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Caspase-3 Deficiency Rescues Peripheral Nervous System Defect in pRb Nullizygous Mice

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A thesis presented to the school of graduate studies and research
in partial fulfillment of the requirements for the degree of
Master of Science

For Emma

ABSTRACT

The retinoblastoma tumour suppressor protein, pRb, is a key regulator of cell cycle and has been implicated in the terminal differentiation of neuronal cells. Mice nullizygous for pRb die by E14.5 from haematopoietic and neurological defects attributed to failed differentiation (Jacks et al., 1992; Lee et al., 1992; Clarke et al., 1992). Previous studies by Macleod et al., (1996) have demonstrated that the loss of p53 protects pRb-deficient central nervous system (CNS) neurons but not peripheral nervous system (PNS) neurons from cell death. Thus, the mechanisms by which PNS neurons undergo apoptosis in response to pRb deficiency remain unknown. In view of the pivotal role of caspase-3 in the regulation of neuronal apoptosis during development, we examined its function in the execution of the widespread neuronal cell death induced by pRb deficiency. Our results support a number of conclusions: First, we show that caspase-3 becomes activated in all neuronal populations undergoing apoptosis. Second, caspase-3 deficiency does not extend the life span of pRb null embryos, as double null mutants exhibit high rates of liver apoptosis resulting in erythropoietic failure. Third, pRb/caspase-3 double mutant neurons of the CNS exhibit widespread apoptosis similar to that seen in pRb mutants alone, thus caspase-3 deficiency does not protect this population from apoptosis. Finally, in contrast to the CNS, neurons of the PNS including those comprising the trigeminal ganglia (TG) and the dorsal root ganglia (DRG) are protected from apoptosis in

pRb/caspase-3 double mutant embryos. Examination of the mechanistic differences between these two cell types revealed that CNS neurons invoke compensatory caspase activity that is not found in PNS neurons. These findings suggest that PNS neurons are dependent upon caspase-3 for the execution of apoptosis and that caspase-3 may serve as a key therapeutic target for neuroprotection following injury of this cell type.

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(And I thought it was done!)

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ABREVIATIONS

Apaf-1	apoptosis activating factor
bFGF	basic fibroblast growth factor
c. elegans	caenorhabditis elegans
CNS	central nervous system
cyt c	cytochrome c
DD	death domain
DED	death effector domain
DHFR	dihydrofolate reductase
DISC	death inducing signalling complex
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
FADD	Fas-associated protein with a death domain
FGF	fibroblast growth factor
FLICE	FADD-homologous ICE/CED-3-like protease (caspase-8)
ICE	interluekin-1b converting enzyme
IL-3	interluekin-3
NGF	nerve growth factor
NT3	neurotrophin-3
PCD	programmed cell death
PNS	peripheral nervous system
pRb	retinoblastoma (protein)
RAIDD	RIPK1 domain containing adapter with death domain
Rb	retinoblastoma (gene)
TG	trigeminal
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
Trk	tyrosine kinase receptor
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling

CHAPTER 1

INTRODUCTION

NEUROGENESIS AND RETINOBLASTOMA

Motor coordination, planning, memory and perception are but a few of the thousands of complex functions carried out by the mature nervous system. These functions depend on the precise formation and intricate inter-connection of many millions of neural cells. Consequently, establishment of the mature pattern is an extremely complex process. Delineating the signaling pathways that control neurogenesis is essential for understanding the defects underlying many developmental abnormalities. Quite possibly, further research in this area may eventually lead to an ability to selectively regenerate neurons following brain injury, disease or trauma. **A long-term research objective of the work in our laboratory is to understand the molecular mechanisms by which neural precursor cells exit the cell cycle and adopt a neuronal phenotype.**

TERMINAL MITOSIS, DIFFERENTIATION, AND NEUROGENESIS

A great deal of work has gone into generating our current understanding of nervous system development. Fate mapping in the cortex has allowed an understanding that cell cycle regulation has a critical role in the initial phases of neuronal development (reviewed in Vaglia et al., 1999). It is now known that cycling neural progenitor cells in the ventricular zones commit to a neuronal fate, withdraw from the cell cycle, and then adopt a neuronal phenotype,

and that this process is regulated by a variety of genes (reviewed in Slack & Miller 1996). The retinoblastoma tumour suppressor protein (pRb) is one of these genes. It has recently been implicated in cell cycle regulation, differentiation and as the developmental cell death of neurons. **As a means to our long-term objective, the present study examines the role of pRb in developmental cell death.**

Some of the early work intending to understand the process of neurogenesis examined the action of extrinsic cues from the developing nervous system. Specifically, basic fibroblast growth factor (bFGF) and neurotrophin-3 (NT3) were found to exert their effects by regulating the proliferation and differentiation, respectively, of precursor cells (Ghosh & Greenberg, 1995). Other work has shown that proliferation and differentiation (or specifically the cell autonomous programs responsible for the differentiation of new neurons from the surrounding ectoderm, and the cell cycle machinery responsible for the decision to undergo terminal mitosis) are intimately coupled. Several lines of evidence support this: first, bromodeoxyuridine labeling, coupled with immunohistochemistry using a ganglion cell specific antibody, showed that chick retina cells in the mitotic layer, begin to differentiate within minutes of S phase of terminal mitosis (McLoon et al., 1989; Waid et al., 1995). This suggests that neuronal cell fate might be determined before or during terminal mitosis. The frequently observed correlation between cell birthday and laminar fate in the cortex prompted another examination of how neuronal phenotypes are determined using heterochronic transplants of developing cortex (McConnell & Kaznowski, 1991). These experiments also linked the processes of determination with terminal cell division. Lastly, the dorsal root ganglion (DRG) precursor neurons of NT3-null mice (NT3-/-) fail to arrest the cell cycle, override the G1 restriction point and die by apoptosis in S phase, reflecting the importance of cell cycle arrest to differentiation (ElShamy et al., 1998). The importance of terminal mitosis in neuronal

development is further indicated by the fact that failure to permanently withdraw from the cell cycle results in failed differentiation and apoptosis (Clarke et al., 1992; Jacks et al, 1992; Lee et al., 1994; Elshamy et al., 1998). Taken together, these results indicate that cell division is required for the acquisition of the differentiated state and that the commitment to a neural phenotype depends on a functional link between the machinery for cell cycle regulation and for differentiation.

While cell cycle regulatory proteins such as pRb have been intensely examined in the arena of oncogenesis, there is little knowledge regarding their regulation in neuronal development. Recently, however, pRb has been implicated in neuronal differentiation and terminal mitosis, and has specifically been shown to restrict cell proliferation, inhibit apoptosis, and promote cell differentiation (reviewed in DiCiommo et al., 2000). **One of the objectives of this work is to investigate how neurons undergo developmental cell death in the absence of functional pRB.**

THE RETINOBLASTOMA PROTEIN (PRB)

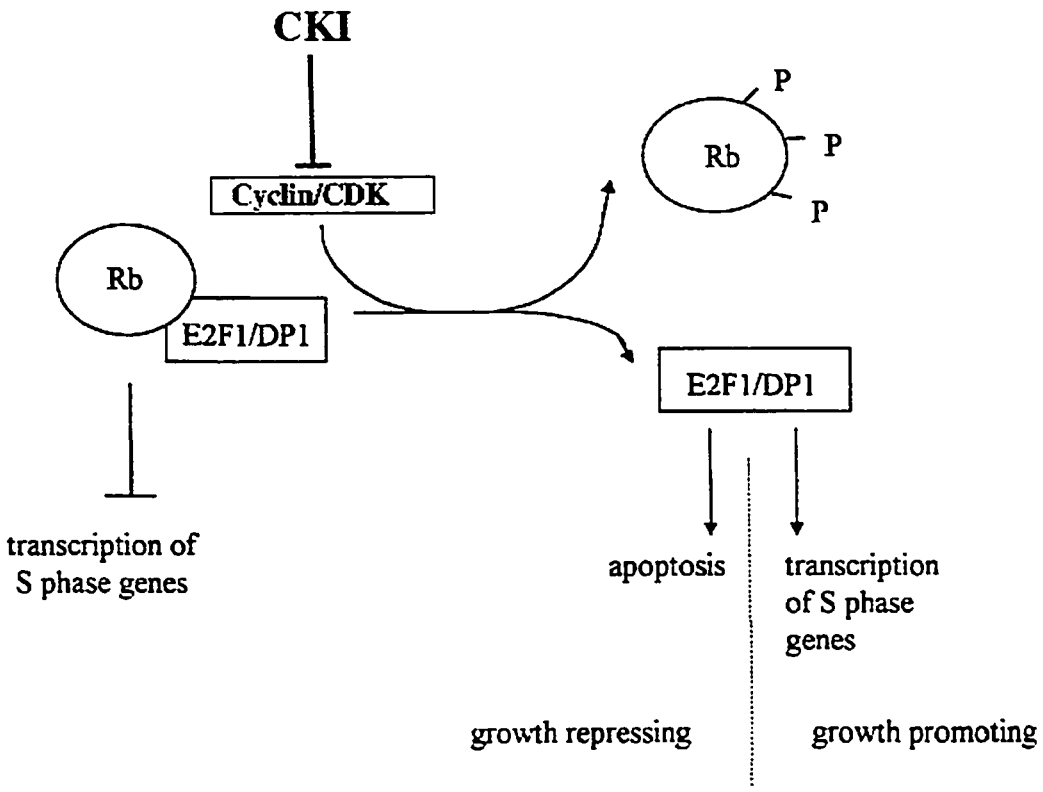
The retinoblastoma gene was the first tumor suppressor gene to be cloned (Dryja et al., 1986; Lee et al., 1987). The Knudson "Two-hit" model of tumour formation was derived from the epidemiological analysis of this gene (Knudsen, 1971). Inactivation of the retinoblastoma (Rb) gene locus is associated with a significant proportion of human cancers, including familial retinoblastoma, osteosarcomas, small-cell lung carcinomas, cervical carcinomas, breast carcinomas, and some forms of leukemias (reviewed in Moppett, 2001). Consequently, the Rb gene has been studied intensively in the field of oncogenesis (reviewed in Mulligan and Jacks, 1998). Its protein product (pRb) is a nuclear phosphoprotein with a relatively long half-life

(>8hrs). It is synthesized throughout the cell cycle and has its activity regulated by cell cycle phosphorylation (Harbour et al., 1999). Other members of the pRb gene family have been identified; these proteins, p107 (Ewen et al., 1991) and p130 (Chen et al., 1996), contain similar pocket domains and are also believed to regulate cell growth (Hannon et al., 1993; Li et al., 1993: Zhu et al., 1993). The following is a discussion of our current understanding of how functional pRb works in wild type cells (see figure 1-1).

To date, at least 110 cellular proteins, including transcription regulators, kinases, phosphatases, kinase regulators, as well as some proteins with unknown or miscellaneous functions, have been reported to associate with pRb (reviewed in Li et al., 2000). This long list of binding partners has sparked a current debate in the literature over which interactions are relevant to its role in development versus tumour suppression. Studies suggest that pRb functions in cell cycle arrest through its role as a transcriptional repressor, while its function in differentiation involves transcriptional activation (Bremner et al., 1995; Weintraub et al., 1995). A consensus of pRb function is that it regulates the cell cycle at the G1/S phase restriction point by controlling entry into S phase (Cobrinik et al., 1992; Hamel et al., 1997; Sherr, 1993). In its underphosphorylated state pRb binds and represses transcription factors, such as E2Fs, thereby disallowing the transactivation of genes responsible for DNA synthesis (Buchkovich et al., 1989; Chen et al., 1989; Decaprio et al., 1989; Ludlow et al., 1990). Phosphorylation by cyclin dependent kinases leads to pRb inactivation (Sherr et al., 1994) and the causative release of bound transcription factors.

Likely the most important physiological role for pRb in cell cycle regulation is in the sequestration of E2F family members. E2Fs (1-6) have been shown to activate a number of genes responsible for DNA synthesis including, for example, DNA polymerase α (Pearson et al., 1991), thymidine kinase (Karlseder et al., 1996), cyclins A and E (DeGregori et al., 1995),

Figure 1-1. Schematic of the Rb / E2F-1 pathway. Underphosphorylated Rb binds E2F-1 in the cell; at given times in the cell cycle (e.g. G1 – S phase transition) Rb becomes phosphorylated by cyclin dependent kinases and undergoes a conformational change that allows it to release E2F-1. E2F-1 then goes on to aid in the transcription of growth promoting or growth repressing genes. (Adapted from Reinke et al., 1999).


CKI
Cyclin/CDK
Rb
E2F1/DP1
transcription of
S phase genes
P
Rb
P
P
E2F1/DP1
apoptosis
transcription
of S phase
genes
growth repressing
growth promoting

and dihydrofolate reductase (DHFR) (Blake et al., 1989; Slansky et al., 1993). For example, E2F-1, inactive when bound with underphosphorylated pRb, has been shown to be a potent inducer of S phase progression in a variety of cell types (Johnson et al., 1993; Qin et al., 1994; Kowalik et al., 1995). E2F-1 has also been implicated in p53-mediated apoptotic induction (Wu et al., 1994; Hiebert et al., 1995; Hsieh et al., 1997). These two functions have been shown to be mechanistically independent, suggesting that cell cycle progression and apoptosis are part of an interactive or communicative pathway that involves pRb, E2F, and p53 (Phillips et al., 1997). There is mounting evidence that this pRb/E2F1 pathway is directly involved in neuronal apoptosis induced by neurotrophin deprivation and in injury situations [Park et al., 1998; Park et al., 1997(a); Park et al., 1997(b)]. Interestingly, embryos mutant for both pRb and E2F-1 demonstrate significant suppression of S phase entry when compared to pRb mutants and also show a significant reduction in apoptosis as measured by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Tsai et al., 1998). This not only supports the evidence for E2F-1's role in S phase progression, but also implicates E2F-1 as a critical mediator of the apoptotic effects. Also, up-regulation of the p53 pathway, required for some forms of cell death, is suppressed in the Rb/E2F-1 double mutants (Tsai et al., 1998). These double mutants do however show defects in cell cycle regulation and apoptosis in a variety of tissues leading to death at approximately E17.0 from anemia, defective skeletal muscle and lung development. This demonstrates that E2F-1 regulation is not the sole function of pRB in development (Tsai et al., 1998).

Transcription activation is believed to be another means by which pRb affects differentiation (Bremner et al., 1995; Weintraub et al., 1995). BRG-1, MyoD, Id-2, and EBP, all regulators of transcription known to play important roles in cell fate determination in several different systems including myogenesis (Weintraub, 1993), haematopoiesis (Porcher et al.,

1996), and neurogenesis (Jan et al., 1993), are also known to interact with pRb and induce differentiation (Reviewed in Morris & Dyson 2001).

pRb and Nervous System Development

The following is some of the evidence for the crucial role for pRb in cell cycle control and differentiation during mammalian nervous system development. First, pRb is expressed at high levels in the developing brain and PNS (Szekely et al., 1993). Second, embryonal carcinoma (P19) cells induced to a neuronal fate significantly upregulate pRb *in vitro* (Slack et al., 1993). Third, mice heterozygous for an Rb mutation, although both viable and fertile, carry an increased risk of developing tumours later in life that are most often related, upon post-mortem examination, to tumours in the brain (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). These tumours are commonly of the pituitary and present as severe wasting of the animal between the ages of 8 and 10 months (Jacks et al., 1992; Lee et al., 1996). Fourth, pRb-null mouse embryos (pRb-/-) display a dramatic neural phenotype consistent with a critical role for pRb in brain development (Jacks et al., 1992). Mice in which the pRb gene has been functionally knocked out by homologous recombination die by E14.5 due to neurological (and haematopoietic) defects attributed to failed terminal differentiation (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). By embryonic day 12.5 (E12.5) onward, ectopic mitoses and massive cell death are observed throughout the developing nervous system, including olfactory epithelium, retina, cortex, sensory ganglia and the hindbrain (Jacks et al., 1992; Lee et al., 1996). The expression of a number of genes, such as the neuron specific βIII tubulin and the neurotrophin receptors TrkA, TrkB, and p75, are significantly decreased in pRb-/- neural tissue, further demonstrating failed differentiation (Lee et al., 1994). Furthermore primary

cultures of pRb-/- neural precursor cells, originally believed to differentiate and survive *in vitro*, were subsequently shown to have an intrinsic cell cycle defect (Slack et al., 1998; Callaghan, et al., 1999). Closer examination of these cells revealed an up to two-day delay in the time-course of cell cycle withdraw, relative to wild type cortical progenitor cells. While this may not effect survival *in vitro*, *in vivo*, where available of growth factor is limiting and differentiation cues are precisely timed, such a delay could be expected to have serious consequences. In fact, this defect can be expected to play a significant role in the dramatic increase in apoptosis in the pRb-/- brain (Callaghan, et al., 1999).

Having accepted the overwhelming evidence for a role for pRb in mammalian nervous system development, further investigations focused on determining the precise developmental time points at which pRb is required during neurogenesis. Functional ablation of pRb in differentiating P19 embryonal carcinoma cells results in apoptosis immediately following commitment to a neuronal fate (Slack et al., 1995). This suggests not only that pRb is essential for the development of the neural lineages but also that the absence of functional pRb activity triggers apoptosis of differentiating neuroectodermal cells. These results were later confirmed *in vivo* when the pRb null mouse was interbred with a "neuronal marker gene mouse" expressing a transgene consisting of the neuron specific Tα1 α-tubulin promoter driving a lacZ reporter gene (Tα1:nlacZ). This reporter gene is induced as progenitor cells commit to a neuronal fate (Gloster et al., 1994; Gloster et al., 1999). Based on the timing of marker gene expression, the numerous nervous system abnormalities were again determined to arise immediately following the commitment decision, shortly after the time at which pRb would normally be expressed (Slack et al., 1998). Thus, not only is the phenotype of pRb nullizygous mice consistent with a role for pRb in regulating terminal mitosis and differentiation of developing neurons but also, in the absence of functional pRb, virtually all neuronal

populations undergo apoptosis immediately following commitment to a neuronal fate. **Thus, to understand the mechanism by which pRb-regulated pathways participate in acute neuronal injury or neurodegeneration, it is first essential to determine the molecular mediators of the apoptotic process induced by pRb deficiency.** Due to the interactive and communicative nature of the pathways of cell cycle control and cell death, examining how these cells die may also provide insight into the mechanism by which neural precursor cells exit the cell cycle and develop a neuronal phenotype. **This could become essential for the development of potential cell replacement therapies in the treatment of acute brain injury or disease, and possibly aid in the elucidation of therapeutic targets for neuroprotection from apoptotic cell death processes.**

APOPTOSIS

In recent years apoptosis has become known as not merely a single form of cell death, but as a fundamental theme in cell biology that has far-reaching implications in the fields of physiology and pathology. Despite ongoing research, the mechanism of apoptosis is not completely understood. Provided below is an explanation of apoptosis and its mechanism and a discussion of its importance to development. (reviewed in Fiskum, 2000; Bredeson, 2000).

What Is Apoptosis?

In normal tissue, cells that are no longer needed are rapidly eliminated without affecting the overall function of the tissue (Kerr et al., 1972). In this process cells undergo an active suicide called programmed cell death (PCD), or apoptosis. The word apoptosis is used, in

contrast to necrosis, to describe the situation in which a cell actively pursues a course toward death as opposed to a widespread passive degradation of damaged tissue (Raff et al., 1993). The morphological changes of apoptosis found in most cell types first involve contraction in cell volume and condensation of the nucleus with the intracellular organelles, such as the mitochondria, which retain their normal morphology. As apoptosis proceeds, blebbing of the plasma membrane occurs and the nucleus becomes fragmented. Finally, the cell itself fragments to form apoptotic bodies that are engulfed by nearby phagocytes. With respect to biochemical changes, it is known that the chromosomes become fragmented into nucleosomal units and DNA forms characteristic ladder patterns when subjected to agarose gel electrophoresis. This 'laddering' can also be detected by TUNEL assay (O'Brien et al., 2001).

Other than pRb deficiency, various stimuli have been reported to induce apoptosis. Some of these include: DNA damaging agents such as radiation or anticancer drugs (Taneja et al., 2001); those mediated by the TNF and Fas receptor family of "death receptors" (see below); and, the deprivation of cytokines that supply survival signals such as nerve growth factor (NGF) (Gotz, 2000), interluekin-3 (IL-3) (Mathieu et al., 2001), and erythropoietin (Siren et al., 2001).

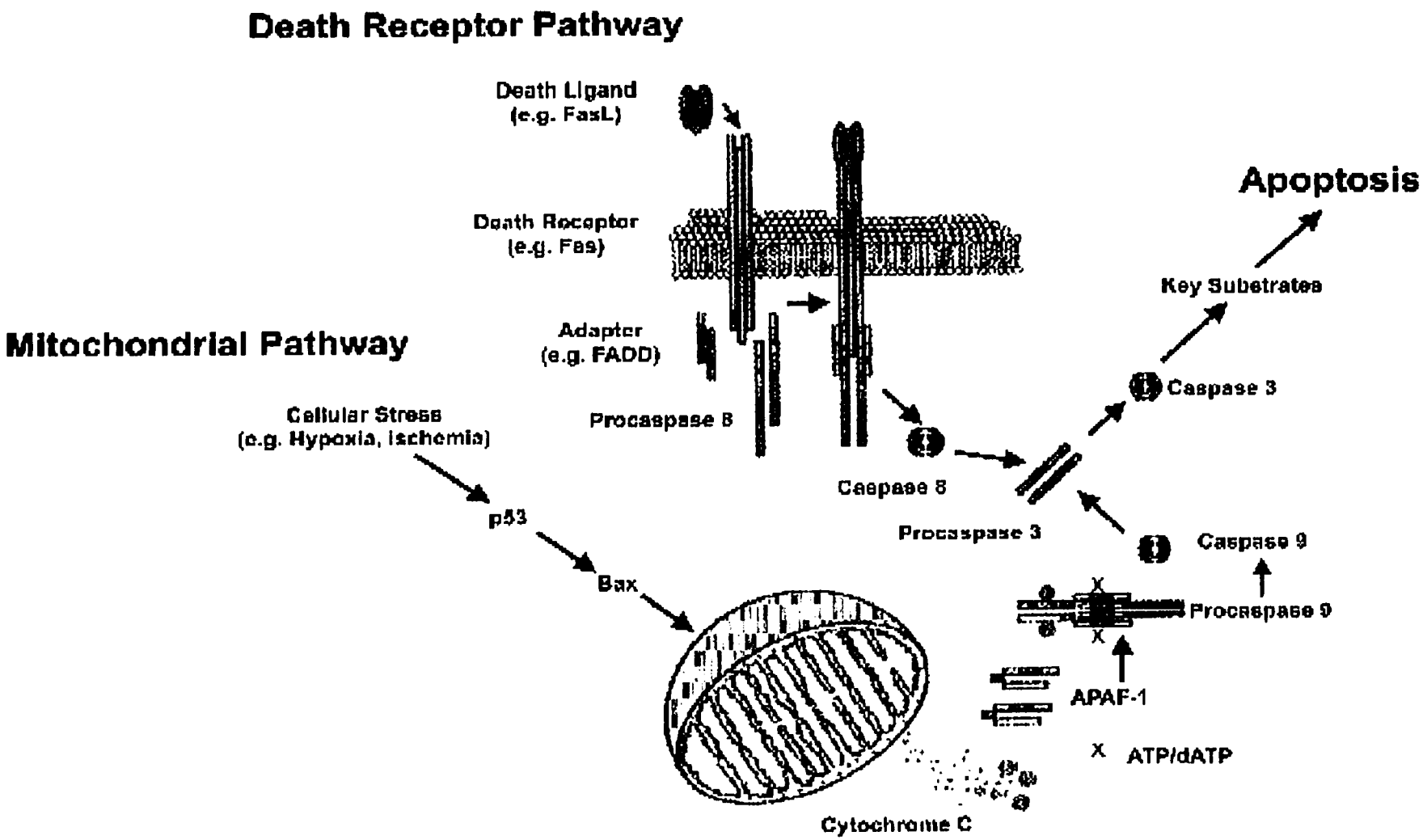
A discussion of apoptosis also begs mention of the tumor suppressor gene p53. Examination of the resistance to apoptosis of cells derived from p53 knockout mice (Morgenbesser et al., 1994) has revealed the importance of p53 in the programmed cell death that follows DNA damage (reviewed in Ko and Prives, 1996). Still other studies have implicated p53 as the key mediator of apoptosis in the context of cell cycle deregulation (reviewed in Ko and Prives, 1996; Morgenbesser et al., 1994). Interestingly however, in terms of brain development, p53 deficiency does not rescue all neuronal populations from cell death. Studies by Macleod et al., (1996) demonstrated striking differences in the p53 requirement for

neuronal apoptosis in the CNS versus the PNS of pRb deficient embryos. TUNEL staining revealed that cell death in the Rb-mutant CNS but not in the PNS was suppressed in the absence of p53. Thus, the mechanism by which the pRb-deficient nervous system undergoes cell death is complicated by the presence of distinct pathways from CNS to PNS. The mechanism remains unknown.

Two Pathways to Apoptosis

Apoptosis requires the activities of many genes first acting to influence a cell's probability of triggering its self-destruction program and then ensuring the coordinated activation and proper execution of the process (reviewed in Hengartner, 2000; Green, 2000). Our present understanding of human apoptotic processes is due, in large measure, to the progress made by Horvitz and colleagues in understanding cell death in the *Caenorhabditis elegans (c. elegans)* (reviewed in Metzstein et al., 1998). Their work has defined a variety of single-gene mutations that have specific effects on programmed cell death and has revealed genes required not only to cause cell death but also to protect cells from dying. Their findings detailed the normal development of the nematode species to such a degree that those cells dieing by apoptosis could be counted and mapped. Two genes, named *ced-3* and *ced-4*, were determined to be vital for the death of all 131 of these apoptotic cells (Ellis & Horvitz, 1986). Another gene, *ced-9*, was determined to antagonize the function of these pro-apoptotic genes and prevent cell death (Hengartner & Horvitz, 1994). This work provided the first molecular players of the apoptotic process, but it was the seminal finding that *ced-3* is the homologue to

Figure 1-2. The Two Pathways to Apoptosis. This is a simplified schematic of the proposed pathways leading to apoptosis. The mitochandrial pathway involves the Bcl2 family and begins with the translocation of Bax to the mitochondrial membrane. This is followed by the release of cyt c into the cytosol. Cyt c and Apaf-1 then activate procaspase-9 in the apoptosome which goes on to activate downstream effector caspases (caspase-3). The death receptor pathway involves the recruitment of procaspase-8 and other adaptor molecules (FADD) to a death receptor (Fas) following its stimulation through ligation of a death ligand (FasL). Caspase-8 then becomes activated and goes on to activate downstream effector caspases (caspase-3) leading eventually to the morphological hallmarks of apoptosis. (Green, 2000).


Death Receptor Pathway
Death Ligand
(e.g. FasL)
Death Receptor
(e.g. Fas)
Adapter
(e.g. FADD)
Procaspase 8
Caspase 8
Procaspase 3
Caspase 3
Key Substrates
Apoptosis
Mitochondrial Pathway
Cellular Stress
(e.g. Hypoxia, Ischemia)
p53
Bax
Caspase 9
Procaspase 9
APAF-1
ATP/dATP
Cytochrome C

human interleukin 1β-converting enzyme (ICE), together with the observation that ICE overexpression induces apoptosis in mammalian cells (Yuan et al., 1993), that led to much of the recent interest in this growing field.

It has since been determined that *ced-9* is homologous to human Bcl-2 (Xue et al., 1997) and that *ced-4* is homologous to mammalian Apaf-1 (Zou et al., 1997), further suggesting an evolutionary conservation of the central cell-death pathways. Two pathways have been defined and characterized and are here described and discussed below (see figure 1-2).

TNF family – The Death Receptor Pathway

The tumor necrosis factor (TNF) family of death triggering signals includes TNF, Fas ligand (FasL), lymphotoxin CD27 ligand, CD30 ligand, CD40 ligand and TNF-related apoptosis inducing ligand (TRAIL) (reviewed in Locksley et al. 2001). Fas (APO-1, CD95) is the receptor for FasL and is a member of the tumour necrosis factor receptor (TNFR) family (reviewed in Locksley et al. 2001; Nagata et al. 1999). This family includes the receptors TNFR1, TNFR2, lymphotoxin-βR, NGFR (p75), CD27, CD30, CD40, death receptor 3 (DR-3), and others (reviewed in Nagata, 1999). These cell surface signaling complexes provide a means to effectively transduce cues in the external environment into cellular demise and essentially define the first apoptotic death pathway.

Binding of ligand to a death receptor leads to receptor oligomerization and subsequent binding of adaptor proteins, which propagate the apoptotic signal through binding of initiator caspases. For example, binding of FasL to Fas initiates receptor trimerization (Nagata & Golstein, 1995). A conformational change then exposes a cytoplasmic, 80 amino acid sequence

that has been designated the "death domain" (DD) and is homologous within the family of receptors (Boldin et al., 1995). Adaptor molecules such as Fas-associated protein with a death domain (FADD also called MORT1) and RAIDD can bind these DD's via their own DD's and subsequently recruit caspase-8 (also known as FLICE) and caspase-10 to the receptor via another domain (the death effector domain - DED) (Chinnaiyan et al., 1995; Duan et al., 1997). The complex of ligand, trimerized receptor, adaptor molecule, and bound caspase is called the "death inducing signaling complex" (DISC) and it is capable of activating the caspase cascade and thereby initiating apoptosis (Boldin et al., 1996). It is important to note that caspase-3 has been shown to be downstream of TNF (Zhao et al., 2001).

The Mitochondrial Pathway

Intracellular signaling events can mediate cell death via this second pathway to apoptosis. Proapoptotic members of the Bcl-2 family of genes trigger the mitochondrial pathway (Antonsson & Martinou, 2000; Adams & Cory, 1998; Reed, 1997). Athough some progress has been made, the specific molecular mediators between cellular stress and Bcl-2 family action are not fully elucidated. In response to environmental cues (such as DNA damage) the "BH3 only" sub-family [so named because they possess only the BH3 Bcl-2 homology (BH)domain], which includes Bid, Bim, Bik, Bad, Harikari, Noxa, and a number of others, initiates the process (reviewed in Lutz, 2000). These proteins engage the Bax sub family (which includes Bax, Bak, and Bok) causing an oligomerization that allows the complex to insert into the mitochondrial membrane (Eskes et al., 2000). The result is a sudden and complete release of cytochrome c (cyt c) from all of the mitochondria in the cell (this becomes fundamentally important downstream with the formation of the apoptosome). Whether the Bax proteins are

loosely associated with the mitochondrial outer membrane or free floating in the cytosol requiring translocation is a question that remains unanswered.

Bcl-2 Family Members

The Bcl-2 family of genes includes both promoters and inhibitors of apoptosis capable of forming homo- and heterodimers whose ratio within the cell is important in determining resistance to programmed cell death (Otter et al., 1998). To a first approximation, the mutual neutralization of the bound pro- and anti- apoptotic Bcl-2 proteins can be seen to suspend apoptosis (reviewed in Gross et al., 1999). Thus, the complexity of the Bcl-2 family interactions collapses into comparing the levels of pro- and anti- family members: Cells with more pro-death proteins are more sensitive to death than cells with an excess of protective family members. The ratio within a cell or cell-line between these two subsets of proteins plays a role in determining the cells' susceptibility to a death signal (for example see Oltvai et al., 1993).

Sequence comparison and structural analysis has provided a clear picture of how these related proteins bind one another (reviewed in Gross et al., 1999). Four BH motifs are found: BH1, BH2, BH3, and BH4. These domains correspond to amphipathic α-helical segments whose presence or absence provides a structural basis for functional analysis. BH1, BH2, and BH3 are present in both anti- and pro-survival subfamilies with increasing support for an understanding that BH3 is a critical death domain, and BH4 is present only in the anti-survival forms (reviewed in Adams & Cory, 1998; Kelekar & Thomson, 1998; Reed, 1998).

The anti-apoptotic members of the Bcl-2 family (including Bcl-2, Bcl-X_L, Mcl-1, A1, and Bcl-w) generally block death by preventing the release of cyt c from the mitochondria

(Moriishi et al., 1999). Due to the nature of their mechanism being related to their interaction with proapoptotic members, they generally have no effect once the apoptotic cascade has already past the mitochondria (Newmeyer et al., 2000). This obviously, however, does not preclude regulation of apoptosis further downstream. Bcl-2 itself can form heterodimers with proapoptotic proteins, such as Bax, Bcl-X_S and Bad, and hence inhibit cell death by preventing, for example, the lethal homodimerization of Bax (Sato et al., 1994; Sedlak et al., 1995). Bcl-X is highly related to Bcl-2 but has long and short alternatively spliced transcripts with dissimilar functions called Bcl-X_L and Bcl-X_S respectively. These can bind Bax, Bad, Bak and Mcl-1, as well as themselves. Bcl-X_L functionally resembles Bcl-2 as a potent inhibitor of apoptosis; however, the short splice variant, Bcl-X_S, antagonizes the cell death inhibition of Bcl-2 and Bcl-X_L (Boise et al., 1993). In contrast to Bcl-2 knockout mice, Bcl-X knockouts are embryonic lethal with massive apoptosis in the brain, spinal chord and haematopoietic system (Motoyama et al., 1995).

In *c. elegans*, it is well established that the Bcl-2 homolog *ced-9* blocks apoptosis by binding to the Apaf-1 homolog *ced-4* and preventing the activation of the caspase protease *ced-3* (Chinnaiyan et al., 1997; Wu et al., 1997). There may be other, yet to be discovered human homologs of Apaf-1 capable of binding Bcl-2 but this process has not been observed in human cells. The great number of molecules and interactions involved provide the cell with options for points to regulate and modulate its susceptibility to apoptosis.

Proteases are also capable of functional modification of Bcl-2 during apoptosis. *In vivo*, the loop domain of Bcl-2 and Bcl-X_L is cleaved by caspases in apoptotic cells (Cheng et al., 1997). The C-terminal product that lacks the BH4 domain acts as a death effector thereby ensuring the inevitability of death (Clem et al., 1998). Bcl-2 may therefore also be a downstream substrate for caspases, suggesting the existence of a feedback loop.

The Apoptosome

As alluded to above, cyt c release is a key step in the apoptotic death via the mitochondrial pathway (reviewed in Deshmukh and Johnson, 1997). Prior to this release, genotoxic damage, cytokine deprivation, or some other cell-death signaling insult that has passed the checkpoint determined by the ratio of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins can lead to mitochondrial dysfunction that culminates in the release of cyt c into the cytosol. This mitochondrial dysfunction also includes a change in the mitochondrial membrane potential (Vander Heiden et al., 1999), the production of reactive oxygen species, and the opening of the permeability transition pore (Gross et al., 1999). The release of cyt c, which follows a variety of death stimuli (Liu et al., 1996; Kluck et al., 1997; Bossy-Wetzel et al., 1998), induces the cytosolic protein Apaf-1 (apoptosis activating factor-1) to oligomerize (Cain et al., 2000; Li et al., 1997; Zou et al., 1997). Then, the Apaf-1/cyt c "apoptosome", in the presence of dATP, is capable of recruiting and activating procaspase-9. Caspase-9 goes on to cleave procaspase-3 to its active form thereby initiating the apoptotic cascade (Zou et al., 1997: Li et al., 1997).

Cells lacking Apaf-1 (Yoshida et al., 1998) or caspase-9 (Kuida et al., 1998) are resistant to apoptosis via the mitochondrial pathway. Yoshida and colleagues (1998) generated murine embryonic stem (ES) cells nullizygous for the Apaf-1 gene (Apaf-1 -/-). These were used in the eventual generation of Apaf-1 -/- mice. The brains of these mice showed an increase in the number of proliferating cells during embryogenesis (concomitant with significantly reduced apoptosis in the effected areas) which resulted in gross cytoarchitectural mutations in the CNS. Kuida and colleagues (1998) carried out much the same procedure generating caspase-9 nullizygous mice by homologous recombination. At the histological level, these caspase-9 -/-

mice exhibited prominent expansions of their proliferative zones within their forebrains and midbrains, as well as stenosis of their cerebral vescicles. These gross anatomical phenotypes, such as protrusions of brain tissue, are consistent with the idea that many of the proliferating cells in the ventricular zones which would normally die by apoptosis triggered by an absence of growth factor during their migration towards an intended destination in the brain, do not die but proliferate because of the absence of a required element of the apoptotic cascade (Apaf-1, or caspase-9).

Caspase Activation

In its simplest presentation, these two pathways to apoptosis are now understood as processes leading to the activation and functioning of a family of cysteine proteinases with specificity for aspartic acid residues called "caspases". Caspases are synthesized as inactive zymogens composed of three domains: an N-terminal prodomain, a p20 domain and a p10 domain. When activated, they appear as a heterotetramer containing two p20/p10 heterodimers (with two active sights) (Earnshaw et al., 1999). The key to understanding apoptosis then is in understanding how stimuli can lead to caspase activation. So far three general mechanisms have been described (reviewed in Hengartner, 2000). The first is proteolytic cleavage by an upstream caspase. This is straightforward and used mostly for the activation of the caspase-3 sub-family known as the effector caspases. The other two mechanisms are equivalent to, and exemplified by, the two pathways to apoptosis described above. The first being activation of caspase-8 due to induced proximity at death receptors and the second being activation of procaspase-9 within the apoptosome. Most of the morphological changes observed by Kerr et al. (1972) are specifically caused by the activation

of these proteases in apoptotic cells (see below *"Caspase Substrates"*). Once activated, they cleave a wide variety of proteins and it is the change in function or loss of function of these key cellular substrates that leads to the death of the cell.

At least 14 distinct caspases have been cloned so far (Chang et al., 2000; Cryns and Yuan, 1998). Most investigators prefer to classify these proteases phylogenically (and functionally) into three groups: a caspase 8 sub-family, an ICE (caspase 1) sub-family and a caspase-3 sub-family. The first group of caspases contain long prodomains and are believed to be the upstream initiator caspases. Among them, caspase-8 and caspase-10 contain tandem repeats of the DED within their prodomain that allows interaction with FADD/MORT-1 and leads to the recruitment of these caspases to death receptors followed by their activation (Salvesen and Dixit, 1999). Another group of caspases, also with relatively long prodomains, includes caspases-1, -2, -4, and -9, each of which contains a "caspase recruitment domain" (CARD). The CARDs of these caspases interact with CARD-containing adaptor molecules. It is likely that these caspases also undergo similar adaptor-mediated aggregation and self-activation, which has the effect of furthering the caspase cascade (Salvesen and Dixit, 1999). By contrast, the third group of caspases, including caspase-3, -6, and -7, have short prodomains and are believed to be downstream effector caspases that depend on the upstream initiator caspases for their activation (Cohen, 1997). This subgroup is of particular importance to this study given that caspase-3 is a key determinant in naturally occurring neuronal apoptosis during embryogenesis (Kuida et al., 1996). Embryos deficient in caspase-3 die as embryos (or perinataly) due to excessive cellularity and duplicated structures in the developing brain. The widespread activation of caspase-3 in the developing nervous system, as well as the finding that the death of sympathetic neurons deprived of NGF could be blocked by a cell permeable pan-caspase inhibitor, bocaspartyl(OMe)-fluoromethylketone (BAF) (Park et al.,

1998), lends further support to the importance of caspase activity in neuronal apoptosis (Urase et al., 1998; reviewed in Deshmukh and Johnson, 1997).

Caspases can thus be considered the executioners of the apoptotic pathway. Eliminating caspase activity by mutation or through the use of pharmacological inhibitors has a significant effect; either slowing down or eliminating apoptosis all together (Earnshaw et al., 1999).

Caspase Substrates

One of the more exciting discoveries identifying important caspase substrates was the finding that caspases activate the nuclease responsible for generating the famous DNA ladder fragments first discovered by Wyllie et al. (1981) and since extensively used as a marker for apoptotic cell death. Liu et al. (1997), Enari et al. (1998), and Sakahira et al. (1998), in an elegant series of experiments, have recently shown that the DNA ladder nuclease is caspase activated (dubbed CAD for caspase activated Dnase). The human homologues are known as DFF-40 and -45 for DNA fragmenting factor of 40 (or 45) kilodaltons (KDa). These exists in living cells as inactive complexes with an inhibitor dubbed "ICAD". The cleavage of the inhibitory subunit by caspase-3 results in the release and activation of the catalytic subunit leading to the classic DNA laddering (reviewed in Nagata, 2000).

Active caspases, probably predominantly caspase-3 and -6, cleave a number of nuclear proteins effectively contributing to the nuclear changes involved in apoptosis. For example, cleavage of the nuclear lamins is responsible for nuclear shrinkage and budding (Buendia et al., 1999; Rao et al., 1996). Acinus, a recently cloned protein shown to be required for chromatin condensation but not DNA fragmentation, is also cleaved by caspases (Sahara et al., 1999). Nuclear scaffold attachment factor, poly-(ADP-ribose) polymerase (PARP), and others are

also cleaved during apoptosis (Liu et al., 1997; Lazebnik et al., 1994, 1995; Gohring et al., 1997; Hirata et al., 1998).

Changes in the external shape or structure of a cell and the appearance of 'blebs' are also linked to caspase activation. A number of structural proteins are cleaved directly by caspases during apoptosis including gelsolin (Kothakota et al., 1997), fodrin (Martin et al., 1995b), actin (Mashima et al., 1995), and Gas2 (Brancolini et al., 1995). Early detachment of cells from surrounding matrix may involve caspase-mediated cleavage of FAK (focal adhesion kinase). Also, the caspase mediated-cleavage of PAK2, a member of the p21-activated kinase family, appears to mediate the active blebbing observed in apoptotic cells (Rudel et al., 1997).

There are many more caspase substrates cleaved during apoptosis that lead to the death of the cell and many others whose function is unclear. For example, caspases were recently shown to cleave pRb *in vitro* (Boutillier et. al. 2000) which may seem significant to the results of our study but likely holds very little relation. This cleavage, if physiologically relevant, may be of importance as a positive feedback mechanism leading to enhanced apoptosis. Although there are apoptotic programs that inevitably lead to death without caspases (Kitanaka et al., 1999; Chautan et al., 1999; Depraetere et al., 1998), understanding how caspases become activated within an apoptotic cell is important for a proper appreciative of apoptotic processes. There also remains much to be determined in reference to events downstream of caspases as well as how the cell cycle machinery comes into play in determining when and how a cell will die. With so much more yet to be examined, cell death will likely remain a vigorous field of study for the foreseeable future.

Apoptosis and the Developing Brain

PCD has long been recognized to occur in most neuronal populations during normal development (reviewed in Oppenheim, 1991). Neurons of the brain and spinal chord including long projection neurons, local circuit neurons, motor neurons, sensory neurons, and autonomic neurons have all been observed to undergo restricted periods of PCD (Reviewed in Raghupathi et al., 2000). Central and peripheral glial cells, although studied less extensively, also undergo PCD (reviewed in Barres et al., 2000). This has been observed extensively during neurulation, in dividing precursor populations, as well as up to the time when synaptic connections are established and hence is not limited to any particular stage of development (Homma et al., 1994). Thus, cell death in the nervous system clearly occurs on a very large scale and consequently must play a fundamental and essential role in normal development.

Possible functions of apoptosis in the nervous system include: The removal of cells that appear to have no function or have already carried out a temporary function as with the glial scaffolding cells in the cortex (Conradt et al., 1999); the death of cells having defective DNA (Dasika et al., 1999) or infected by viruses (Medana et al., 2000; Aleman et al., 2001); error correction including death of cells with inappropriate synaptic connections or aberrant pathway projections; and, systems-matching between interconnected groups of neurons to allow for proper pattern formation and morphogenesis (reviewed in Milligan et al., 2000).

Hamburger and Levi-Montalcini's pioneering work in developmental cell death focused on the role of target-derived trophic factors. They showed that the location of the limb buds in chick embryos directly affects the size of their adjacent spinal ganglia, a result that subsequently led to the discovery of NGF (Hamburger and Levi-Montalcini, 1949). This seminal finding became one of the most influential concepts in nervous system development

of this century (reviewed in Levi-Montalcini, 1998). Accordingly, the predominant view of cell death is that it occurs in order to match the size of a neuronal population with the magnitude of their targets and to eliminate neurons with erroneous connections (Raff, et al., 1996; Gorden et al., 1995; Cowan, et al., 1984).

Some recent work seemed to further this predominant view. For example, mutant mice that overexpress the anti-apoptotic gene Bcl-2 or those that lack the pro-apoptotic gene Bax (White et al., 1998) exhibit increases in selected neuronal sub-populations without gross malformations of the nervous system (Sawanda et al., 2000; Phillips et al., 2000; Martinou et al., 1994). This reduced apoptosis, consistent with the idea of target or 'social' control of survival and death, seemed to exclude a significant role for cell death in the total morphogenesis of the nervous system.

Given the prevailing view, it was therefore surprising that mutant mice deficient in a number of other pro-apoptotic genes showed severe malformations of the nervous system due to the reduction of developmental cell death. Specifically caspase-3 (Kuida et al., 1996), caspase-9 (Kuida et al., 1998; Hakem et al., 1998), and Apaf-1 (Cecconi, et al., 1998) knockouts have significant malformations of the brain. Moreover, mice deficient in the protein kinases Jnk1 and Jnk2 showed pronounced neurulation defects preceded by a severe reduction of cell death prior to the closure of the hindbrain neural tube (Kuan et al., 1999). These results suggest a significant role for cell death in the morphogenesis of the developing brain. Subsequent elucidation of the role of these proteins has clarified this seeming discrepancy to some extent, by drawing a distinction between the cell death of post-mitotic neurons and that of progenitor or founder cells within the nervous system.

Beginning with the observation of apoptosis in the proliferative ventricular zones (VZ) (Thomaidou et al., 1997) it became intuitively likely that those molecules more directly

involved with the apoptotic death of post-mitotic neurons will have less effect on the morphogenesis of the brain than those known to affect the cell death of progenitors. One significant experiment by Roth et al. (2000) introduced a caspase-3 mutation into Bcl-X-deficient mice in an attempt to determine whether the caspase-3 deficiency would abolish the phenotype of the upstream Bcl-X_L deficiency as predicted by the epistatic relationship of their molecular counterparts in *c. elegans* (Hengartner et al., 1992). Their results, which also compared the expression patterns and null-mutation phenotypes of the Bax, Bcl-X, and caspase-3 mice, indicated that caspase-3's pivitol role in normal brain development is due to its distinct effect on apoptosis of neuronal precursor cells. Although caspase-3 deficiency results in decreased apoptosis of post-mitotic neurons in the developing cortex [given the normal brain structures of Bax deficient mice (White et al., 1998)], it is the exponential expansion of progenitors that results in the duplicated structures, marked dysplasia and significant brain malformations in caspase-3 knockouts (Roth et al., 2000). Caspase-3 then is seemingly critical for establishing the size of the initial neuronal stem cell pool during normal brain development.

Objectives and Aims

In view of the pivotal role of caspase-3 in the regulation of neuronal apoptosis during nervous system development, we examined its function in executing the wide-spread neuronal cell death induced by pRb deficiency. The overall objective of this work is to understand the molecular mechanisms regulating developmental apoptosis within the mammalian nervous system. The specific questions this work aims to answer include: A. Is caspase-3 involved in the apoptotic processes induced by pRb deficiency? B. Is caspase-3 important to this process. C. Are there differences in the mechanism of cell death between populations of cells within the

mammalian nervous system? D. Can other caspases compensate for the loss of caspase-3 to facilitate cell death?

CHAPTER 2

MATERIALS AND METHODS

TRANSGENIC MICE - GENERATION

All animals were treated in accordance with procedures outlined in the "Guide for the Care and Use of Experimental Animals" endorsed by the Medical Research Council of Canada. Housed no more than three per cage, the animals were acclimatized to a 12 hour day/night cycle and given free access to water and Purina laboratory chow.

The pRb-deficient transgenic mice, originally generated by Jacks and colleagues (1992), were purchased from The Jackson Laboratories (Bar Harbor, ME) and maintained on a C57BL6 genetic background. These mice were created using the positive-negative selection method of Mansour et al. (1988) to incorporate two different stop codon sites and a new PstI restriction site into exon 3 of the Rb gene. Termination of translation at either stop codon creates an abhorrent N-terminal pRb peptide. This was achieved by disrupting one allele of the pRb gene in murine embryonic stem cells by inserting a bacterial neomycin gene (neo) expression cassette into the third intron while ligating the herpes simplex virus thymidine kinase (HSV-tk) gene upstream of the pRb sequences. Neo-positive clones survive the application of anti-biotic and HSV-tk-negative clones were counter-selected against the non-viable ES cells that acquired the targeting vector randomly. This positive-negative selection method ensured that the exogenous pRb sequences were acquired by homologous recombination and the presence of the point mutations in the third exon were further verified by DNA sequencing by polymerase chain reaction (PCR). ES cells heterozygous for the desired pRb mutation were then injected into C57BL/6 embryos at the blastocyst-stage, which

were subsequently inserted into pseudo-pregnant females to create chimeric animals. Incorporation of the mutation into the germline of chimeras was determined by breeding (Jacks et al., 1992). Our breeding protocol began with mice heterozygous for the pRb mutation (verified by PCR – see below). These mice are both viable and fertile although they carry an increased risk of acquiring adenocarcinoma of the pituitary or other histopathological tumours (Jacks et al., 1992). This is also consistent with and actually confirmed the role of pRb in the Knudson "two-hit model of tumour-genesis" (Knudson, 1971).

Transgenic mice carrying a null mutation for caspase-3 were obtained from Dr. D.W. Nicholson (Merck Frosst, Canada) (Cregan et al, 1999; Keramis et al., 2000). The mice were originally created by homologous recombination using the same positive-negative selection methods described above for the pRb mutation (Kuida et al., 1996). The phenotype of these caspase-3 deficient mice was similar to those described by others (Kuida et al., 1996; Woo et al., 1999). All caspase-3 deficient mice were maintained on C57BL/6 background to maintain genetic uniformity.

TRANSGENIC MICE – BREEDING

Mice deficient for both pRb and caspase-3 were generated by mating mice heterozygous for pRb ([pRb +/- : caspase-3 +/+]) with mice heterozygous for caspase-3 ([pRb +/+ : caspase-3 +/-]). Progeny found by PCR to carry both mutations, [pRb +/- : caspase-3 +/-], were then mated to produce double knockout embryos ([pRb -/- : caspase-3 -/-]). The breeding protocol is shown in appendix A. As the F2 generation offspring have all of the desired genotypes in our study, littermates were used for all experiments. The four desired genotypes pRb -/-, caspase-3 -/-, wild type, and double knockout (pRb-/- : caspase-3 -/-), were determined and verified by polymerase chain reaction .

DNA Isolation

DNA was isolated from tissue samples using standard proteinases K, phenol:chloroform isolation protocol (Laird, 1991). Tail clippings from adult mice were used. Two tissue samples were taken from each of the embryos. One of the embryonic tissue samples was placed immediately into proteinase K solution at 37°C to begin the DNA extraction, the other was placed into a 1 ml eppendorf tube and frozen at –20°C for later genotype confirmation. All of the embryos used for histological analysis were double-checked for genotype.

Polymerase Chain Reaction (PCR)

PRb-deficient mice were genotyped by PCR to verify the presence of exon 3 mutations using primers RX3 and PGK3' as previously described (Jacks et al., 1992). PGK3' recognizes a DNA sequence within the promoter region of the PGK-neo cassette inserted into intron three of the mutant allele. If this primer recognizes the mutant sequence the resulting product is slightly larger than in the wildtype band [(they are both ~400 base pairs (bps)]. The wild-type PCR product is created using the RX3 primer and a second primer, RI3, that recognizes a similar loci in intron 3 of the wildtype gene (see figure 3-1). Primer sequences: RX3 (5'-AATTGCGGCCGCATCTGCATCTTTATCGC-3'), RI3 (5'-CCCATGTTCGGTCCCTAG-3'), PGK3' (5'-GAAGAACGAGATCAGCAG-3'). PCR reaction conditions were set as follows: 94°C - 2 min (1 cycle); 94°C-30 sec; 60°C - 1 min, 72°C - 1 min, (30 cycles); 72°C - 5 min (1 cycle). A separate reaction was used for wildtype and mutant.

Caspase-3 genotyping was carried out by PCR in the usual PCR reaction buffer containing .25mM MgCl2 and 5% DMSO (Cregan et al., 1999). The primers for the wild type caspase-3 alleles were CTAAGTTAACCAAACTGAGCACCGA (sense) and ATGAATGAA

GGCAGCATAGTACTCC (antisense). For the detection of the targeted allele, the same sense and the following antisense primers: GTCGATCCACTAGTTCTAGA GCGGC were used. Conditions were set as follows: 94°C - 2 min (1 cycle); 94°C - 30 sec; 60°C - 1 min, 72°C - 1 min, (30 cycles); 72°C - 5 min (1 cycle). The wild-type allele has a PCR reaction product of 1733 base pairs (bp), and the mutant allele has a product of 1605 bps (see figure 3-1). The PCR products were electrophoresed through agarose gel in the presence of ethydium bromide intercalating label and photographed under ultra-violet light (see figure 3-1).

Tissue Sample Preparation

At E13.5 days of gestation, pregnant mice were sacrificed with an interperitoneal injection of Somnotol (1ml/Kg body weight). Embryos were not used at later time points due to the high rate of lethality found at E14.5 onward. Embryos were then removed and tissue samples of individual embryos were taken for genotyping prior to fixation in 4% paraformaldehyde (PFA) for 4 hours. The embryos themselves, after fixation, were washed in PBS and then cryoprotected in 10% sucrose overnight at 4°C. The tissue was then fast frozen in tissue tech solution via direct CO_2 application and then sectioned on a cryostat at 14 μM. Series of 20 microscope slides were captured at one time with subsequent sections being placed onto adjacent slides. This allowed for a wide range of sections to be present on any given slide such that virtually every slide had a section (or two) containing portions of the dorsal root ganglion (DRG), a section containing trigeminal ganglion (Tg), and more than one section containing representative CNS tissue. Immunostaining or TUNEL assay followed (see below).

Determination Of Cell Death

TUNEL staining

For terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, frozen sections were treated with acetone/methanol (1:1) for 1 minute followed by three washes with 1xPBS. Sections were then incubated for 1 hour at 37°C with 75 ml of a cocktail (Boehringer Mannheim, Indianapolis, IN) consisting of 0.5 ml terminal transferase, 0.95 ml biotin-16-dUTP, 6.0 ml CoCl2, 15.0 ml 5x TdT buffer, and 52.55 ml distilled water. After 3 washes in 1xPBS, sections were incubated with a streptavidin Cy2 secondary antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). After 3 washes with 1xPBS, sections were counterstained with Hoechst and examined with a Zeiss Axioskop fluorescent microscope. For cell counting the following regions in the nervous system of [wild type], [Rb-/-], [Caspase-3-/-], [Rb-/-:Caspase-3 -/-] littermates were selected: forebrain at the medial aspect of the ganglionic eminence; hindbrain at the caudal pons adjacent to the fourth ventricle, trigeminal ganglion, caudal and rostral DRG. At 40X magnification images were captured using Northern Eclipse software and a defined 100 square micron area at the centre of the ganglia was counted for each specimen. The data were expressed as the number of TUNEL-positive cells as a percentage of the total cell count as determined by Hoechst staining.

Fluoro-jade staining

To evaluate neuronal degeneration including both apoptotic and necrotic modes of cell death Fluoro-jade labelling was used (Schmued et al.,1997; Noraberg et al., 1999). Fluoro-Jade staining was performed on sections immunolabeled for ΔC-APP (see immunostaining for

details). Briefly, sections were washed in three changes of PBS (10 minutes each) after immunolabeling for ΔC-APP, followed by a brief 1 minute rinse in distilled water. The sections were then stained with 0.00001% Fluoro-Jade in 0.1% acetic acid for 20 minutes at room temperature. After washing in three changes of distilled water (1 minute each), the slides were dried on a slide warmer and cover slips were mounted with D.P.X. neutral mounting media (Aldrich Chemical Company, Inc.).

IMMUNOSTAINING

Caspase Activation

Active caspase-3 was detected immunohistochemically by using three different antibodies. These antibodies were anti-caspase-3 from PharMingen, and anti-neoepitope and anti-active caspase-3 antibody from Merck Frosst. Among the three antibodies, the former two selectively recognize the p17 fragment of caspase-3, the larger subunit of the active enzyme, whereas the third was directed against the catalytically active (p17/p12) conformer. Fresh frozen sections (14 μM) were incubated with the primary antibodies [anti-caspase-3 (PharMingen), 1:1000; anti-neoepitope (Merck Frosst), 1/1000 and anti-active caspase-3 (Merck Frosst), 1/2000] at 4°C for 48 hours. After three washes with PBS (10 min each), the sections were incubated with CY3-labeled donkey anti-rabbit IgG (1:800; Amersham, Buckinghamshire, UK) for 2 hours at room temperature.

Detection of the Caspase-Cleaved Fragment of APP in Apoptotic Neurons

The polyclonal antibody recognizing the caspase-generated APP fragment α-ΔCcsp-APP was previously described (Gervais et al., 1999). Fresh frozen sections were incubated with the

α-ΔCcsp-APP (Δ-APP)antibody at a dilution of 1/500 overnight at 4°C followed by a CY3 donkey anti-rabbit IgG (as above).

Protein Gene Product 9.5 (PGP9.5)

A monoclonal antibody directed against PGP 9.5, a neuronal specific element of the ubiquitin pathway, was used as a neuronal marker, staining neuronal cell bodies and axons of neurons in the CNS and periphery, small nerve fibres in the peripheral tissues and neuroendocrine cells in the pituitary, thyroid, and pancreas (Wilson et al., 1988). The polyclonal antibody to PGP 9.5 (Cedarlane Laboratories Ltd.) was diluted at 1:500 followed by a CY3 anti-rabbit secondary.

B-III Tubulin

Class III beta-tubulin was used as an early neuronal marker that is normally induced at the time of neuronal commitment (Gloster et al., 1994). A monoclonal antibody directed against this protein previously described (Caccamo et al., 1989) was a gift from Dr. David Brown, University of Ottawa. The hybridoma supernatant was diluted at 1:10 in 5% goat serum and after 3 PBS washes was followed by a secondary antibody, Alexa fluor 488 anti-mouse (Molecular Probes) at 1:2000.

Trk A

As a differentiation marker for peripheral neurons an antibody directed against Trk A, kindly supplied by Dr. Louis Riechardt, was used (Clary et al., 1994). This polyclonal antibody was diluted at 1:200 and was followed by a CY2 goat anti-rabbit secondary (1:500).

CHAPTER 3

RESULTS

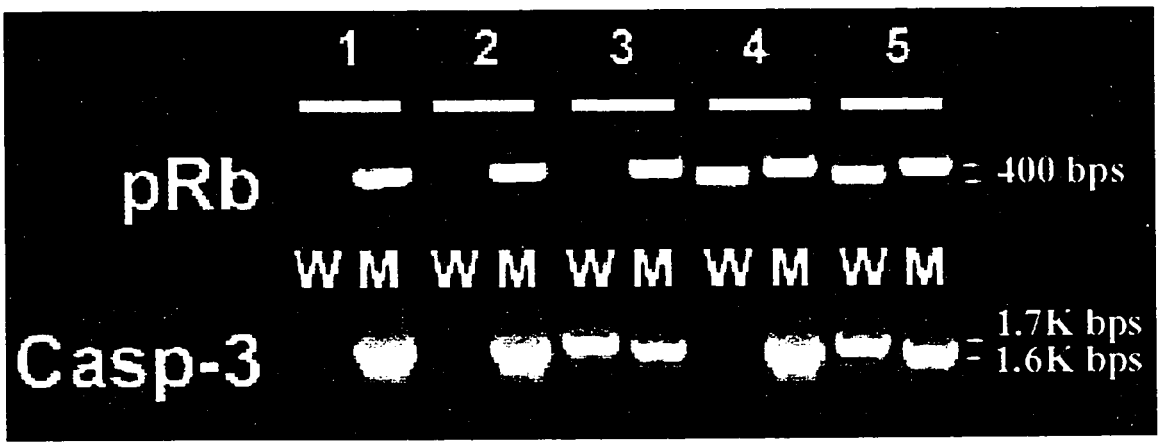
GENOTYPING

Figure 3-1 is a representative photograph of an ethydium bromide agarose gel showing the PCR amplifications of pRb and caspase-3 mutant and wildtype genes for five different embryos. Each gel was run with the amplification product of the wildtype primers adjacent to the amplification product using the mutant primers for a given gene (pRb or caspase-3) and a given embryo. The expected fragment size for each of the products is as follows: pRb wildtype allele; ~400 bps: pRb mutant allele; ~400 bps: caspase-3 wildtype allele; 1733 bps: caspase-3 mutant allele; 1605 bps. The five embryos in the figure (1 through 5) have the following genotypes 1. (Rb -/- : caspase-3 -/-), 2.(Rb -/- : caspase-3 -/-), 3.(Rb -/- : Caspase-3 +/-), 4. (Rb +/- : caspase-3+/-) 5. (Rb +/- : caspase-3 +/-).

CASPASE-3 IS INDUCED IN THE RB DEFICIENT NERVOUS SYSTEM.

Our first goal was to determine whether caspase-3 is first expressed and second activated in neural tissue that is undergoing apoptosis in response to pRb deficiency. Using three different antibodies for caspase-3 that selectively recognize the products of caspase-3 activation [p17 fragment or the active (p17/p12) tetramer (Rasper et al., 1998; Cohen, 1997; Nicholson et al., 1995)], we examined the brain, spinal cord, and spinal ganglia of embryos

Figure 3-1. Representative PCR Gel for pRb and caspase-3 genotyping. The five embryos in the figure (1 through 5) have the following genotypes (pRb:caspase-3) 1.(-/- : -/-), 2.(-/- : -/-), 3.(-/- : +/-), 4. (+/- : +/-) 5. (+/- : +/-). For pRb plate: Primer sequences: RX3 (5'-AATTGCGGCCGCATCTG CATCTTTATCGC-3'), RI3 (5'-CCCATGTTCGGTCCCTAG-3'), PGK3' (5'-GAAGAACGAGATCAGCAG-3'). PCR reaction conditions were set as follows: 94°C - 2 min (1 cycle); 94°C-30 sec; 60°C - 1 min, 72°C - 1 min, (30 cycles); 72°C - 5 min (1 cycle). For caspase-3 plate: The primers for the wild type caspase-3 alleles were CTAAGTTAACCAAACTGAGCACCGA (sense) and ATGAATGAAGGCAGCATAGTACTCC (antisense). For the detection of the targeted allele, the same sense and the following antisense primers: GTCGATCCACTAGTTCTAGAGCGGC were used. Conditions were set as follows: 94°C - 2 min (1 cycle); 94°C-30 sec; 60°C - 1 min, 72°C - 1 min, (30 cycles); 72°C - 5 min (1 cycle). The expected fragment size for each of the products is as follows: pRb wildtype allele; 400 bps: pRb mutant allele; 400 bps: caspase-3 wildtype allele; 1733 bps: caspase-3 mutant allele; 1605 bps.


1
2
3
4
5
pRb
= 400 bps
W M W M W M W M W M
Casp-3
1.7K bps
= 1.6K bps

from the four representative genotypes in our study (pRb null, caspase-3 null, wild type, and double null for both caspase-3 and pRb). Figure 3-2 shows the striking increase in activated caspase-3 in pRb null embryos relative to wild type in developing neural tissue regions known to be undergoing high rates of apoptosis. This was true for both CNS and PNS structures. Both central neurons of the hindbrain region (figures 3-2C) and peripheral neurons of the dorsal root ganglion (DRG) (figure 3-2G) show high rates of expression of active caspase-3.

Healthy wild type littermates also exhibited some active caspase-3 expression in the PNS and CNS structures examined, which was comparable to the level of apoptotic cell death normally observed in these regions at this time (figure 3-2 A,E). This further demonstrates the involvement of caspase-3 in the naturally occurring cell death of neurons during development. Embryos carrying a null mutation for caspase-3 show no detectable immunostaining for active caspase-3, indicating the specificity of the antibody (figure 3-2 B,D,F,H).

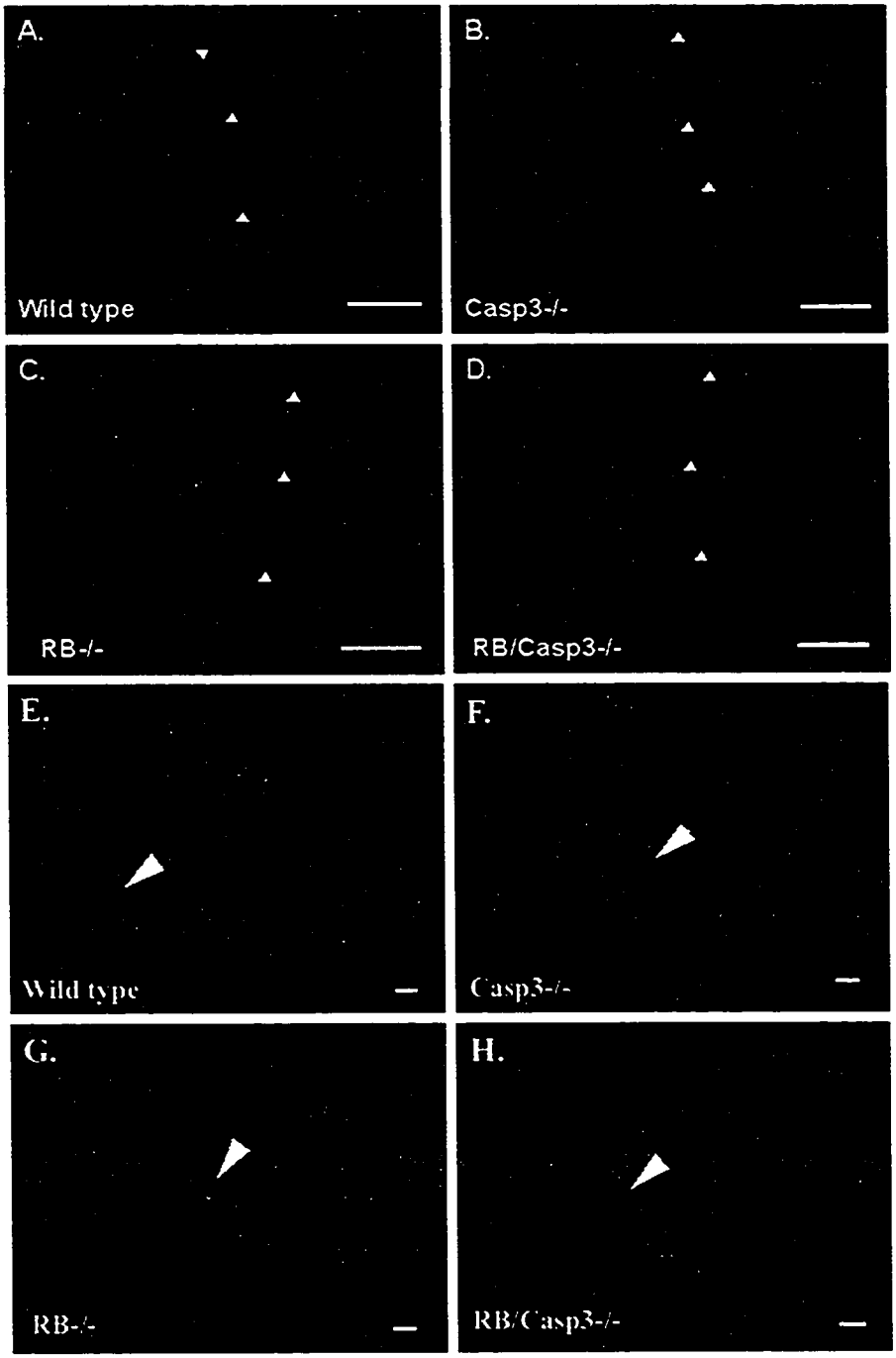
These results suggest that the widespread apoptosis in the nervous system of pRb nullizygous embryos might depend on the presence of active caspase-3 for its progression. We therefore next asked if apoptosis induced by pRb deficiency could progress in the absence of caspase-3 by generating double null embryos and examining their nervous systems for cell death.

CASPASE-3 DEFICIENCY RESCUES THE PERIPHERAL NERVOUS SYSTEM DEFECT IN PRB-DEFICIENT MICE.

Mice carrying a heterozygous null mutation for pRb were interbred with mice heterozygous for a targeted mutation for caspase-3. The progeny of these mice (pRb +/-: caspase-3 +/-) were viable and fertile and were then interbred to generate double null embryos

Figure 3-2. Caspase 3 is activated in the Rb deficient mouse nervous system.

Active caspase-3 immunostaining of DRG (A-D) and hindbrain (E-H) from E13.5 embryos. Extensive caspase-3 activation is seen throughout the developing nervous system of the pRb-/- embryo (C,G), is present at low levels in wild type tissue (A, E), and is undetectable in caspase-3 $^{-/-}$ (B,F) or pRb $^{-/-}$ / caspase-3 $^{-/-}$ (D,H) samples. Arrows indicate DRG, arrowheads point to the pons. Scale bars are 300 μm.


A.
Wild type
B.
Casp3-/-
C.
RB-/-
D.
RB/Casp3-/-
E.
Wild type
F.
Casp3-/-
G.
RB-/-
H.
RB/Casp3-/-

(pRb -/-: caspase-3 -/-). The frequencies of the genotypes obtained from these crosses roughly coincide with the expected frequencies obtained by Mendelian analysis (Table 1). Embryos were routinely examined at E13.5 just prior to the death of most pRb -/- embryos at a time when apoptosis was detectable in virtually all neuronal populations. Gross morphological comparison of pRb deficient and pRb/caspase-3 deficient embryos did not reveal any striking morphological differences other than those previously described for pRb or caspase-3 null embryos alone. Furthermore, caspase-3 deficiency did not rescue the haematopoietic defect manifest in pRb deficiency, as evident by the pale color due to massive apoptosis in the developing liver (see below). pRb/caspase-3-deficient embryos also exhibited the typical hunchback appearance due to swelling in the region of the fourth ventricle as previously described for the pRb knockout (Jacks et al., 1992; Lee et al., 1992; Clarke et al., 1992). Thus, gross examination of pRb/caspase-3 deficient embryos revealed embryonic defects typically seen by E13.5 in pRb deficiency alone and survival was not extended due to the haematopoietic failure in these embryos.

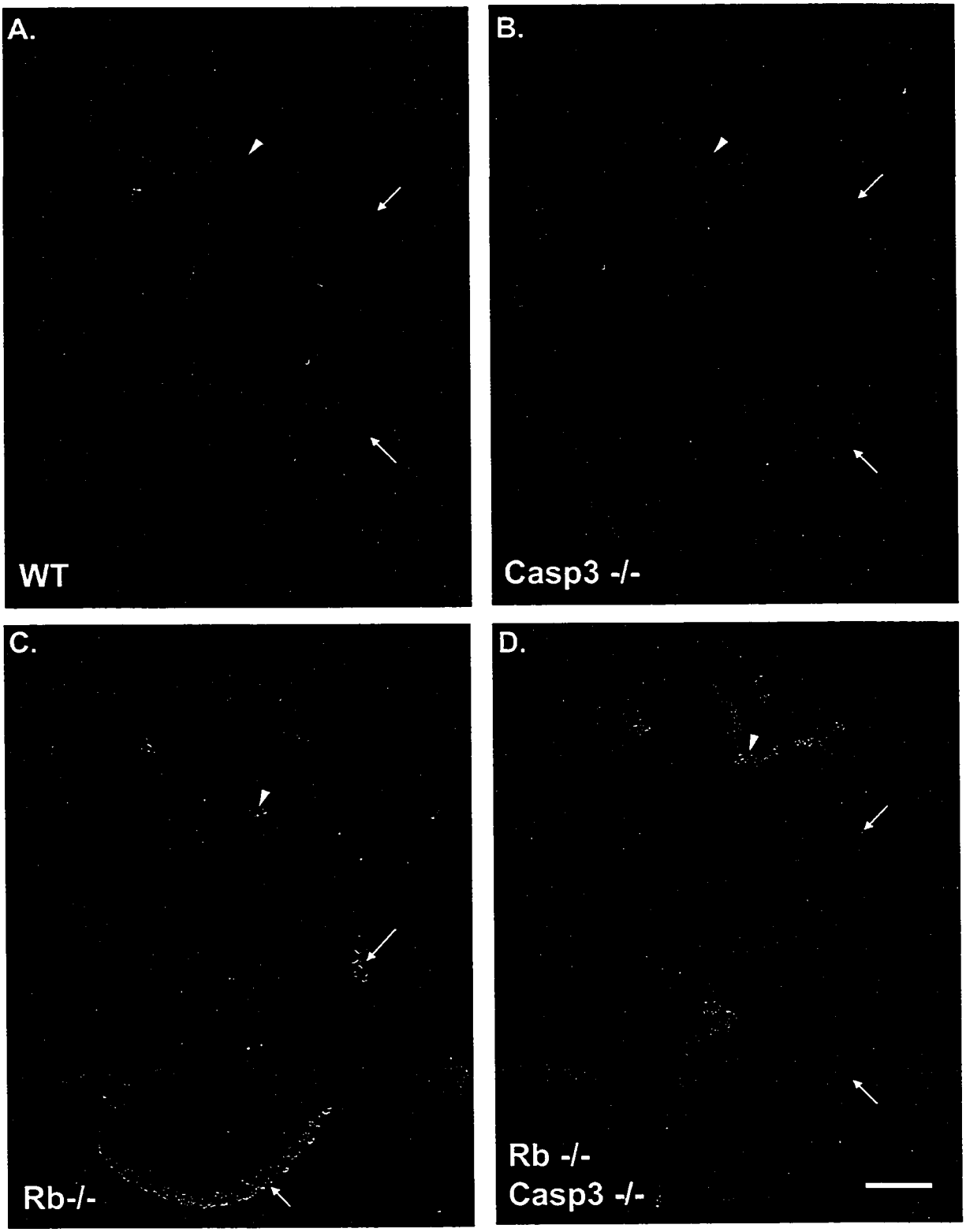
To determine whether any of the pRb-deficient cell populations were protected from apoptosis in the absence of caspase-3, littermates of the following genotypes were examined: [wild type], [caspase-3 -/-];[pRb -/-]; and [pRb -/-: caspase-3 -/-]. Embryos were removed at E13.5, and tissue was sectioned and stained with the TUNEL reagent for the detection of apoptotic cells (figure 3-3). Wild type and caspase-3-deficient embryos exhibited very low levels of TUNEL positive cells (figure 3-3 A, B), while pRb-deficient embryos exhibited intense TUNEL staining in the liver, CNS including the forebrain, hindbrain, spinal cord and the peripheral nervous system (figure 3-3C). This is the typical phenotype of pRb-deficient embryos (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). In contrast, examination of the pRb-null phenotype on a caspase-3-deficient background revealed a striking protection of a

Table 3-1. Genotype Frequency of Offspring from interbreeding Rb $^{+/-}$ / CASP3 $^{+/-}$ mice

Genotype of offspring (expected frequency, %)	No. of E13.5 Embryos	:quency (%)
RB $^{+/+}$ / CASP3 $^{+/+}$ (6.25)	11	6.9
RB $^{+/+}$ / CASP3 $^{+/-}$ (12.5)	20	12.5
RB $^{+/+}$ / CASP3 $^{-/-}$ (6.25)	10	6.3
RB $^{+/-}$ / CASP3 $^{+/+}$ (12.5)	21	13.1
RB $^{+/-}$ / CASP3 $^{+/-}$ (25)	46	28.7
RB $^{+/-}$ / CASP3 $^{-/-}$ (12.5)	18	11.2
RB $^{-/-}$ / CASP3 $^{+/+}$ (6.25)	5	3.1
RB $^{-/-}$ / CASP3 $^{+/-}$ (12.5)	22	13.7
RB $^{-/-}$ / CASP3 $^{-/-}$ (6.25)	7	4.4

E13.5 embryos (n=160) were examined.

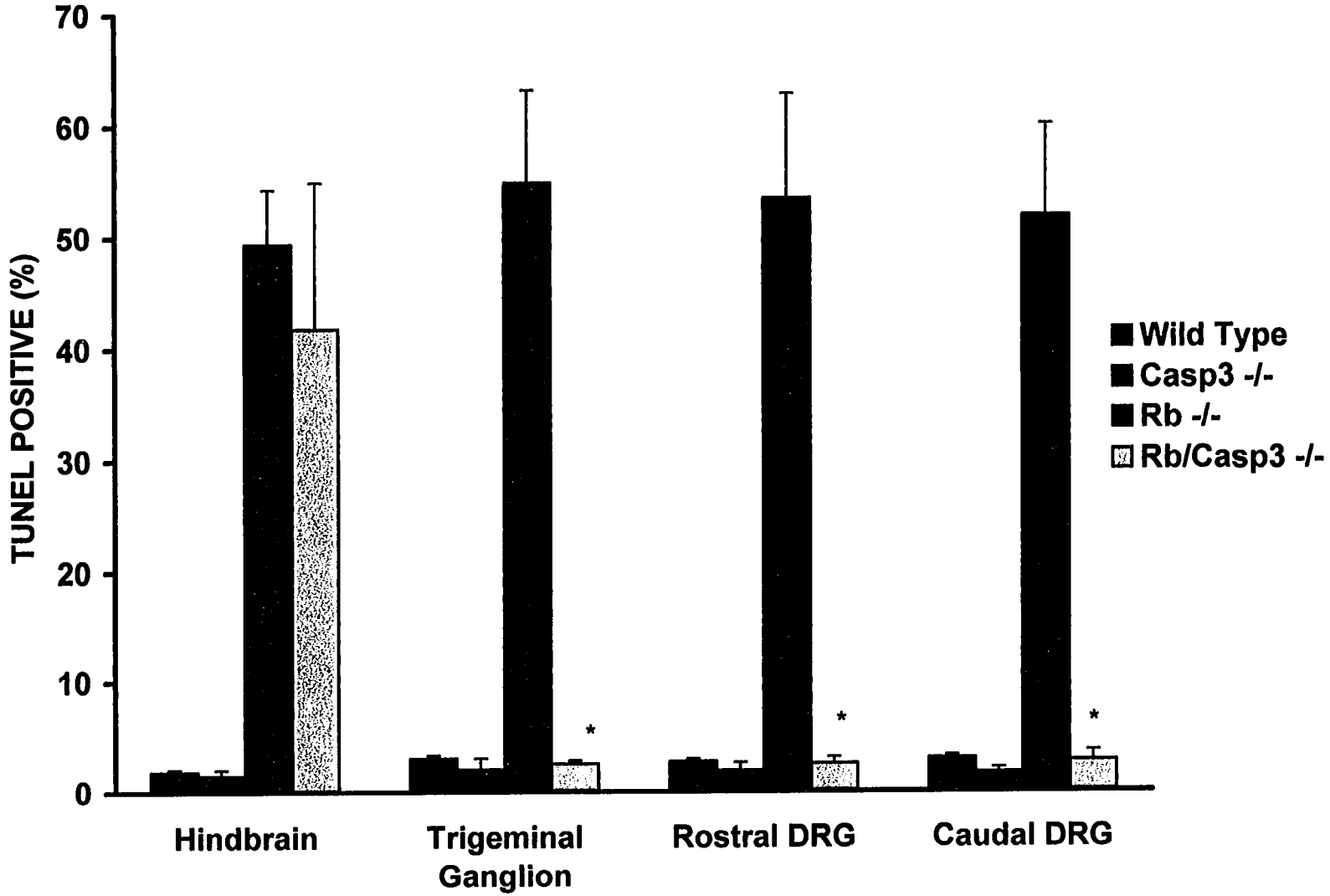
Figure 3-3. Caspase 3 deficiency protects neurons of the PNS but not the CNS or liver from apoptosis induced by pRb deficiency. Sections from (A) Wild type, (B) caspase-3 $^{-/-}$, (C) pRb$^{-/-}$, and (D) pRb $^{-/-}$ / caspase-3 $^{-/-}$ were stained for TUNEL and whole embryo images were captured. Arrows indicate rostral and caudal DRG. Scale bar, 1.5 mm.


A.
WT
B.
Casp3 -/-
C.
Rb-/-
D.
Rb -/-
Casp3 -/-

very specific cell population (figure 3-3 D). Consistent with the pale color of pRb/caspase-3 null embryos, there was intense apoptosis in the liver, indicating that the haematopoietic defect that causes the early death of the embryo was not rescued by the absence of caspase-3. Similarly, the CNS, including the hindbrain and forebrain structures, exhibited massive apoptosis at levels similar to pRb-deficient embryos. Thus, apoptosis in structures including the liver and the CNS could not be rescued by the absence of caspase-3. In contrast, neurons of the peripheral nervous system were significantly protected from cell death (as evident by the absence of TUNEL positive cells in the DRG) despite the advanced phenotype of this particular E13.5 pRb/caspase-3 double null embryo. Note that there are very few apoptotic cells in the DRG, yet the levels of apoptosis in the liver and hindbrain are striking in this particular embryo. While these experiments suggest that the absence of caspase-3 protects neurons of the peripheral nervous system from apoptosis, a closer examination of the structures was carried out.

To assess the progression of neuronal apoptosis in the absence of caspase-3, specific structures were examined in detail including: A. the forebrain at the medial aspect of the ganglionic eminence; B. the hindbrain at the caudal pons adjacent to the fourth ventricle; C. the trigeminal ganglia; and D. the rostral and caudal DRG. Sections were stained with a neuronal marker PGP 9.5 (protein gene product of 9.5 KDa) (Wilson et al., 1988) to identify the ganglia in the peripheral nervous system and then labeled with two markers for cell death including the TUNEL reagent for detection of apoptotic cells and Fluoro-jade (Schmued et al., 1997; Noraberg et al., 1999) for the detection of both apoptotic and necrotic cells. The number of TUNEL positive cells were counted in these specific regions and expressed as a percent of total cell count in the field. The cell counts for each region are summarized in figure 3-4.

Figure 3-4. Caspase-3 deficiency results in decreased TUNEL labelling in the PNS but not the CNS of the pRb -/- mouse embryo. TUNEL positive cells in the hindbrain, trigeminal ganglion, caudal and rostral DRG, were quantified by examination at 40X magnification. The mean is expressed as a percentage of TUNEL positive cells/total cell number as determined by Hoechst staining within a 100 square micron area for each group. Counts were obtained from 3 independent embryos (n=3) and error bars indicate S.E. * $P < 0.001$.


70
60
50
40
30
20
10
0
TUNEL POSITIVE (%)
Wild Type
Casp3 -/-
Rb -/-
Rb/Casp3 -/-
*
*
*
Hindbrain
Trigeminal Ganglion
Rostral DRG
Caudal DRG

While there were few TUNEL positive cells in wild type and caspase-3 deficient forebrains (figure 3-5 A-D), pRb-deficient brains exhibited many apoptotic cells and this enhanced rate of apoptosis was not affected by the absence of caspase-3 (figure 3-5 E-H). Similarly, the hindbrain, in the region of the caudal pons adjacent to the fourth ventricle, pRb-deficient neurons revealed an accelerated rate of cell death as evident by the high levels of TUNEL positive cells at a rate of 50 ± 5% (figure 3-5 E,F). pRb/caspase-3 double knock out mice revealed a high percentage of TUNEL positive cells similar to those lacking pRb alone including the large apoptotic clusters found throughout this region (figure 3-5 I-P). These results demonstrate that pRb deficient CNS neurons lacking caspase-3 are not protected from programmed cell death and that these cells exhibit DNA fragmentation (as detected by TUNEL labeling) that is typical of apoptosis.

While cells of the CNS exhibited widespread cell death despite the absence of caspase-3, neurons of the peripheral nervous system appeared to be protected from apoptosis. In the absence of pRb, neurons of the trigeminal ganglia exhibit massive apoptosis as evident by the high percentage of TUNEL positive cells of 55 ± 8% (figure 3-6 E,F). In contrast, when pRb deficient neurons were examined on a caspase-3 null background the level of neuronal apoptosis was dramatically reduced to 2.5 ± 0.3% similar to wild type levels (figure 3-6 G, H). Thus, neurons of the trigeminal appear to be protected from apoptosis in the absence of caspase-3.

Massive cell death in the developing DRG is one of the hallmarks of the pRb phenotype. In the absence of both pRb and caspase-3 a dramatic protection from apoptosis in the DRG was found (figure 3-7 M-P) such that these ganglia remained intact with very few apoptotic cells. Specifically, in the absence of pRb alone, 53 ± 9 % of the cells in the rostral DRG are

Figure 3-5. Caspase 3 deficiency does not rescue apoptosis in the CNS of pRb deficient embryos. Frozen sections of (A,B,I,J) Wild type, (C,D,K,L) caspase-$3^{-/-}$, (E,F,M,N) $pRb^{-/-}$, and (G,H,O,P) pRb $^{-/-}$ / caspase-3 $^{-/-}$ mouse E13.5 brain were stained for TUNEL (B,J,D,L,F,N,H,P) and counterstained with PGP 9.5 (A,I,C,K,E,M,G,O) to view neuronal cell bodies and axons. Scale bar, 150 μm.

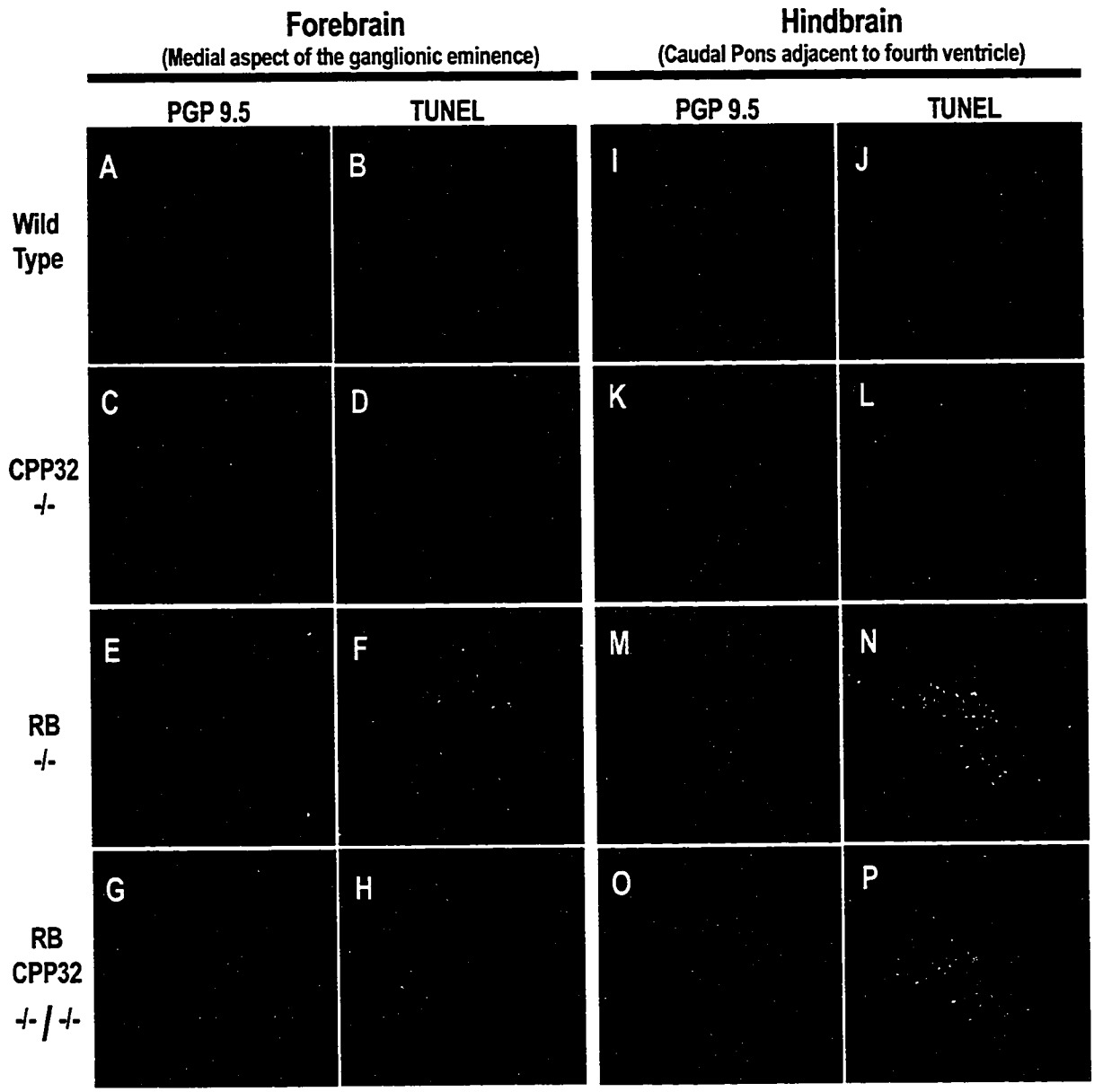
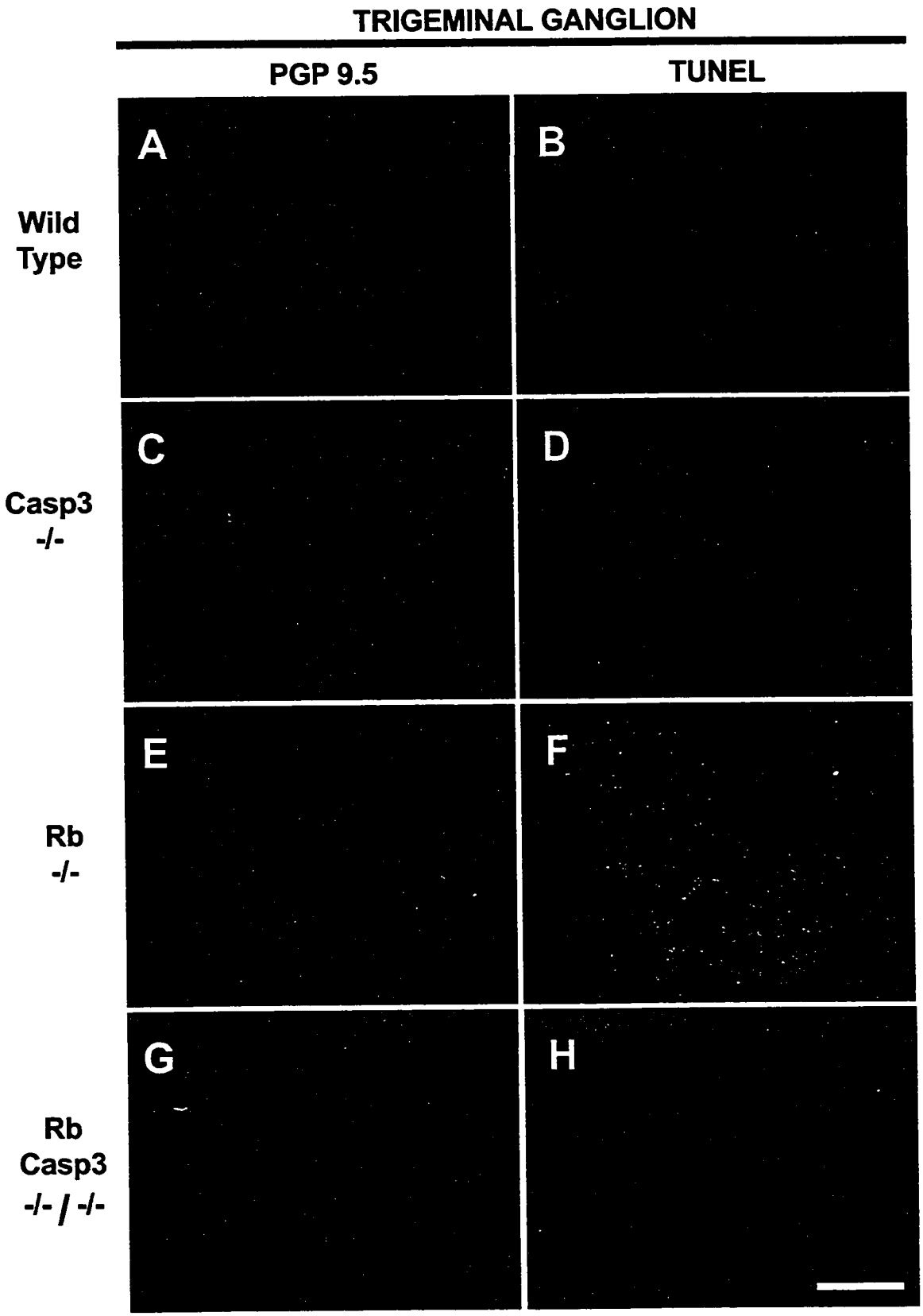

Forebrain
(Medial aspect of the ganglionic eminence)
Hindbrain
(Caudal Pons adjacent to fourth ventricle)
PGP 9.5
TUNEL
PGP 9.5
TUNEL
Wild Type
CPP32 -/-
RB -/-
RB CPP32 -/-/-
A
B
I
J
C
D
K
L
E
F
M
N
G
H
O
P

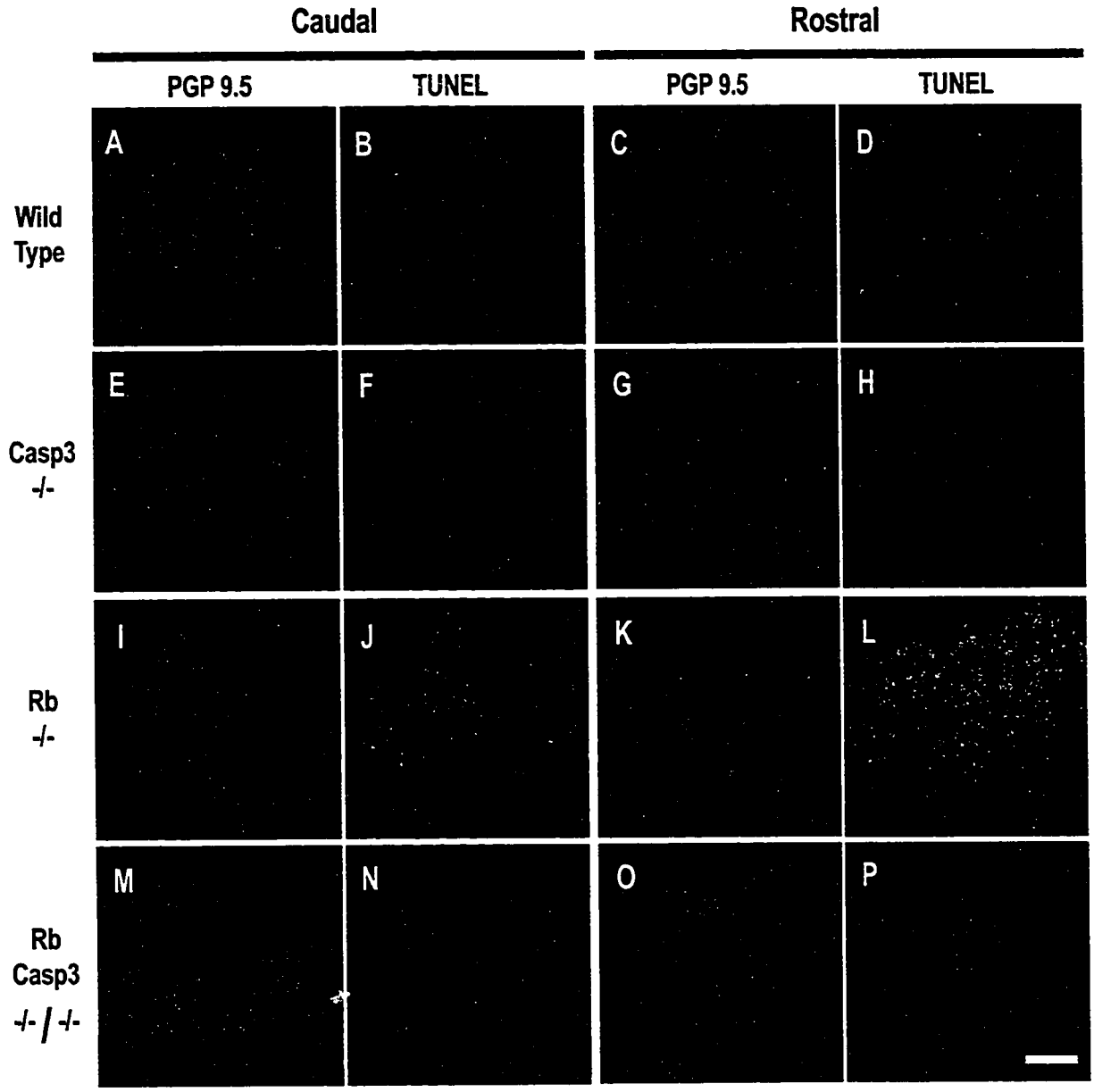
Figure 3-6. Caspase-3 is required for apoptosis in neurons of the pRb deficient trigeminal ganglia. Frozen sections from (A,B) Wild type, (C,D) caspase-3 $^{-/-}$, (E,F) pRb$^{-/-}$, and (G,H) pRb $^{-/-}$ / caspase-3 $^{-/-}$ were stained for TUNEL and counterstained with PGP 9.5 to view neuronal cell bodies and axons. Scale bar, 160 μm.


TRIGEMINAL GANGLION
PGP 9.5
TUNEL
A
B
C
D
E
F
G
H
Wild Type
Casp3 -/-
Rb -/-
Rb Casp3 -/- / -/-

TUNEL positive while caudal DRG were found to be 52 ± 8% TUNEL positive (figure 3-7 I-L). When both pRb and caspase-3 are absent the rate of neuronal cell death is dramatically reduced to 2.7 ± 0.9% in caudal and 2.5 ± 0.6% in rostral DRG (figure 3-7 M-P). These findings suggest that caspase-3 deficient DRG are protected from neuronal cell death induced by the loss of pRb. Thus, histological examination and cell counting within specific regions of the pRb-deficient embryo reveals high rates of cell death throughout the entire nervous system. In contrast, mice deficient for both caspase-3 and pRb exhibit massive cell death in the liver and the CNS, however, apoptosis in the PNS appears to be abated, exhibiting rates similar to those observed in wild type littermates.

While the peripheral nervous system including the DRG and the trigeminal ganglia exhibit TUNEL staining at levels similar to wild type, one possibility is that these cells may nevertheless die by a more necrotic mechanism due to the absence of caspase-3 and this caspase-independent mechanism may not be readily detectable by TUNEL staining. To rule out this possibility, embryos were stained with Fluoro-jade for the detection of all dying cells, including necrotic as well as apoptotic death pathways (Schmued et al., 1997; Noraberg et al., 1999). To confirm protection from cell death in the peripheral nervous system, sections from wild type, pRb -/-, caspase-3 -/- and pRb/caspase-3 double null were double-stained for caspase activation and fluoro-jade to identify dying cells. Caspase activity was detected using a polyclonal antibody directed against the neoepitope, designated ΔC-APP, which is generated by caspase-mediated cleavage of amyloid precursor protein (APP) and has been found in neurons undergoing apoptosis by multiple death stimuli (Gervais et al., 1999). Sections were then double labeled with fluoro-jade to identify dying cells. The results in figure 3-8 demonstrate that pRb deficient DRG exhibit abundant caspase activity coincident with striking positive staining for Fluoro-jade suggesting that the majority of neurons of pRb-deficient

Figure 3-7. pRb deficient DRG's do not undergo apoptosis in the absence of caspase-3. Frozen sections containing caudal and rostral DRGs from (A-D) Wild type. (E-H) caspase-3 $^{-/-}$, (I-L) pRb$^{-/-}$, and (M-P) pRb $^{-/-}$ / caspase-3 $^{-/-}$ were stained for TUNEL and counterstained with PGP 9.5 to view neuronal cell bodies and axons. Scale bar, 150 μm.


Caudal
Rostral
PGP 9.5
TUNEL
PGP 9.5
TUNEL
Wild Type
Casp3 -/-
Rb -/-
Rb Casp3 -/- / -/-
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

DRG are undergoing cell death (figure 3-8 A,B). In contrast, DRG from pRb/caspase-3 double null embryos exhibit no detectable levels of ΔC-APP and little positive staining for Fluoro-jade (figure.3-8 C, D). These staining levels are equivalent to that detected in healthy wild type or caspase-3 deficient littermates. Thus, consistent with the results from TUNEL staining, pRb deficient DRG appear to be protected from apoptosis when caspase-3 is absent.

CASPASE-3 DEFICIENCY RESTORES PAN-NEURONAL GENE EXPRESSION IN DRG OF PRB-NULL EMBRYOS

Although our data so far suggest that neurons of the peripheral nervous system are protected from apoptosis we questioned whether the surviving cells expressed neuronal differentiation markers. Previous studies indicated that pRb-deficient DRG exhibit reduced expression of neuronal genes such as β-III tubulin and Trk A (Lee et al., 1994). We therefore examined the expression of these markers to determine whether the absence of caspase-3 could restore panneuronal gene expression in pRb-deficient peripheral neurons. The antibody directed against the nerve growth factor receptor, Trk A, stained peripheral neurons in wild type and caspase-3 deficient embryos (figure 3-9A, B). As previously described pRb-deficient DRG exhibit very low levels of Trk A immunostaining (figure 3-9 C) relative to wild type littermates (figure 3-9 A). In contrast, Trk A immunoreactivity was restored to wild type levels in pRb/caspase-3 double null embryos (figure 3-9 D). Similar results were obtained for β-III tubulin, where by pRb-deficient embryos exhibited very little β-III tubulin immunoreactivity in the DRG relative to wild type littermates as described previously (Lee et al., 1994). Consistent with results obtained with Trk A, β-III tubulin immunoreactivity was restored to wild type

Figure 3-8. Fluoro-Jade labelling demonstrates caspase-3 requirement for neuronal cell death in the pRb-deficient peripheral nervous system. C-APP (A,C) and Fluoro-jade (B,D) double labelling of degenerating DRG neurons in pRb-/- (A,B) and pRb/caspase-3 double knockout embryos (C,D). Arrows indicate DRG. Scale bar, 260 μm.

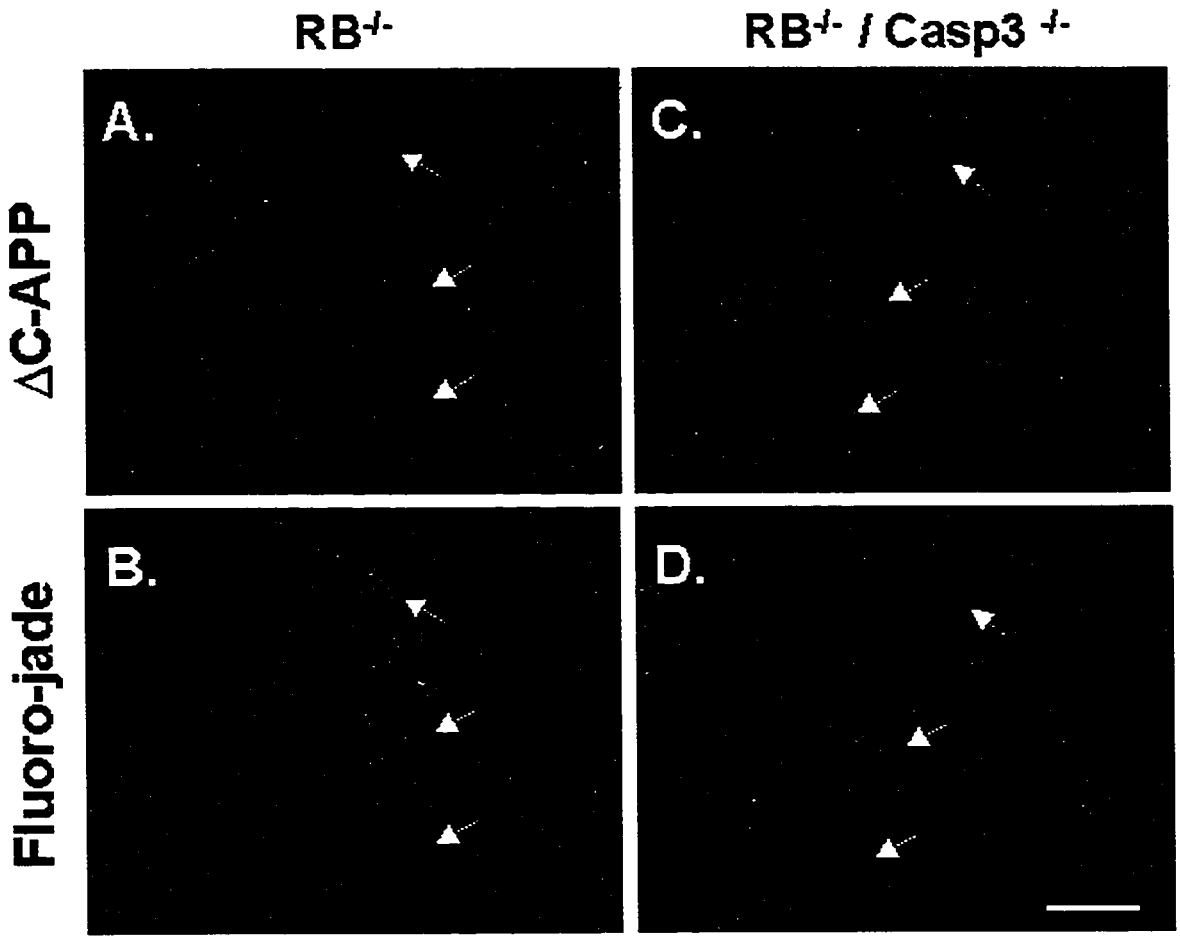
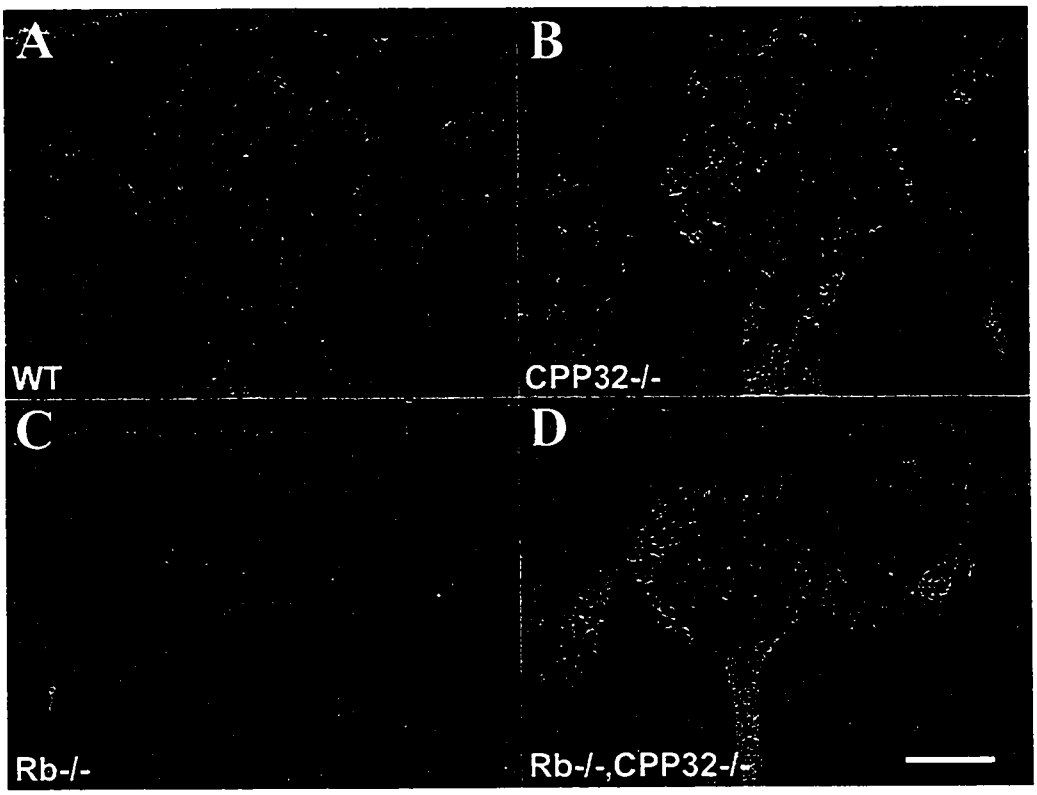
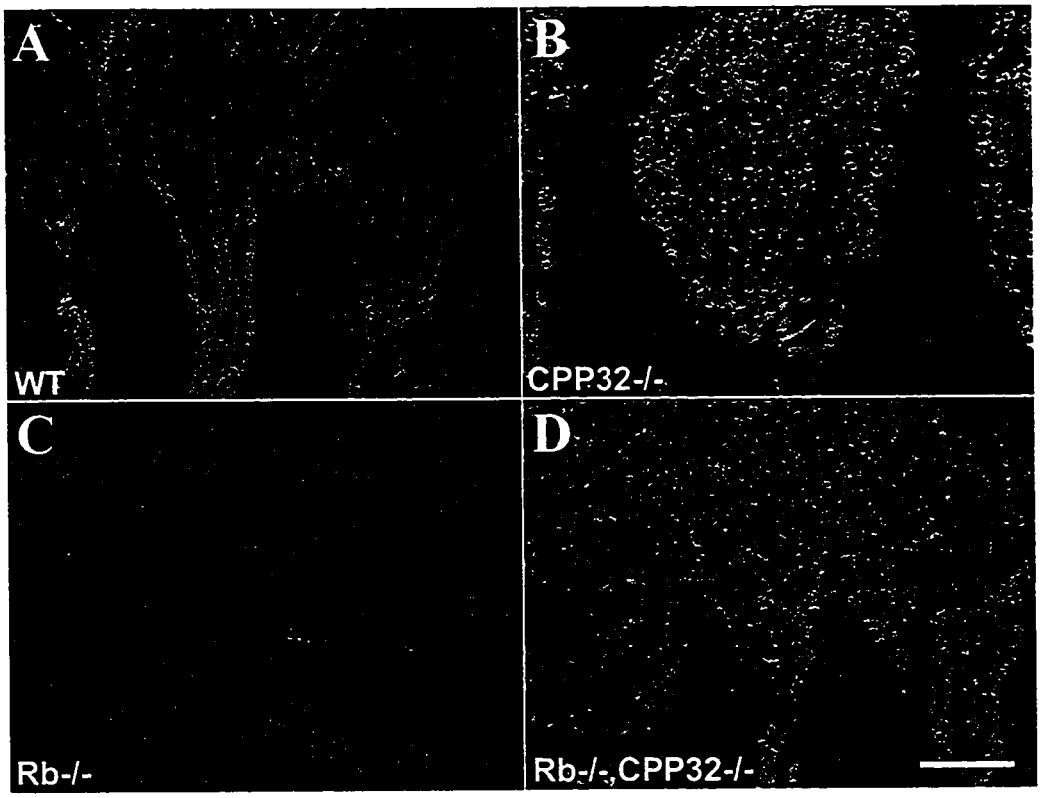

RB-/-
RB-/- / Casp3 -/-
ΔC-APP
Fluoro-jade
A.
B.
C.
D.

Figure 3-9. pRb-/-:caspase-3-/- compound mutants exhibit normal levels of Trk A and Tuj-1 expression. ***Top Panel:*** Trk A is highly expressed in wild type (Top: A), and caspase-3 -/- (Top: B) DRG by E13.5. This staining is dramatically reduced in pRb deficient DRG neurons (Top: C), however Trk A staining is restored to wild type levels when caspase-3 is also absent (pRb-/-,caspase-3 -/-) (Top: D). ***Lower Panel:*** Tuj-1 is also highly expressed in wild type (Lower: A), and caspase-3 -/- (Lower: B) DRG by E13.5. This staining is dramatically reduced in pRb deficient DRG neurons (Lower: C), however Tuj-1 staining is restored to wild type levels when caspase-3 is also absent (pRb-/-,caspase-3 -/-) (Lower: D). Scale bar is 150 μm.

TrkA



Tuj-1



levels when both pRb and caspase-3 were absent (figure 3-9). Thus, examination of pan-neuronal gene expression indicates that the absence of caspase-3 in the pRb-deficient peripheral nervous system not only reduces apoptosis but also allows cells to continue to differentiate, consistent with the interpretation that caspase-3 deficiency rescues the apoptotic phenotype in the pRb deficient peripheral nervous system.

pRb/Caspase-3 deficient CNS neurons exhibit compensatory caspase activity

Since our data demonstrate that the mechanisms regulating neuronal cell death in the CNS are significantly different from those of the PNS we sought to determine the molecular mechanisms involved. We questioned whether there may be any difference in compensatory caspase activation between the different regions of the developing nervous system by examining the appearance of a caspase cleavage product. We chose the ΔC-APP cleavage product of Alzheimer's amyloid-β precursor protein (APP). APP is directly and efficiently cleaved by caspases during apoptosis and it has been previously determined that caspase-3 is the predominant caspase involved in generating ΔC-APP (Gervais et al., 1999). Interest in APP has arisen from its implication in the pathogenesis of Alzheimers disease. Hippocampal neurons are known to die by apoptosis during the early stages of Alzheimers disease (Smale et al., 1995; Su et al., 1997). Accumulation of the caspase cleavage product of APP with amyloid-β peptide (Aβ) at senile plaques or near sites of neuronal degeneration is suspected to contribute to the susceptibility of these neurons to premature death (Gervais et al., 1999).

Due to the very limiting amounts of CNS and PNS tissue available from pRb/caspase-3 compound mutants, APP cleavage was monitored immunohistochemically (figure 3-10 & 3-

11). As described above, a polyclonal antibody was used for the detection of the neoepitope, designated as ΔC-APP, which is generated by caspase-mediated cleavage of amyloid precursor protein (APP) and has been detected in neurons undergoing apoptosis by multiple death stimuli (Gervais et al., 1999). Wild type embryos exhibited low levels of the product ΔC-APP in the developing nervous system consistent with the ongoing naturally occurring cell death that occurs at this time (figure 3-10 A, 3-11 A, E). Slightly less cleavage of ΔC-APP was detected in the caspase-3 null embryos consistent with the impairment of developmental cell death previously reported (Kuida et al., 1996) (figure.3-10 B, 3-11 B, F). However, it should be noted that the cleavage product of APP was also detected when caspase-3 was absent. This indicates that APP can be cleaved by other caspases in the absence of caspase-3. pRb deficient embryos showed extensive cleavage of APP throughout the developing nervous system (figure 3-10 C; 3-11 C,G). In the pRb/caspase-3 compound null embryos, very little cleavage product was detected in the PNS, suggesting that there was no detectable compensatory caspase activation to facilitate the cleavage of APP (figure 3-10 D, 3-11 H). In contrast, neurons of the CNS exhibited intense positive staining for ΔC-APP (figure 3-10 D, 3-11 D), consistent with the interpretation that other caspase activity was upregulated to compensate for the absence of caspase-3. This compensatory caspase activity was not detected in the PNS. Thus, the rescue of the peripheral nervous system defect in pRb null mice may be, at least in part, accounted for by the dependence on caspase-3 to execute neuronal apoptosis in this tissue.

Figure 3-10. Cleavage of the caspase substrate, APP, in E13.5 mouse embryos. Immunodetection of ΔC-APP in (A) Wild type, (B) caspase-3 $^{-/-}$, (C) pRb$^{-/-}$, and (D) pRb $^{-/-}$ / caspase-3 $^{-/-}$ E13.5 mouse embryos. The caspase-3 cleavage product, ΔC-APP, is detected in all neuronal populations undergoing apoptosis in the pRb null mouse (C). ΔC-APP is detected in the CNS but is significantly reduced in the PNS of pRb $^{-/-}$ / caspase-3 $^{-/-}$ compound mutant embryos. Arrows indicate DRG. Scale bar, 1.5 mm.

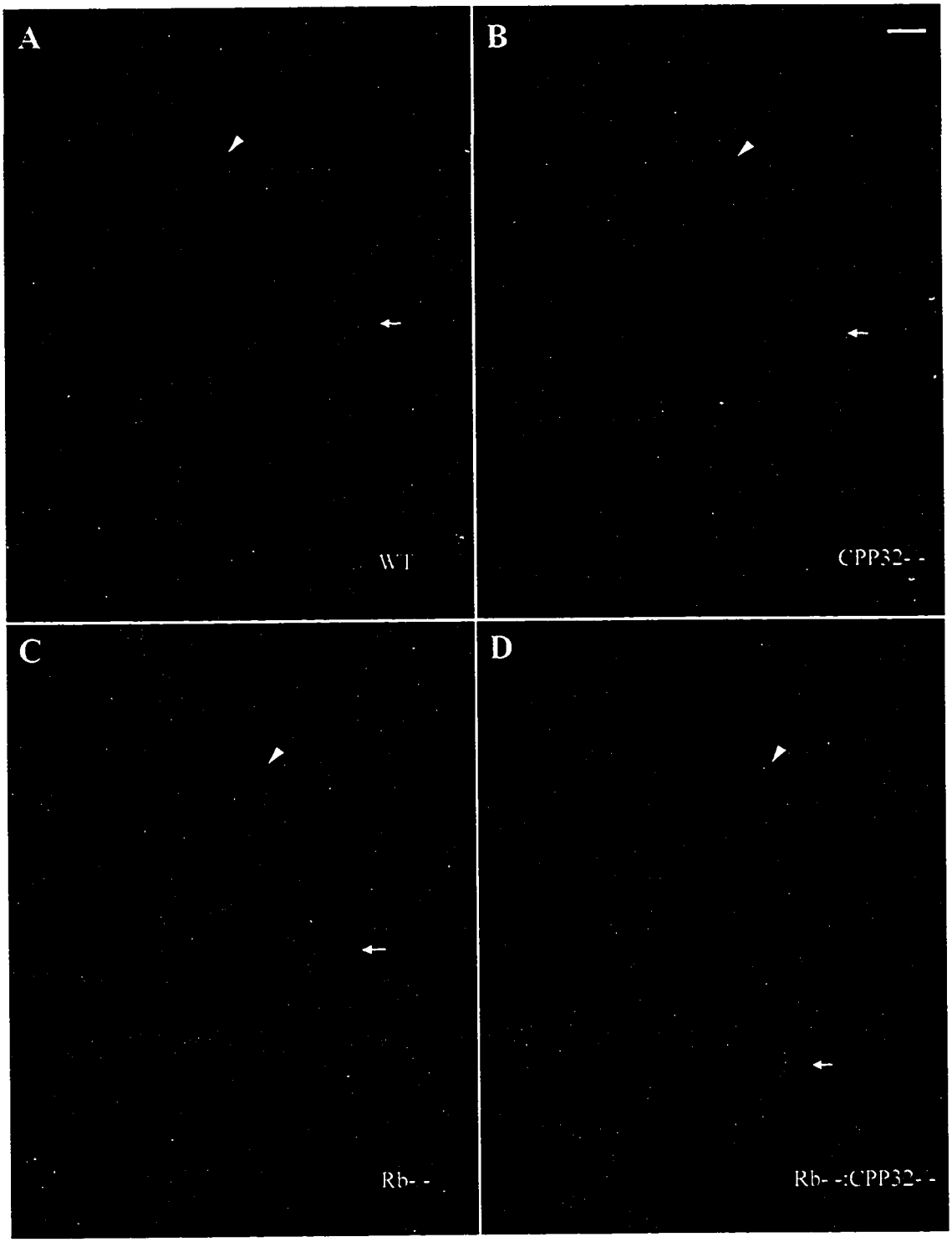
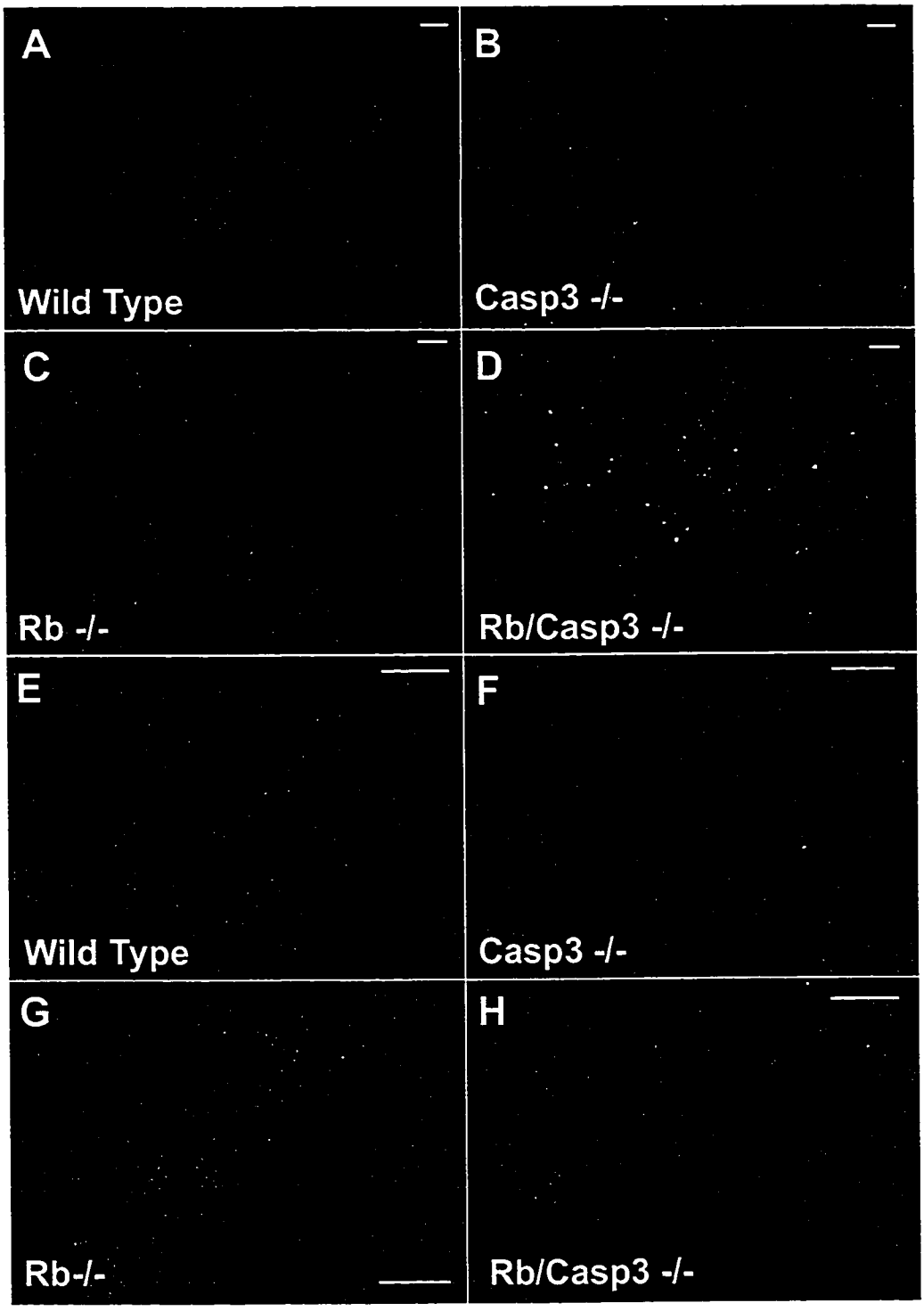

A
WT
B
CPP32-/-
C
Rb-/-
D
Rb-/-:CPP32-/-

Figure 3-11. APP is cleaved in the CNS but not PNS neurons of pRb/caspase-3 double null mutants. Immunodetection of cleaved caspase substrate, APP, in hindbrain (A-D) and DRG (E-H) from E13.5 embryos. (A,E) Wild type, (B,F) caspase-3 $^{-/-}$, (C,G) pRb$^{-/-}$, and (D,H) pRb $^{-/-}$ / caspase-3 -/-. Extensive APP is seen throughout the developing nervous system of the pRb-/- embryo, including hindbrain(C) and DRG (G), and is present at low levels in corresponding wild type tissue (A, E). ΔC-APP is abundant in pRb $^{-/-}$ / caspase-3 -/- hindbrain (D) showing compensatory caspase activation but is undetectable above control levels in pRb $^{-/-}$ / caspase-3 $^{-/-}$ DRG (H) showing the lack of compensatory caspase-mediated ΔC-APP cleavage. Scale bars are 300 μm.


A
Wild Type
B
Casp3 -/-
C
Rb -/-
D
Rb/Casp3 -/-
E
Wild Type
F
Casp3 -/-
G
Rb-/-
H
Rb/Casp3 -/-

CHAPTER 4

GENERAL DISCUSSION

The results of these studies support a number of conclusions. First, caspase-3 becomes activated in neuronal populations undergoing apoptosis in pRb-deficient embryos. Second, caspase-3 deficiency does not extend the life span of the pRb-null embryo, because double null mutants exhibit high rates of apoptosis in the developing liver and the CNS. Third, loss of caspase-3 results in the protection of very specific neuronal populations; neurons of the trigeminal ganglion and of the dorsal root ganglia are protected from apoptosis induced by pRb disruption. Finally, the presence of cleaved APP in the CNS of double null mutants suggests that neurons of the CNS are capable of activating other caspases in the absence of caspase-3, whereas no such activity is observed in PNS structures. Thus, neurons of the peripheral nervous system may be dependent on caspase-3 for the execution of apoptosis; a fact that suggests a possible target for neuroprotection of PNS cells following injury.

Although active caspase-3 was shown to be present in the pRb-deficient mouse CNS and PNS by immunostaining (figure 3-1), our results suggest that, except in the case of very specific populations of PNS neurons, apoptosis can proceed without functional caspase-3. This suggests that PNS cells are incapable of carrying out their apoptotic programs in the absence of caspase-3 or that most other neuronal populations can compensate for its absence by, for example, upregulation of another effector caspase.

Interestingly, the life span of the pRB/caspase-3 double null embryos is not extended relative to the life span of pRb-null embryos. Thus, loss of caspase-3 results in the protection

of specific neuronal populations from apoptosis induced by pRb disruption but does not protect the embryo from lethality. This again suggests that the absence of caspase-3 does not prevent all cell death invoked by pRb deficiency. Whether the lethality is caused by the high rate of apoptosis in the CNS or the haematopoietic abnormalities is yet to be determined. Given that the double null embryos were significantly whiter in appearance than their littermates at E14, the extensive damage to the developing liver and the consequent haematopoietic failure is likely a significant contribution to the lethality.

The pRb-deficient embryo dies between E13.5 to E14.5 exhibiting neurological as well as haematopoietic defects (Jacks et al., 1992; Lee et al., 1992;Clarke et al., 1992). The widespread apoptosis in virtually all neuronal populations examined provides an ideal model for the study of apoptosis in the developing nervous system. Presently, the signal triggering apoptosis of neurons in pRb-null embryos remains poorly understood. While cell cycle deregulation can cause apoptosis *in vivo*, presumably as a result of enhanced proliferation, such a defect does not cause neuronal cell death *in vitro* where growth factors are present in unlimited supply (Slack et al., 1998; Callaghan et al., 1999). Thus, the precise trigger evoking neuronal cell death *in vivo* in response to pRb disruption remains unclear. Enhanced proliferation of neural progenitors causing an intrinsic delay in differentiation may lead to cell death *in vivo*, where differentiation cues are precisely timed and growth factors are limiting. The limitation of trophic support in the pRb-deficient nervous system has been previously suggested (Maandag, et al., 1994; Lee et al., 1994; Williams et al., 1994) thus apoptosis in pRb null mice may represent a model for naturally occurring cell death during development.

The molecular mechanisms evoking cell death in the pRb-deficient nervous system appears to be quite complex and depends on the neuronal cell type being examined. p53 has been implicated as the molecular switch triggering the demise of pRb-deficient cells in the

developing lens (Morgenbesser et al., 1994), however the mechanisms regulating neuronal apoptosis appear to be less clear. The generation of p53/pRb double null embryos did not result in global neuroprotection nor was the life span of the embryos increased. While there was significant protection in the CNS, no such protection was observed in the peripheral nervous system (MacLeod et al., 1998). These studies suggest that the molecular mechanisms regulating neuronal apoptosis in the peripheral nervous system are clearly distinct from those of the CNS and are presently unknown.

Interestingly, a recent study examining the role of caspase-3 in the demise of Bcl-X_L deficient neurons has revealed significant protection in all neuronal populations including the CNS (Roth et al., 2000). The Bcl-X_L null phenotype is strikingly similar to that of the pRb knockout, exhibiting widespread neuronal apoptosis and early embryonic lethality due to failed liver haematopoiesis. However, at E12.5 caspase-3 deficiency appears to protect all neuronal populations, even the large apoptotic clusters appearing in the Bcl-X_L-deficient CNS were absent when caspase-3 was also disrupted. In contrast, in the present study, the absence of caspase-3 resulted in the protection of only PNS neurons, not those of the CNS. This suggests that although the Bcl-X_L and pRb-null mice exhibit similar phenotypes, the molecular pathways induced by pRb deficiency in the CNS may differ significantly from those triggered by the absence of Bcl-X_L.

Due to the importance of caspase-3 in regulating neuronal cell death in development we asked whether it plays a pivotal role in neuronal apoptosis in response to pRb deficiency. Our results demonstrate that the majority of cell populations, in particular, the liver and neurons of the CNS, do not require caspase-3 to execute apoptosis or even DNA fragmentation (figure 3-4). Previous studies have indicated that p53 is a key determinant in neuronal apoptosis occurring in the CNS (MacLeod et al., 1996). Studies in our lab have previously shown that

cells induced to die by a p53-dependent mechanism or by direct upregulation of p53 exhibit high levels of caspase-3 activation. While caspase-3 appears to be a key component of the p53-mediated cell death pathway, its absence delays but does not protect such neurons from apoptosis (Cregan et al., 1999; Kerameris et al., 2000). Similar results from other groups have demonstrated that despite the involvement of caspases in neuronal cell death, pharmacological blockers of caspase activity could not protect CNS neurons from apoptosis (Miller et al., 1997; Johnson et al., 1999). One possibility is that CNS neurons may be capable of activating compensatory caspase activity. Recent studies examining Fas-mediated apoptosis have demonstrated alternative caspase activation in caspase-3-null hepatocytes that is not normally found in wild type cells (Zheng et al., 2000). While there was no difference in levels of caspase transcripts, biochemical analysis revealed that caspase-3 null hepatocytes activate caspase 6 and 7 preceding cell death. Thus it is likely that the lack of protection by caspase-3 disruption in hepatocytes may be accounted for by this compensatory caspase activation.

These results again indicate that the molecular determinants that regulate apoptosis in the peripheral nervous system are distinct from those of the CNS. Unlike CNS neurons, neurons of the DRG and trigeminal ganglion in pRb-nullizygous mice are protected from apoptosis when caspase-3 is also inactivated by homologous recombination. While CNS neurons continue to undergo apoptosis, neurons of the PNS continue their development, as evident by the continued expression of TrkA and βIII-tubulin, until the death of the embryos due to haematopoeitic failure. These data are consistent with the interpretation that PNS neurons are protected from apoptosis in the absence of caspase-3.

Since compensatory caspase activation has been demonstrated in hepatocytes (Zheng et al., 2000), we asked whether this might account for the differences in neuroprotection observed in the pRb/caspase-3 deficient nervous system. Due to the minute amount of

available tissue from pRb/caspase-3 double null nervous system, caspase activation was examined immunohistochemically using an antibody directed against the caspase cleaved substrate ΔC-APP. From this we determined that, in the absence of caspase-3, pRb-deficient neurons of the CNS exhibit extensive cleavage of the caspase substrate while no such cleavage product was found in peripheral neurons. This suggests that CNS neurons can induce compensatory caspase activation while neurons of the periphery lack this activity. The difference in the ability of these different populations to induce caspase activation may account for the observed protection in dorsal root and trigeminal neurons. Taken together, our results indicate that PNS neurons may be dependent on caspase-3 activity to execute neuronal cell death, implicating caspase-3 as a key target for therapeutic intervention in the treatment of injured peripheral neurons.

The focus of the upcoming research will attempt to further answer two primary questions. The first involves an undertaking to determine which molecule is compensating for the absence of caspase-3 in the CNS. Is it another caspase and if so, which one(s)? The absence of the caspase-generated APP fragment in the dorsal roots of double null embryos is not surprising given the absence of caspase-3. However, the significant cleavage in CNS structures suggests an alternative cleavage pathway and alternative apoptotic machinery that can more readily be utilized in the CNS than the PNS. We have already begun by conducting western analysis of CNS from the four representative genotypes in our study for the presence of activated species of a variety of other caspases including caspase-2, -7, -6, -8 and -9. Preliminary results indicate the presence of caspases-2, -6, and -7. Caspase-2 exhibited the typical intermediate N-terminal cleavage product of approximately 35-38 kDa seen after activation specifically in the Rb/Caspase-3 double knockout. This suggests that caspase-2 may become activated and cleave substrates common to caspase-3 to execute apoptosis in CNS

neurons. Indeed, previous studies have shown that the caspases-2, -3, and -7 have similar activities and are capable of cleaving common substrates such as DEVD (Thornberry et al., 1997). Also, caspase-2 and -3 activation was found in cortical neurons induced to die by camptothecin, a p53-dependent process (Stefanis et al., 1999; Keramaris et al., 2000).

The second question involves determining the extent to which pRb-null DRG actually progress in their differentiation in the absence of caspase-3. We have begun to answer this question with the two differentiation markers (TrkA and Tuj1). Although, these results indicate that these two cell type specific markers are more prevalent in the double knockout versus the pRb, the gross morphology of the double knockout DRG seldom resemble wild-type structure, hence, it will be interesting to determine the level of protection. Are these neurons restored in functionality as well? One possible experiment would involve culturing DRG neurons from the representative embryos in our study for further analysis however, at less than 15 d.p.c., these embryos are far too underdeveloped to make this a possibility. Although expensive, time-consuming, and difficult, generating cell type specific conditional knockouts using the cre/lox delivery system to functionally ablate pRb and/or caspase-3 from the PNS or CNS at predetermined time points might prove fruitful towards this end.

CONCLUSIONS

Apoptosis is involved in many homeostatic processes in multicellular organisms, both during development and in the mature being. Dysregulation of apoptosis can lead to pathological states involving cell accumulation (such as cancer) or cell loss (such as neurodegeneration). Major efforts are underway to modulate the processes involved in apoptotic cell death in an attempt to treat a wide range of human diseases. Pro-apoptotic

interventions have already been employed in cancer therepy (Bcl-2 expression and functional modulation) (reviewed in Konopleva et al., 1999). Other groups are employing anti-apoptotic strategies aimed at reducing tissue damage in autoimmune diseases, stroke, myocardial infarction and hepatitis. The therapeutic opportunities that await knowledge gained from these mechanistic studies are astounding.

It has been estimated that half of the neurons produced during embryogenesis die via this apoptosis before reaching adulthood resulting in the optimization of synaptic connections (Oppenheim, 1991, Burek & Oppenheim, 1999). Understanding how neurons are programmed to die is important not only as a basic biological process, but also because of the potential for elucidating novel therapeutic targets for virtually all neurodegenerative disease (Nijhawan, et. al., 2000). Although it is not definitively determined whether apoptotic cell death is the primary cause of loss of function in a variety of neurological disorders, it is likely that intervening in the cascade of programmed cell death, or even totally halting apoptosis, could have a significant impact on the symptoms of some of the most debilitating diseases known. Insight into the pathways that determine whether a cell will live or die holds great promise of increasing our understanding of human disease, development and health.

The results of these studies reveal that the requirement for caspase-3 in apoptosis of the pRb-deficient nervous system is highly cell type dependent. Caspase-3 disruption protects pRb deficient neurons from programmed cell death only in the peripheral nervous system. This protection was not apparent for other cell types including pRb-deficient neurons of the CNS or the developing liver that exhibited rates of apoptosis similar to that manifest by the pRb-null mutation alone. Thus, caspase-3 is likely an essential regulator of PNS apoptosis and may serve as a key therapeutic target for maintaining the survival of injured PNS neurons. Any

neurodegenerative disorder of the PNS that involves apoptotic processes might significantly benefit from this result.

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Appendix A

BREEDING PROTOCOL

We began by mating mice heterozygous for Rb with mice heterozygous for caspase-3 to build a colony of double heterozygous mice for later breeding:

F1: [pRB +/- : CASPASE-3 +/+] X [pRB +/+ : CASPASE-3 +/-]
OFFSPRING:

Genotype	Expected frequency	Observed frequency
[pRb +/+ : caspase-3 +/+]	25%	25%
[pRb +/+ : caspase-3 +/-]	25%	25%
[pRb +/- : caspase-3 +/+]	25%	25%
[pRb +/- : caspase-3 +/-]	25%	25%

The double het's, [pRb +/- : caspase-3 +/-], were then crossed to produce the desired genotypes for our study:

F2: [pRB +/- : CASPASE-3 +/-] X [pRB +/- : CASPASE-3 +/-]
OFFSPRING:

Genotype	Expected frequency	Observed frequency (n = 160)
[pRb +/+ : caspase-3 +/+]	1/16 = 6.25%	6.9

[pRb +/+ : caspase-3 +/-]	2/16 = 12.5%	12.5
[pRb +/+ : caspase-3 -/-]	1/16 = 6.25%	6.3
[pRb +/- : caspase-3 +/+]	2/16 = 12.5%	13.1
[pRb +/- : caspase-3 +/-]	4/16 = 25%	28.7
[pRb +/- : caspase-3 -/-]	2/16 = 12.5%	11.2
[pRb -/- : caspase-3 +/+]	1/16 = 6.25%	3.1
[pRb -/- : caspase-3 +/-]	2/16 = 12.5%	13.7
[pRb -/- : caspase-3 -/-]	1/16 = 6.25%	4.4

The double het's generated in this generation were subsequently utilized to maintain the aging colony.