. J Cell Biol 1999;146(5):955-66.

122. Klesse LJ, Parada LF. p21 ras and phosphatidylinositol-3 kinase are required for survival of wild-type and NF1 mutant sensory neurons. J Neurosci 1998;18(24):10420-8.

123. Virdee K, Xue L, Hemmings BA, Goemans C, Heumann R, Tolkovsky AM. Nerve growth factor-induced PKB/Akt activity is sustained by phosphoinositide 3-kinase dependent and independent signals in sympathetic neurons. Brain Res 1999;837(1-2):127-42.

124. Hetman M, Kanning K, Cavanaugh JE, Xia Z. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. J Biol Chem 1999;274(32):22569-80.

125. Dolcet X, Egea J, Soler RM, Martin-Zanca D, Comella JX. Activation of phosphatidylinositol 3-kinase, but not extracellular-regulated kinases, is necessary to mediate brain-derived neurotrophic factor-induced motoneuron survival. J Neurochem 1999;73(2):521-31.

126. Williams EJ, Doherty P. Evidence for and against a pivotal role of PI 3-kinase in a neuronal cell survival pathway. Mol Cell Neurosci 1999;13(4):272-80.

127. Holgado-Madruga M, Moscatello DK, Emlet DR, Dieterich R, Wong AJ. Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. Proc Natl Acad Sci U S A 1997;94(23):12419-24.

128. Korhonen JM, Said FA, Wong AJ, Kaplan DR. Gab1 mediates neurite outgrowth, DNA synthesis, and survival in PC12 cells. J Biol Chem 1999;274(52):37307-14.

129. Andjelkovic M, Suidan HS, Meier R, Frech M, Alessi DR, Hemmings BA. Nerve growth factor promotes activation of the alpha, beta and gamma isoforms of protein kinase B in PC12 pheochromocytoma cells. Eur J Biochem 1998;251(1-2):195-200.

130. Ashcroft M, Stephens RM, Hallberg B, Downward J, Kaplan DR. The selective and inducible activation of endogenous PI 3-kinase in PC12 cells results in efficient NGF-mediated survival but defective neurite outgrowth. Oncogene 1999;18(32):4586-97.

131. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997;91(2):231-41.

132. del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 1997;278(5338):687-9.

133. LaCasse EC, Baird S, Korneluk RG, MacKenzie AE. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene 1998;17(25):3247-59.

134. Rabizadeh S, Oh J, Zhong LT, Yang J, Bitler CM, Butcher LL, Bredesen DE. Induction of apoptosis by the low-affinity NGF receptor. Science 1993;261(5119):345-8.

135. Barrett GL, Bartlett PF. The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. Proc Natl Acad Sci U S A 1994;91(14):6501-5.

136. Davey F, Davies AM. TrkB signalling inhibits p75-mediated apoptosis induced by nerve growth factor in embryonic proprioceptive neurons. Curr Biol 1998;8(16):915-8.

137. Bamji SX, Majdan M, Pozniak CD, Belliveau DJ, Aloyz R, Kohn J, Causing CG, Miller FD. The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. J Cell Biol 1998;140(4):911-23.

138. Wiese S, Metzger F, Holtmann B, Sendtner M. The role of p75NTR in modulating neurotrophin survival effects in developing motoneurons. Eur J Neurosci 1999;11(5):1668-76.

139. Sedel F, Bechade C, Triller A. Nerve growth factor (NGF) induces motoneuron apoptosis in rat embryonic spinal cord in vitro. Eur J Neurosci 1999;11(11):3904-12.

140. Friedman WJ. Neurotrophins induce death of hippocampal neurons via the p75 receptor. J Neurosci 2000;20(17):6340-6.

141. Casaccia-Bonnefil P, Carter BD, Dobrowsky RT, Chao MV. Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. Nature 1996;383(6602):716-9.

142. Soilu-Hanninen M, Ekert P, Bucci T, Syroid D, Bartlett PF, Kilpatrick TJ. Nerve growth factor signaling through p75 induces apoptosis in Schwann cells via a Bcl-2-independent pathway. J Neurosci 1999;19(12):4828-38.

143. Frade JM, Barde YA. Genetic evidence for cell death mediated by nerve growth factor and the neurotrophin receptor p75 in the developing mouse retina and spinal cord. Development 1999;126(4):683-90.

144. Van der Zee CE, Ross GM, Riopelle RJ, Hagg T. Survival of cholinergic forebrain neurons in developing p75NGFR-deficient mice. Science 1996;274(5293):1729-32.

145. Majdan M, Lachance C, Gloster A, Aloyz R, Zeindler C, Bamji S, Bhakar A, Belliveau D, Fawcett J, Miller FD and others. Transgenic mice expressing the intracellular domain of the p75 neurotrophin receptor undergo neuronal apoptosis. J Neurosci 1997;17(18):6988-98.

146. Yoon SO, Casaccia-Bonnefil P, Carter B, Chao MV. Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. J Neurosci 1998;18(9):3273-81.

147. Casademunt E, Carter BD, Benzel I, Frade JM, Dechant G, Barde YA. The zinc finger protein NRIF interacts with the neurotrophin receptor p75(NTR) and participates in programmed cell death. Embo J 1999;18(21):6050-61.

148. Mukai J, Hachiya T, Shoji-Hoshino S, Kimura MT, Nadano D, Suvanto P, Hanaoka T, Li Y, Irie S, Greene LA and others. NADE, a p75NTR-associated cell death executor, is involved in signal transduction mediated

by the common neurotrophin receptor p75NTR. J Biol Chem 2000;275(23):17566-70.

149. Chittka A, Chao MV. Identification of a zinc finger protein whose subcellular distribution is regulated by serum and nerve growth factor. Proc Natl Acad Sci U S A 1999;96(19):10705-10.

150. Yamashita T, Tucker KL, Barde YA. Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. Neuron 1999;24(3):585-93.

151. Canossa M, Twiss JL, Verity AN, Shooter EM. p75(NGFR) and TrkA receptors collaborate to rapidly activate a p75(NGFR)-associated protein kinase. Embo J 1996;15(13):3369-76.

152. Volonte C, Ross AH, Greene LA. Association of a purine-analogue-sensitive protein kinase activity with p75 nerve growth factor receptors. Mol Biol Cell 1993;4(1):71-8.

153. Khursigara G, Orlinick JR, Chao MV. Association of the p75 neurotrophin receptor with TRAF6. J Biol Chem 1999;274(5):2597-600.

154. Ye X, Mehlen P, Rabizadeh S, VanArsdale T, Zhang H, Shin H, Wang JJ, Leo E, Zapata J, Hauser CA and others. TRAF family proteins interact with the common neurotrophin receptor and modulate apoptosis induction. J Biol Chem 1999;274(42):30202-8.

155. Salehi AH, Roux PP, Kubu CJ, Zeindler C, Bhakar A, Tannis LL, Verdi JM, Barker PA. NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis. Neuron 2000;27(2):279-88.

156. Lumsden A, Krumlauf R. Patterning the vertebrate neuraxis. Science 1996;274(5290):1109-15.

157. Rubenstein JL, Beachy PA. Patterning of the embryonic forebrain. Curr Opin Neurobiol 1998;8(1):18-26.

158. Tanabe Y, Jessell TM. Diversity and pattern in the developing spinal cord. Science 1996;274(5290):1115-23.

159. Edlund T, Jessell TM. Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. Cell 1999;96(2):211-24.

160. Shimamura K, Rubenstein JL. Inductive interactions direct early regionalization of the mouse forebrain. Development 1997;124(14):2709-18.

161. Hallonet M, Hollemann T, Wehr R, Jenkins NA, Copeland NG, Pieler T, Gruss P. Vax1 is a novel homeobox-containing gene expressed in the developing anterior ventral forebrain. Development 1998;125(14):2599-610.

162. Martinez S, Wassef M, Alvarado-Mallart RM. Induction of a mesencephalic phenotype in the 2-day-old chick prosencephalon is preceded by the early expression of the homeobox gene en. Neuron 1991;6(6):971-81.

163. Ericson J, Muhr J, Placzek M, Lints T, Jessell TM, Edlund T. Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning within the neural tube. Cell 1995;81(5):747-56.

164. Dale JK, Vesque C, Lints TJ, Sampath TK, Furley A, Dodd J, Placzek M. Cooperation of BMP7 and SHH in the induction of forebrain ventral midline cells by prechordal mesoderm. Cell 1997;90(2):257-69.

165. Schier AF. Genetics of neural development in zebrafish. Curr Opin Neurobiol 1997;7(1):119-26.

166. Sampath K, Rubinstein AL, Cheng AM, Liang JO, Fekany K, Solnica-Krezel L, Korzh V, Halpern ME, Wright CV. Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. Nature 1998;395(6698):185-9.

167. Mehler MF, Mabie PC, Zhang D, Kessler JA. Bone morphogenetic proteins in the nervous system. Trends Neurosci 1997;20(7):309-17.

168. Lee KJ, Jessell TM. The specification of dorsal cell fates in the vertebrate central nervous system. Annu Rev Neurosci 1999;22:261-94.

169. McMahon AP, Bradley A. The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. Cell 1990;62(6):1073-85.

170. Meyers EN, Lewandoski M, Martin GR. An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. Nat Genet 1998;18(2):136-41.

171. Rubenstein JL, Shimamura K, Martinez S, Puelles L. Regionalization of the prosencephalic neural plate. Annu Rev Neurosci 1998;21:445-77.

172. Crossley PH, Martin GR. The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. Development 1995;121(2):439-51.

173. Furuta Y, Piston DW, Hogan BL. Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. Development 1997;124(11):2203-12.

174. Golden JA. Holoprosencephaly: a defect in brain patterning. J Neuropathol Exp Neurol 1998;57(11):991-9.

175. Crossley PH, Martinez S, Ohkubo Y, Rubenstein JL. Coordinate expression of Fgf8, Otx2, Bmp4, and Shh in the rostral prosencephalon during development of the telencephalic and optic vesicles. Neuroscience 2001;108(2):183-206.

176. Ohkubo Y, Chiang C, Rubenstein JL. Coordinate regulation and synergistic actions of BMP4, SHH and FGF8 in the rostral prosencephalon regulate morphogenesis of the telencephalic and optic vesicles. Neuroscience 2002;111(1):1-17.

177. Ghosh A, Greenberg ME. Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. Neuron 1995;15(1):89-103.

178. Johe KK, Hazel TG, Muller T, Dugich-Djordjevic MM, McKay RD. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev 1996;10(24):3129-40.

179. Bonni A, Sun Y, Nadal-Vicens M, Bhatt A, Frank DA, Rozovsky I, Stahl N, Yancopoulos GD, Greenberg ME. Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science 1997;278(5337):477-83.

180. Gross RE, Mehler MF, Mabie PC, Zang Z, Santschi L, Kessler JA. Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. Neuron 1996;17(4):595-606.

181. Li W, Cogswell CA, LoTurco JJ. Neuronal differentiation of precursors in the neocortical ventricular zone is triggered by BMP. J Neurosci 1998;18(21):8853-62.

182. Hoodless PA, Haerry T, Abdollah S, Stapleton M, O'Connor MB, Attisano L, Wrana JL. MADR1, a MAD-related protein that functions in BMP2 signaling pathways. Cell 1996;85(4):489-500.

183. Reddi H. Bone morphogenetic proteins. Adv Dent Res 1995;9(3 Suppl):13.

184. Wozney JM, Rosen V, Byrne M, Celeste AJ, Moutsatsos I, Wang EA. Growth factors influencing bone development. J Cell Sci Suppl 1990;13:149-56.

185. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA. Novel regulators of bone formation: molecular clones and activities. Science 1988;242(4885):1528-34.

186. Hogan BL. Bone morphogenetic proteins in development. Curr Opin Genet Dev 1996;6(4):432-8.

187. Celeste AJ, Iannazzi JA, Taylor RC, Hewick RM, Rosen V, Wang EA, Wozney JM. Identification of transforming growth factor beta family members present in bone-inductive protein purified from bovine bone. Proc Natl Acad Sci U S A 1990;87(24):9843-7.

188. Cunningham NS, Jenkins NA, Gilbert DJ, Copeland NG, Reddi AH, Lee SJ. Growth/differentiation factor-10: a new member of the transforming growth factor-beta superfamily related to bone morphogenetic protein-3. Growth Factors 1995;12(2):99-109.

189. Dunn NR, Winnier GE, Hargett LK, Schrick JJ, Fogo AB, Hogan BL. Haploinsufficient phenotypes in Bmp4 heterozygous null mice and modification by mutations in Gli3 and Alx4. Dev Biol 1997;188(2):235-47.

190. Padgett RW, St Johnston RD, Gelbart WM. A transcript from a Drosophila pattern gene predicts a protein homologous to the transforming growth factor-beta family. Nature 1987;325(6099):81-4.

191. Storm EE, Huynh TV, Copeland NG, Jenkins NA, Kingsley DM, Lee SJ. Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily. Nature 1994;368(6472):639-43.

192. Storm EE, Kingsley DM. GDF5 coordinates bone and joint formation during digit development. Dev Biol 1999;209(1):11-27.

193. Urist MR. Bone: formation by autoinduction. Science 1965;150(698):893-9.

194. Winnier G, Blessing M, Labosky PA, Hogan BL. Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev 1995;9(17):2105-16.

195. Zhang Z, Lu S, Wang J. [Distribution and effectiveness of endogenic bone morphogenetic protein (BMP) in bone defect]. Zhonghua Wai Ke Za Zhi 1996;34(10):596-8.

196. Huber TL, Zhou Y, Mead PE, Zon LI. Cooperative effects of growth factors involved in the induction of hematopoietic mesoderm. Blood 1998;92(11):4128-37.

197. Kawabata M, Chytil A, Moses HL. Cloning of a novel type II serine/threonine kinase receptor through interaction with the type I transforming growth factor-beta receptor. J Biol Chem 1995;270(10):5625-30.

198. Attisano L, Wrana JL, Cheifetz S, Massague J. Novel activin receptors: distinct genes and alternative mRNA splicing generate a repertoire of serine/threonine kinase receptors. Cell 1992;68(1):97-108.

199. Mathews LS, Hammer RE, Behringer RR, D'Ercole AJ, Bell GI, Brinster RL, Palmiter RD. Growth enhancement of transgenic mice expressing human insulin-like growth factor I. Endocrinology 1988;123(6):2827-33.

200. Rosenzweig BL, Imamura T, Okadome T, Cox GN, Yamashita H, ten Dijke P, Heldin CH, Miyazono K. Cloning and characterization of a human type II receptor for bone morphogenetic proteins. Proc Natl Acad Sci U S A 1995;92(17):7632-6.

201. ten Dijke P, Yamashita H, Ichijo H, Franzen P, Laiho M, Miyazono K, Heldin CH. Characterization of type I receptors for transforming growth factor-beta and activin. Science 1994;264(5155):101-4.

202. ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin CH, Miyazono K. Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. J Biol Chem 1994;269(25):16985-8.

203. Yamashita H, ten Dijke P, Franzen P, Miyazono K, Heldin CH. Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-beta. J Biol Chem 1994;269(31):20172-8.

204. Massague J, Andres J, Attisano L, Cheifetz S, Lopez-Casillas F, Ohtsuki M, Wrana JL. TGF-beta receptors. Mol Reprod Dev 1992;32(2):99-104.

205. Wrana JL, Attisano L, Wieser R, Ventura F, Massague J. Mechanism of activation of the TGF-beta receptor. Nature 1994;370(6488):341-7.

206. Chen YG, Liu F, Massague J. Mechanism of TGFbeta receptor inhibition by FKBP12. Embo J 1997;16(13):3866-76.

207. Chen YG, Massague J. Smad1 recognition and activation by the ALK1 group of transforming growth factor-beta family receptors. J Biol Chem 1999;274(6):3672-7.

208. Macias-Silva M, Hoodless PA, Tang SJ, Buchwald M, Wrana JL. Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. J Biol Chem 1998;273(40):25628-36.

209. Tamaki K, Souchelnytskyi S, Itoh S, Nakao A, Sampath K, Heldin CH, ten Dijke P. Intracellular signaling of osteogenic protein-1 through Smad5 activation. J Cell Physiol 1998;177(2):355-63.

210. Thomsen GH. Xenopus mothers against decapentaplegic is an embryonic ventralizing agent that acts downstream of the BMP-2/4 receptor. Development 1996;122(8):2359-66.

211. Liu F, Pouponnot C, Massague J. Dual role of the Smad4/DPC4 tumor suppressor in TGFbeta-inducible transcriptional complexes. Genes Dev 1997;11(23):3157-67.

212. Souchelnytskyi S, Nakayama T, Nakao A, Moren A, Heldin CH, Christian JL, ten Dijke P. Physical and functional interaction of murine and Xenopus Smad7 with bone morphogenetic protein receptors and transforming growth factor-beta receptors. J Biol Chem 1998;273(39):25364-70.

213. Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K. Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science 1995;270(5244):2008-11.

214. Shibuya H, Iwata H, Masuyama N, Gotoh Y, Yamaguchi K, Irie K, Matsumoto K, Nishida E, Ueno N. Role of TAK1 and TAB1 in BMP signaling in early Xenopus development. Embo J 1998;17(4):1019-28.

215. Moriguchi T, Kuroyanagi N, Yamaguchi K, Gotoh Y, Irie K, Kano T, Shirakabe K, Muro Y, Shibuya H, Matsumoto K and others. A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. J Biol Chem 1996;271(23):13675-9.

216. Shirakabe K, Yamaguchi K, Shibuya H, Irie K, Matsuda S, Moriguchi T, Gotoh Y, Matsumoto K, Nishida E. TAK1 mediates the ceramide signaling to stress-activated protein kinase/c-Jun N-terminal kinase. J Biol Chem 1997;272(13):8141-4.

217. Kimura N, Matsuo R, Shibuya H, Nakashima K, Taga T. BMP2-induced apoptosis is mediated by activation of the TAK1-p38 kinase pathway that is negatively regulated by Smad6. J Biol Chem 2000;275(23):17647-52.

218. Lou J, Tu Y, Li S, Manske PR. Involvement of ERK in BMP-2 induced osteoblastic differentiation of mesenchymal progenitor cell line C3H10T1/2. Biochem Biophys Res Commun 2000;268(3):757-62.

219. Mulder KM. Role of Ras and Mapks in TGFbeta signaling. Cytokine Growth Factor Rev 2000;11(1-2):23-35.

220. Lai CF, Cheng SL. Signal transductions induced by bone morphogenetic protein-2 and transforming growth factor-beta in normal human osteoblastic cells. J Biol Chem 2002;277(18):15514-22.

221. Massague J. The transforming growth factor-beta family. Annu Rev Cell Biol 1990;6:597-641.

222. McDonald VL, Dick KO, Malik N, Shoyab M. Selection and characterization of a variant of human melanoma cell line, A375 resistant to growth inhibitory effects of oncostatin M (OM): coresistant to interleukin 6 (IL-6). Growth Factors 1993;9(3):167-75.

223. Daopin S, Li M, Davies DR. Crystal structure of TGF-beta 2 refined at 1.8 A resolution. Proteins 1993;17(2):176-92.

224. Scheufler C, Sebald W, Hulsmeyer M. Crystal structure of human bone morphogenetic protein-2 at 2.7 A resolution. J Mol Biol 1999;287(1):103-15.

225. ten Dijke P, Miyazono K, Heldin CH. Signaling via hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors. Curr Opin Cell Biol 1996;8(2):139-45.

226. ten Dijke P, Ichijo H, Franzen P, Schulz P, Saras J, Toyoshima H, Heldin CH, Miyazono K. Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. Oncogene 1993;8(10):2879-87.

227. ten Dijke P, Franzen P, Yamashita H, Ichijo H, Heldin CH, Miyazono K. Serine/threonine kinase receptors. Prog Growth Factor Res 1994;5(1):55-72.

228. Liu F, Ventura F, Doody J, Massague J. Human type II receptor for bone morphogenic proteins (BMPs): extension of the two-kinase receptor model to the BMPs. Mol Cell Biol 1995;15(7):3479-86.

229. Massague J. TGF-beta signal transduction. Annu Rev Biochem 1998;67:753-91.

230. Ebisawa T, Tada K, Kitajima I, Tojo K, Sampath TK, Kawabata M, Miyazono K, Imamura T. Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation. J Cell Sci 1999;112 (Pt 20):3519-27.

231. Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K, Inoue K, Hori S, Kakizuka A, Ichijo H. ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. Genes Dev 2002;16(11):1345-55.

232. Gilboa L, Nohe A, Geissendorfer T, Sebald W, Henis YI, Knaus P. Bone morphogenetic protein receptor complexes on the surface of live cells: a new oligomerization mode for serine/threonine kinase receptors. Mol Biol Cell 2000;11(3):1023-35.

233. Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P. The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. J Biol Chem 2002;277(7):5330-8.

234. Macias-Silva M, Abdollah S, Hoodless PA, Pirone R, Attisano L, Wrana JL. MADR2 is a substrate of the TGFbeta receptor and its phosphorylation is required for nuclear accumulation and signaling. Cell 1996;87(7):1215-24.

235. Kim J, Johnson K, Chen HJ, Carroll S, Laughon A. Drosophila Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic. Nature 1997;388(6639):304-8.

236. Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. Embo J 1998;17(11):3091-100.

237. Shi Y, Wang YF, Jayaraman L, Yang H, Massague J, Pavletich NP. Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. Cell 1998;94(5):585-94.

238. Zawel L, Dai JL, Buckhaults P, Zhou S, Kinzler KW, Vogelstein B, Kern SE. Human Smad3 and Smad4 are sequence-specific transcription activators. Mol Cell 1998;1(4):611-7.

239. Liu F, Hata A, Baker JC, Doody J, Carcamo J, Harland RM, Massague J. A human Mad protein acting as a BMP-regulated transcriptional activator. Nature 1996;381(6583):620-3.

240. Feng XH, Zhang Y, Wu RY, Derynck R. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF-beta-induced transcriptional activation. Genes Dev 1998;12(14):2153-63.

241. Shah NM, Groves AK, Anderson DJ. Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. Cell 1996;85(3):331-43.

242. Janknecht R, Wells NJ, Hunter T. TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. Genes Dev 1998;12(14):2114-9.

243. Pouponnot C, Jayaraman L, Massague J. Physical and functional interaction of SMADs and p300/CBP. J Biol Chem 1998;273(36):22865-8.

244. Topper JN, DiChiara MR, Brown JD, Williams AJ, Falb D, Collins T, Gimbrone MA, Jr. CREB binding protein is a required coactivator for Smad-dependent, transforming growth factor beta transcriptional responses in endothelial cells. Proc Natl Acad Sci U S A 1998;95(16):9506-11.

245. Yamaguchi K, Nagai S, Ninomiya-Tsuji J, Nishita M, Tamai K, Irie K, Ueno N, Nishida E, Shibuya H, Matsumoto K. XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. Embo J 1999;18(1):179-87.

246. Ninomiya-Tsuji J, Kishimoto K, Hiyama A, Inoue J, Cao Z, Matsumoto K. The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. Nature 1999;398(6724):252-6.

247. Yao Z, Zhou G, Wang XS, Brown A, Diener K, Gan H, Tan TH. A novel human STE20-related protein kinase, HGK, that specifically activates the c-Jun N-terminal kinase signaling pathway. J Biol Chem 1999;274(4):2118-25.

248. Wang XS, Diener K, Manthey CL, Wang S, Rosenzweig B, Bray J, Delaney J, Cole CN, Chan-Hui PY, Mantlo N and others. Molecular cloning and characterization of a novel p38 mitogen-activated protein kinase. J Biol Chem 1997;272(38):23668-74.

249. Takatsu Y, Nakamura M, Stapleton M, Danos MC, Matsumoto K, O'Connor MB, Shibuya H, Ueno N. TAK1 participates in c-Jun N-terminal kinase signaling during Drosophila development. Mol Cell Biol 2000;20(9):3015-26.

250. Ichijo H. From receptors to stress-activated MAP kinases. Oncogene 1999;18(45):6087-93.

251. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 2001;81(2):807-69.

252. Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 1999;79(1):143-80.

253. Tibbles LA, Woodgett JR. The stress-activated protein kinase pathways. Cell Mol Life Sci 1999;55(10):1230-54.

254. Yang DD, Kuan CY, Whitmarsh AJ, Rincon M, Zheng TS, Davis RJ, Rakic P, Flavell RA. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature 1997;389(6653):865-70.

255. Kuan CY, Yang DD, Samanta Roy DR, Davis RJ, Rakic P, Flavell RA. The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. Neuron 1999;22(4):667-76.

256. Harper SJ, LoGrasso P. Signalling for survival and death in neurones: the role of stress-activated kinases, JNK and p38. Cell Signal 2001;13(5):299-310.

257. Kawasaki H, Morooka T, Shimohama S, Kimura J, Hirano T, Gotoh Y, Nishida E. Activation and involvement of p38 mitogen-activated protein kinase in glutamate-induced apoptosis in rat cerebellar granule cells. J Biol Chem 1997;272(30):18518-21.

258. Mielke K, Herdegen T. JNK and p38 stresskinases--degenerative effectors of signal-transduction-cascades in the nervous system. Prog Neurobiol 2000;61(1):45-60.

259. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995;270(5240):1326-31.

260. Adams RH, Porras A, Alonso G, Jones M, Vintersten K, Panelli S, Valladares A, Perez L, Klein R, Nebreda AR. Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. Mol Cell 2000;6(1):109-16.

261. Allen M, Svensson L, Roach M, Hambor J, McNeish J, Gabel CA. Deficiency of the stress kinase p38alpha results in embryonic lethality: characterization of the kinase dependence of stress responses of enzyme-deficient embryonic stem cells. J Exp Med 2000;191(5):859-70.

262. Mudgett JS, Ding J, Guh-Siesel L, Chartrain NA, Yang L, Gopal S, Shen MM. Essential role for p38alpha mitogen-activated protein kinase in placental angiogenesis. Proc Natl Acad Sci U S A 2000;97(19):10454-9.

263. Tamura K, Sudo T, Senftleben U, Dadak AM, Johnson R, Karin M. Requirement for p38alpha in erythropoietin expression: a role for stress kinases in erythropoiesis. Cell 2000;102(2):221-31.

264. Martin-Blanco E. p38 MAPK signalling cascades: ancient roles and new functions. Bioessays 2000;22(7):637-45.

265. Nebreda AR, Porras A. p38 MAP kinases: beyond the stress response. Trends Biochem Sci 2000;25(6):257-60.

266. Ono K, Han J. The p38 signal transduction pathway: activation and function. Cell Signal 2000;12(1):1-13.

267. Morooka T, Nishida E. Requirement of p38 mitogen-activated protein kinase for neuronal differentiation in PC12 cells. J Biol Chem 1998;273(38):24285-8.

268. Iwasaki S, Hattori A, Sato M, Tsujimoto M, Kohno M. Characterization of the bone morphogenetic protein-2 as a neurotrophic factor. Induction of neuronal differentiation of PC12 cells in the absence of mitogen-activated protein kinase activation. J Biol Chem 1996;271(29):17360-5.

269. Paralkar VM, Weeks BS, Yu YM, Kleinman HK, Reddi AH. Recombinant human bone morphogenetic protein 2B stimulates PC12 cell differentiation: potentiation and binding to type IV collagen. J Cell Biol 1992;119(6):1721-8.

270. Iwasaki S, Iguchi M, Watanabe K, Hoshino R, Tsujimoto M, Kohno M. Specific activation of the p38 mitogen-activated protein kinase signaling pathway and induction of neurite outgrowth in PC12 cells by bone morphogenetic protein-2. J Biol Chem 1999;274(37):26503-10.

271. Okamoto S, Krainc D, Sherman K, Lipton SA. Antiapoptotic role of the p38 mitogen-activated protein kinase-myocyte enhancer factor 2 transcription factor pathway during neuronal differentiation. Proc Natl Acad Sci U S A 2000;97(13):7561-6.

272. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. A gene encoding an antigen recognized

by cytolytic T lymphocytes on a human melanoma. Science 1991;254(5038):1643-7.

273. De Plaen E, Arden K, Traversari C, Gaforio JJ, Szikora JP, De Smet C, Brasseur F, van der Bruggen P, Lethe B, Lurquin C and others. Structure, chromosomal localization, and expression of 12 genes of the MAGE family. Immunogenetics 1994;40(5):360-9.

274. Rogner UC, Wilke K, Steck E, Korn B, Poustka A. The melanoma antigen gene (MAGE) family is clustered in the chromosomal band Xq28. Genomics 1995;29(3):725-31.

275. Muscatelli F, Walker AP, De Plaen E, Stafford AN, Monaco AP. Isolation and characterization of a MAGE gene family in the Xp21.3 region. Proc Natl Acad Sci U S A 1995;92(11):4987-91.

276. Dabovic B, Zanaria E, Bardoni B, Lisa A, Bordignon C, Russo V, Matessi C, Traversari C, Camerino G. A family of rapidly evolving genes from the sex reversal critical region in Xp21. Mamm Genome 1995;6(9):571-80.

277. Lurquin C, De Smet C, Brasseur F, Muscatelli F, Martelange V, De Plaen E, Brasseur R, Monaco AP, Boon T. Two members of the human MAGEB gene family located in Xp21.3 are expressed in tumors of various histological origins. Genomics 1997;46(3):397-408.

278. Lucas S, Brasseur F, Boon T. A new MAGE gene with ubiquitous expression does not code for known MAGE antigens recognized by T cells. Cancer Res 1999;59(16):4100-3.

279. Hara I, Hara S, Miyake H, Yamanaka K, Nagai H, Gohji K, Arakawa S, Kamidono S. Expression of MAGE genes in testicular germ cell tumors. Urology 1999;53(4):843-7.

280. De Smet C, De Backer O, Faraoni I, Lurquin C, Brasseur F, Boon T. The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. Proc Natl Acad Sci U S A 1996;93(14):7149-53.

281. Pold M, Zhou J, Chen GL, Hall JM, Vescio RA, Berenson JR. Identification of a new, unorthodox member of the MAGE gene family. Genomics 1999;59(2):161-7.

282. Kubu CJ, Goldhawk DG, Barker PA, Verdi JM. Identification of the translational initiation codon in human MAGED1. Genomics 2000;70(1):150-2.

283. Maruyama K, Usami M, Aizawa T, Yoshikawa K. A novel brain-specific mRNA encoding nuclear protein (necdin) expressed in neurally differentiated embryonal carcinoma cells. Biochem Biophys Res Commun 1991;178(1):291-6.

284. Aizawa T, Maruyama K, Kondo H, Yoshikawa K. Expression of necdin, an embryonal carcinoma-derived nuclear protein, in developing mouse brain. Brain Res Dev Brain Res 1992;68(2):265-74.

285. Hayashi Y, Matsuyama K, Takagi K, Sugiura H, Yoshikawa K. Arrest of cell growth by necdin, a nuclear protein expressed in postmitotic neurons. Biochem Biophys Res Commun 1995;213(1):317-24.

286. Uetsuki T, Takagi K, Sugiura H, Yoshikawa K. Structure and expression of the mouse necdin gene. Identification of a postmitotic neuron-restrictive core promoter. J Biol Chem 1996;271(2):918-24.

287. Taniura H, Matsumoto K, Yoshikawa K. Physical and functional interactions of neuronal growth suppressor necdin with p53. J Biol Chem 1999;274(23):16242-8.

288. Taniura H, Taniguchi N, Hara M, Yoshikawa K. Necdin, a postmitotic neuron-specific growth suppressor, interacts with viral transforming proteins and cellular transcription factor E2F1. J Biol Chem 1998;273(2):720-8.

289. Ferguson KL, Callaghan SM, O'Hare MJ, Park DS, Slack RS. The Rb-CDK4/6 signaling pathway is critical in neural precursor cell cycle regulation. J Biol Chem 2000;275(43):33593-600.

290. Jordan BW, Dinev D, LeMellay V, Troppmair J, Gotz R, Wixler L, Sendtner M, Ludwig S, Rapp UR. Neurotrophin receptor-interacting mage

homologue is an inducible inhibitor of apoptosis protein-interacting protein that augments cell death. J Biol Chem 2001;276(43):39985-9.

291. Masuda Y, Sasaki A, Shibuya H, Ueno N, Ikeda K, Watanabe K. Dlxin-1, a novel protein that binds Dlx5 and regulates its transcriptional function. J Biol Chem 2001;276(7):5331-8.

292. Sasaki A, Masuda Y, Iwai K, Ikeda K, Watanabe K. A RING finger protein Praja1 regulates Dlx5-dependent transcription through its ubiquitin ligase activity for the Dlx/Msx-interacting MAGE/Necdin family protein, Dlxin-1. J Biol Chem 2002;277(25):22541-6.

293. Matsuda T, Suzuki H, Oishi I, Kani S, Kuroda Y, Komori T, Sasaki A, Watanabe K, Minami Y. The receptor tyrosine kinase Ror2 associates with the melanoma-associated antigen (MAGE) family protein Dlxin-1 and regulates its intracellular distribution. J Biol Chem 2003;278(31):29057-64.

294. Williams ME, Strickland P, Watanabe K, Hinck L. UNC5H1 induces apoptosis via its juxtamembrane region through an interaction with NRAGE. J Biol Chem 2003;278(19):17483-90.

295. Nijhawan D, Honarpour N, Wang X. Apoptosis in neural development and disease. Annu Rev Neurosci 2000;23:73-87.

296. Lindsay RM. Neurotrophic growth factors and neurodegenerative diseases: therapeutic potential of the neurotrophins and ciliary neurotrophic factor. Neurobiol Aging 1994;15(2):249-51.

297. Friedman WJ, Greene LA. Neurotrophin signaling via Trks and p75. Exp Cell Res 1999;253(1):131-42.

298. Bothwell M. p75NTR: a receptor after all. Science 1996;272(5261):506-7.

299. Barrett GL. The p75 neurotrophin receptor and neuronal apoptosis. Prog Neurobiol 2000;61(2):205-29.

300. Verdi JM, Ip N, Yancopoulos GD, Anderson DJ. Expression of trk in MAH cells lacking the p75 low-affinity nerve growth factor receptor is sufficient

to permit nerve growth factor-induced differentiation to postmitotic neurons. Proc Natl Acad Sci U S A 1994;91(9):3949-53.

301. Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV. High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature 1991;350(6320):678-83.

302. Barker PA, Barbee G, Misko TP, Shooter EM. The low affinity neurotrophin receptor, p75LNTR, is palmitoylated by thioester formation through cysteine 279. J Biol Chem 1994;269(48):30645-50.

303. Frade JM, Rodriguez-Tebar A, Barde YA. Induction of cell death by endogenous nerve growth factor through its p75 receptor. Nature 1996;383(6596):166-8.

304. von Schack D, Casademunt E, Schweigreiter R, Meyer M, Bibel M, Dechant G. Complete ablation of the neurotrophin receptor p75NTR causes defects both in the nervous and the vascular system. Nat Neurosci 2001;4(10):977-8.

305. Saburi S, Nadano D, Akama TO, Hirama K, Yamanouchi K, Naito K, Tojo H, Tachi C, Fukuda MN. The trophinin gene encodes a novel group of MAGE proteins, magphinins, and regulates cell proliferation during gametogenesis in the mouse. J Biol Chem 2001;276(52):49378-89.

306. Barker PA, Salehi A. The MAGE proteins: emerging roles in cell cycle progression, apoptosis, and neurogenetic disease. J Neurosci Res 2002;67(6):705-12.

307. Verdi JM, Anderson DJ. Neurotrophins regulate sequential changes in neurotrophin receptor expression by sympathetic neuroblasts. Neuron 1994;13(6):1359-72.

308. Wolinsky EJ, Landis SC, Patterson PH. Expression of noradrenergic and cholinergic traits by sympathetic neurons cultured without serum. J Neurosci 1985;5(6):1497-508.

309. Groves AK, George KM, Tissier-Seta JP, Engel JD, Brunet JF, Anderson DJ. Differential regulation of transcription factor gene expression and

phenotypic markers in developing sympathetic neurons. Development 1995;121(3):887-901.

310. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem 1985;150(1):76-85.

311. Mazzoni IE, Kenigsberg RL. Thrombin indirectly affects cholinergic cell expression in primary septal cell cultures in a manner distinct from nerve growth factor. Neuroscience 1991;45(1):195-204.

312. Allendoerfer KL, Shelton DL, Shooter EM, Shatz CJ. Nerve growth factor receptor immunoreactivity is transiently associated with the subplate neurons of the mammalian cerebral cortex. Proc Natl Acad Sci U S A 1990;87(1):187-90.

313. Koh S, Higgins GA. Differential regulation of the low-affinity nerve growth factor receptor during postnatal development of the rat brain. J Comp Neurol 1991;313(3):494-508.

314. Mackarehtschian K, Lau CK, Caras I, McConnell SK. Regional differences in the developing cerebral cortex revealed by ephrin-A5 expression. Cereb Cortex 1999;9(6):601-10.

315. Niinobe M, Koyama K, Yoshikawa K. Cellular and subcellular localization of necdin in fetal and adult mouse brain. Dev Neurosci 2000;22(4):310-9.

316. Paterno GD, Gillespie LL, Julien JP, Skup D. Regulation of neurofilament L, M and H gene expression during retinoic acid-induced neural differentiation of P19 embryonal carcinoma cells. Brain Res Mol Brain Res 1997;49(1-2):247-54.

317. Pyle SJ, Roberts KG, Reuhl KR. Delayed expression of the NFH subunit in differentiating P19 cells. Brain Res Dev Brain Res 2001;132(1):103-6.

318. Tanaka Y, Kawahata K, Nakata T, Hirokawa N. Chronological expression of microtubule-associated proteins (MAPs) in EC cell P19 after neuronal induction by retinoic acid. Brain Res 1992;596(1-2):269-78.

319. Farinas I. Neurotrophin actions during the development of the peripheral nervous system. Microsc Res Tech 1999;45(4-5):233-42.

320. Buck CR, Martinez HJ, Black IB, Chao MV. Developmentally regulated expression of the nerve growth factor receptor gene in the periphery and brain. Proc Natl Acad Sci U S A 1987;84(9):3060-3.

321. Meinecke DL, Rakic P. Low-affinity p75 nerve growth factor receptor expression in the embryonic monkey telencephalon: timing and localization in diverse cellular elements. Neuroscience 1993;54(1):105-16.

322. Murray SS, Bartlett PF, Cheema SS. Differential loss of spinal sensory but not motor neurons in the p75NTR knockout mouse. Neurosci Lett 1999;267(1):45-8.

323. Roux PP, Barker PA. Neurotrophin signaling through the p75 neurotrophin receptor. Prog Neurobiol 2002;67(3):203-33.

324. Greferath U, Bennie A, Kourakis A, Barrett GL. Impaired spatial learning in aged rats is associated with loss of p75-positive neurons in the basal forebrain. Neuroscience 2000;100(2):363-73.

325. Roux PP, Colicos MA, Barker PA, Kennedy TE. p75 neurotrophin receptor expression is induced in apoptotic neurons after seizure. J Neurosci 1999;19(16):6887-96.

326. Casaccia-Bonnefil P, Gu C, Khursigara G, Chao MV. p75 neurotrophin receptor as a modulator of survival and death decisions. Microsc Res Tech 1999;45(4-5):217-24.

327. Valente T, Auladell C. Expression pattern of Zac1 mouse gene, a new zinc-finger protein that regulates apoptosis and cellular cycle arrest, in both adult brain and along development. Mech Dev 2001;108(1-2):207-11.

328. Yamamoto Y, Henderson CE. Patterns of programmed cell death in populations of developing spinal motoneurons in chicken, mouse, and rat. Dev Biol 1999;214(1):60-71.

329. Ernfors P, Hallbook F, Ebendal T, Shooter EM, Radeke MJ, Misko TP, Persson H. Developmental and regional expression of beta-nerve growth factor receptor mRNA in the chick and rat. Neuron 1988;1(10):983-96.

330. Lee KF, Bachman K, Landis S, Jaenisch R. Dependence on p75 for innervation of some sympathetic targets. Science 1994;263(5152):1447-9.

331. Lee KF, Davies AM, Jaenisch R. p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. Development 1994;120(4):1027-33.

332. Yan Q, Johnson EM, Jr. An immunohistochemical study of the nerve growth factor receptor in developing rats. J Neurosci 1988;8(9):3481-98.

333. Schatteman GC, Gibbs L, Lanahan AA, Claude P, Bothwell M. Expression of NGF receptor in the developing and adult primate central nervous system. J Neurosci 1988;8(3):860-73.

334. Kordower JH, Mufson EJ. Nerve growth factor receptor-immunoreactive neurons within the developing human cortex. J Comp Neurol 1992;323(1):25-41.

335. DeFreitas MF, McQuillen PS, Shatz CJ. A novel p75NTR signaling pathway promotes survival, not death, of immunopurified neocortical subplate neurons. J Neurosci 2001;21(14):5121-9.

336. Hartikka J, Hefti F. Comparison of nerve growth factor's effects on development of septum, striatum, and nucleus basalis cholinergic neurons in vitro. J Neurosci Res 1988;21(2-4):352-64.

337. Koh S, Oyler GA, Higgins GA. Localization of nerve growth factor receptor messenger RNA and protein in the adult rat brain. Exp Neurol 1989;106(3):209-21.

338. Meikrantz W, Gisselbrecht S, Tam SW, Schlegel R. Activation of cyclin A-dependent protein kinases during apoptosis. Proc Natl Acad Sci U S A 1994;91(9):3754-8.

339. D'Sa-Eipper C, Leonard JR, Putcha G, Zheng TS, Flavell RA, Rakic P, Kuida K, Roth KA. DNA damage-induced neural precursor cell apoptosis requires p53 and caspase 9 but neither Bax nor caspase 3. Development 2001;128(1):137-46.

340. Freeman RS, Estus S, Johnson EM, Jr. Analysis of cell cycle-related gene expression in postmitotic neurons: selective induction of Cyclin D1 during programmed cell death. Neuron 1994;12(2):343-55.

341. Simonati A, Rosso T, Rizzuto N. DNA fragmentation in normal development of the human central nervous system: a morphological study during corticogenesis. Neuropathol Appl Neurobiol 1997;23(3):203-11.

342. Strom RC, Williams RW. Cell production and cell death in the generation of variation in neuron number. J Neurosci 1998;18(23):9948-53.

343. Haydar TF, Kuan CY, Flavell RA, Rakic P. The role of cell death in regulating the size and shape of the mammalian forebrain. Cereb Cortex 1999;9(6):621-6.

344. Pompeiano M, Blaschke AJ, Flavell RA, Srinivasan A, Chun J. Decreased apoptosis in proliferative and postmitotic regions of the Caspase 3-deficient embryonic central nervous system. J Comp Neurol 2000;423(1):1-12.

345. Cecconi F, Alvarez-Bolado G, Meyer BI, Roth KA, Gruss P. Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. Cell 1998;94(6):727-37.

346. Sommer L, Rao M. Neural stem cells and regulation of cell number. Prog Neurobiol 2002;66(1):1-18.

347. Caviness VS, Jr. Neocortical histogenesis in normal and reeler mice: a developmental study based upon [3H]thymidine autoradiography. Brain Res 1982;256(3):293-302.

348. Weinberg RA. The retinoblastoma gene and gene product. Cancer Surv 1992;12:43-57.

349. Field SJ, Tsai FY, Kuo F, Zubiaga AM, Kaelin WG, Jr., Livingston DM, Orkin SH, Greenberg ME. E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell 1996;85(4):549-61.

350. MacGrogan D, Saint-Andre JP, Dicou E. Expression of nerve growth factor and nerve growth factor receptor genes in human tissues and in prostatic adenocarcinoma cell lines. J Neurochem 1992;59(4):1381-91.

351. Lomen-Hoerth C, Shooter EM. Widespread neurotrophin receptor expression in the immune system and other nonneuronal rat tissues. J Neurochem 1995;64(4):1780-9.

352. Altman J, Bayer SA. Horizontal compartmentation in the germinal matrices and intermediate zone of the embryonic rat cerebral cortex. Exp Neurol 1990;107(1):36-47.

353. Reynolds BA, Tetzlaff W, Weiss S. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci 1992;12(11):4565-74.

354. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 1992;255(5052):1707-10.

355. Kilpatrick TJ, Bartlett PF. Cloning and growth of multipotential neural precursors: requirements for proliferation and differentiation. Neuron 1993;10(2):255-65.

356. Temple S, Davis AA. Isolated rat cortical progenitor cells are maintained in division in vitro by membrane-associated factors. Development 1994;120(4):999-1008.

357. Davis AA, Temple S. A self-renewing multipotential stem cell in embryonic rat cerebral cortex. Nature 1994;372(6503):263-6.

358. Pfrieger FW, Barres BA. Synaptic efficacy enhanced by glial cells in vitro. Science 1997;277(5332):1684-7.

359. Meyer G, Schaaps JP, Moreau L, Goffinet AM. Embryonic and early fetal development of the human neocortex. J Neurosci 2000;20(5):1858-68.

360. Qian X, Davis AA, Goderie SK, Temple S. FGF2 concentration regulates the generation of neurons and glia from multipotent cortical stem cells. Neuron 1997;18(1):81-93.

361. Qian X, Shen Q, Goderie SK, He W, Capela A, Davis AA, Temple S. Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron 2000;28(1):69-80.

362. Munoz-Sanjuan I, Brivanlou AH. Neural induction, the default model and embryonic stem cells. Nat Rev Neurosci 2002;3(4):271-80.

363. Kubu CJ, Orimoto K, Morrison SJ, Weinmaster G, Anderson DJ, Verdi JM. Developmental changes in Notch1 and numb expression mediated by local cell-cell interactions underlie progressively increasing delta sensitivity in neural crest stem cells. Dev Biol 2002;244(1):199-214.

364. Finlay BL, Slattery M. Local differences in the amount of early cell death in neocortex predict adult local specializations. Science 1983;219(4590):1349-51.

365. Wood JG, Martin S, Price DJ. Evidence that the earliest generated cells of the murine cerebral cortex form a transient population in the subplate and marginal zone. Brain Res Dev Brain Res 1992;66(1):137-40.

366. Takahashi T, Nowakowski RS, Caviness VS, Jr. The leaving or Q fraction of the murine cerebral proliferative epithelium: a general model of neocortical neuronogenesis. J Neurosci 1996;16(19):6183-96.

367. Takahashi T, Nowakowski RS, Caviness VS, Jr. Interkinetic and migratory behavior of a cohort of neocortical neurons arising in the early embryonic murine cerebral wall. J Neurosci 1996;16(18):5762-76.

368. Price DJ, Aslam S, Tasker L, Gillies K. Fates of the earliest generated cells in the developing murine neocortex. J Comp Neurol 1997;377(3):414-22.

369. Massague J, Weis-Garcia F. Serine/threonine kinase receptors: mediators of transforming growth factor beta family signals. Cancer Surv 1996;27:41-64.

370. Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature 1997;390(6659):465-71.

371. Derynck R, Zhang Y, Feng XH. Smads: transcriptional activators of TGF-beta responses. Cell 1998;95(6):737-40.

372. Shibuya H, Yamaguchi K, Shirakabe K, Tonegawa A, Gotoh Y, Ueno N, Irie K, Nishida E, Matsumoto K. TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction. Science 1996;272(5265):1179-82.

373. Hanafusa H, Ninomiya-Tsuji J, Masuyama N, Nishita M, Fujisawa J, Shibuya H, Matsumoto K, Nishida E. Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression. J Biol Chem 1999;274(38):27161-7.

374. Ishitani T, Ninomiya-Tsuji J, Nagai S, Nishita M, Meneghini M, Barker N, Waterman M, Bowerman B, Clevers H, Shibuya H and others. The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta-catenin and transcription factor TCF. Nature 1999;399(6738):798-802.

375. Fann MJ, Patterson PH. Depolarization differentially regulates the effects of bone morphogenetic protein (BMP)-2, BMP-6, and activin A on sympathetic neuronal phenotype. J Neurochem 1994;63(6):2074-9.

376. Liem KF, Jr., Tremml G, Roelink H, Jessell TM. Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. Cell 1995;82(6):969-79.

377. Sasai Y, Lu B, Steinbeisser H, De Robertis EM. Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus. Nature 1995;377(6551):757.

378. Wilson PA, Hemmati-Brivanlou A. Induction of epidermis and inhibition of neural fate by Bmp-4. Nature 1995;376(6538):331-3.

379. Mujtaba T, Mayer-Proschel M, Rao MS. A common neural progenitor for the CNS and PNS. Dev Biol 1998;200(1):1-15.

380. White PM, Morrison SJ, Orimoto K, Kubu CJ, Verdi JM, Anderson DJ. Neural crest stem cells undergo cell-intrinsic developmental changes in sensitivity to instructive differentiation signals. Neuron 2001;29(1):57-71.

381. Zou H, Niswander L. Requirement for BMP signaling in interdigital apoptosis and scale formation. Science 1996;272(5262):738-41.

382. Smith A, Graham A. Restricting Bmp-4 mediated apoptosis in hindbrain neural crest. Dev Dyn 2001;220(3):276-83.

383. Mabie PC, Mehler MF, Kessler JA. Multiple roles of bone morphogenetic protein signaling in the regulation of cortical cell number and phenotype. J Neurosci 1999;19(16):7077-88.

384. Panchision DM, Pickel JM, Studer L, Lee SH, Turner PA, Hazel TG, McKay RD. Sequential actions of BMP receptors control neural precursor cell production and fate. Genes Dev 2001;15(16):2094-110.

385. Chang MY, Son H, Lee YS, Lee SH. Neurons and astrocytes secrete factors that cause stem cells to differentiate into neurons and astrocytes, respectively. Mol Cell Neurosci 2003;23(3):414-26.

386. Kalyani AJ, Piper D, Mujtaba T, Lucero MT, Rao MS. Spinal cord neuronal precursors generate multiple neuronal phenotypes in culture. J Neurosci 1998;18(19):7856-68.

387. Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. Embo J 1998;17(8):2215-23.

388. Zhang D, Mehler MF, Song Q, Kessler JA. Development of bone morphogenetic protein receptors in the nervous system and possible roles in regulating trkC expression. J Neurosci 1998;18(9):3314-26.

389. Kendall SE, Goldhawk DE, Kubu C, Barker PA, Verdi JM. Expression analysis of a novel p75(NTR) signaling protein, which regulates cell cycle progression and apoptosis. Mech Dev 2002;117(1-2):187-200.

390. Krishan A. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol 1975;66(1):188-93.

391. McBurney MW, Jones-Villeneuve EM, Edwards MK, Anderson PJ. Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. Nature 1982;299(5879):165-7.

392. Glozak MA, Rogers MB. Retinoic acid- and bone morphogenetic protein 4-induced apoptosis in P19 embryonal carcinoma cells requires p27. Exp Cell Res 2001;268(2):128-38.

393. McBurney MW, Rogers BJ. Isolation of male embryonal carcinoma cells and their chromosome replication patterns. Dev Biol 1982;89(2):503-8.

394. Sun Y, Nadal-Vicens M, Misono S, Lin MZ, Zubiaga A, Hua X, Fan G, Greenberg ME. Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. Cell 2001;104(3):365-76.

395. Salvatore AM, Cozzolino M, Gargano N, Galanti S, Levi A, Alema S. Neuronal differentiation of P19 embryonal cells exhibits cell-specific regulation of neurotrophin receptors. Neuroreport 1995;6(6):873-7.

396. Rodriguez-Leon J, Merino R, Macias D, Ganan Y, Santesteban E, Hurle JM. Retinoic acid regulates programmed cell death through BMP signalling. Nat Cell Biol 1999;1(2):125-6.

397. Takeda K, Ichijo H. Neuronal p38 MAPK signalling: an emerging regulator of cell fate and function in the nervous system. Genes Cells 2002;7(11):1099-111.

398. Hou ST, Xie X, Baggley A, Park DS, Chen G, Walker T. Activation of the Rb/E2F1 pathway by the nonproliferative p38 MAPK during Fas (APO1/CD95)-mediated neuronal apoptosis. J Biol Chem 2002;277(50):48764-70.

399. Wang S, Nath N, Minden A, Chellappan S. Regulation of Rb and E2F by signal transduction cascades: divergent effects of JNK1 and p38 kinases. Embo J 1999;18(6):1559-70.

400. Smith RA, Walker T, Xie X, Hou ST. Involvement of the transcription factor E2F1/Rb in kainic acid-induced death of murine cerebellar granule cells. Brain Res Mol Brain Res 2003;116(1-2):70-9.

401. Nath N, Wang S, Betts V, Knudsen E, Chellappan S. Apoptotic and mitogenic stimuli inactivate Rb by differential utilization of p38 and cyclin-dependent kinases. Oncogene 2003;22(38):5986-94.

402. Vermeulen K, Berneman ZN, Van Bockstaele DR. Cell cycle and apoptosis. Cell Prolif 2003;36(3):165-75.

403. Yagi K, Furuhashi M, Aoki H, Goto D, Kuwano H, Sugamura K, Miyazono K, Kato M. c-myc is a downstream target of the Smad pathway. J Biol Chem 2002;277(1):854-61.

404. Nakamura Y, Ozaki T, Koseki H, Nakagawara A, Sakiyama S. Accumulation of p27 KIP1 is associated with BMP2-induced growth arrest and neuronal differentiation of human neuroblastoma-derived cell lines. Biochem Biophys Res Commun 2003;307(1):206-13.

405. Wimmel A, Wiedenmann B, Rosewicz S. Autocrine growth inhibition by transforming growth factor beta-1 (TGFbeta-1) in human neuroendocrine tumour cells. Gut 2003;52(9):1308-16.

406. Shimozaki K, Nakashima K, Niwa H, Taga T. Involvement of Oct3/4 in the enhancement of neuronal differentiation of ES cells in neurogenesis-inducing cultures. Development 2003;130(11):2505-12.

407. Qi X, Li TG, Hao J, Hu J, Wang J, Simmons H, Miura S, Mishina Y, Zhao GQ. BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. Proc Natl Acad Sci U S A 2004;101(16):6027-32.

408. Kaushal S, Schneider JW, Nadal-Ginard B, Mahdavi V. Activation of the myogenic lineage by MEF2A, a factor that induces and cooperates with MyoD. Science 1994;266(5188):1236-40.

409. Molkentin JD, Black BL, Martin JF, Olson EN. Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. Cell 1995;83(7):1125-36.

410. Lyons GE, Micales BK, Schwarz J, Martin JF, Olson EN. Expression of mef2 genes in the mouse central nervous system suggests a role in neuronal maturation. J Neurosci 1995;15(8):5727-38.

411. Jones-Villeneuve EM, McBurney MW, Rogers KA, Kalnins VI. Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. J Cell Biol 1982;94(2):253-62.

412. Kobayashi T, Shinozaki A, Momoi T, Arahata K, Tsukahara T. Identification of an interleukin-1 beta converting enzyme-like activity that increases upon treatment of P19 cells with retinoic acid as the proteasome. J Biochem (Tokyo) 1996;120(4):699-704.

413. Mukasa T, Khoroku Y, Tsukahara T, Momoi MY, Kimura I, Momoi T. Wortmannin enhances CPP32-like activity during neuronal differentiation of P19 embryonal carcinoma cells induced by retinoic acid. Biochem Biophys Res Commun 1997;232(1):192-7.

414. Fujita E, Soyama A, Kawabata M, Momoi T. BMP-4 and retinoic acid synergistically induce activation of caspase-9 and cause apoptosis of P19 embryonal carcinoma cells cultured as a monolayer. Cell Death Differ 1999;6(11):1109-16.

415. Glozak MA, Rogers MB. Specific induction of apoptosis in P19 embryonal carcinoma cells by retinoic acid and BMP2 or BMP4. Dev Biol 1996;179(2):458-70.

416. Marazzi G, Wang Y, Sassoon D. Msx2 is a transcriptional regulator in the BMP4-mediated programmed cell death pathway. Dev Biol 1997;186(2):127-38.

417. Glozak MA, Rogers MB. BMP4- and RA-induced apoptosis is mediated through the activation of retinoic acid receptor alpha and gamma in P19 embryonal carcinoma cells. Exp Cell Res 1998;242(1):165-73.

418. Heller LC, Li Y, Abrams KL, Rogers MB. Transcriptional regulation of the Bmp2 gene. Retinoic acid induction in F9 embryonal carcinoma cells and Saccharomyces cerevisiae. J Biol Chem 1999;274(3):1394-400.

419. Helvering LM, Sharp RL, Ou X, Geiser AG. Regulation of the promoters for the human bone morphogenetic protein 2 and 4 genes. Gene 2000;256(1-2):123-38.

420. Sakurai H, Miyoshi H, Mizukami J, Sugita T. Phosphorylation-dependent activation of TAK1 mitogen-activated protein kinase kinase kinase by TAB1. FEBS Lett 2000;474(2-3):141-5.

421. Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K, Gotoh Y. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 1997;275(5296):90-4.

422. Goswami M, Uzgare AR, Sater AK. Regulation of MAP kinase by the BMP-4/TAK1 pathway in Xenopus ectoderm. Dev Biol 2001;236(2):259-70.

423. Fukuchi-Shimogori T, Grove EA. Neocortex patterning by the secreted signaling molecule FGF8. Science 2001;294(5544):1071-4.

424. Eagleson GW, Dempewolf RD. The role of the anterior neural ridge and Fgf-8 in early forebrain patterning and regionalization in Xenopus laevis. Comp Biochem Physiol B Biochem Mol Biol 2002;132(1):179-89.

425. Lavelle D, DeSimone J, Hankewych M, Kousnetzova T, Chen YH. Decitabine induces cell cycle arrest at the G1 phase via p21(WAF1) and the G2/M phase via the p38 MAP kinase pathway. Leuk Res 2003;27(11):999-1007.

426. Polager S, Ginsberg D. E2F mediates sustained G2 arrest and down-regulation of Stathmin and AIM-1 expression in response to genotoxic stress. J Biol Chem 2003;278(3):1443-9.

427. Han J, Jiang Y, Li Z, Kravchenko VV, Ulevitch RJ. Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. Nature 1997;386(6622):296-9.

428. Kato Y, Kravchenko VV, Tapping RI, Han J, Ulevitch RJ, Lee JD. BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. Embo J 1997;16(23):7054-66.

429. Zhao M, New L, Kravchenko VV, Kato Y, Gram H, di Padova F, Olson EN, Ulevitch RJ, Han J. Regulation of the MEF2 family of transcription factors by p38. Mol Cell Biol 1999;19(1):21-30.

430. Yang SH, Galanis A, Sharrocks AD. Targeting of p38 mitogen-activated protein kinases to MEF2 transcription factors. Mol Cell Biol 1999;19(6):4028-38.

431. Johnson JE, Zimmerman K, Saito T, Anderson DJ. Induction and repression of mammalian achaete-scute homologue (MASH) gene expression during neuronal differentiation of P19 embryonal carcinoma cells. Development 1992;114(1):75-87.

432. Black BL, Ligon KL, Zhang Y, Olson EN. Cooperative transcriptional activation by the neurogenic basic helix-loop-helix protein MASH1 and members of the myocyte enhancer factor-2 (MEF2) family. J Biol Chem 1996;271(43):26659-63.

433. Mao Z, Nadal-Ginard B. Functional and physical interactions between mammalian achaete-scute homolog 1 and myocyte enhancer factor 2A. J Biol Chem 1996;271(24):14371-5.

434. Novitch BG, Spicer DB, Kim PS, Cheung WL, Lassar AB. pRb is required for MEF2-dependent gene expression as well as cell-cycle arrest during skeletal muscle differentiation. Curr Biol 1999;9(9):449-59.

435. Peschiaroli A, Figliola R, Coltella L, Strom A, Valentini A, D'Agnano I, Maione R. MyoD induces apoptosis in the absence of RB function through a p21(WAF1)-dependent re-localization of cyclin/cdk complexes to the nucleus. Oncogene 2002;21(53):8114-27.

436. Israsena N, Kessler JA. Msx2 and p21(CIP1/WAF1) mediate the proapoptotic effects of bone morphogenetic protein-4 on ventricular zone progenitor cells. J Neurosci Res 2002;69(6):803-9.

437. Morgenbesser SD, Williams BO, Jacks T, DePinho RA. p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. Nature 1994;371(6492):72-4.

438. Ferguson KL, Slack RS. The Rb pathway in neurogenesis. Neuroreport 2001;12(9):A55-62.

439. Tsai RY, McKay RD. A nucleolar mechanism controlling cell proliferation in stem cells and cancer cells. Genes Dev 2002;16(23):2991-3003.

440. Nahle Z, Polakoff J, Davuluri RV, McCurrach ME, Jacobson MD, Narita M, Zhang MQ, Lazebnik Y, Bar-Sagi D, Lowe SW. Direct coupling of the cell cycle and cell death machinery by E2F. Nat Cell Biol 2002;4(11):859-64.

441. Ferguson KL, Vanderluit JL, Hebert JM, McIntosh WC, Tibbo E, MacLaurin JG, Park DS, Wallace VA, Vooijs M, McConnell SK and others. Telencephalon-specific Rb knockouts reveal enhanced neurogenesis, survival and abnormal cortical development. Embo J 2002;21(13):3337-46.

442. Storm EE, Rubenstein JL, Martin GR. Dosage of Fgf8 determines whether cell survival is positively or negatively regulated in the developing forebrain. Proc Natl Acad Sci U S A 2003;100(4):1757-62.

443. Baker JC, Beddington RS, Harland RM. Wnt signaling in Xenopus embryos inhibits bmp4 expression and activates neural development. Genes Dev 1999;13(23):3149-59.

444. Wilson SI, Rydstrom A, Trimborn T, Willert K, Nusse R, Jessell TM, Edlund T. The status of Wnt signalling regulates neural and epidermal fates in the chick embryo. Nature 2001;411(6835):325-30.

445. Bainter JJ, Boos A, Kroll KL. Neural induction takes a transcriptional twist. Dev Dyn 2001;222(3):315-27.

446. Chenn A, Walsh CA. Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. Science 2002;297(5580):365-9.

447. Gomez-Skarmeta J, de La Calle-Mustienes E, Modolell J. The Wnt-activated Xiro1 gene encodes a repressor that is essential for neural development and downregulates Bmp4. Development 2001;128(4):551-60.

448. Driever W, Solnica-Krezel L, Schier AF, Neuhauss SC, Malicki J, Stemple DL, Stainier DY, Zwartkruis F, Abdelilah S, Rangini Z and others. A genetic screen for mutations affecting embryogenesis in zebrafish. Development 1996;123:37-46.

449. Solnica-Krezel L, Stemple DL, Mountcastle-Shah E, Rangini Z, Neuhauss SC, Malicki J, Schier AF, Stainier DY, Zwartkruis F, Abdelilah S and others. Mutations affecting cell fates and cellular rearrangements during gastrulation in zebrafish. Development 1996;123:67-80.

450. Kim CH, Oda T, Itoh M, Jiang D, Artinger KB, Chandrasekharappa SC, Driever W, Chitnis AB. Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. Nature 2000;407(6806):913-6.

451. Houart C, Caneparo L, Heisenberg C, Barth K, Take-Uchi M, Wilson S. Establishment of the telencephalon during gastrulation by local antagonism of Wnt signaling. Neuron 2002;35(2):255-65.

452. Uren A, Reichsman F, Anest V, Taylor WG, Muraiso K, Bottaro DP, Cumberledge S, Rubin JS. Secreted frizzled-related protein-1 binds directly to Wingless and is a biphasic modulator of Wnt signaling. J Biol Chem 2000;275(6):4374-82.

453. Kiecker C, Niehrs C. A morphogen gradient of Wnt/beta-catenin signalling regulates anteroposterior neural patterning in Xenopus. Development 2001;128(21):4189-201.

454. Lupo G, Harris WA, Barsacchi G, Vignali R. Induction and patterning of the telencephalon in Xenopus laevis. Development 2002;129(23):5421-36.

455. Nordstrom U, Jessell TM, Edlund T. Progressive induction of caudal neural character by graded Wnt signaling. Nat Neurosci 2002;5(6):525-32.

456. Lagutin OV, Zhu CC, Kobayashi D, Topczewski J, Shimamura K, Puelles L, Russell HR, McKinnon PJ, Solnica-Krezel L, Oliver G. Six3 repression

of Wnt signaling in the anterior neuroectoderm is essential for vertebrate forebrain development. Genes Dev 2003;17(3):368-79.

457. Meneghini MD, Ishitani T, Carter JC, Hisamoto N, Ninomiya-Tsuji J, Thorpe CJ, Hamill DR, Matsumoto K, Bowerman B. MAP kinase and Wnt pathways converge to downregulate an HMG-domain repressor in Caenorhabditis elegans. Nature 1999;399(6738):793-7.

458. Mirkovic I, Charish K, Gorski SM, McKnight K, Verheyen EM. Drosophila nemo is an essential gene involved in the regulation of programmed cell death. Mech Dev 2002;119(1):9-20.

459. Hyodo-Miura J, Urushiyama S, Nagai S, Nishita M, Ueno N, Shibuya H. Involvement of NLK and Sox11 in neural induction in Xenopus development. Genes Cells 2002;7(5):487-96.

460. Lee PL, Johnson DE, Cousens LS, Fried VA, Williams LT. Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. Science 1989;245(4913):57-60.

461. Dionne CA, Crumley G, Bellot F, Kaplow JM, Searfoss G, Ruta M, Burgess WH, Jaye M, Schlessinger J. Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. Embo J 1990;9(9):2685-92.

462. Johnson DE, Williams LT. Structural and functional diversity in the FGF receptor multigene family. Adv Cancer Res 1993;60:1-41.

463. Negri S, Oberson A, Steinmann M, Sauser C, Nicod P, Waeber G, Schorderet DF, Bonny C. cDNA cloning and mapping of a novel islet-brain/JNK-interacting protein. Genomics 2000;64(3):324-30.

464. Mohammadi M, Dionne CA, Li W, Li N, Spivak T, Honegger AM, Jaye M, Schlessinger J. Point mutation in FGF receptor eliminates phosphatidylinositol hydrolysis without affecting mitogenesis. Nature 1992;358(6388):681-4.

465. Peters KG, Marie J, Wilson E, Ives HE, Escobedo J, Del Rosario M, Mirda D, Williams LT. Point mutation of an FGF receptor abolishes

phosphatidylinositol turnover and Ca2+ flux but not mitogenesis. Nature 1992;358(6388):678-81.

466. Kouhara H, Hadari YR, Spivak-Kroizman T, Schilling J, Bar-Sagi D, Lax I, Schlessinger J. A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. Cell 1997;89(5):693-702.

467. Wang JK, Xu H, Li HC, Goldfarb M. Broadly expressed SNT-like proteins link FGF receptor stimulation to activators of Ras. Oncogene 1996;13(4):721-9.

468. Klint P, Claesson-Welsh L. Signal transduction by fibroblast growth factor receptors. Front Biosci 1999;4:D165-77.

469. Fukuchi-Shimogori T, Grove EA. Emx2 patterns the neocortex by regulating FGF positional signaling. Nat Neurosci 2003;6(8):825-31.

470. Grove EA, Fukuchi-Shimogori T. Generating the cerebral cortical area map. Annu Rev Neurosci 2003;26:355-80.

471. Ye W, Shimamura K, Rubenstein JL, Hynes MA, Rosenthal A. FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. Cell 1998;93(5):755-66.

472. Garel S, Huffman KJ, Rubenstein JL. Molecular regionalization of the neocortex is disrupted in Fgf8 hypomorphic mutants. Development 2003;130(9):1903-14.

473. Carl M, Loosli F, Wittbrodt J. Six3 inactivation reveals its essential role for the formation and patterning of the vertebrate eye. Development 2002;129(17):4057-63.

474. Tian E, Kimura C, Takeda N, Aizawa S, Matsuo I. Otx2 is required to respond to signals from anterior neural ridge for forebrain specification. Dev Biol 2002;242(2):204-23.

475. Bachiller D, Klingensmith J, Kemp C, Belo JA, Anderson RM, May SR, McMahon JA, McMahon AP, Harland RM, Rossant J and others. The

organizer factors Chordin and Noggin are required for mouse forebrain development. Nature 2000;403(6770):658-61.

476. Barth KA, Kishimoto Y, Rohr KB, Seydler C, Schulte-Merker S, Wilson SW. Bmp activity establishes a gradient of positional information throughout the entire neural plate. Development 1999;126(22):4977-87.

477. Hammerschmidt M, Kramer C, Nowak M, Herzog W, Wittbrodt J. Loss of maternal Smad5 in zebrafish embryos affects patterning and morphogenesis of optic primordia. Dev Dyn 2003;227(1):128-33.

478. Aybar MJ, Mayor R. Early induction of neural crest cells: lessons learned from frog, fish and chick. Curr Opin Genet Dev 2002;12(4):452-8.

479. Nguyen VH, Trout J, Connors SA, Andermann P, Weinberg E, Mullins MC. Dorsal and intermediate neuronal cell types of the spinal cord are established by a BMP signaling pathway. Development 2000;127(6):1209-20.

480. Anderson RM, Lawrence AR, Stottmann RW, Bachiller D, Klingensmith J. Chordin and noggin promote organizing centers of forebrain development in the mouse. Development 2002;129(21):4975-87.

481. Mehler MF, Mabie PC, Zhu G, Gokhan S, Kessler JA. Developmental changes in progenitor cell responsiveness to bone morphogenetic proteins differentially modulate progressive CNS lineage fate. Dev Neurosci 2000;22(1-2):74-85.

482. Zhu G, Mehler MF, Mabie PC, Kessler JA. Developmental changes in progenitor cell responsiveness to cytokines. J Neurosci Res 1999;56(2):131-45.

483. Kretzschmar M, Doody J, Massague J. Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. Nature 1997;389(6651):618-22.

484. Bernier SM, Goltzman D. Effect of protein and steroidal osteotropic agents on differentiation and epidermal growth factor-mediated growth of the CFK1 osseous cell line. J Cell Physiol 1992;152(2):317-27.

485. Ganan Y, Macias D, Duterque-Coquillaud M, Ros MA, Hurle JM. Role of TGF beta s and BMPs as signals controlling the position of the digits and the areas of interdigital cell death in the developing chick limb autopod. Development 1996;122(8):2349-57.

486. Vainio S, Karavanova I, Jowett A, Thesleff I. Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. Cell 1993;75(1):45-58.

487. Neubuser A, Peters H, Balling R, Martin GR. Antagonistic interactions between FGF and BMP signaling pathways: a mechanism for positioning the sites of tooth formation. Cell 1997;90(2):247-55.

488. Kettunen P, Thesleff I. Expression and function of FGFs-4, -8, and -9 suggest functional redundancy and repetitive use as epithelial signals during tooth morphogenesis. Dev Dyn 1998;211(3):256-68.

489. Christen B, Beck CW, Lombardo A, Slack JM. Regeneration-specific expression pattern of three posterior Hox genes. Dev Dyn 2003;226(2):349-55.

490. Williams JG. STAT signalling in cell proliferation and in development. Curr Opin Genet Dev 2000;10(5):503-7.

491. Nakashima K, Taga T. Mechanisms underlying cytokine-mediated cell-fate regulation in the nervous system. Mol Neurobiol 2002;25(3):233-44.

492. Nakashima K, Yanagisawa M, Arakawa H, Taga T. Astrocyte differentiation mediated by LIF in cooperation with BMP2. FEBS Lett 1999;457(1):43-6.

493. Takizawa T, Ochiai W, Nakashima K, Taga T. Enhanced gene activation by Notch and BMP signaling cross-talk. Nucleic Acids Res 2003;31(19):5723-31.

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