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Cell Cycle Related Signalling in Neuronal Death

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Cell Cycle Related Signaling in Neuronal Death

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Dedicated to my parents

Without their love and open arms I never would have found my path

And to my wife

Who gives me the strength to walk it

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Abstract

Evidence indicates that neuronal loss in the course of neurodegenerative disease often occurs through programmed death processes. The development of effective therapeutic treatments for such diseases requires detailed knowledge of the intracellular signaling pathways controlling these death processes. Previous work has identified cyclin-dependent kinases, a family of kinases normally involved in the control of cell division, as potential regulators of death in neurons. For instance, a number of events that occur during the G1 to S transition in proliferating cells, such as cyclin D/cdk4 activation and phosphorylation of its target, pRb, have been detected in dying neurons and appear to be essential for death. In the cell cycle, pRb phosphorylation is followed by activation of the transcription factor E2F1. It is not known if E2F1 is also involved in neuronal death. I found that E2F1 expression in neurons induced apoptotic death dependent on Bax but independent of p53. Also, E2F1 mRNA and protein levels increase in neurons induced to die by exposure to low concentrations of K^+, and neurons from E2F1 null mice are resistant to this death. These results are consistent with participation of endogenous E2F1 in neuronal death signaling.

Cdk5 is a member of the cyclin-dependent kinase family which does not have a function in the cell cycle. Instead cdk5, together with its binding partner's p35 and p39, is involved in a variety of neuronal functions. Cleavage of p35 into a smaller p25 form has been shown to convert cdk5 into a death promoting kinase. However, it is not yet clear under which circumstances cdk5 signals death, and there is also some contrasting evidence suggesting cdk5 is a pro-survival factor. By targeting dominant negative cdk5 expression to either the nuclear or cytoplasmic compartments I show that cdk5 performs

a pro-death function within the nucleus but a pro-survival function within the cytoplasm. The nuclear pro-death signal is relevant only when p25 is produced early, as it is following glutamate induced death, and not when it is produced late as a result of caspase activation, as it is following DNA damage.

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List of Abbreviations

Aβ	β-amyloid peptide
AIF	apoptosis inducing factor
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
Apaf-1	apoptotic peptidase activating factor 1
APP	amyloid precursor protein
BDNF	brain-derived neurotrophic factor
BH	Bcl-2 homology domain
BIR	baculovirus inhibitor repeat
CAD	caspase activated DNase
CAK	CDK activating kinase
CENPA	centromere protein A
CREB	cAMP response element binding protein
CDK	cyclin-dependent kinase
CGN	cerebellar granule neuron
CKI	CDK inhibitors
CNS	central nervous system
DARPP-32	dopamine- and cAMP-regulated phosphoprotein, molecular mass 32 kDa
DED	death effector domain
DISC	death inducing signaling complex
DR	death receptor
E2F	E2 promotor binding factor
endoG	endonuclease G
FADD	Fas-associated protein with death domain
HDAC	histone deacetylase
HD	Huntington's disease
Htt	huntingtin
IAP	inhibitor of apoptosis
ICAD	inhibitor of CAD
ICE	interleukin 1β converting enzyme
JNK	c-Jun N-terminal kinase
MAD2L1	mitotic arrest deficient 2-like 1
MCM	mini chromosome maintenance
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NES	nuclear exclusion sequence
NGF	nerve growth factor
NLS	nuclear localization sequence
NMDA	N-methyl-D-aspartate
NTF	neurofibrillary tangle
NT-3	neurotrophin-3

NT-4	neurotrophin-4
PARP	poly (ADP-Ribose) polymerase
PD	Parkinson's disease
PKA	protein kinase A
PP-1	protein phosphatase-1
PS1	presenilin 1
PS2	presenilin 2
PTPC	permeability transition pore complex
pRb	retinoblastoma protein
SMAC	second mitochondrial activator of caspases
SMC2L1/4L1	structural maintenance of chromosomes 2/4 like 1
SOD1	superoxide dismutase 1
SV40Tag	SV40 T antigen
TNF	tumour necrosis factor
TNΦα	tumor necrosis factor-α
TRAIL	TNF-α related apoptosis-inducing ligand
TUNEL	terminal transferase-mediated dUTP-biotin nick end labeling

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Chapter 1. Introduction

The aim of this initial chapter is to first give the reader an appreciation for the importance of programmed neuronal death in the proper development of the nervous system and in the inappropriate neuronal loss seen in multiple neurodegenerative disorders. I will then outline the conserved signaling elements which control activation of programmed cell death in mammalian cells. The data implicating cell cycle related proteins as upstream activators of these conserved death elements in neuronal death will then be discussed in order to present to the reader the rationale for the experiments undertaken in the following studies.

1.1 Mechanisms of Cell Death

The modes by which cells can die have traditionally been defined as either necrotic or programmed. Necrotic death is considered to be a passive, energy independent, form of death in which a cell is rapidly overcome by powerful external factors such as physical trauma or extreme ionic or osmotic imbalances. Programmed cell death on the other hand involves an active, energy dependent, self-destructive response by cells to certain intrinsic or extrinsic cues. Programmed death is a normal physiological occurrence in virtually all multicellular organisms. In fact, the vast majority of cells produced from normal mitotic events are destined to undergo programmed death (Vaux and Korsmeyer, 1999). This type of death process occurs at very high rates during embryonic development, also the time of greatest cell division. In

humans, for example, removal of the vestigial tail and loss of the webbing between digits both occur as a result of programmed cell death (Kelley, 1970; Fallon and Simandl, 1978; Sapunar et al., 2001).

More recently, the sharp distinction between necrosis and programmed cell death has blurred somewhat, and it is becoming more widely recognized that cells die through a variety of mechanisms which lie on a continuum from purely necrotic to purely programmed (Clarke, 1990; Sperandio et al., 2000; Assuncao Guimaraes and Linden, 2004). In fact, even some types of necrotic death have been shown to involve active cellular signaling and can be triggered by intrinsic cues (Leist and Jaattela, 2001; Syntichaki and Tavernarakis, 2003). For these reasons the debates which were once common over what name should be ascribed to a particular instance of cellular death have given way to an acceptance that nomenclature is not as important as determining the intracellular signaling pathways that might contribute to the death process. In these pages I will use the term 'neuronal death' to refer to the process under investigation. It should be understood that for the most part I am using this term to refer to forms of programmed cell death.

The best studied type of programmed cell death is apoptosis. This term was coined by Kerr, Wyllie, and Currie in 1972 to denote a "... mechanism of controlled cell deletion, which is complementary to mitosis in the regulation of animal cell populations" (Kerr et al., 1972). It was soon found that apoptosis could also be a characteristic feature of pathological cell death (Wyllie et al., 1973; Wyllie et al., 1980). Cells undergoing apoptosis display unique morphological characteristics (Figure 1). In early phases there is condensation of chromatin along the periphery of the nuclear envelope and compaction

Figure 1. **Morphological features of apoptosis and necrosis.** The structural changes that occur during apoptosis differ sharply from those occurring during necrotic cell death. Necrotic death occurs as a result of physical trauma or extreme changes in extracellular conditions. Cells in the early stages of necrotic death appear swollen and intracellular organelles are dilated. In later stages there is a breakdown of plasma membrane integrity and leakage of cellular contents into the extracellular space. Apoptotic cells display distinct cellular changes, particularly in the nucleus. During apoptosis chromatin becomes condensed along the nuclear periphery and the cytoplasm is generally compacted slightly. In latter stages the nuclei fragment but remain contained, and the plasma membrane begins to bleb. Eventually these blebbings brake off into apoptotic bodies that are engulfed by phagocytic cells.

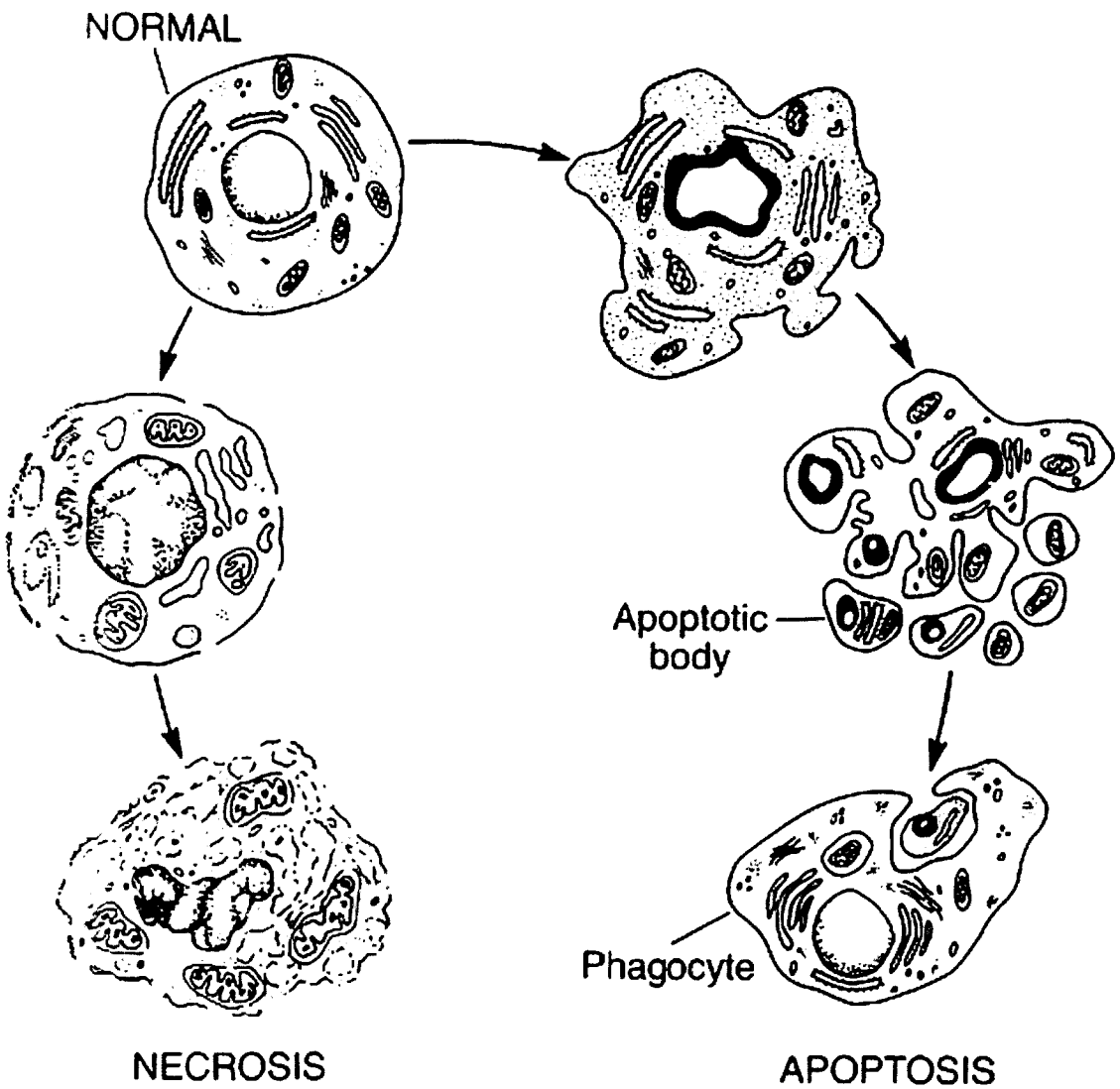

Figure 1

of the cytoplasm. This is followed by nuclear fragmentation and blebbing of the plasma membrane into apoptotic bodies which are then phagocytosed by nearby cells. In contrast, necrosis is characterized by organelle dilation, extensive vacuolation, progressive swelling, and finally the breakdown of the plasma membrane. This breakdown results in the spillage of cellular contents into the intracellular space and the activation of inflammatory responses. Because necrosis is caused by very powerful stimuli, adjacent groups of cells are usually affected together. Apoptosis, however, typically affects isolated cells and does not induce an inflammatory response (Kerr et al., 1972; Walker et al., 1988).

Apoptosis and other forms of programmed cell death were first described within the context of developing organisms. Indeed, programmed cell death is now well known to be a major force which contributes to the formation of mature tissues throughout development in virtually all metazoans (Saunders, 1966; Lockshin, 1981; Snow, 1987; Hurle, 1988; Sanders and Wride, 1995). The development of the vertebrate nervous system is one of the most striking examples of developmental cell death. It has been observed that on average about 50%, and in some regions up to 80%, of post-mitotic neurons generated during embryonic development are lost through a process of programmed death (Oppenheim, 1991).

1.2 Cell Death in Nervous System Development

The earliest observation of neuronal degeneration during nervous system development was made by the French cytologist R. Collin in 1906 when he briefly described the histolysis of spinal motor nuclei in chick embryos (Collin, 1906). Developmental neuronal loss was again observed as part of the much more comprehensive reports from Ernst (Ernst, 1926) and Glucksmann (Glucksmann, 1930), which classified cell loss in numerous embryonic tissues in addition to the nervous system. These early remarks were impressive in their detail but lacked any interpretation as to the relevance of these cell death events to the process of development and did not in any way address the cause of the death.

It was from the relatively new school of experimental embryology that these answers would come. Viktor Hamburger investigated whether the developing limb, or more particularly, the nerve target tissue within the limb, was required for proper neural development. Five days following ablation of the wing bud of the chick embryo, Hamburger found marked hypoplasia of both the dorsal root ganglia and spinal motor nuclei (Hamburger, 1934). His assessment of this hypoplasia was that the target tissues must somehow dictate the size of the neuron populations by stimulating differentiation (or recruitment) from a pool of undifferentiated cells. This interpretation turned out to be only half correct.

Dr. Rita Levi-Montalcini, working during World War II in a makeshift laboratory in her own bedroom, replicated Hamburger's experiments, but believed that

the loss of neurons was due to a degeneration which occurred only after the onset of differentiation (Levi-Montalcini and Levi, 1942, 1944). This proposal was a distinct diversion from Hamburger's idea of recruitment and, more importantly, marked the first introduction of the concept that neuronal death following differentiation may be regulated by peripheral targets.

In order to resolve these differences of interpretation Hamburger invited Levi-Montalcini to join his laboratory. Working together they showed conclusively that the reduction in cell number in central neuronal populations following limb ablation was due to a degeneration of differentiated neurons, and not due to differences in proliferation or differentiation (Hamburger and Levi-Montalcini, 1949). Levi-Montalcini's interpretations had been more accurate. Later evidence would show that this death displayed the classical features of apoptosis. They also noticed that even under non-experimental conditions a large percentage of dorsal ganglia and spinal motor neurons degenerated about the same time they are establishing contacts with their targets (Hamburger and Levi-Montalcini, 1949). This suggested that competition for a tissue generated survival signal might regulate neuronal survival during normal development. Hamburger and Levi-Montalcini hypothesized that the developing tissues release a *factor* which is retrogradely transported by the neurons to their cell bodies and promotes their survival. This concept laid down the foundation for the understanding of naturally occurring developmental neuronal death.

Because neither Hamburger nor Levi-Montalcini possessed the skills that would be necessary for the purification of this nerve growth promoting signal, in 1953 they joined forces with the biochemist Stanley Cohen. Through a fortuitous series of

experiements, they noticed that the submaxillary salivatory gland of adult mice produced a nerve growth promoting activity (Cohen, 1960). Rabbit antiserum raised against submaxillary extracts completely blocked the growth stimulatory properties and also caused almost complete degeneration of sympathetic ganglia (Levi-Montalcini and Booker, 1960), underlying the importance of this factor in promoting fiber growth and in maintaining survival. These antiserum results also demonstrated that the survival/growth promoting agent was a protein. This protein species became known as Nerve Growth Factor (NGF).

The exact nature of this protein factor was vigorously pursued for the next number of years. Through an arduous series of biochemical purifications NGF was eventually purified and its amino acid composition determined (Cohen, 1960; Angeletti and Bradshaw, 1971). Injection of anti-NGF specific antibodies into newborn mice results in a striking reduction in the number of neurons in both the sympathetic and dorsal root ganglia (Levi-Montalcini, 1972). Moreover, the administration of purified NGF to embryonic or newborn mice can prevent the naturally occurring death of sympathetic and dorsal root neurons. These studies conclusively showed that NGF is required for the maintenance of developing neurons. Interestingly, adult neurons no longer demonstrate such a requirement since removal of NGF does not lead to death of mature neurons.

The above series of experiments by Hamburger, Levi-Montalcini, and Cohen established concepts which now form foundations for much of developmental neurobiology and the biology of cell death. In the context of developmental neurobiology their work established that degeneration of neurons in the developing nervous system occurs as a result of competition for target generated survival signals. To

cell death they provided the concept that extrinsic factors could suppress an intrinsic program of cellular degeneration. Each of these concepts has been extensively supported and explored in numerous different contexts. The significance of these achievements was recognized by awarding of the Nobel Prize in Medicine to Rita Levi-Montalcini and Stanley Cohen in 1986.

Since the discovery of NGF, additional vertebrate proteins with a high degree of sequence similarity have been cloned, thus placing NGF as the proto-typical member of a family of neurotrophins (Kaplan and Miller, 2000). Within mammalian species this family includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). NGF is responsible mainly for the maintenance of peripheral neuronal populations (i.e. sympathetic and dorsal root ganglia neurons) and has very limited effects on central nervous system (CNS) neurons. Conversely, BDNF, NT-3, and NT-4 maintain mainly central populations. All mature neurotrophins are in fact cleaved forms of larger precursor proteins (pro-neurotrophins). Both mature and pro-neurotrophins are secreted and have distinct biological activities (Lee et al., 2001). Neurotrophins are capable of binding to at least two different receptors. Each neurotrophin binds to the p75 receptor, a member of the tumor necrosis factor (TNF) receptor superfamily (Johnson et al., 1986; Radeke et al., 1987). Additionally, each member of the neurotrophin family binds to a unique member of the Trk receptor family; NGF binds to TrkA, BDNF and NT4/5 bind to TrkB, while NT-3 binds to TrkC. All Trk receptors are single transmembrane proteins with intracellular tyrosine kinase domains. Together, these two different classes of receptors direct and modulate the responses of neurons to extracellular binding of neurotrophin. These responses can actually be quite

varied and include not only neuronal survival, but also neurite growth, differentiation, and synaptic plasticity.

1.3 Cell Death In Nervous System Degeneration

The first era of research into neurodegenerative diseases was predominantly descriptive in nature. Tremendous effort in this area established a unifying theme; that most degenerative neurological conditions result from the selective loss of specific neuronal populations, rather than a general widespread neuronal dropout. For example, Parkinson's disease results from a selective loss of neurons in the substantia nigra pars compacta, while Alzheimer's disease is caused by a progressive degeneration of the medial temporal areas, prefrontal cortex, and basal forebrain. In each case there is relative sparing in all other parts of the nervous system. Little was known about the mechanisms which contribute to this selective loss until the advent of more experimental approaches, the most significant of which was the development of *in vitro* tissue culture techniques. These approaches soon revealed that a number of possible environmental conditions can bring about the demise of neuronal populations. These include oxidative stress, metabolic disturbances, calcium toxicity, and excitotoxicity. Not long after it had become recognized that programmed neuronal death played an important role in ensuring the proper development of the nervous system, researchers began to speculate that inappropriate activation of programmed death may also be the cause of neuronal loss in neurodegenerative situations. In the following section I will outline the evidence implicating apoptosis as a causative event in various neurodegenerative conditions. For each condition this evidence generally comes in one or more of the following forms: tissue culture manipulations, animal models, and human postmortem studies. The

rationale for including this information is to make clear the importance of achieving a greater understanding of the molecular pathways controlling programmed neuronal death. The design of effective therapeutic targets for neurodegenerative diseases requires very detailed knowledge of these pathways. It is important to note that the conditions listed below are not the only neurological diseases with features of programmed neuronal loss, but rather a selection of those to which most research attention has been paid.

Alzheimer's Disease

Alzheimer's disease (AD) is a progressive condition which begins with mild impairments of declarative memory and progresses to severe failure of short and long term memory and loss of cognitive function (Goetz, 2003). The pathology of AD is confined in the early stages to the limbic structures of the medial temporal lobe (amygdala and hippocampus). Neurons of the basal forebrain, the main cholinergic input to the hippocampus and the entire neocortex, are also lost. In later stages of the disease the frontal and prefrontal cortices shrink dramatically due to widespread neuronal loss. The histopathological characteristics of AD are neuritic plaques, consisting largely of the β-amyloid peptide (Aβ), and neurofibrillary tangles (NFTs) containing hyperphosphorylated forms of the protein tau (Goedert et al., 1996).

When mature cultures of hippocampal neurons are grown in the presence of Aβ the neurons begin to display neuritic retraction followed by neuronal death (Yankner et al., 1990; Pike et al., 1991; Pike et al., 1992). Such Aβ treatment also sensitizes cultured neurons to other death stimuli including excitotoxicity and glucose deprivation (Koh et al., 1990; Copani et al., 1991; Mattson et al., 1992). Death induced by Aβ was found to

be associated with some typical characteristics of apoptosis. For example, in late stages of death the neurons become small and condensed with irregularly shaped cell bodies (Loo et al., 1993; Watt et al., 1994). DNA from Aβ-treated neurons show the typical apoptotic ladder pattern on gel electrophoresis, a result of the regular cleavage at internucleosomal sites (Forloni et al., 1993; Loo et al., 1993). Other typical features of apoptosis observed include cleavage of poly (ADP-Ribose) polymerase (PARP), caspase activation, TUNEL (terminal transferase-mediated dUTP-biotin nick end labeling) staining, and plasma membrane inversion (Ivins et al., 1998; Harada and Sugimoto, 1999; Ivins et al., 1999; Ding et al., 2005).

The findings summarized above clearly established that Aβ can bring about an apoptotic like death of cultured neurons, suggesting that the neuronal loss seen in AD is a direct consequence of apoptotic signaling by endogenous aggregates of Aβ. If this is the case one would expect to find signs of apoptosis in tissue from AD patients. Numerous investigators have in fact detected apoptotic neurons in post-mortem analysis of AD brains (Lassmann et al., 1995; Smale et al., 1995; Anderson et al., 1996; Lassmann, 1996; Troncoso et al., 1996; Su et al., 1997). Although also present in age matched control brains, apoptotic signs are much more frequent in AD tissue. These studies have varied widely in their estimate of the frequency of apoptotic neurons in AD, with some reporting that as many as 80% of neurons show some sign of apoptotic DNA fragmentation, while others report that only a very small fraction of neurons are in fact undergoing apoptosis. These differences are likely due, at least in part, to methodological variations. However, since any histopathological analysis of AD brains represents only a single snapshot of the disease, generally at a very late stage of progression, the conclusions which can be made

from such studies are limited. Also, it is important to consider that AD is a disease which progresses slowly over a period of two to five decades, with clinical signs occurring only once 60-80% of neurons in the entorhinal cortex and hippocampus have been lost. Thus, the rate at which neurons die is likely to be slow and gradual. Such gradual neuronal dropout is also in itself suggestive of a programmed or apoptotic-like death, since necrosis is generally considered to be an acute response to overpowering toxic stimuli.

Numerous studies involving genes with genetic linkage to AD have also suggested that apoptosis plays a central role in the disease. Mutations in the gene encoding the amyloid precursor protein (APP), the protein from which Aβ is produced, have been linked to multiple familial AD pedigrees (Price and Sisodia, 1998; Tanzi and Bertram, 2005). Expression of APP containing these AD-linked mutations in neurons results in apoptosis (Yamatsuji et al., 1996b; Yamatsuji et al., 1996a; Zhao et al., 1997). Overexpression of presenilin 2 (PS2), mutations in which have also been linked to familial AD, sensitizes PC12 cells to a variety of apoptotic stimuli (Deng et al., 1996; Wolozin et al., 1996). Expression of disease-linked PS2 mutants also enhances the basal rate of apoptosis in these neurons (Wolozin et al., 1996), while antisense knockdown of PS2 reduces neuronal death induced by NGF withdrawal, glutamate, or APP overexpression (Vito et al., 1996; Wolozin et al., 1996). AD-linked mutants of presenilin 1 (PS1) promote neurotoxicity in a manner that can be inhibited by Bcl-2 expression, suggesting a reliance on core apoptotic signaling (Guo et al., 1996; Guo et al., 1997). Although the mechanisms by which mutant PS1 and PS2 activate death are not well understood, some evidence suggests that they compromise ER calcium homeostasis and increase oxidative stress (Ito et al., 1994; Guo et al., 1996; Terro et al., 2002), two events

which are thought to contribute to AD pathology (LaFerla, 2002; Takuma et al., 2005). These findings suggest that AD may result, at least in some familial forms, from the programmed death of sensitized neurons. However, given that expression of these AD-linked genes is rather ubiquitous throughout the brain, they do not address the specificity of neuronal loss seen in AD.

Parkinson's Disease

Parkinson's Disease (PD) is a slowly progressive disease affecting the extra-pyramidal motor system. Selective loss of neuromelanin pigmented neurons of the substantia nigra pars compacta results in a dopamine imbalance within the striatum. The net effect of this imbalance is the classic symptoms of PD including resting tremor, rigidity, bradykinesia, and postural instability. Whether or not the cell death within the pars compacta occurs by apoptosis is as yet unclear. Several studies have detected structural and biochemical changes that are consistent with apoptotic death (Mochizuki et al., 1996; Anglade et al., 1997; Tatton et al., 1998). Yet others have failed to find such evidence (Dragunow et al., 1995; Kosel et al., 1997; Banati et al., 1998), and some reports instead implicate autophagic death as the main cause of neuronal loss in PD (Anglade et al., 1997; Stefanis et al., 2001; Marino and Lopez-Otin, 2004).

Examination of mice treated with the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which induces PD like symptoms, revealed apoptotic nuclei in the substantia nigra (Tatton and Kish, 1997). Moreover, mice null for Bax, a major death promoting constituent of the core apoptotic machinery, are resistant to MPTP treatment (Vila et al., 2001). Familial PD can arise from mutations in the α-synuclein

gene, and α-synuclein protein is a major constituent of Lewy bodies, another pathological hallmark seen in most PD cases. Interestingly, disease-inducing mutants of α-synuclein can cause apoptosis of cultured neuroblastoma cells (El-Agnaf et al., 1998). Whether or not neuronal loss in PD occurs by apoptotic mechanisms, this loss is likely to be 'programmed' to some degree in that it depends upon various cellular signaling pathways. This is demonstrated by the fact that a large number of studies have shown that modulating cellular signals can provide protection in the MPTP model (Dauer and Przedborski, 2003).

Amyotrophic Lateral Sclerosis

Selective loss of upper and lower motor neurons with concomitant paralysis of somatic musculature is the defining profile of amyotrophic lateral sclerosis (ALS). The majority of cases are sporadic, although there are some familial forms. The most common of the familial forms is caused by a mutation in the cytosolic copper/zinc superoxide dismutase 1 (SOD1) gene. Spinal cord from ALS patients has shown an increased number of TUNEL positive ventral horn neurons (Troost et al., 1995). Activated forms of caspases-3 and -7 have also been detected in patient tissues (Martin, 1999). Further studies have reported decreased levels of the antiapoptotic Bcl-2 and increased levels of the proapoptotic Bax (Mu et al., 1996), suggesting that an imbalance in the core apoptotic machinery may lead to death. Studies of SOD1 mutants associated with ALS have also supported a role for apoptosis in the disease. Mutation of SOD1 may predispose neurons to apoptosis since expression of wild-type SOD1 protects cultured neurons from apoptosis while expression of ALS-mutant SOD1 induces

apoptosis (Rabizadeh et al., 1995; Durham et al., 1997). Transgenic expression of human ALS-linked SOD1 mutations in mice replicates both the clinical and histopathological hallmarks of ALS including the imbalances between Bcl-2 and Bax expression (Gurney et al., 1994; Wong et al., 1995; Bruijn et al., 1997). Deregulation of Bax may be particularly important in disease progression since forced expression of Bcl-2, which antagonizes Bax apoptotic activity, mitigates the motor neuron degeneration and prolongs the life of SOD1 mutant mice (Kostic et al., 1997).

Huntington's Disease

Selective loss of neurons in the caudate nucleus of the basal ganglia is the major characteristic of Huntington's disease (HD). Apoptosis, determined by positive TUNEL staining with respect to control, has been observed in this region in HD brains (Dragunow et al., 1995; Portera-Cailliau et al., 1995; Thomas et al., 1995). HD is caused by an expansion in the number of CAG repeats in the *huntingtin* (htt) gene on chromosome 4. This CAG repeat results in the expression of Htt protein containing a polyglutamine tract. The htt knockout mouse is embryonic lethal and displays extensive abnormal apoptosis (Zeitlin et al., 1995), implying that the normal function of Htt is to suppress apoptotic signaling. Transgenic mice expressing the disease causing CAG repeat of htt develop striatal degeneration and behavioral deficits similar to human HD (Mangiarini et al., 1996). In such mice, inhibition of either caspase-1 or -3 is sufficient to prevent pathology and extend lifespan (Ona et al., 1999; Chen et al., 2000). Caspase-8 may also be required for HD neuronal loss since its inhibition prevents CAG repeat-induced death *in vitro* and

its activated form is detected in the affected brain regions of HD patients (Sanchez et al., 1999).

Together these results are consistent with a model in which mutant Htt triggers the core apoptotic machinery, the end stage of which is caspase activation. However, the role of caspases in HD is likely not so simple. The Htt protein itself has been shown to be a caspase substrate. Caspase-3 can cleave Htt, resulting in the production of shorter fragments containing the polyglutamine tract. These shorter fragments show increased toxicity compared to the full length protein (Goldberg et al., 1996; Wellington et al., 1998; Wellington et al., 2000). The implication of these findings is that caspases may not simply be the end stage executioners of death but may play an additional role in magnifying the death signal.

Stroke

Neuronal death brought about by brain ischemia is distinct from the types of death in the previously discussed neurodegenerative conditions in that it occurs within minutes to days after the onset of an acute death trigger (the cessation of oxygen and glucose supply), rather than occurring over many years as the result of undefined chronic stimuli. After the onset of ischemia, neurons in the most severely affected areas undergo a nearly immediate necrotic death. There is also a more gradual loss of neurons in the region outside this core, where neurons are subjected to a less severe insult. This death can take place over a rather wide time span of 12hrs up to many days (Dirnagl et al., 1999; Graham and Chen, 2001). This delayed death may occur by a programmed apoptotic means (Mattson et al., 2000). Early results showed that damage due to experimental

stroke in rats can be reduced by the protein synthesis inhibitor cycloheximide (Linnik et al., 1993; Du et al., 1996). Also, DNA from the ischemic brain tissue showed signs of apoptotic-like fragmentation (Linnik et al., 1993; MacManus et al., 1993; MacManus et al., 1994; Du et al., 1996). Interestingly, subsequent reports which more closely examined the morphology of dying neurons following ischemia found less similarity with typical apoptotic cells. For example, the dying neurons did not appear to degenerate in neatly packaged membrane-bound bodies. Many dying neurons instead displayed punctured, leaking membranes (MacManus and Buchan, 2000), suggesting the death was not as neat an affair as in classical apoptosis. Moreover, DNA cleavage during ischemic neuronal death does not result in clean, blunt ends (MacManus et al., 1997; MacManus et al., 1999b).

Despite these differences to classical apoptosis, substantial evidence has accumulated implicating distinct components of the apoptotic machinery in ischemia-induced delayed neuronal death. Activated caspases can be detected in mice following stroke and their inhibition can decrease infarct size by 40% to 55% (Cheng et al., 1998; Namura et al., 1998; Krajewski et al., 1999; Velier et al., 1999; Le et al., 2002). Activation of the extrinsic caspase pathway by Fas/FasL has been reported in ischemia-induced neuronal death (Martin-Villalba et al., 1999; Velier et al., 1999) and appears to require Bid and/or Bax (Krajewski et al., 1995; Plesnila et al., 2001). Further reports indicate that inhibition of additional, caspase-independent cell death pathways, such as apoptosis inducing factor (AIF), also protects against ischemic neuronal loss (Zhu et al., 2003; Cregan et al., 2004a; Plesnila et al., 2004). These results, as well as others not

mentioned here, suggest that much of the delayed neuronal death following ischemia occurs in a manner dependent on distinct cell signaling pathways.

1.4 Conserved Cell Death Signaling Pathways

Soon after apoptosis was identified as an essential feature of development, attempts to uncover its genetic and molecular controls began. Progress on this front was slow until the introduction of the nematode *C. elegans* as a research tool. Pioneering work by John Sulston, Robert Horvitz and their colleagues comprehensively mapped the lineage and fate of every cell in the developing worm (Sulston, 1976; Sulston and Horvitz, 1977). Each mature *C. elegans* contains exactly 959 cells, produced from an original pool of 1090. During development exactly 131 cells are eliminated through programmed cell death. Invariably the same 131 cells undergo programmed death in each individual organism.

Remarkably, the vast majority of the 131 *C. elegans* cells which undergo programmed death are neurons (105 of 131). This situation is paralleled in vertebrates, perhaps to the greatest degree in humans, where a large overproduction of early neurons is followed by a wave of massive apoptosis. Furthermore, a striking similarity also exists in the signaling pathways which control programmed neuronal death in mammals and nematodes (Putcha and Johnson, 2004). These findings not only underline the evolutionary importance of programmed cell death in nervous system development, but also point to *C. elegans* as an excellent tool for unraveling the mechanisms of neuronal death in development and disease.

Mutational screens by Horvitz's lab identified a series of genes required for the normal occurrence of programmed cell death in *C. elegans*. The first two of these genes were *ced-3* and *ced-4* (Ellis and Horvitz, 1986). Loss of either of these genes resulted in

the complete absence of developmental programmed cell death. Despite these extra cells the worms appeared phenotypically normal. A third gene, *ced-9*, was later identified which, when overactive due to a gain-of-function mutation, also prevents all normal programmed cell death events (Hengartner et al., 1992). Inactivation of *ced-9* causes widespread death of cells which normally survive, and such embryos are not viable. Ensuing biochemical studies revealed that the three proteins are part of a multiprotein complex in which CED-9 antagonizes the ability of CED-4 to activate the protease potential of CED-3 (Hengartner, 1996; Spector et al., 1997). The displacement of CED-9 from the complex results in CED-4-mediated activation of CED-3 and subsequent death. CED-9 displacement is promoted by the presence of a fourth protein, EGL-1, which itself bears sequence similarity to CED-9 (Conradt and Horvitz, 1998). The identification of this basic genetic pathway in worms, followed closely by the cloning of vertebrate homolgues to each of these four proteins, paved the way for a surge of data, throughout the 1990s and into the 21st century, from mammalian systems and a dramatic advancement in our understanding of the molecular pathways of programmed cell death in human development and disease.

Ced-3 and the Caspases

The first identified mammalian homologue to *ced-3* was the interleukin 1β converting enzyme (ICE), which is required for the production of the mature cytokine interleukin 1β, and is involved in inflammatory responses (Yuan et al., 1993). Further members of this family were identified and together they became known as the caspases, a name derived from their functional characteristics: they all contain cysteine in their

active site, cleave at regions containing aspartate residues, and are proteases (Alnemri et al., 1996). ICE, as the first identified member, became known as caspase-1. To date there are at least 14 members of the caspase family. While caspase-1 and caspase-11 are generally thought to be limited to roles in inflammation, other members of the family act as the executioners of cell death. Expression of caspases is sufficient to induce apoptotic death and their inhibition can prevent death (Bump et al., 1995; Nicholson et al., 1995; Tewari et al., 1995).

Once activated caspases cleave a wide variety of substrates (more than 280 have been identified), the net effect of which is the disassembly of the cell and the morphological appearance of apoptosis (Fischer et al., 2003b). In order to orchestrate this disassembly caspases need to inactivate certain proteins which may interfere with death, while simultaneously activate other proteins essential for the execution of the cell death program. Cleavage of DNA repair enzymes results in their inactivation and prevents the cell from attempting to repair the DNA fragmentation characteristic of programmed death (Casciola-Rosen et al., 1995; Song et al., 1996; Zhan et al., 2002). Caspases also silence various pro-survival kinases and transcription factors including Akt, Raf-1, and cAMP response element binding protein (CREB) (Widmann et al., 1998; Francois et al., 2000), thereby reinforcing the cell death decision. The DNA fragmentation of programmed death is directly dependent on caspase activity. The DNase CAD (caspase activated DNase) is normally kept in an inactive state by binding of ICAD (inhibitor of CAD). During death ICAD is cleaved by caspase-3, allowing CAD to cleave DNA into internucleosomal segments (Enari et al., 1998). A second major nuclear event in apoptosis, chromatin condensation, is dependent on caspase-mediated

activation of the nuclear enzymes, acinus and helicard (Sahara et al., 1999; Kovacsovics et al., 2002). Although some overlap may exist, substrate specificity varies amongst individual caspases and is determined by short sequences near the target site (Nicholson and Thornberry, 1997).

Much like other proteases, all caspases are synthesized as proenzymes (30-50 kD) with extremely low activity. The proenzymes contain three distinct domains: a large subunit domain, a small subunit domain, and an N-terminal prodomain which can be either short or long. The active enzyme is produced by cleavage at sites located between these domains followed by heterodimeric association of two large (20 kD) and two small (10 kD) subunits into a tetramer (Walker et al., 1994; Wilson et al., 1994; Rotonda et al., 1996). These cleavage sites strongly resemble consensus caspase target sequences, consistent with the finding that caspases either autoactivate or are activated by other family members (Salvesen and Dixit, 1997; Colussi et al., 1998; Srinivasula et al., 1998). This distinction in activation is also used to classify caspases as either initiators, those which autoactivate, and effectors, those which are activated by initiators. Initiator caspases contain long prodomain sequences which seem to facilitate autoactivation through interaction with other proteins. The initiator caspases include caspase-2, -8, -9, and -10, while the effector class includes caspase-3, -6, and -7. Because the activation of an initiator caspase triggers an irreversible sequence of downstream caspase activation, it is not surprisingly a tightly regulated event which generally requires the interaction and assembly of multi-protein complexes initiated by the death-inducing stimulus. Two main pathways of initiator caspase activation have been identified; the intrinsic pathway (also

called the mitochondrial pathway), and the extrinsic pathway (also called the death receptor pathway).

The Intrinsic Death Pathway and the Bcl-2 Family

Death stimuli arising from intracellular events such as DNA damage, nutrient deficiency, and oxidative stress lead to caspase activation through the intrinsic death pathway (Figure 2A). This pathway hinges upon the release of critical death regulators from the mitochondria (Wang, 2001). When present in the cytoplasm these regulators cause the formation of a multi-protein caspase activation complex. One of these proteins is cytochrome c, a normal constituent of the electron transport chain (Liu et al., 1996; Kluck et al., 1997). Once released from the mitochondria, cytochrome *c* binds and activates Apaf-1 (apoptotic peptidase activating factor 1), a homologue of *ced-4* (Zou et al., 1997). Interaction with cytochrome *c* allows Apaf-1 to bind both dATP and the inactive proenzyme form of caspase-9 (Li et al., 1997a). Together this complex is referred to as the apoptosome. Binding of Apaf-1 to pro-caspase-9 results in a large increase in its catalytic activity and facilitates autoprocessing to active caspase-9 (Jiang and Wang, 2000), which in turn goes on to cleave and activate caspase-3 and other effector caspases (Li et al., 1997a).

The release of cytochrome c into the cytoplasm is governed by the Bcl-2 protein family, members of which share homology with either CED-9 or EGL-1. Members such as Bcl-2 and Bcl-xL share homology to CED-9 and promote survival (Boise et al., 1993; Hengartner and Horvitz, 1994a, b), while members such as Bax, Bak, Bad, Bid, Bim, Noxa, and Puma are homologues of EGL-1 and promote death (Oltvai et al., 1993;

Figure 2. **The Intrinsic and Extrinsic Conserved Death Pathways. A.** Activation of the intrinsic death pathway impinges upon BH3-only proteins of the Bcl-2 family. These small proteins promote oligomerization of Bax and Bak at the mitochondria. Oligomerization is antagonized by the prosurvival proteins of the Bcl-2 family such as Bcl-2 and Bcl-xL. Bax/Bak oligomers promote the loss of mitochondrial membrane potential and lead to the release of mitochondrial proteins into the cytoplasm. One of these is cytochrome c, which binds Apaf-1 and causes the assembly of the Apoptosome with the initiator caspase pro-caspase 9. Autoactivation of caspase 9 ensues followed by the cleavage and activation of effector caspases such as caspase 3. Smac is another mitochondrial protein released along with cytochrome c. Smac binds IAP proteins and prevents their ability to inhibit caspases. **B.** The extrinsic death pathway is initiated by death ligands binding to death receptors. Binding of Fas ligand to the Fas receptor promotes interaction between the intracellular death domain (DD) of the receptor with the DD of the adaptor protein FADD. A second domain on FADD, the death effector domain (DED), binds another DED on pro-caspase 8. Association of multiple copies of pro-caspase 8 leads to its auto activation, followed by cleavage and activation of effector caspases like caspase 3. Caspase 8 can also co-opt the intrinsic death pathway by cleaving BID to a truncated form (tBID). Full length BID is normally cytoplasmic, but tBID translocates to the mitochondria to promote cytochrome c release.

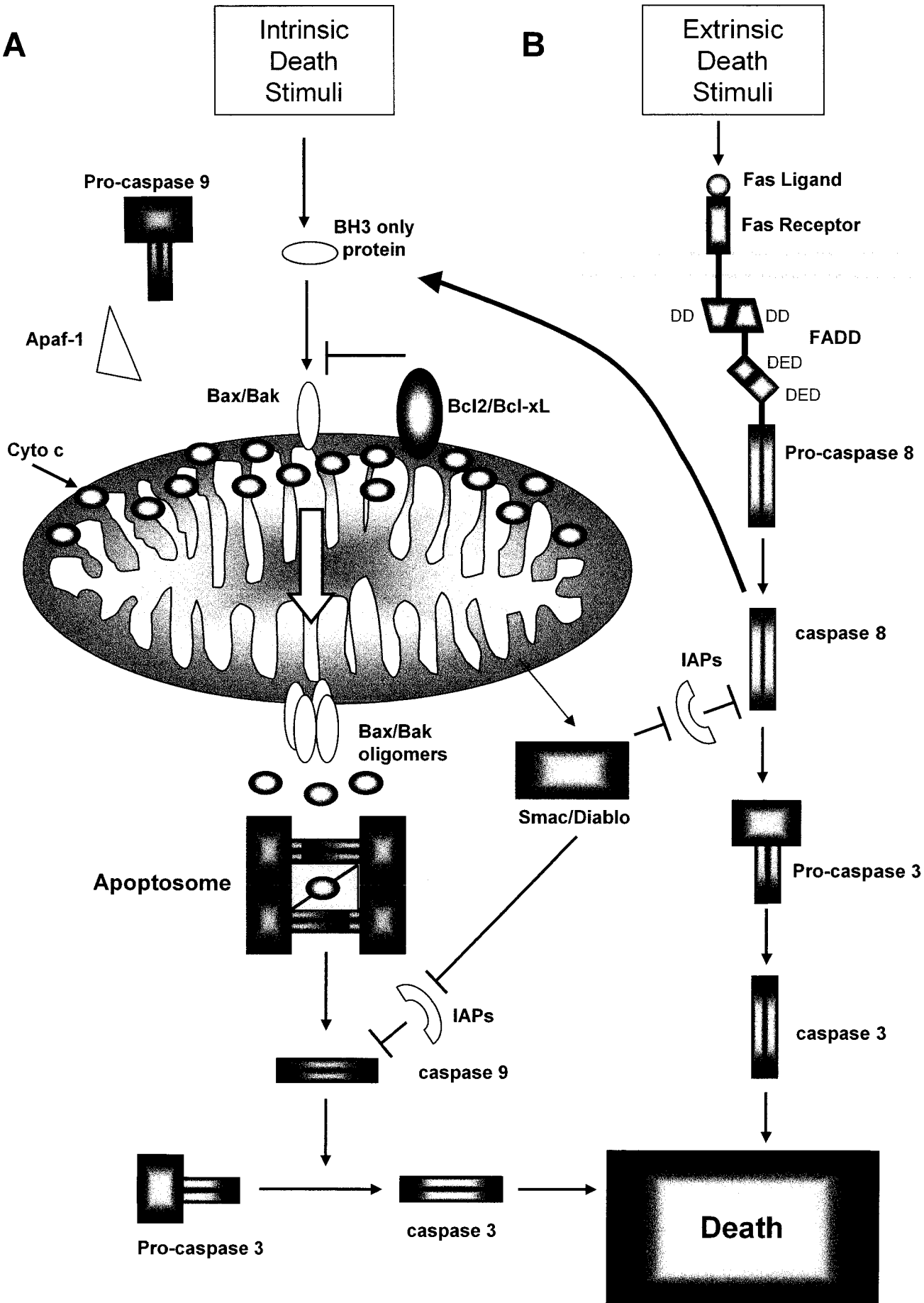
A
Intrinsic Death Stimuli
B
Extrinsic Death Stimuli
Pro-caspase 9
BH3 only protein
Fas Ligand
Fas Receptor
DD
DD
FADD
DED
DED
Pro-caspase 8
Apaf-1
Bax/Bak
Bcl2/Bcl-xL
Cyto c
caspase 8
IAPs
Bax/Bak oligomers
Smac/Diablo
Pro-caspase 3
Apoptosome
IAPs
caspase 3
caspase 9
Pro-caspase 3
caspase 3
Death

Figure 2

Huang and Strasser, 2000). All Bcl-2 family proteins contain one or more of four homologous regions, termed Bcl-2 homology domains (BH1-4). Pro-survival members show conservation in all four of these regions. Maintenance of only the BH3 region seems to be required for pro-death activity and the most potent death inducers contain only this BH domain. Nearly all pro-death and pro-survival family members also contain a conserved C-terminal transmembrane region used for targeting to the mitochondrial outer membrane. By the simplest model currently employed, the ratio of pro-survival to pro-death proteins at the mitochondria determines cytochrome *c* release. Bcl-2 and Bcl-xL appear to be constitutively localized to the mitochondrial outer membrane. Bax, and other pro-death members, tend to be localized to the cytoplasm or loosely attached to membranes. In response to an intrinsic death signal Bax inserts into the outer mitochondrial membrane where it interacts in an antagonistic manner with Bcl-2/Bcl-xL leading to the release of cytochrome *c*. A more complex picture arose when it was found that the presence of either Bax or Bak is required for all instances of death through the intrinsic pathway (Lindsten et al., 2000; Wei et al., 2001). The activation or expression of pro-death BH3 domain molecules (Bid, Bim, Bad, Noxa, Puma) induce changes in Bax/Bak that allow them to promote cytochrome *c* release (Cheng et al., 2001). In this model the pro-survival function of Bcl-2 and Bcl-xL is dependent on their ability to bind and sequester the BH3 proteins away from Bax/Bak.

The precise mechanism by which Bax/Bak mediate cytochrome *c* release is still actively being investigated. One model holds that oligomers of these proteins directly form pores in the outer mitochondrial membrane (Martinou and Green, 2001). When Bax inserts into the membrane it does so as oligomerized multimers (Antonsson et al.,

2001; Mikhailov et al., 2001; Nechushtan et al., 2001). Furthermore, Bax/Bak and other Bcl-2 family members share structural homology to certain bacterial toxins which are known to form helical pores (Muchmore et al., 1996). There is in fact evidence that Bax can form channels in artificial membranes and induce the release of cytochrome *c* from liposomes (Antonsson et al., 2000; Saito et al., 2000). The alternative explanation is that Bcl-2 family members modulate cytochrome *c* release indirectly through interaction with components of the permeability transition pore complex (PTPC) (Zamzami and Kroemer, 2001). This hypothesis is based primarily on the findings that Bcl-2 and Bax physically interact with components of the PTPC (Marzo et al., 1998; Shimizu et al., 1999), and that inhibition of PTPC constituents can provide protection from cell death (Sullivan et al., 1999; Budd et al., 2000; Tafani et al., 2000).

Cytochrome *c* is not the only molecule released from mitochondria during programmed cell death. At least three additional mitochondrial proteins are involved in regulating nuclear and cytoplasmic death events downstream of Bcl-2 family regulation of mitochondrial permeability. SMAC (second mitochondrial activator of caspases, also called Diablo in mice) is released alongside cytochrome *c* and strongly facilitates caspase activation by binding and suppressing IAPs (inhibitors of apoptosis) (Du et al., 2000). IAPs are a unique group of proteins capable of binding to and inhibiting the active forms of caspases (Salvesen and Duckett, 2002).

Another important polypeptide released by mitochondria during programmed cell death has been implicated in DNA degradation. Most attention had focused on CAD as the sole nuclease responsible for DNA fragmentation associated with apoptotic death. However, in some cells with depleted CAD activity there was still evidence of

internucleosomal DNA digestion (Li et al., 2001). Studies showed that this residual nuclease activity was provided by endonuclease G (endoG), an enzyme normally localized to the mitochondria but released in response to intrinsic death cues. Because endoG activation occurs independently, although usually concurrently, from caspase activity, it has become an important component of what is now termed caspase-independent cell death (discussed below).

In many cells in which caspase activity has been abolished, either using pharmacological inhibitors or genetic knockouts, death is only slightly delayed or not affected at all. For example, in cortical neuron cultures, direct caspase inhibition, or inhibition through loss of caspase-9 or Apaf-1, results in only transient protection (Stefanis et al., 1999; Cregan et al., 2002). Conversely, inhibiting the intrinsic death pathway at the level of the mitochondria, by either inhibiting events upstream from the Bcl-2 family or removing BH3-only proteins, results in a much greater sustained protection (Miller et al., 1997b; Xiang et al., 1998; Cregan et al., 1999a; Cheng et al., 2001). These results suggested that a death promoting pathway exists downstream of the mitochondria that is independent of the conserved apoptosome-caspase pathway. Part of this death may occur as a result of mitochondrial failure, but it is also now clear that active components released from the mitochondria stimulate death pathways distinct from the caspases (Chipuk and Green, 2005). EndoG represents one such component, and it clearly contributes to caspase-independent DNA fragmentation. Yet, perhaps the most important caspase-independent signal is AIF. AIF is a flavoprotein localized to the mitochondria in healthy cells. Within the mitochondria AIF likely acts as an oxidoreductase since it contains a FAD domain and has redox activity (Miramar et al.,

2001). Harlequin mutant mice, which have 80% reduced levels of AIF, are highly sensitive to oxidative stress, indicating that AIF's function as an oxidoreductase is essential for normal cellular metabolism (Klein et al., 2002). However, in response to death stimuli AIF is released from mitochondria, whereupon it translocates to the nucleus to promote apoptotic nuclear morphology (Joza et al., 2001; Cregan et al., 2002; Yu et al., 2002). Exogenous expression of AIF is sufficient to induce nuclear signs of apoptosis (Susin et al., 1999; Joza et al., 2001; Cregan et al., 2002). In addition, injection of neutralizing antibodies towards AIF can prevent death caused by a variety of triggers (Susin et al., 1999; Braun et al., 2001; Cregan et al., 2002; Wang et al., 2002; Yu et al., 2002), and Harlequin mice display resistance to neurotoxic stimuli (Cheung et al., 2005). Importantly, AIF appears to be responsible for the death that occurs even when caspase activity is prevented, as evidenced by the observation that AIF inhibition further extends the lifespan of Apaf-1 deficient neurons undergoing death (Cregan et al., 2002). AIF induced caspase-independent cell death pathways may be important in embryonic development given that AIF-deficient murine embryos do not develop because of a defect in cavitation, the earliest developmental step requiring programmed cell death (Joza et al., 2001).

The Extrinsic Death Pathway

Metazoan cells require the ability to undergo programmed death in response to cues from neighbouring or distant cells. To perform this task the extrinsic death pathway employs a conserved family of plasma membrane receptors and cytoplasmic adaptor proteins in cooperation to induce caspase activation (Figure 2B). The transmembrane

receptors belong to the tumor necrosis factor-α (TNF-α) receptor family and, in addition to TNF-α receptor 1, include the Fas receptor, the p75 neurotrophin receptor, and the DR4 and DR5 receptors (Thorburn, 2004). Each member of this receptor family contains a conserved death domain (DD) within its cytoplasmic region. In response to extracellular binding of the appropriate ligand, the DD is capable of binding proteins with homologous death domains. For example, Fas ligand binding to the Fas receptor results in the binding of FADD (Fas-associated protein with death domain) to the Fas cytoplasmic region through DD-DD interactions (Chinnaiyan et al., 1995). A second distinct domain on FADD termed the death effector domain (DED), then facilitates binding to procaspase-8 through a DED. Formation of this multi-protein complex (called the DISC or death inducing signaling complex) leads to the processing of procaspase-8 into active caspase-8 (Muzio et al., 1996). Activation of caspase-8 appears to result from the clustering of multiple procaspase-8 zymogens and subsequent autocleavage (Salvesen and Dixit, 1999). Other death receptors also function to activate caspase-8, although they do so through a different and more complex array of adaptor proteins. Similar to what happens in the intrinsic pathway, caspase-8 activation through death receptors leads to the processing and activation of effector caspases like caspase-3 and -7 (Srinivasula et al., 1996).

In some cell types the activation of caspase-8 via death receptors requires an amplification loop through the intrinsic pathway in order to produce sufficient caspase-3/7 activity to commit to cell death. In these cell types Bcl-2 expression can prevent death induced by Fas and other death receptor ligands. Crosstalk between the extrinsic and intrinsic pathways is mediated by caspase-8 cleavage of Bid. Caspase-8-cleaved Bid

translocates to the mitochondria where it promotes cytochrome *c* release (Li et al., 1998; Luo et al., 1998b). The subsequent activation of caspase-3 through Apaf-1 and caspase-9 provides positive feedback amplification to secure the death response.

1.5 Cell Cycle Overview

The cell cycle refers to the repeating pattern of stages through which dividing cells pass (Figure 3). These stages begin with the first gap phase (G1), which is then followed by the DNA synthesis phase (S). Once DNA replication is complete cells enter a second gap phase (G2), followed by the mitotic phase (M). Cells which have stopped dividing and are post-mitotic are said to be in the Go phase. Transition between these phases is controlled in large part by a conserved family of proteins known as cyclin-dependent kinases (CDKs) (Pines, 1993; Morgan, 1997). These are proline-directed serine/threonine kinases with, as their name implies, a strict dependence on binding to a cyclin protein for activity. Each CDK is activated by a unique cyclin that is expressed selectively at a particular point in the cell cycle. For example, at the beginning of the G1-S transition, a critical point which determines a commitment to cell division, cells upregulate cyclin-D which then binds to and activates cdk4 and/or cdk6 (cdk4/6). Cyclin-D/cdk4/6 complexes promote S phase entry by phosphorylating the retinoblastoma protein (pRb) (Ekholm and Reed, 2000; Massague, 2004). Phosphorylation of pRb disrupts its ability to bind and inhibit the E2F transcription factor, resulting in activation of E2F-mediated transcription. E2F target genes encode proteins required for DNA replication and for control of subsequent cell cycle stages (Stevaux and Dyson, 2002). As examples of the latter, cyclin-E, cyclin-A, cyclin-B, and cdk1 are all upregulated in an E2F-dependent manner (DeGregori et al., 1995; Hurford et al., 1997; Lavia and Jansen-Durr, 1999; Ishida et al., 2001). A rise in cyclin-E levels

Figure 3. **Overview of the Cell Cycle.** The mammalian cell cycle is composed of four distinct phases: two transitory gap phases (G1 and G2), the DNA synthesis phase (S), and the mitotic phase (M). Beginning in G1, cells respond to extracellular growth factors by upregulating the level of cyclin D. D-type cyclins bind to and activate cdk4 and cdk6. The major target of cyclin D/cdk4/6 complexes is pRb. Once pRb is phosphorylated by cyclin D/cdk4/6 it loses its ability to inhibit the transcription factor E2F. E2F target genes include those required for initiation of DNA synthesis as well as those required for regulation of subsequent cell cycle stages. One of the major E2F target genes is cyclin E, which together with cdk2 further phosphorylates pRb and commits cells to enter S-phase. Transition from G1 to S phase is strongly controlled by cyclin-dependent kinase inhibitors (CKIs). Those of the INK4 family (p15, p16, p18, and p19) inhibit the activity of cyclin D/cdk4/6, while Cip/Kip family members (p21, p27, p57) inhibit cyclin E/cdk2. In mid to late S phase cdk2 begins to associate more with cyclin A and this complex is responsible for the termination of DNA replication and exit from S phase. The final two transitions of the cell cycle, from G2 to M and from M back to G1, are governed by the cyclin B/cdk1 complex.

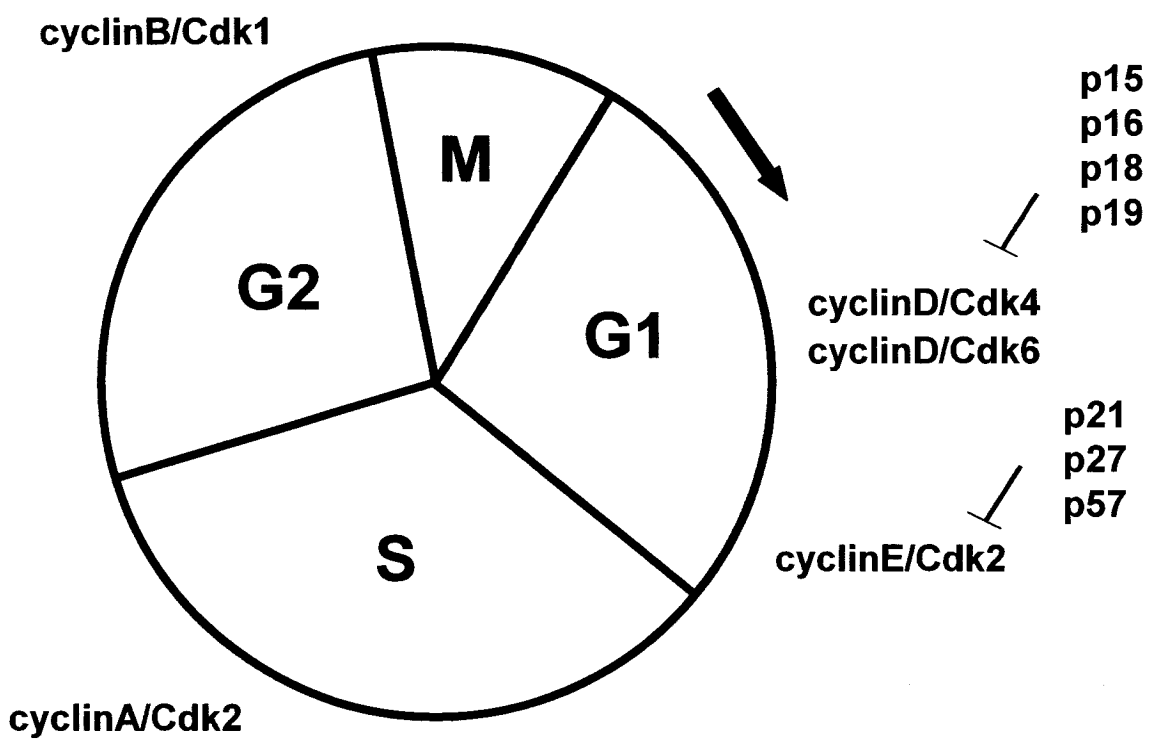
cyclinB/Cdk1
M
G2
G1
S
cyclinA/Cdk2
p15
p16
p18
p19
cyclinD/Cdk4
cyclinD/Cdk6
p21
p27
p57
cyclinE/Cdk2

Figure 3

results in more cyclin-E/cdk2 complexes, increased cdk2 activity, and further phosphorylation of pRb to commit cells to S phase. Cyclin-A together with cdk2 is required for termination of DNA synthesis and transition from S phase to G2. One of the means by which cyclin-A/cdk2 accomplishes this is by interacting with and phosphorylating E2F to induce its dissociation from DNA, effectively silencing its transactivation ability (Dynlacht et al., 1997). The final cell cycle transition, from G2 to M phase, is regulated largely by cyclin-B/cdk1 complexes (Smits and Medema, 2001). Cyclin-B/cdk1 has numerous targets through which it influences virtually all of the structural reorganization of mitosis. For example, cyclin-B/cdk1 phosphorylation of lamin subunits leads to the breakdown of the nuclear lamina (Peter et al., 1990).

In order to prevent abnormal proliferation CDKs are subject to multiple layers of regulation. In addition to regulation by cyclin binding, CDKs are also subject to regulation by CDK inhibitor (CKI) proteins. These proteins belong to one of two distinct families: the INK4a family (p16, p15, p18, p19), and the Cip/Kip family (p21, p27, p57). INK4a family members selectively inhibit the cyclin-D binding kinases cdk4 and cdk6, and are therefore specific only for early G1 phase. Cip/Kip members inhibit cdk1 and cdk2 kinases and can therefore have effects at multiple stages of the cell cycle (Coqueret, 2003).

Another major method of CDK regulation is through phosphorylation on highly conserved sites (Lew and Kornbluth, 1996). Phosphorylation of cdk1 (also known as Cdc2), the prototypical CDK, at Tyr-15 inhibits activity (Gould and Nurse, 1989; Krek and Nigg, 1991), while phosphorylation at Thr-161 promotes activity (Solomon et al., 1992). All identified cell cycle CDKs contain phosphorylation sites homologous to Tyr-

15 and Thr-161 of cdk1. Inhibitory phosphorylation on N-terminal Tyr residues is carried out by Wee1 kinases (Featherstone and Russell, 1991; Parker and Piwnica-Worms, 1992; McGowan and Russell, 1995; Mueller et al., 1995). Activating phosphorylation of Thr residues is performed by the CDK activating kinase (CAK) (Desai et al., 1992; Solomon et al., 1992). These phosphorylation events occur only once the CDK is bound to its cyclin partner. Inhibitory Tyr phosphorylation is dominant over Thr phosphorylation such that the dual phosphorylated kinase is still inactive. The phosphorylation state of these critical Tyr residues is also controlled by Cdc25 phosphatases. Cdc25 removes inhibitory phosphates, thereby promoting CDK activity.

The overriding theme of the work presented in Chapters 2 and 3 is to describe how the reactivation of cell cycle pathways can be a critical step on the road to neuronal death. Because the transition between G1 and S phases is absolutely critical in any decision to re-enter the cell cycle, and E2F is the key determinant of this transition, I will concentrate on reviewing E2F and the mechanisms by which it controls the G1/S transition. As mentioned above, CDK-mediated phosphorylation of pRb, and the resultant release and activation of E2F, constitutes the major control point of this transition. Further details of this control circuit are outlined below.

1.6 E2F1 and the E2F Family

The roots of our understanding of E2F lie in studies of adenovirus replication. The name E2F itself means E2 promotor binding factor, indicating the ability to activate the adenovirus E2 promoter (Kovesdi et al., 1987; La Thangue and Rigby, 1987; Yee et al., 1987). Early studies using gel shift assays found that E2F activity could be separated into free and complexed forms. The retinoblastoma protein (pRb) was identified as a component of complexed E2F based on the ability of the adenovirus E1A protein, which binds and sequesters pRb, to promote the formation of free E2F and increase E2 transactivation (Yee et al., 1987; Bagchi et al., 1991; Bandara and La Thangue, 1991; Chellappan et al., 1991). Since pRb was originally identified as a tumor suppressor, it was soon realized that E2F was a key effector of cell division downstream of pRb inactivation. The mechanisms by which pRb and E2F control cell proliferation have therefore, been of major interest in the field of cancer research. Thousands of studies have been directed at unraveling these mechanisms and we now know a great deal about the functions of E2F. These functions include not only a major role in controlling cell division but also in cell death, differentiation, DNA repair, and even in inhibition of cell division. A number of excellent reviews summarizing E2F's various roles can be found (Dyson, 1998; Helin, 1998; Lavia and Jansen-Durr, 1999; Phillips and Vousden, 2001; Stevaux and Dyson, 2002; Trimarchi and Lees, 2002; Dimova and Dyson, 2005).

Functional E2F is actually a heterodimer consisting of one member of a family of E2F proteins and one DP protein (Girling et al., 1993). The DP proteins stimulate

transcription indirectly by potentiating the activity of E2F (Bandara et al., 1993; Helin et al., 1993b). In mammals there are currently 8 identified members of the E2F family (E2F1-8) and three DP family members (DP1-3). See Figure 4 for a schematic representation of the E2F family. E2F and DP proteins contain homologous DNA-binding and dimerization domains. E2F1-3 contain a C-terminal transactivation domain that is not found in the DP proteins. E2F4-8 do not contain functional transactivation domains and are thought to act as dominant inhibitors of E2F transcription (Morkel et al., 1997; Trimarchi et al., 1998; de Bruin et al., 2003a; Di Stefano et al., 2003; Logan et al., 2005; Maiti et al., 2005). E2F7 and 8 are also unique in that they lack DP dimerization domains but seem to make up for this by possessing a second DNA binding region (de Bruin et al., 2003a; Di Stefano et al., 2003; Logan et al., 2005; Maiti et al., 2005). The pRb family has also extended to include three different members; pRb, p107, and p130 (Ewen et al., 1991; Hannon et al., 1993). Embedded within the transactivation domains of E2F1-5 is a short sequence critical for binding to a member of the pRb family (Helin et al., 1993a). This short sequence fits directly into the pocket domain of the pRb protein, resulting in the physical shielding of the transactivation domain (Flemington et al., 1993; Helin et al., 1993a), providing a simple model to explain the inhibition of E2F transcription by pRb. Because E2F6-8 each lack the transactivation domain, they also lack this pRb interaction sequence and are not thought to associate with any pRb protein.

Functional divisions exist between E2F family members. E2F1-3 are considered the "activators", while E2F4-8 are generally considered inhibitors of E2F transcription. E2F6 and 8 represent a relatively new subclass of the inhibitory E2F family and their role in transcriptional repression and cell cycle control is not as well understood. Functional

Figure 4. **Structure of the E2F Protein Family.** E2F family members contain several distinct domains. E2F1-6 contain a single DNA binding domain and a single DP-dimerization domain. E2F7 and 8 are unique in that do not have a dimerization domain and contain two separate DNA binding domains. Transactivation domains are found in E2F1-4 but those in E2F4 and 5 do not appear functional in expression assays. Embedded within the transactivation domain is a sequence responsible for binding to members of the pRb family. Localization can be regulated by binding to DP or pRb proteins but also through nuclear localization sequences, in the case of E2F1-3, and nuclear exclusion sequences, in the case of E2F4 and 5.

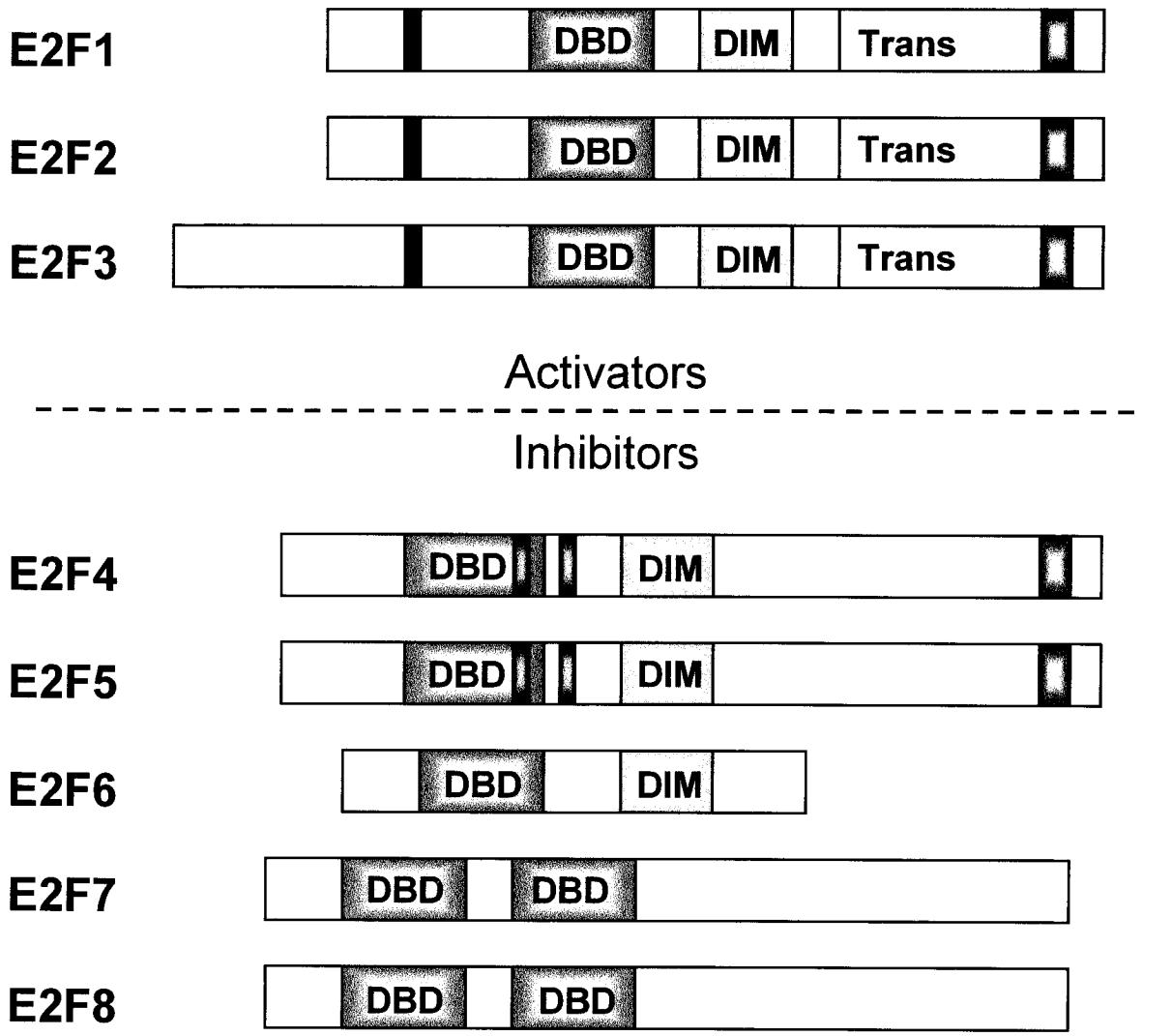
E2F1
DBD
DIM
Trans
E2F2
DBD
DIM
Trans
E2F3
DBD
DIM
Trans
Activators
Inhibitors
E2F4
DBD
DIM
E2F5
DBD
DIM
E2F6
DBD
DIM
E2F7
DBD
DBD
E2F8
DBD
DBD
Nuclear Localization Signal
Nuclear Exclusion Signal
pRb Family Binding Region
DBD
DNA Binding Domain
DIM
Dimerization Domain
Trans
Transactivation Domain

Figure 4

divisions are mirrored by preferences in the pairing of E2F and pRb proteins; E2F1-3 preferentially bind to pRb, E2F-5 binds to p130, and E2F4 binds primarily to p107 but is also able to bind p130, and pRb (Dyson, 1998). Complexes of p130/E2F4/5 seem most common in non-dividing cells while pRb/E2F1-3 complexes and free E2F1-3 predominate in cells traversing the G1-S phase transition (Ikeda et al., 1996; Moberg et al., 1996). The most abundant E2F species in the majority of cells, including neurons, is E2F4, and its overall levels remain quite stable throughout the cycle.

As mentioned, E2Fs can act as either transcriptional activators or transcriptional repressors. The predominant activators are E2F1-3 and the predominant repressors are E2F4 and E2F5. Transcription of E2F-responsive genes can occur due either to direct activation of transcription, or via loss of active repression (Ross et al., 2001). Active repression is mediated mainly by E2F4 and E2F5 through their association with p130 and p107 (Harbour and Dean, 2000; Nielsen et al., 2001; Vandel et al., 2001). Loss of repression occurs when p130 and p107 become phosphorylated and dissociate from E2F4/5. In total, three distinct mechanism of gene control can occur at E2F target sites: direct transcriptional activation by free E2F1-3, direct transcriptional repression by complexed E2F4/5, or indirect transcriptional activation due to loss of repression. Direct activation by E2F1-3 is required for S phase while direct repression by E2F4/5 is required for quiescence or to maintain cells in a differentiated state.

Activating E2Fs

In overexpression assays E2F1-3 are potent transcriptional activators and efficiently drive quiescent cells into the cell cycle (Johnson et al., 1993; Lees et al., 1993;

Qin et al., 1994; Lukas et al., 1996). Activation of endogenous E2F1-3 requires the phosphorylation of pRb. There are 16 distinct CDK consensus phosphorylation sites on pRb. In response to mitotic signals pRb is first phosphorylated by cyclin D-associated kinases, and subsequently, by cyclin E-associated kinases (Mittnacht, 1998). Primary phosphorylation by cyclin D kinases is thought to cause an initial low level of E2F activation, which upregulates cyclin E, thus promoting a feed forward signal to further phosphorylate pRb and establish strong E2F transcription (Ohtani et al., 1995).

The molecular mechanisms of E2F transactivation following pRb phosphorylation have not been clearly established. Nevertheless, an outline of these mechanisms has emerged. The transactivation domains of the activator E2Fs are able to promote transcription by at least two means. The first is by promoting histone acetylation and the second is by recruitment and activation of components of the basal transcriptional machinery. Activating E2Fs interact with a variety of histone acetyltransferase complexes including PCAF/GCN5, p300/CBP, and Tip60 (Trouche et al., 1996; Fry et al., 1999; Lang et al., 2001; Taubert et al., 2004). These interactions lead to the acetylation of histone proteins near E2F target sites and allow easy access to target genes by the transcriptional machinery. This machinery is also actively recruited by the E2F transactivation domain. E2F1 interacts through its transactivation domain with the TATA binding protein (TBP) and TFIIH, which in turn promote the formation of the RNA polymerase II pre-initiation complex (Emili and Ingles, 1995; Pearson and Greenblatt, 1997).

Attempts to identify E2F-regulated genes have resulted in the identification of many hundreds of targets (Bracken et al., 2004). Many, but not all, of these genes have

known functions in controlling either DNA synthesis or transition through cell cycle stages beyond S phase (DeGregori et al., 1995). Targets with roles in DNA synthesis include those whose products orchestrate assembly of the pre-replication complex at origins of replication such as mini chromosome maintenance proteins (MCMs), Orc1, and Cdc6 (Ohtani et al., 1996; Yan et al., 1998; Ohtani et al., 1999). Some E2F targets are required for the synthesis of DNA precursors (dihydrofolate reductase) or for the direct synthesis of DNA (thymidine kinase, DNA polymerase α, proliferating cell nuclear antigen, and ribonucleotide reductase (Blake and Azizkhan, 1989; Ogris et al., 1993; Dou et al., 1994; Li et al., 1994; DeGregori et al., 1995; Wade et al., 1995; Chabes et al., 2004).

Some of the most strongly induced E2F target genes are those involved in further control of cell cycle transition. As mentioned previously, cyclin E is induced by E2F leading to an autoactivating feedback loop. Activating E2Fs also control expression of the S-phase promoting products c-Myb and cdk2 (DeGregori et al., 1995; Campanero et al., 1999). E2F-directed gene expression also functions to control cell cycle events beyond S phase. For instance, one important target is cyclin A, which is required for timely S phase exit. Cyclin A/cdk2 targets include the activating E2Fs themselves. Phosphorylation by cyclin A/cdk2 inhibits E2F DNA binding activity and negatively regulates transactivation (Krek et al., 1994; Xu et al., 1994), effectively providing an autoinhibitory feedback loop that allows E2F activity to be stopped once DNA synthesis is completed. Interestingly, this feedback loop works in a completely opposite way to the cyclin E/cdk2 feedback loop.

E2F1-3 also target genes with functions in G2 and M phases such as cyclin B1 and B2, cdk1, and cdc25a, all of which work cooperatively to orchestrate numerous mitotic events (Ishida et al., 2001; Muller et al., 2001; Ren et al., 2002). Other targets also include SMC2L1/4L1 (structural maintenance of chromosomes 2/4 like 1), CENPA (centromere protein A), and MAD2L1 (mitotic arrest deficient 2-like 1), which are involved in chromosome condensation and segregation (Ren et al., 2002). Importantly, E2F target sites are present in the promoters of each of the activating E2Fs themselves, providing another feed forward activating mechanism (Lavia and Jansen-Durr, 1999; Ren et al., 2002).

An important problem which emerged after observing the variety and number of different E2F targets is how to determine which particular E2F is responsible for mediating the expression of which genes. Knockout strategies have been useful in addressing this question. E2F1 or E2F2 knockouts display only small tissue specific variations over wild type animals and isolated cells from these animals are able to proliferate quite normally (Field et al., 1996; Leone et al., 2001). E2F3 deficient animals, however, are more severely affected. The majority die *in utero* with proliferation defects and those that do survive succumb prematurely (Humbert et al., 2000; Cloud et al., 2002). Furthermore, E2F3 deficient mouse embryonic fibroblasts (MEFs) show a dramatic reduction in their ability to respond to mitogenic signals (Humbert et al., 2000). These data indicated that E2F3 plays a predominant role in the control of E2F transcription. However, the presence of E2F1 may be able to at least partially compensate in the absence of E2F3 since E2F3/E2F1 double mutant embryos show an exacerbation of phenotype over single $E2F3^{-/-}$ embryos (Wu et al., 2001). Cells from triply deficient

mutants lacking all three activating E2Fs were completely unable to induce DNA synthesis and could not proliferate. This observation clearly established E2F1-3 as the major, if not the sole, cellular mediators of cell cycle progression at S phase (Wu et al., 2001).

Repressor E2Fs

E2F4 or E2F5 expression does not strongly induce transcription and cannot promote cell cycle re-entry (Muller et al., 1997; Verona et al., 1997). Loss of both E2F4 and E2F5 renders cells completely resistant to G1 arrest mediated by $p16^{INK4a}$ (Gaubatz et al., 2000). The highest levels of E2F4/5 are found in quiescent cells (Ikeda et al., 1996; Moberg et al., 1996). E2F4 and E2F5 are thought to maintain and promote cell cycle exit through their recruitment of pRb, p107 and p130 to E2F promoters. At these promoters pRb family members actively engage histone and DNA modifying enzymes which cooperate to silence transcription. Some of the enzymes recruited by pRb proteins include histone deacetylases (HDACs), SWI/SNF factors, and methyltransferases (Brehm et al., 1998; Luo et al., 1998a; Magnaghi-Jaulin et al., 1998; Harbour and Dean, 2000; Zhang et al., 2000; Nielsen et al., 2001; Vandel et al., 2001).

Not only does the function of E2F4 and E2F5 diverge sharply from that of E2F1-3, but also the sequences are quite distinct (Figure 4). E2F4/5 lack the domain that is amino-terminal to the DNA binding region. In E2F1-3 this region contains a nuclear localization sequence (NLS). E2F4/5 are also unique in that they contain a nuclear exclusion sequence (NES) just C-terminal to the DNA binding region. As expected, these structural differences result in divergent expression profiles. E2F1-3 are

constitutively translocated to the nucleus whereas the location of E2F4/5 change throughout the cell cycle (Verona et al., 1997). In G0, E2F4/5 are found in the nucleus but with the transition to S phase almost all of the E2F4/5 becomes localized to the cytoplasm (Lindeman et al., 1997; Muller et al., 1997). Nuclear localization of E2F4/5 is dependent on association with other nuclear proteins, possibly DP1/2 or p107/p130 (Lindeman et al., 1997).

Cells that are deficient in both E2F4 and E2F5 display a severe defect in their ability to induce cell cycle exit in response to various signals, including $p16^{INK4a}$ expression, contact inhibition, and DNA damage (Gaubatz et al., 2000). These cells can still respond normally to stimuli which promote cell division and they proliferate normally (Lindeman et al., 1997; Gaubatz et al., 2000; Rempel et al., 2000). Taken together, these results indicate that E2F4/5 are required for cell cycle exit yet dispensable for proliferation.

E2F1 In Apoptosis

The generation of E2F1 knockout mice unexpectedly led to the attribution of an entirely novel function to E2F1. Surprisingly, these mice did not display any major defects in cellular proliferation. Instead, the main phenotypes seemed to result from abnormally increased proliferation. E2F1 deficiency leads to both an excess of mature T cells, due to a developmental defect in thymocyte apoptosis, and to the development of a range of tumors in adulthood (Field et al., 1996). These observations suggested that E2F1 may also play a role in negatively regulating growth. Consistent with this, separate studies also observed that overexpression of E2F1 *in vitro* causes high levels of death by

apoptosis (Qin et al., 1994; Shan and Lee, 1994; Kowalik et al., 1995; Asano et al., 1996). It is now generally accepted that the apoptotic ability of E2F1 allows proliferating cells to couple misregulated proliferative signals, which are potentially harmful, to a preventative apoptosis program.

E2F1-induced death can occur in either a p53-dependent, or p53-independent manner. In many cell types E2F1 expression leads to an increase in p53 expression (Hiebert et al., 1995), and cells that are deficient for p53 are often resistant to E2F1 apoptosis (Wu and Levine, 1994; Kowalik et al., 1995). E2F1 was shown to induce p53-dependent apoptosis through the induction of $p19^{ARF}$ (Bates et al., 1998; Zhang et al., 1998). $p19^{ARF}$ indirectly leads to stabilization of p53 by disrupting the interaction between MDM2 and p53. MDM2 normally keeps p53 at low levels in the cell by targeting it for degradation (Momand et al., 1992; Wu et al., 1993). When MDM2 is bound by $p19^{ARF}$ it is no longer able to perform this function (Pomerantz et al., 1998). The result is the activation of the p53-mediated death involving Bax and the intrinsic death pathway. Death induced by E2F1 in this way requires the presence of an intact transactivation domain.

Other studies have found that E2F1 mutants lacking the transactivation domain are still capable of inducing death (Hsieh et al., 1997; Phillips et al., 1999). Furthermore, wild type or transactivation-deficient E2F1 are still capable of inducing death in p53 null cell types (Hsieh et al., 1997). However, functional DNA binding is still required for the apoptotic potential of E2F1 (Phillips et al., 1997; Phillips et al., 1999). This p53-independent death induced by E2F1 may be mediated by p73, a family member of p53, since its disruption prevents such death (Irwin et al., 2000; Lissy et al., 2000).

Alternatively, or perhaps in parallel, E2F1 can also promote p53-independent death by downregulating pro-survival signals originating from the tumor necrosis factor receptor (Phillips et al., 1999).

Whether E2F1 is the only member of the E2F family that is capable of inducing cell death is still somewhat of an open question. The earliest data to address this question reported that the overexpression of E2F2 and E2F3 failed to induce apoptosis (DeGregori et al., 1997). This result was later supported by results from the same group (Kowalik et al., 1998). Further evidence was obtained by another group who reported that cells lacking E2F1 were resistant to c-Myc induced death but those lacking E2F2 or E2F3 showed no such resistance (Leone et al., 2001). However, at least two groups have reported that E2F2 and E2F3 are equally as capable at inducing cell death as E2F1 (Vigo et al., 1999; Trimarchi and Lees, 2002), and that E2F1 null and E2F3 null cells are protected from apoptotic stimuli (Trimarchi and Lees, 2002). The controversy over whether or not E2F1 is the only E2F family member capable of signaling apoptosis may have been recently solved with the observation that although E2F3 overexpression can induce apoptosis, it cannot do so in E2F1 null cells (Denchi and Helin, 2005).

The above evidence clearly established an important function for E2F1 (and possibly for E2F2 and E2F3) in death of proliferating cells under a variety of circumstances. Whether or not E2F1 was also involved in promoting the death of post-mitotic neurons, however, was not known until the publication of the data presented here in Chapter 2.

1.7 Cyclin-Dependent Kinase 5: The Neuron Specific CDK

Cdk5 was originally identified based on its nearly 60% homology to cdk1 (Lew et al., 1992; Meyerson et al., 1992). Cdk5 is expressed in many different tissues but, interestingly, its activity is highest when purified from brain extracts (Tsai et al., 1993). Moreover, although cdk5 could be found to interact with cyclin D1, it did not seem to be activated by this interaction, and it is unable to perform a cell cycle related function (Meyerson et al., 1992; Xiong et al., 1992). Why would a member of the CDK family be selectively active in a predominantly post-mitotic environment? This question generated a great deal of interest in the neurobiological community and spawned a prolific series of publications which have contributed greatly to our understanding of the function of cdk5 in the nervous system.

Cdk5, like other CDKs, requires association with a regulatory co-factor for enzymatic activity. Since known cyclins were unable to activate cdk5 the hunt was on to discover the identity of this necessary co-factor. Using extracts of brain protein, the cdk5 activator was identified as p35 (Ishiguro et al., 1994; Lew et al., 1994; Tsai et al., 1994). P35 is expressed predominantly in brain tissue, explaining the localization of cdk5 activity. Shortly after the identification of p35 a homologous protein, p39, was cloned and also found to be capable of activating cdk5 (Tang et al., 1995). The expression of p39 is also highest in neurons although it does not completely overlap with that of p35 (Zheng et al., 1998; Wu et al., 2000a). P35 and p39 share approximately 57% amino acid identity. Although p35 and p39 do not display any significant primary sequence

homology to cyclins, it has been proposed that their mature structure bears significant similarity (Tang et al., 1997; Chou et al., 1999; Tarricone et al., 2001). Both p35/p39 and cdk5 are very highly conserved amongst other eukaryotes including fly, worm, and yeast (Dhavan and Tsai, 2001).

The p35 sequence contains consensus cdk5 phosphorylation sites (identified as XS/TPXK/R, where X is a neutral or basic amino acid) that are actively phosphorylated (Patrick et al., 1998). Phosphorylation of p35 by cdk5 is thought to regulate the stability and half-life of p35 since mutants lacking these sites are much more long lived (Patrick et al., 1998). The inhibition of cdk5 activity also increases the half-life of p35 several fold. Together this suggests that cdk5/p35 complexes maintain an autoinhibitory feedback mechanism to regulate activity. Protein levels of p35 are controlled through ubiquitination and degradation by the proteosome pathway (Patrick et al., 1998; Saito et al., 1998). Phosphorylation by cdk5 signals p35 for ubiquitination and initiates degradation (Patrick et al., 1998; Saito et al., 2003). Interestingly, the phosphorylated form of p35 predominates in the fetal mouse brain while the unphosphorylated form is more common in adult brain (Saito et al., 2003). Overall levels of p35 increase during development in line with the switch from phosphorylated to unphosphorylated forms.

Cdk5 activity can be regulated by phosphorylation events but in a manner distinct from that of mitotic CDKs. Cdk5 contains conserved residues similar to cdk1 Thr14, Tyr15 and Thr161 (Thr14, Tyr15, and Ser 159 in cdk5). Thr14 and Tyr15 of cdk1 are phosphorylated by Wee1/Myt1 kinases and these events are inhibitory. Despite the conservation of Thr14 and Tyr15 in cdk5, these sites can no longer be phosphorylated by Wee1 (Poon et al., 1997). However, Tyr15 can be phosphorylated by c-Abl (Zukerberg

et al., 2000). Strangely, this phosphorylation appears to stimulate rather than inhibit cdk5 activity (Zukerberg et al., 2000). Thr14 of cdk5 can be phosphorylated by a kinase present in bovine thymus cytosol (Matsuura and Wang, 1996). In this case the reported result is to inhibit cdk5 kinase activity. How exactly phosphorylation of immediately adjacent amino acids results in opposite effects is unknown and requires further investigation. The Ser159 site of cdk5 has been reported to be phosphorylated by the CDK activating kinase (CAK). While one report suggested that CAK leads to activation of cdk5 (Rosales et al., 2003), other reports have concluded that Ser159 is dispensable for activity (Poon et al., 1997) and that its phosphorylation actually disrupts cdk5-p35 interaction (Tarricone et al., 2001).

Despite this biochemical data little was known about the function of cdk5 within the nervous system until the development of genetic knockouts. These experiments would lead to the discovery that cdk5 plays an essential role in establishing proper brain architecture during development. Cdk5 null mice die *in utero* or shortly after birth with conspicuous aberrations in the layering of numerous brain structures including the cerebral cortex, hippocampus, cerebellar cortex, and olfactory cortex (Ohshima et al., 1996; Gilmore et al., 1998). In the cerebrum the normal cellular layering is completely inverted with later born neurons failing to migrate past previously born neurons (Ohshima et al., 1999). These phenotypes, as well as complete survival, can be restored by expressing cdk5 under the p35 promoter (Tanaka et al., 2001). Deletion of p35 also causes inversion of the cerebral cortex, but with less severe disordering of hippocampal and cerebellar structures than the cdk5 mutant (Chae et al., 1997; Hallows et al., 2003). As a result, p35 null mice actually survive and can breed, although one group reports an

increased susceptibility to seizures (Chae et al., 1997). One reason for the reduced severity of the p35 knockout is compensation by p39. Targeted deletion of both p35 and p39 produces prenatal/perinatal lethality and developmental abnormalities almost indistinguishable from cdk5 deficiency (Ko et al., 2001). Loss of p39 alone does not produce any detectable brain abnormalities, suggesting that p35 can completely compensate in its absence. The phenotype of p35 nulls suggests that p39 is only partially capable of compensating for loss of p35.

Once this function in nervous system development was found for cdk5, the molecular mechanisms by which it acts in neurons was pursued. The migration defect in the cortex of $p35^{-/-}$ and $cdk5^{-/-}$ mice stimulated an investigation of cdk5 in control of neurite outgrowth, cytoskeletal dynamics, cell-cell adhesion, and transport, all of which are known to regulate migration. Inhibition or downregulation of cdk5, p35, or p39 in cultured neurons hinders normal extension of axons (Nikolic et al., 1996). Overexpression of cdk5 and p35 results in longer axon length, and exogenous expression of p35 or p39 in fibroblasts causes the production of lemellipodia and filopodia (Nikolic et al., 1996). Moreover cdk5 and p35 are abundant in axonal growth cones, where they co-localize with F-actin (Nikolic et al., 1996). Cdk5 likely acts as a central control switch in growth cone extension by interacting with Rac, a Rho family GTPase (Nikolic et al., 1998). This interaction results in the phosporylation and activation of PAK1, which dynamically regulates actin polymerization (Joneson et al., 1996), a necessary event in growth cone motility.

Cdk5 can also phosphorylate the microtubule associated proteins MAP1B and Tau (Baumann et al., 1993; Paudel et al., 1993; Ishiguro et al., 1994; Paglini et al., 1998).

Phosphorylation of MAP1B by cdk5 appears to promote axonal elongation (Paglini et al., 1998), whereas phosphorylation of Tau interferes with the ability of Tau to promote microtubule assembly (Wada et al., 1998; Patrick et al., 1999; Evans et al., 2000). It has been proposed that cdk5 phosphorylation of Tau occurs as a pathological event in various disease states, leading to microtubule destabilization and neurofibrillary tangles (see section below on cdk5 in neuronal death).

It is possible that inappropriate adhesion is at the root of the migration defects of cdk5/p35 deficiency. N-cadherin is a transmembrane protein that promotes adhesion in multiple cell types and suppression of its activity is known to promote migration (Wheelock and Johnson, 2003). Through its intracellular domain N-cadherin binds β-catenin, and this interaction is required for N-cadherin-mediated cell-cell adhesion (Hirano et al., 1992). Binding studies showed that β-catenin directly interacts with p35 (Kwon et al., 2000). This association allows cdk5 to phosphorylate β-catenin, disrupting its binding to N-cadherin (Kesavapany et al., 2001). In this way cdk5 activity can prevent N-cadherin mediated cellular adhesion. Loss of any one or more of the functions listed here for cdk5 (cytoskeletal dynamics or adhesion) may in part explain the dramatic migration defect of cdk5 and p35 null mice.

In addition to the above functions cdk5 has also been implicated as a key mediator of pre- and post-synaptic signaling. Cdk5, along with its regulators p35 and p39, are all expressed at high levels in adult neurons and they are highly enriched in synaptic compartments. Cdk5 phosphorylates several different synaptic proteins. One of these is MUNC18, a negative regulator of vesicle-SNARE interactions (Hata et al., 1993). Phosphorylation by Cdk5 antagonizes this function of MUNC18, thereby facilitating

vesicle fusion and secretion (Shuang et al., 1998; Fletcher et al., 1999). Another pre-synaptic protein, dynamin I, is a cdk5 substrate involved in synaptic vesicle endocytosis. In this case cdk5 phosphorylation of dynamin I is essential for priming the synaptic vesicle endocytosis program (Tan et al., 2003). In a somewhat similar manner, cdk5 also influences endocytosis through phosphorylation of synaptojanin I (Lee et al., 2004) and amphiphysin I (Floyd et al., 2001; Tomizawa et al., 2003). These synaptic functions of cdk5 may underlie a generalized role in learning and memory (Cheng and Ip, 2003; Fischer et al., 2003a; Fischer et al., 2005; Ohshima et al., 2005).

Yet another process in which cdk5 has been implicated is the post-synaptic control of dopamine signaling. Cocaine treatment blocks dopamine transporters and increases the amount of dopamine at synapses. Long lasting changes in gene expression induced by chronic cocaine administration are mediated through intracellular signaling initiated at the dopamine receptor. Protein kinase A (PKA) plays a critical role in this signaling by linking the receptor to downstream effectors such as CREB. PKA activity is antagonized through dephosphorylation by protein phosphatase-1 (PP-1). One of the targets of PKA is DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, molecular mass 32 kDa), and phosphorylation by PKA on Thr-34 converts DARPP-32 into a potent PP-1 inhibitor (Blank et al., 1997). Therefore, through DARPP-32 PKA is able to promote its own activity. One of the effects of chronic cocaine treatment is to increase the post-synaptic levels of p35 and cdk5 (Bibb et al., 2001; McClung and Nestler, 2003). Inhibition of cdk5 potentiates the behavioural effects of cocaine administration in rodents (Bibb et al., 2001). Cdk5 is able to dampen post-synaptic dopamine signaling by phosphorylating DARPP-32 on Thr-75 (Bibb et al., 2001). This

phosphorylation has the opposite effect of Thr-34 phosphorylation and effectively converts DARP-32 into an activator of PP-1, leading to decreased PKA activity. These findings implicate cdk5 in long term synaptic modifications underlying drug addiction.

It should briefly be noted that cdk5 has now been shown to be involved in a diverse range of processes outside of the nervous system. As mentioned above, although cdk5 is most highly expressed in neurons, it is also easily detectable in other tissues. Its activity, on the other hand, was not readily detected in non-neuronal cells because of the lack of p35/p39 expression. However, more recent results have found that p35/p39 are occasionally expressed in non-neuronal cells where they regulate important cdk5 activities. For example, pancreatic beta cells express p35 and p39, and high levels of cdk5 activity can be purified from these cells (Lilja et al., 2004; Ubeda et al., 2004; Wei et al., 2005). In these cells p35/cdk5 complexes were shown to be negative regulators of glucose-stimulated insulin secretion (Wei et al., 2005). As another example, cdk5 activity has been reported to be essential for irreversible cell cycle exit, or senescence. Some transformed cells undergo senescence in response to expression of wild type pRb. This event was shown to require cdk5 phosphorylation of ezrin and Rac1, proteins which orchestrate structural changes associated with senescence (Yang et al., 2003a; Yang and Hinds, 2003; Alexander et al., 2004). Other recent work has implicated cdk5 as a critical signal in the dispersal of non-innervated acetylcholine receptor clusters at the developing neuromuscular junction (Lin et al., 2005). A variety of other non-neuronal functions for cdk5 have also been described (Dhavan and Tsai, 2001). Section 1.9 below outlines the substantial evidence implicating cdk5 as a mediator of neuronal death and also reviews the reports which suggest it may be capable of performing a pro-survival function.

1.8 Cell Cycle Pathways in Neurodegeneration

Neurons are highly differentiated post-mitotic cells that are thought to have permanently exited the cell cycle. However, over the last 10 to 15 years numerous studies have revealed that reactivation of the cell cycle is often a common and required event during neuronal death (Copani et al., 2001; Becker and Bonni, 2004; Greene et al., 2004; Herrup et al., 2004). This data will be summarized in the following section with an emphasis on components of the G1-S phase transition that point towards the potential involvement of E2F1 in the death process.

Early Evidence

A possible link between cell division and cell death was first considered in the context of proliferating cells when it was observed that cells undergoing apoptosis display membrane and nuclear changes similar to cells in the early to mid stages of mitosis (Vermeulen et al., 2003). Since then a wide array of proteins originally identified on the basis of their ability to modulate the cell cycle have also been shown to have roles in apoptosis of proliferating cells (Evan et al., 1995; Vermeulen et al., 2003; Senderowicz, 2004). These include CDKs and their upstream regulators and downstream effectors, such as c-Myc, Ras, p53, pRb, E2F and others (Evan et al., 1995). The ability of proteins that are involved in cell-cycle control to modulate cell death provides the cell with the ability to escape inappropriate proliferative (and potentially oncogenic) signals by inducing death.

The earliest direct suggestion that cell cycle deregulation could compromise neuronal survival came with the development of SV40 T antigen (SV40Tag) transgenic mice in the laboratory of Harry Orr. These mice were engineered to express SV40Tag selectively in Purkinje neurons of the cerebellum. Interestingly, SV40Tag transgenic mice developed ataxia early in life that was directly related to the degeneration of Purkinje neurons after differentiation (Feddersen et al., 1992). SV40Tag is known to interact with pRb and disrupt its ability to sequester E2F (Cao et al., 1992). Degeneration of SV40Tag expressing Purkinje neurons suggested that pRb may play some role in maintaining their survival. Indeed this appeared to be the case since the pRb-SV40Tag interaction was required for this effect (Feddersen et al., 1995). E2F1 is also likely involved since its modulation can influence the Purkinje neuron survival (Athanasiou et al., 1998). These results from Orr's group hinted at a previously unknown capacity for pRb/E2F in the control of neuronal survival.

The generation of whole embryo pRb null mice added further support to this nascent concept. Mice lacking pRb in all tissues die prior to E16 with multiple abnormalities, the most conspicuous of which are massive ectopic mitoses and neuronal death throughout the central and peripheral nervous systems, as well as defective erythropoiesis (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Many neurons appear to enter apoptosis after re-entering S phase (Lee et al., 1994), suggesting the involvement of E2F. Indeed, additional loss of E2F1 prevents the widespread neuronal apoptosis and S phase entry seen in $pRb^{-/-}$ embryos (Tsai et al., 1998). This death was also dependent on p53 in central neurons but not in peripheral neurons (Macleod et al., 1996), suggesting the activation of a p53-dependent E2F1 death pathway in the CNS.

Together, these mouse knockout models were considered some of the strongest evidence that deregulated cell cycle control leads to neuronal death. However, the basis for this conclusion was called into question with the development of conditional pRb knockouts. If pRb expression is maintained in the developing placenta alone, the massive neuronal death seen in complete pRb knockouts is prevented (de Bruin et al., 2003b; Wu et al., 2003). With this observation it appeared that the neuronal death seen in whole embryo $pRb^{-/-}$ mice is an indirect effect of placental dysfunction. Consistent results were observed when pRb was deleted in specific neuronal populations at defined developmental periods (Lipinski et al., 2001; Ferguson et al., 2002; MacPherson et al., 2003). These studies showed that pRb deficient neurons, developing within an animal expressing wild type pRb in non-neural tissues, undergo ectopic S-phase entry but do not succumb to apoptosis. These newer findings necessitated an entirely new interpretation of the original whole embryo pRb knockout data. Nevertheless, the early pRb knockout findings were of central importance in initiating interest in the cell cycle/neuronal death hypothesis.

The above observations prompted a few authors to speculate about a general coupling of cell cycle to neuronal death and even that inappropriate initiation of this coupling may trigger neurodegeneration (Heintz, 1993; Ferrari and Greene, 1994; Rubin et al., 1994; Herrup and Busser, 1995). Such speculation instigated a series of controlled investigations using a variety of *in vitro* and animal death models. These experiments would more concretely establish a function for certain cell cycle components in neuronal death.

Evidence from In Vitro and Animal Models

The first model in which an association between cell cycle proteins and death was observed was the NGF deprivation model of cultured sympathetic and PC12 cells. Freeman et al. observed that expression of the cyclin D1 gene is induced following NGF deprivation (Freeman et al., 1994). Since this discovery, induction of cyclin D protein or message has been observed in numerous culture and animal models, including low $[K^+]$-induced death of cultured cerebellar granule neurons (CGNs) (Boutillier et al., 1999; Padmanabhan et al., 1999; Sakai et al., 1999), β-amyloid exposure of cultured cortical neurons (Copani et al., 1999), cisplatin toxicity in rats and PC12 cells (Gill and Windebank, 1998; Fischer et al., 2001), mutant mouse models of ALS (Nguyen et al., 2003), animal models of stroke (Timsit et al., 1999; Osuga et al., 2000), and kainic acid administration (Giardina et al., 1998; Timsit et al., 1999; Park et al., 2000a). Inhibition of Ras, which is thought to be an upstream activator of cyclin D expression in proliferating cells, is sufficient to prevent NGF deprivation induced death of PC12 cells (Ferrari and Greene, 1994). The significance of cyclin D1 in neuronal death is demonstrated by the observations that deletion or downregulation of cyclin D1 protects neurons from hypoxia (Rashidian et al., 2005) and kainic acid (Ino and Chiba, 2001).

As mentioned above, the binding partners for cyclin D are cdk4 and cdk6, and together these complexes are the main initiators of transition from G1 to S phase. Although proliferating cells contain relatively high levels of cdk4 or cdk6, most neurons have downregulated their expression (Angelastro et al., 2000; Greene et al., 2004). Therefore, for increased cyclin D to be functional in neurons it must also be accompanied by increased cdk4 or cdk6. In fact in many of the models listed above for cyclin D

induction, upregulation of cdk4 has also been observed (Gill and Windebank, 1998; Giovanni et al., 1999; Padmanabhan et al., 1999; Osuga et al., 2000; Ino and Chiba, 2001; Nguyen et al., 2003). Cdk6 expression has not been examined in most cases. An increase in cyclin D-associated kinase activity has also been demonstrated in the ALS model (Nguyen et al., 2003), the K^+ deprivation model (Padmanabhan et al., 1999), and in DNA damage induced death of cultured cortical neurons (Park et al., 1998a). Increased pRb phosphorylation, an indirect marker of CDK activation, has also been observed in death induced by DNA damage (Park et al., 1998a), K^+ deprivation (Padmanabhan et al., 1999), β-amyloid exposure (Giovanni et al., 1999; Copani et al., 2001), cerebral ischemia (Osuga et al., 2000), and other models.

The question of whether cdk4 is functionally relevant in neuronal death has been addressed using a variety of methods. The first method employed was pharmacological inhibition using drugs such as flavopiridol, olomucine, and roscovitine. These inhibitors, in particular flavopiridol, were very effective in blocking many different types of *in vitro* neuronal death (Park et al., 1996; Park et al., 1997b; Park et al., 1998a; Giovanni et al., 1999; Padmanabhan et al., 1999; Rideout et al., 2003). Flavopiridol treatment was also found to prevent ischemia-induced cyclin D upregulation, pRb phosphorylation, and neuronal degeneration (Osuga et al., 2000; Wang et al., 2002). Data using these agents was insufficient since they lack appropriate specificity and can also inhibit other signals which regulate survival. A second, more specific method to inhibit cdk4 has been to overexpress the CKIs $p16^{INK4a}$, $p21^{WAF}$, and $p27^{KIP1}$. Expression of either of these CKIs is sufficient to prevent death in a number of models (Park et al., 1997a; Park et al., 1998a; Rideout et al., 2003). Endogenous inhibition of CDKs may be essential to neuronal

maintenance *in vivo* since mice lacking p19INK4d and p27Kip1 die prematurely with extensive post-mitotic neuronal loss and neurological deficits (Zindy et al., 1999). Furthermore levels of p16INK4a and p27KIP1 decrease in models of ischemia prior to an increase in cyclin D1 and cdk2 kinase activity (Katchanov et al., 2001). A final means of CKD inhibition has been through the use of dominant negatives (DNs). Viral-mediated delivery of DNcdk4 or DNcdk6 significantly protect neurons from death induced by DNA damage (Park et al., 1998a), β-amyloid exposure (Giovanni et al., 1999), and proteosome inhibition (Rideout et al., 2003). Moreover, neurons cultured from DNcdk4 expressing transgenic mice are resistant to hypoxia treatment (Rashidian et al., 2005). However, potential non-specific effects of CKI and DN expression necessitate further work using more specific means of inhibition such as gene knockout and/or siRNA strategies.

Although cyclin D and cdk4/6 have been the most widely implicated, they are far from the only cell cycle proteins found to be involved in neuronal death. Cyclin E protein is upregulated in neurons exposed to β-amyloid (Copani et al., 1999), or low [K^+] (Padmanabhan et al., 1999). Kinase activity of cyclin E/cdk2 complexes is increased in low [K^+] treated cultured neurons (Padmanabhan et al., 1999) and following ischemia in striatal neurons (Katchanov et al., 2001). Cdk2 activity is required for some forms of neuronal death including death induced by β-amyloid (Copani et al., 1999) and proteosome inhibition (Rideout et al., 2003). In each of these models in which cyclin E and/or cdk2 have been implicated, cyclin D/cdk4/6 complexes have also been shown to be involved. It is not clear yet whether, as in actively dividing cells, cyclin D regulated activity precedes and promotes the activity of cyclin E complexes.

The mitotic phase of the cell cycle is controlled in large part by cyclin B and its partner cdk1. An important question is whether or not neurons, once stimulated to re-activate the cell cycle, are able to progress as far as M phase. Increased cyclin B levels have been associated with death in a limited number of death models; *in vitro* dopamine exposure (Shirvan et al., 1997) and activation of the p75 neurotrophin receptor (Frade, 2000). Interestingly, cyclin D upregulation did not occur in either instance. Cdk1 is elevated in CGNs *in vivo* undergoing developmental death, and *in vitro* in response to low [K^+] (Konishi et al., 2002). Most significantly, inhibition of cdk1 by expression of a dominant negative protects CGNs from low [K^+] (Konishi et al., 2002).

A major question of interest in the field of cell cycle and neuronal death has been how activation of these cell cycle components leads to death. It is now clear that in many cases cell cycle dysregulation leads to death through activation of conserved programmed cell death pathways, in particular the intrinsic death pathway. Inhibition of CDKs prevents death while at the same time preventing activation of caspases (Stefanis et al., 1999; Keramaris et al., 2000; Morris et al., 2001). Disrupting the function of Apaf-1 or pro-apoptotic members of the Bcl-2 family is often protective in the same models in which CDK inhibition is protective (Deckwerth et al., 1998; Xiang et al., 1998; Putcha et al., 1999; Keramaris et al., 2000; Fortin et al., 2001; Putcha et al., 2001). Furthermore, CDK inhibition also prevents mitochondrial events associated with death including membrane depolarization and Bax translocation (Stefanis et al., 1999). Precise mechanisms linking cell cycle re-activation to conserved death signals in neurons was lacking until after the report, contained here as Chapter 2, that E2F1 plays a required role in the death process. Subsequent studies established that E2F1 acts as a direct link

between CDKs, pRb phosphorylation, and activation of the intrinsic death pathway. This data is summarized in further detail in Chapter 4.

Evidence From Human Studies

The implications of cell cycle in *in vitro* neuronal death paradigms are made more significant by *in vivo* findings of ectopic expression of many cell cycle markers in a variety of neurodegenerative conditions. Expression of nearly all the major cyclins and CDKs has been detected in diseased human post-mortem brains. The majority of these studies have looked at AD brains but other conditions have also been examined. Cyclin D1 is aberrantly expressed in temporal lobe structures in AD (Busser et al., 1998) and in motorneurons of ALS patients (Ranganathan and Bowser, 2003). Increased cdk4 was also detected in susceptible neurons of AD patients (McShea et al., 1997; Busser et al., 1998), suggesting functional cyclinD/cdk4 complexes could be formed. In the cell cycle these complexes regulate the subsequent appearance and activation of cyclin E/cdk2 complexes. This may also happen in AD since elevations of cyclin E have been detected (Nagy et al., 1997). However, to date there are no reports of increased or aberrant expression of cdk2 in AD or any other disorder. The mitotic cyclin, cyclin B, as well as its activating partner cdk1, have been found to be upregulated in AD (Vincent et al., 1997; Busser et al., 1998; Vincent et al., 2001; Yang et al., 2003b), Down's syndrome, Pick's disease, progressive supranuclear palsy (Husseman et al., 2000), and stroke (Smith et al., 1999). Together, this evidence presents an association between cell cycle reactivation and neurodegenerative disease, particularly in AD. However, further research is required to determine whether this relationship is causative or merely

correlative. In the case of AD, it will be important to test whether cell cycle inhibition is protective in some of the more recent animal models.

In summary, multiple lines of evidence illustrate that cell cycle proteins are activated within neurons in response to various death triggers. In some cases their activity is essential for the death process, and that this may contribute to neurological disease. While a coupling of cell cycle regulation with apoptosis makes certain sense in a proliferating system, it is not clear why postmitotic neurons would utilize cell cycle proteins to induce death. One rationale is that neurons respond to certain pathological stimuli by attempting to re-enter the cell cycle but this re-entry is fatal because the biochemical state of the neuron is incompatible with cell division. Alternatively, it may simply be that some cell cycle proteins possess dual abilities, and that in neurons they are activated solely and specifically for their ability to induce death. However, consistent with the former hypothesis, there is evidence that under stress conditions neurons can proceed quite deep into the cell cycle. For example, treatment of neurons with β-amyloid or DNA damaging agents can lead to the upregulation of DNA polymerase-β and initiation of DNA synthesis (Wu et al., 2000b; Copani et al., 2002). Even more indicative of cell cycle commitment is the finding that some neurons in susceptible regions of AD brains have actually traversed S phase and duplicated their chromosomes (Yang et al., 2001).

1.9 Cdk5 in Neuronal Death and Survival

As mentioned previously, cdk5 has been shown to be essential for normal brain development and is involved in regulating numerous subcellular functions within neurons. However, similar to the mitotic CDKs, growing evidence now suggests that the inappropriate activation of cdk5 also evokes neuronal death. One important mechanism underlying cdk5-mediated death signaling involves the activation of calpain, a Ca^{2+}-dependent cysteine protease. Several reports have observed calpain-mediated cleavage of the cdk5 activator p35 to a smaller p25 form in *in vitro* models of cell death (Patrick et al., 1999; Kusakawa et al., 2000; Lee et al., 2000). The p25–cdk5 complex appears to be a more stable complex and is thought to produce a prolonged and inappropriately localized activation of cdk5 (Patrick et al., 1999). Further supporting the importance of p25–cdk5 in cell death is the observation that p25 overexpression induces neuronal death in culture, whereas the inhibition of cdk5 activity by antisense or dominant-negative strategies blocks cell death induced by β-amyloid and oxidative stress (Alvarez et al., 1999; Gong et al., 2003). The conversion of p35 to p25 has been reported for *in vitro* death models as well as *in vivo* models of ALS and Niemann-Pick type C disease, and in the post-mortem analysis of AD brains (Patrick et al., 1999; Nguyen et al., 2001; Bu et al., 2002). It is important to note that other researchers detect no evidence for p25 formation or cdk5 deregulation in AD (Yoo and Lubec, 2001; Tandon et al., 2003).

Several mechanisms have been proposed to account for the death-inducing potential of p25-activated cdk5. First, calpain-mediated generation of p25 has been

associated with the phosphorylation of Tau in *in vitro* AD models (Patrick et al., 1999). Hyperphosphorylated Tau is a major component of the neurofibrillary tangles found in AD and other neurodegenerative conditions and is thought to contribute to disruption of the cytoskeletal network. Overexpression of p25 in mice results in hyperphosphorylation of Tau and cytoskeletal abnormalities similar to those observed in AD pathology (Cruz et al., 2003). A second potential target of the p25–cdk5 complex is the NR2A subunit of the N-methyl-D-aspartate (NMDA) receptor. Wang *et al.* have shown that ischemia-induced death of CA1 pyramidal cells correlated with p25 expression and cdk5 activation (Wang et al., 2003). The phosphorylation of NR2A by cdk5 results in increased NMDA receptor activity following ischemia. Expression of dominant-negative cdk5 improved the survival of CA1 neurons following global ischemia (Wang et al., 2003).

A third potential mechanism by which p25 contributes to death is highlighted by observations that cdk5 activity might be targeted to the nucleus, suggesting the regulation of death- or survival-related transcription factors. Gong *et al.* showed that oxidative stress induces nuclear p25–cdk5 activity, resulting in the inhibition of myocyte enhancing factor 2 (MEF2) survival activity (Gong et al., 2003). Cdk5 inhibits MEF2 function through phosphorylation on Ser444 (Gong et al., 2003). Cdk5 has also been shown to phosphorylate and activate p53 (another nuclear protein) (Zhang et al., 2002), although the relevance of this event to death has not been clearly established.

Strong evidence also exists implicating cdk5 in the degeneration of midbrain dopamine neurons in PD. Firstly, cdk5 is accumulated in the Lewy bodies that are observed in dopaminergic neurons of postmortem PD brains (Brion and Couck, 1995; Nakamura et al., 1997). In addition, several reports suggest that cdk5 is ectopically

expressed in neonatal dopaminergic rat neurons undergoing programmed cell death (Henchcliffe and Burke, 1997; Neystat et al., 2001; El-Khodor et al., 2003). A similar increase in cdk5 levels and activity has been observed in the MPTP mouse model of PD (Smith et al., 2003). Most importantly, expression of DNcdk5 protects dopaminergic neurons from MPTP-induced degeneration *in vivo* and CDK inhibition strategies also improved the behavioral deficits that were associated with MPTP treatment (Smith et al., 2003).

In addition to the role described for cdk5 in neuronal death, there is also data suggesting cdk5 performs a pro-survival function within neurons. For example, p35/cdk5 can inhibit the pathogenic activity of JNK. Expression of p35/cdk5 suppresses UV-induced JNK activation in HEK293 cells (Li et al., 2002). Furthermore, cdk5-deficient cortical neurons display increased JNK phosphorylation and display greater death in response to apoptotic stimuli (Li et al., 2002). Cdk5 has also been identified as a positive activator of the phosphatidylinositol 3-kinase/Akt signaling, a powerful pro-survival pathway (Li et al., 2003). These reports suggest that loss of p35/cdk5 complexes (perhaps in parallel with increased p25/cdk5) could result in the transduction of a death signal. This assumes that the substrates of p25/cdk5 and p35/cdk5 are distinct, which could be achieved through a difference in substrate specificities or cellular localization.

1.10 Statement of Problems and Objectives

Based on the material presented in the preceding sections I have identified two general areas which remain unexplored in neuronal death research.

1. Previously reported data have strongly linked the cyclin D/cdk4/6 – pRb pathway to the induction of neuronal death. Precisely how this pathway evokes neuronal death is not known. In the cell cycle one of the main effects of pRb phosphorylation by cyclin D/cdk4/6 is the disinhibition of activator E2Fs. Expression of E2F1 in proliferating cells has also been shown to induce apoptosis. Whether or not E2F1 is involved in neuronal death still remains to be explored. Consequently, I propose the following objective.

Determine whether exogenous E2F1 expression is sufficient to induce neuronal death, and whether endogenous expression is required for death.

This objective is addressed in Chapter 2.

2. Deregulation of cdk5 signaling through conversion of p35 to p25 has been proposed as a causative event in numerous neurpathological conditions including AD, PD, ALS, and stroke. p25/cdk5 complexes have been shown to actively promote neuronal death induced by β-amyloid, excitotoxicity, and oxidative stress. Each of these death models is

thought to involve calcium-mediated signaling and is not thought to have a strict reliance on conserved death signals such as the intrinsic/mitochondrial pathway. The function of cdk5 in neuronal death occurring through the intrinsic death pathway has not been investigated. Moreover, some evidence suggests that cdk5 can act as a pro-survival factor in neurons. Regulation by p35 versus p25 appears to be important in determining whether cdk5 activity promotes survival or death, possibly through localizing cdk5 to different cellular compartments. However, the precise means through which cdk5 can perform both a pro-survival and pro-death function have not been examined. With these points in mind I propose the following objectives:

Determine whether p25 regulated cdk5 activity is required in a model of delayed neuronal death in which death is dependent on the intrinsic pathway,

and

Determine whether differential localization of cdk5 by p35 and p25 determines its ability to act as either a pro-survival or pro-death signal.

These objectives are explored in Chapter 3.

Chapter 2.

O'Hare, M.J. *et al.* (2000) Induction and Modulation of Cerebellar Granule Neuron Death by E2F1. *J. Biol. Chem.* 275(33):25358-64

This first manuscript examines the ability of exogenous E2F1 expression to induce death of post-mitotic neurons and explores the involvement of endogenous E2F1 in neuronal death evoked by low concentrations of K^+.

All of the experiments were performed by M.J. O'Hare, with the partial exception of the RT-PCR shown in Fig. 8, which was performed by S.T. Hou. Q. Xu assisted in some of the culture preparations. S.P. Cregan, E.J. Morris, and R.S. Slack provided valuable insight with respect to the experimental direction of the project and critical review of the manuscript. The manuscript was written by M.J. O'Hare with guidance and editing by D.S. Park.

Induction and Modulation of Cerebellar Granule Neuron Death by E2F-1

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SUMMARY

Growing evidence suggests that certain cell cycle regulators also mediate neuronal death. Of relevance, cyclin D1 associated kinase activity is increased and the retinoblastoma protein (Rb), a substrate of the cyclin D1/Cdk4/6 complex, is phosphorylated during K^+ deprivation evoked death of cerebellar granule neurons (CGNs). Cyclin dependent kinase (CDK) inhibitors block this death suggesting a requirement for the cyclin D1/Cdk4/6-Rb pathway. However, the downstream target(s) of this pathway are not well defined. The transcription factor E2F-1 is regulated by Rb and is reported to evoke death in proliferating cells when overexpressed. Accordingly, we examined whether E2F-1 was sufficient to evoke death of CGNs and whether it was required for death evoked by low K^+. We show that adenoviral mediated expression of E2F-1 in CGNs results in apoptotic death which is independent of p53, dependent upon Bax, and associated with caspase 3-like activity. In addition, we demonstrate that levels of E2F-1 mRNA and protein increase during K^+ deprivation evoked death. The increase in E2F-1 protein is blocked by the CDK inhibitor flavopiridol. Finally, E2F-1 deficient neurons are modestly resistant to death induced by low K^+. These results indicate that E2F-1 expression is sufficient to promote neuronal apoptosis and that endogenous E2F-1 modulates the death of CGNs evoked by low K^+.

INTRODUCTION

Increasing evidence suggests that apoptosis may be involved in a number of neurological disorders, including Alzheimer's disease (Loo et al., 1993; Cotman and Anderson, 1995; Smale et al., 1995) and stroke (MacManus et al., 1993; Nitatori et al., 1995; Namura et al., 1998; Dirnagl et al., 1999). A greater understanding of the cellular signaling pathways which regulate neuronal apoptosis may lead to novel therapeutic targets for these and other neuropathologies. However, the pathways which regulate neuronal death are not fully delineated.

The cell cycle is regulated through a complex series of events controlled through the actions of cyclin dependent kinases (CDKs) and their obligate binding partners, the cyclins (Pines, 1993). Interestingly, a growing body of work has implicated these CDKs in some instances of neuronal death. This evidence includes both *in vivo* and *in vitro* upregulation of the activity and/or the expression of cyclin/CDK complexes during neuronal death. For example, levels of cyclin D and B, and activity of Cdk2 and Cdk4, increase during PC12 cell death evoked by trophic factor deprivation (Gao and Zelenka, 1995; Yan and Ziff, 1995). Increased levels of these cell cycle proteins have also been found in Alzheimer's (McShea et al., 1997; Vincent et al., 1997; Busser et al., 1998) and ischemic (Li et al., 1997b; Timsit et al., 1999) brain tissue. In addition, cyclin-D1 associated kinase activity increases during DNA damage (Park et al., 1998a) and low K^+ induced neuronal death (Padmanabhan et al., 1999). Finally, pharmacological CDK inhibitors and/or expression of dominant negative Cdk4/6 inhibit death of neurons by

DNA damage (Park et al., 1997b; Park et al., 1998a), β-amyloid (Giovanni et al., 1999), and K^+ deprivation (Padmanabhan et al., 1999).

Although the above evidence implicates cyclin-D1/Cdk4/6 in neuronal death, the downstream effector(s) of the activated complex are unclear. It has been demonstrated that the tumor suppressor Rb, a substrate of Cdk4/6, is phosphorylated in response to β-amyloid (Giovanni et al., 1999), K+ deprivation (Padmanabhan et al., 1999), and cisplatin induced death (Gill and Windebank, 1998). Rb is also capable of protecting neurons from DNA damage induced death when overexpressed (Park et al., 2000b). While this evidence suggests Rb involvement, the manner by which Rb controls neuronal death is not fully delineated.

The function of Rb within the nervous system is complex, and it likely plays a role in both cell cycle control and differentiation (Weinberg, 1995; Slack and Miller, 1996). Although Rb interacts with a large number of proteins, its regulation of E2F members is best characterized (Dyson, 1998). E2F-1 is a transcription factor that, along with its heterodimeric partner DP1/2, plays a pivotal role in the G1/S transition of the cell cycle (Nevins, 1992; Wu et al., 1996). Binding of Rb to the E2F-1/DP complex inhibits transcriptional activity either through sequestration of active E2F-1 complexes or formation of active transcriptional repressor complexes. Phosphorylation of Rb by CDKs results in loss of Rb-E2F-1 binding, loss of repression, and promotion of transcriptional activity (Dyson, 1998). Interestingly, several groups have reported that E2F-1 can induce apoptosis in proliferating cells when overexpressed (Kowalik et al., 1995; Asano et al., 1996; Fueyo et al., 1998), and this death can be inhibited by Rb (Hsieh et al., 1997).

Previous evidence demonstrated that dominant negative DP-1 can block neuronal death evoked by DNA damage (Park et al., 2000b) and β-amyloid (Giovanni et al., 1999). These results have led us to hypothesize that E2F-1 may also be capable of signaling apoptosis in neurons. In order to further test this model and extend our observations of E2F-1 involvement in neuronal death, we determined whether E2F-1 expression alone was sufficient to evoke death in primary cultures of cerebellar granule neurons (CGNs). We presently show that expression of E2F-1 evokes death of CGNs in a manner dependent upon Bax and independent of p53. We also examined whether E2F-1 was required for death of CGNs evoked by K^+ withdrawal, a model of death known to activate the Cdk4/6-Rb pathway (Padmanabhan et al., 1999). We demonstrate increased E2F-1 mRNA and protein levels following K^+ withdrawal and a delay of death in E2F-1 deficient CGNs.

MATERIALS AND METHODS

Cell Culture/K^+ Deprivation. Primary CGN cultures were prepared from 7-9 day post-natal mice as previously described (Cregan et al., 1999a). Neurons were maintained in complete media (EMEM media (Sigma) containing 2mM glutamine, 25mM glucose, 0.02mg/ml gentamycin (Sigma), 10% dialyzed FBS) supplemented with K^+ to a final concentration of 25mM. Equal number of neurons (in any given experiment) were plated in 24 well dishes coated with poly-D-lysine (Sigma) at a density of 500, 000 cells/well. The anti-mitotic cytosine-arabinoside (10μM final, Sigma), which kills dividing cells, was added 18-24 hours after plating to reduce the amount of glia cells in the culture. Cytosine-arabinoside administered at these doses had no toxic effects on CGNs. Recombinant adenovirus expressing human E2F-1 or LacZ (multiplicity of infection (MOI) of 6 or 10 as indicated) was added to cell suspensions immediately before plating. K^+ deprivation was performed 7 days after plating by washing each well twice with 750 μl of complete media (final K^+ concentration of 5 mM) and then incubating in 1mL of complete media (5 mM K^+ final). Control cells were washed similarly with 5 mM K^+ media and then maintained in 1mL of complete media containing 25 mM K^+. Cells exposed to flavopiridol were washed as above with complete media containing 5 mM K^+ and 1μM flavopiridol and then maintained in 1mL of this same media for the indicated times.

Knockout Mice. CD-1 mice were obtained from Charles River laboratories. Bax and p53 deficient neurons were obtained from pups derived from heterozygous parents maintained

on a C57BL6 genetic background. E2F-1 deficient neurons were obtained from pups produced by mating heterozygous or heterozygous and homozygous mice from a B6/129 background. For each experiment neurons from heterozygote or wild type littermates were used as control. Each animal was genotyped prior to dissection. Genotyping of Bax mice was performed as previously described (Knudson et al., 1995a). E2F-1 was genotyped using GGATATGATTCTTGGACTTCTTGG (E2F-1-5'), CTAAATCTGAC CACCAAACGC (E2F-1-3'), and CAAGTGCCAGCGGGGCTGCTAAAG (PGKB) primers. The primers were used in one PCR reaction to amplify an untargeted 170-bp fragment and a targeted 230-bp fragment. PCR conditions are as follows: 94°C, 1 min (1 cycle): 94°C, 5 sec; 92°C, 45 sec; 55°C, 1 min; 72°C, 2 min (30 cycles); 72°C, 5 min. p53 animals were genotyped using GTATCTGGAAGACAGGCAGAC (O-p53-1) and TGTACTTGTAGTGGATGGTGG (O-p53-2) primers to detect the wild type allele (450 bp) and TATACTCAGAGCCGGCCT (O-p53-X7) and TTCCTCGTGCTTTACGGT ATC (O-neo-2) primers to detect the targeted allele (533bp). PCR conditions are as follows: 94°C, 5min (1 cycle): 94°C, 1 min; 55°C, 1 min; 72°C, 1 min; 72°C, 1 min (30 cycles).

Cell Survival: At indicated time points media was aspirated and cells were lysed in 200 µl of lysis buffer (0.1X PBS, pH 7.4, 0.4 mM Na_2HPO_4, 0.15 mM KH_2P0_4, 13.5 mM NaCl, 0.25 mM KCl, 0.5% Triton X-100, 2 mM $MgCl_2$, and 0.5 g/100ml cetyldimethylethylammonium bromide). This solution disrupts cell membranes while nuclei remain intact and distinguishable under light microscopy (Rukenstein et al., 1991; Padmanabhan et al., 1999; Stefanis et al., 1999). 10 µl was loaded onto a hemacytometer

and the number of healthy intact nuclei were counted while those displaying blebbing, disruption of nuclear membrane, or phase bright bodies were excluded. The percentage of surviving neurons is expressed relative to the number of neurons in uninfected or non-K+ deprived wells plated at the same time as the experimental wells. Each data point is the mean ±SEM from three samples. Alternatively, neurons were fixed in 0.2% glutaraldehyde and incubated with Hoescht 33258 (0.25 μg/ml) for 30 minutes at room temperature. After washing with 1X PBS the percentage of shrunken and condensed nuclei was assessed. Each data point is the mean ±SEM from three samples.

Caspase Activity Assay. At the indicated time points cells were washed 3 times in cold 1X PBS and then processed in a lysis buffer as previously described (Stefanis et al., 1996). Lysates were incubated on ice for 20 minutes and sonicated for 3 seconds. The samples were then centrifuged for 15 minutes at 12,000 rpm on an Eppendorf tabletop centrifuge. Supernatant was collected and protein concentration was assayed using the Bradford reagent (Biorad). Equivalent protein (3.5μg) for each sample was incubated with DEVD-AFC (BIOMOL) in a buffer as described (Stefanis et al., 1996) and the increase in fluorescence at 505 nm was measured using an excitation wavelength of 400 nm. Measurements were performed on Perkin Elmer LS 50B spectrometer with a slit width setting of 10 and integration time of 3 seconds. DEVD-AFC cleavage activity was determined by plotting the change in fluorescence over time. The slope of the curve was then determined and set as DEVD-AFC cleavage activity (in arbitrary units).

Western Blot. At the indicated time points following either infection or K+ deprivation, neurons were washed in 1X PBS and then collected in solubilization buffer (0.0625 M Tris, 2.5 mM EDTA, 2.5 mM EGTA, 10% glycerol, 2% SDS, 0.001% bromophenol blue, and 5% β-mercaptoethanol). Protein was loaded onto SDS-polyacrylamide gels and transferred to nitrocellulose membranes as previously described. Membranes were then probed with anti-E2F-1 (Santa Cruz) or anti-β-actin (Sigma) antibody.

Immunofluorescence and β-gal staining. Neuronal cultures were fixed in 4% PFA for 30 min at 4°C. After washing three times for 10 minutes in 1X PBS, polyclonal E2F-1 antibody (1:500 in 1X PBS, Santa Cruz) was added to cells for 1 hour at room temperature. After washing with 1X PBS, Cy3-conjugated secondary antibody (1:200; Amersham) was added to cells for 1 hour and then washed with 1X PBS. Cells were then washed three times in 1X PBS and visualized by fluorescent microscopy. β-galactosidase staining was performed as described (Cregan et al., 1999a).

RT-PCR. Total RNA was extracted from rat brains using TRIzol reagent (GibcoBRL, Life Technologies). First strand cDNAs were reverse transcribed from 4 μg of total RNA. The same amount of cDNAs were subsequently used for PCR amplification for a total of 25 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min of each cycle. These optimal amplification conditions and cycle numbers were determined experimentally to ensure specific and linear signal generation. Expression of β-actin mRNA was used as a standard to quantify the relative amount of expression of E2F-1 as described previously (Hou et al., 1997). The mouse specific E2F-1 PCR primers

(GACTGTGACTTTGGGACC and TGTTCACCTTCATTCCC) and actin primers (AACACCCCAGCCATGTACGTAG and GTGTTGGCATAGAGGTCTTTACGG) were used to generate an E2F-1 product of 434 bp and β-actin fragment of 509 bp. The PCR products were fractionated on a 2% agarose gel, photographed, and quantified by scanning laser densitometry. The authenticity of the PCR products were confirmed by cloning into a pCR2.1 vector (Invitrogen) and sequencing using an automated ABI373A DNA sequencer.

RESULTS

Overexpression of E2F-1 evokes death of CGNs.

We first examined whether expression of E2F-1 was sufficient to induce death of CGNs. CGNs cultured from neonates (CD-1) were infected with recombinant adenoviral constructs expressing E2F-1 or LacZ as a control. As shown in Figure 1, four days following infection, expression of E2F-1 resulted in 70% reduction in survival compared to control non-infected neurons, while infection with LacZ resulted in only 9% reduction in survival. Western blot analyses confirmed increased expression of E2F-1 in E2F-1 infected cultures (Fig 2H). With higher exposure of the identical blot a low level of endogenous E2F-1 was observed in control cultures (data not shown). E2F-1 expression was also confirmed by immunocytochemistry (Fig 2A-D). We find that 62% of the neurons in E2F-1 infected cultures present at day 4 stained for E2F-1 (Fig 2G). The lower number of E2F-1 positive neurons compared to dying neurons, as assessed by nuclear counts, is likely a result of some E2F-1 expressing cells having died and lifted off the plate prior to fixation. Staining for β-galactosidase verified 75% expression in LacZ infected cultures (fig 2E-G). CGNs infected with E2F-1 expressing adenovirus died with nuclear signs of apoptosis (Fig 3 A, B). Quantification of Hoescht staining shows that 40% of the neurons in E2F-1 infected cultures, and 5% of LacZ infected cultures, displayed condensed and/or fragmented nuclei (Fig 3C).

E2F-1 induces caspase-3-like activity and neuronal death in a Bax-dependent manner

Neuronal death evoked by numerous death stimuli is dependent upon Bax. Of

Figure 1: Adenovirus mediated overexpression of E2F-1 leads to death of CGNs. Neurons cultured from 7-9 day old CD-1 mice were infected (MOI 10) at time of plating with either β-galactosidase expressing (LacZ) or E2F-1 expressing adenovirus vectors. Each data point is the mean ± SEM from 3 independent experiments and is reported relative to the number of neurons present in uninfected cultures. * Indicates significance ($p<0.01$, independent t-test) relative to LacZ treated cultures at each time point.

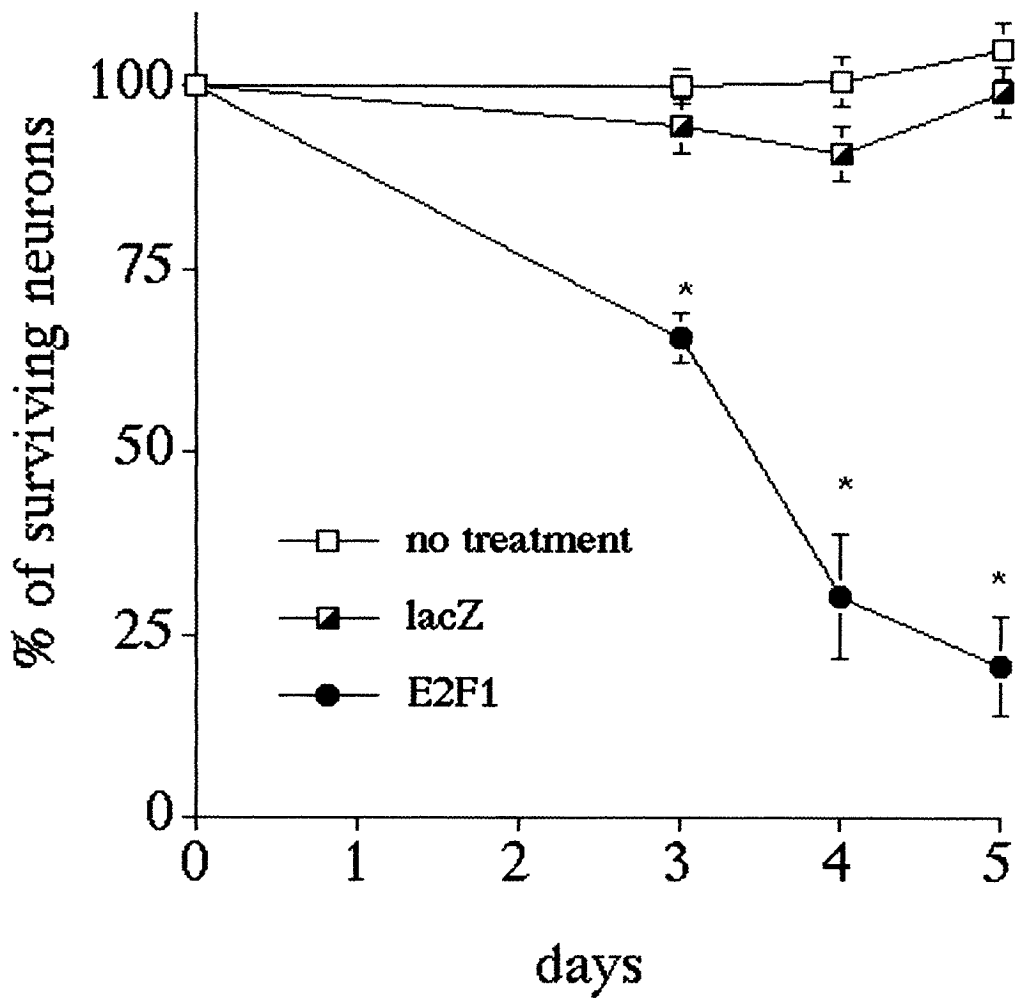
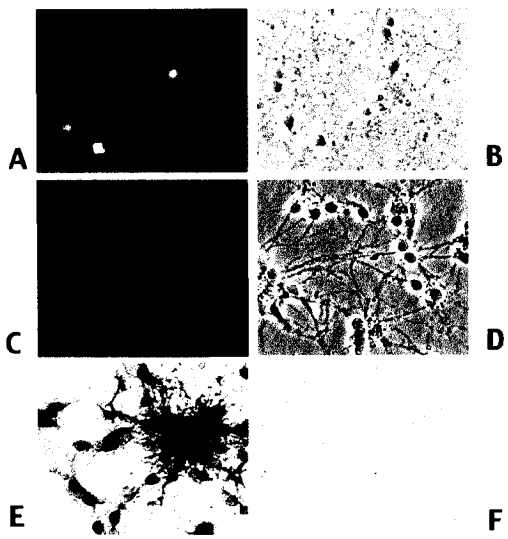
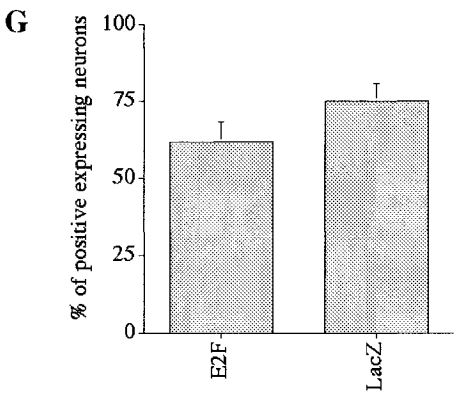

100
75
50
25
0
% of surviving neurons
0
1
2
3
4
5
days
no treatment
lacZ
E2F1
*
*
*

Figure 2: Expression of E2F-1 and β-galactosidase in CGNs.

Neurons were infected (MOI 10) with E2F-1 (A, B) or LacZ (C, D) at the time of plating and fixed after 4 days in culture. Cultures were stained for E2F-1 (A-D) and visualized by immunofluorescence (A, C) or phase microscopy (B, D). Alternatively, cultures infected with LacZ (E) or non-infected control cultures (F) were analyzed by X-gal staining. (G) Quantification of the proportion of neurons expressing E2F-1 or LacZ as determined from 3 independent cultures. (H) Western blot analysis of neuronal cultures infected with E2F-1 or LacZ.


A
B
C
D
E
F


G
% of positive expressing neurons
100
75
50
25
0
E2F
LacZ

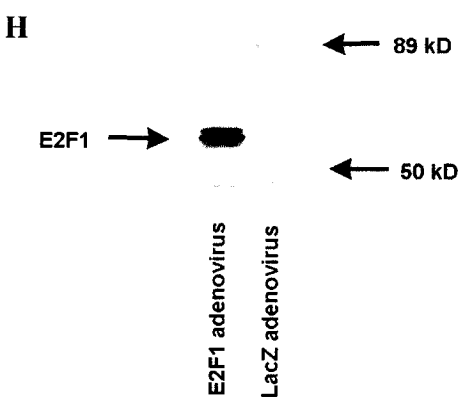
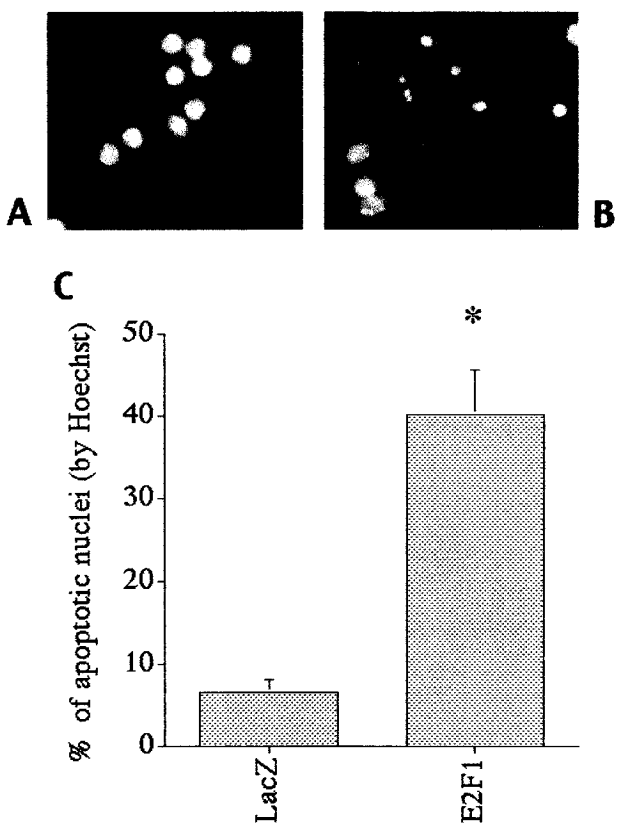

H
89 kD
E2F1
50 kD
E2F1 adenovirus
LacZ adenovirus

Figure 3: Hoescht staining demonstrates condensed/fragmented nuclei in E2F-1 infected but not LacZ infected neurons (MOI 10).

Neurons were infected with LacZ (A) or E2F-1 (B) and analyzed for Hoescht staining (A, B) four days after infection. (C) Quantification of apoptotic nuclei (mean ± SEM) from 4 independent cultures infected with either E2F-1 or LacZ. * Indicates significance ($p<0.001$) by independent t-test.


A
B
C
% of apoptotic nuclei (by Hoechst)
50
40
30
20
10
0
*
LacZ
E2F1

relevance, several reports have indicated that CGN death evoked by either low K^+ concentration or p53 overexpression require Bax activity (Miller et al., 1997b; Cregan et al., 1999a; McGinnis et al., 1999). Accordingly, we examined whether Bax was required for E2F-1 mediated neuronal death by comparing the effects of E2F-1 expression in neuronal cultures obtained from Bax deficient mice and littermate controls. As shown in Figure 4, Bax deficient neurons expressing E2F-1 were significantly more resistant to death as compared to Bax wild type littermates (88% survival for Bax (-/-) vs. 36% for Bax (+/+), 4 days following infection). Bax deficient neurons infected with E2F-1 displayed healthy soma and intact processes typical of healthy neurons while neurons from Bax wild type littermates infected with E2F-1 show degenerating processes and apoptotic bodies (Fig 4B-D).

The resistance of Bax deficient neurons to E2F-1 induced death indicates that Bax acts downstream of the E2F-1 death signal. One model by which Bax mediates death involves its ability to facilitate the release of cytochrome-c from the mitochondria which leads to formation of the apoptosome complex and subsequent caspase activation (Adams and Cory, 1998; Green and Reed, 1998). To test whether Bax-dependent E2F-1 induced death was associated with caspase activation, we measured caspase-3-like activity in Bax null and wild type littermates infected with E2F-1 or LacZ adenovirus. Caspase-3-like activity was measured by DEVD-AFC cleavage activity. CGNs from Bax (+/+) and (+/-) mice showed a 7-8 fold increase in DEVD-AFC cleavage activity 4 days after infection with E2F-1 (Fig 5). DEVD-AFC cleavage activity, however, is not detected in Bax deficient CGNs expressing E2F-1. These results indicate that death of CGNs induced by

Figure 4: E2F-1 evoked death of CGNs is a Bax-dependent event.

(A) Neurons from Bax deficient and heterozygote littermates were cultured and infected (MOI 10) with either β-gal or E2F-1 expressing adenovirus. Each point is the mean ± SEM from 3 independent cultures. * Indicates significance ($p<0.05$) relative to LacZ treated cultures by independent t-test. (B-D) Phase photomicrographs of cultures from Bax (-/+) mice infected with LacZ (B), E2F-1 (C), and cultures from Bax (-/-) mice infected with E2F-1 expressing virus (D).

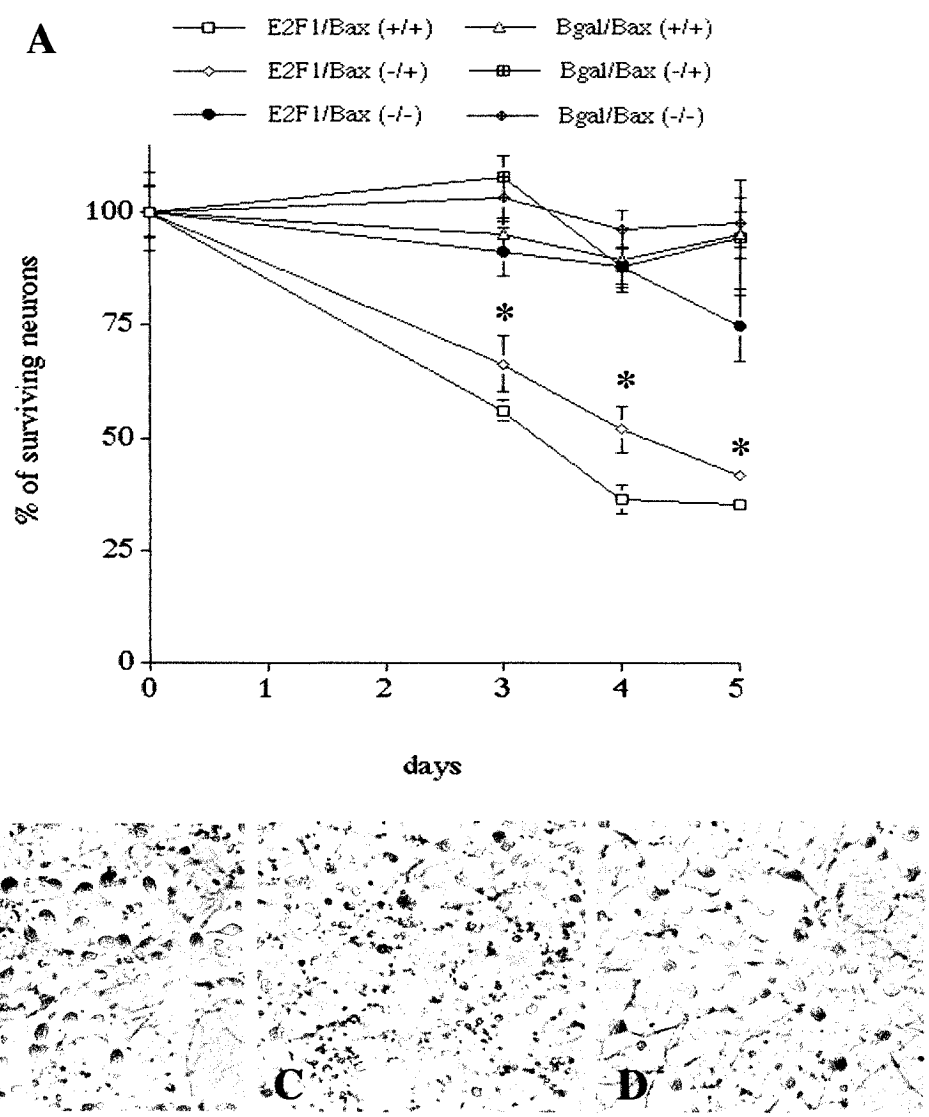
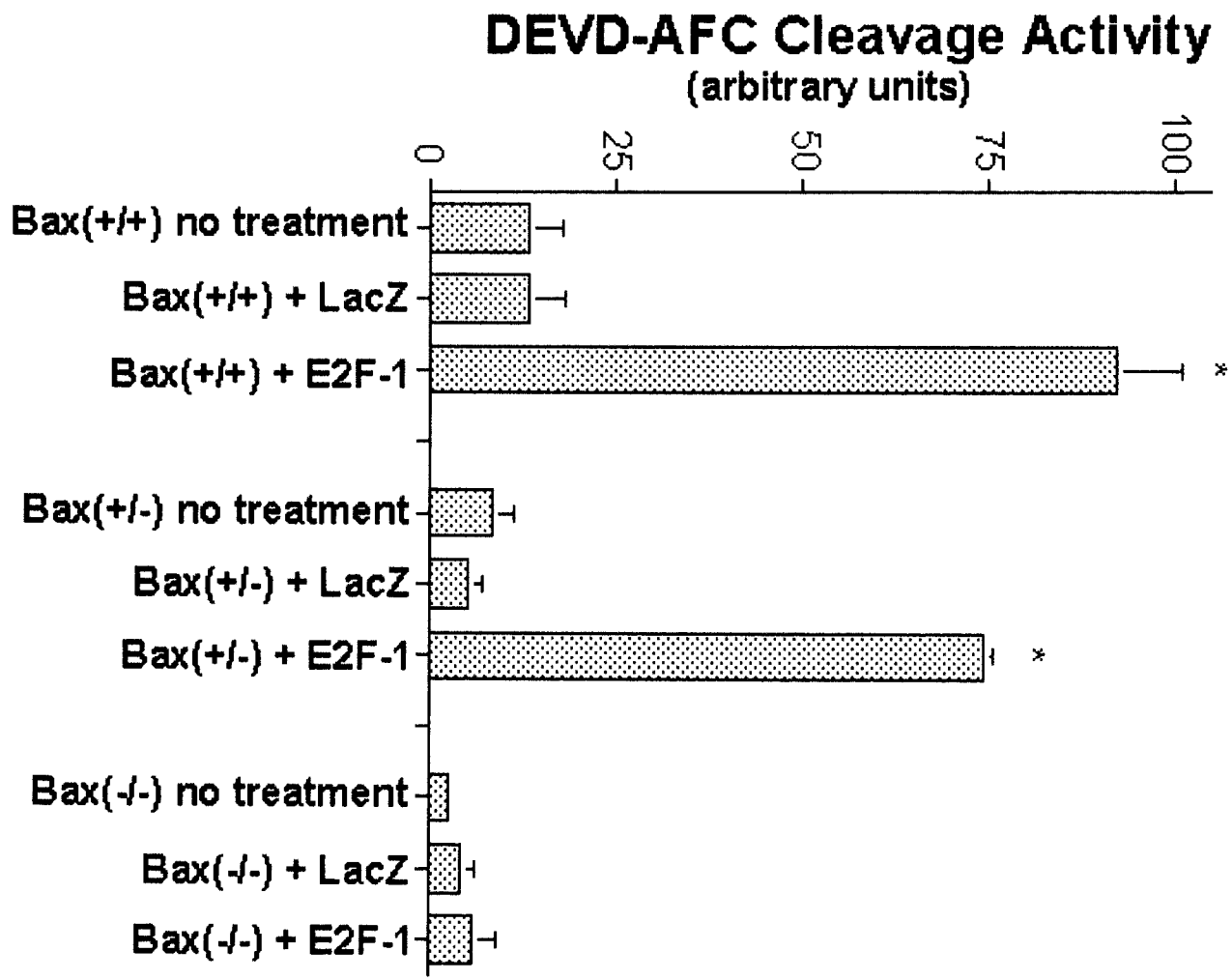

A
E2F1/Bax (+/+)
Bgal/Bax (+/+)
E2F1/Bax (-/+)
Bgal/Bax (-/+)
E2F1/Bax (-/-)
Bgal/Bax (-/-)
% of surviving neurons
100
75
50
25
0
0
1
2
3
4
5
days
*
*
*
B
C
D

Figure 5: E2F-1 mediated caspase activation is dependent on Bax.

In vitro DEVD-AFC cleavage activity was measured in CGN cultures from Bax deficient, heterozygous, and wild type litter mates uninfected or infected (MOI 10) with LacZ or E2F-1 as indicated. Each point is the mean ± SEM from 3 independent cultures. * Indicates significance ($p<0.01$) by independent t-test.


DEVD-AFC Cleavage Activity
(arbitrary units)
0
25
50
75
100
Bax(+/+) no treatment
Bax(+/+) + LacZ
Bax(+/+) + E2F-1
Bax(+/-) no treatment
Bax(+/-) + LacZ
Bax(+/-) + E2F-1
Bax(-/-) no treatment
Bax(-/-) + LacZ
Bax(-/-) + E2F-1
*
*

E2F-1 expression is associated with increased caspase-3-like activity and that this activity is dependent on Bax.

Death of CGNs induced by expression of E2F-1 is not dependent on p53.

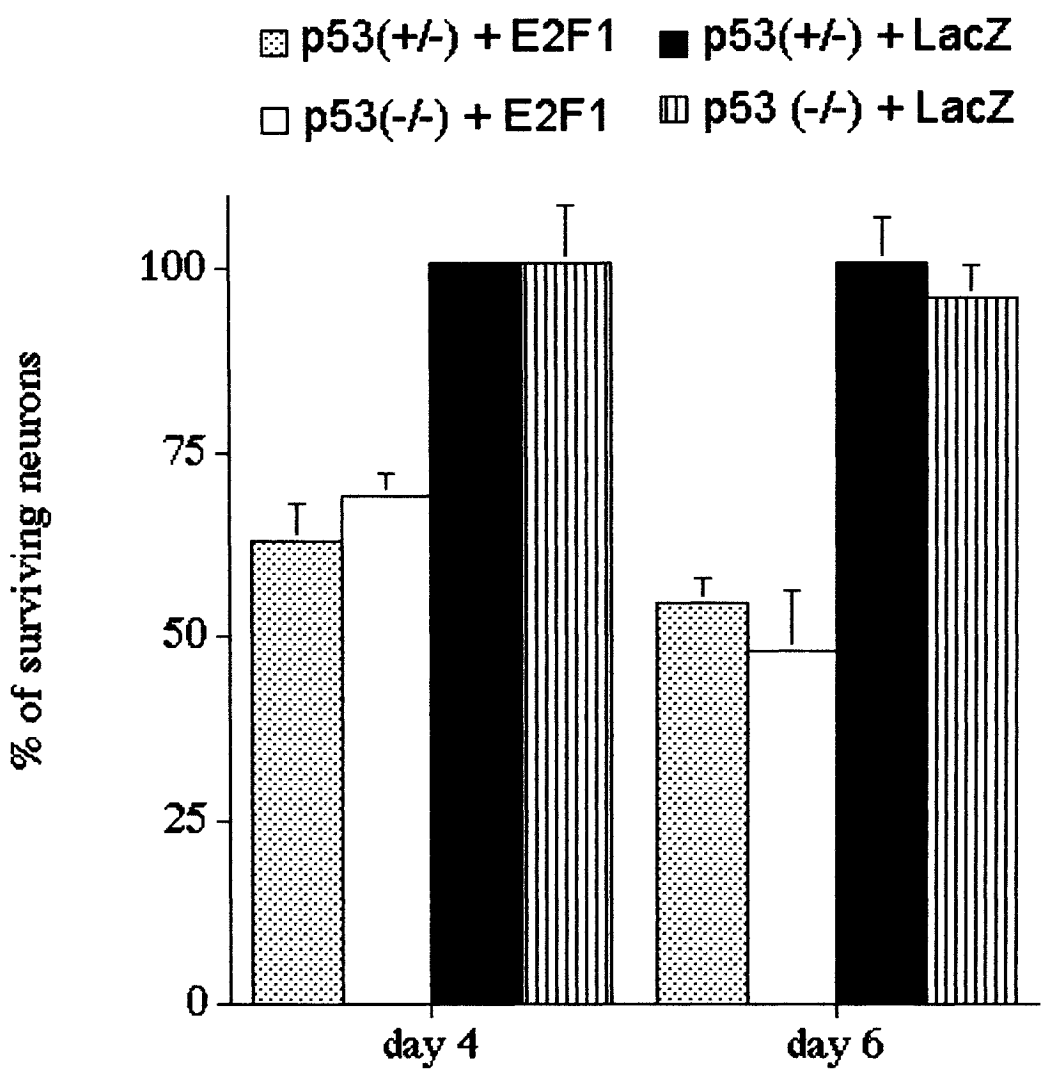
Multiple reports in proliferating cells have indicated that E2F-1 mediated death can be a p53-dependent (Qin et al., 1994; Wu and Levine, 1994; Kowalik et al., 1995) or independent (Hsieh et al., 1997; Phillips et al., 1997) phenomenon. In addition, E2F-1-dependent neuronal death *in vivo* due to Rb deficiency can be either p53-dependent or p53-independent according to neuronal type (Morgenbesser et al., 1994; Macleod et al., 1996; Tsai et al., 1998). In order to establish the relevance of p53 in E2F-1 induced neuronal death, we examined whether p53 (-/-) neurons were resistant to E2F-1 expression. We found that neurons from p53 deficient and heterozygous mice displayed no significant differences in survival in response to E2F-1 expression (Fig 6). This indicates that p53 is not required for death of CGNs induced by E2F-1 expression and that the activation of Bax mediated death signals occurs independently of p53.

E2F1 involvement in death of neurons evoked by K^+ deprivation.

The above studies indicate that E2F-1 expression is sufficient to cause neuronal death. We next asked whether E2F-1 was involved in a CGN model of death in which E2F-1 itself is not the death stimulus. To address this question we examined the requirement for E2F-1 in K^+ deprivation induced death of CGNs. In this death paradigm, CGNs die apoptotically when switched from media containing a high K^+ concentration (25 mM) to media with a low K^+ concentration (5 mM) (Gallo et al. , 1987; D. Mello et

Figure 6: p53 is not required for E2F-1 induced death.

CGNs from p53 deficient and heterozygous littermates were cultured and infected (MOI 6) with either E2F-1 or LacZ adenovirus. Each point is the mean ± SEM from 4 independent experiments and is expressed relative to the number of neurons present in untreated control cultures.


p53(+/-) + E2F1
p53(+/-) + LacZ
p53(-/-) + E2F1
p53 (-/-) + LacZ
% of surviving neurons
100
75
50
25
0
day 4
day 6

al., 1993; Schulz et al., 1996). Moreover, similar to our results with E2F-1 overexpression, K^+ deprivation induced death is dependent upon Bax and induces caspase-3-like activity (Miller et al., 1997b; Marks et al., 1998).

We first determined whether E2F-1 was required for death of CGNs evoked by K^+ deprivation. CGNs from E2F-1 deficient animals displayed a small but significant delay of death compared to neurons from heterozygous E2F-1 littermates. The effect was most significant 12 h following deprivation, with 68% of E2F-1 (-/-) neurons surviving versus 39% survival for E2F-1 (+/-) (Fig 7). Consistent with this observed protection, E2F-1 transcripts increase during CGN death induced by K^+ withdrawal, as determined by semi-quantitative RT-PCR. We detected increases in E2F-1 message as early as 1 h following induction of K^+ deprivation (Fig 8A). As an internal control, β-actin message was co-amplified with E2F-1. The densitometric ratio of E2F-1 to β-actin increases approximately 3-fold at 1 h and plateaus 12 h following K^+ deprivation (Fig 8B). Combined data from 3 independent experiments shows that E2F-1 transcript levels increase 4-fold over baseline within 6 h of K^+ deprivation (Fig 8C). Because the E2F-1 gene is itself regulated by E2F activity (Lavia and Jansen-Durr, 1999), our data suggests a deregulation of E2F-1 transcriptional complexes.

To determine if the increase in E2F-1 message is associated with an increase in E2F-1 protein levels we performed Western blot analysis of protein isolated from CGNs deprived of K^+. Prior to K^+ deprivation E2F-1 protein levels are low but detectable. However, as early as 6 h following the lowering of K^+ concentration we detected an increase in total E2F-1 protein (Fig 9). To investigate the involvement of CDKs in the increase in E2F-1 expression, we treated K^+ deprived CGNs with flavopiridol.

Figure 7: E2F-1 deficiency delays neuronal death following K^+ deprivation.

Neurons from E2F-1 deficient and heterozygous littermates were cultured and deprived of K^+ as described in materials and methods. Each point is the mean ± SEM from 4 independent experiments. * Indicates significance ($p<0.05$) by independent t-test.

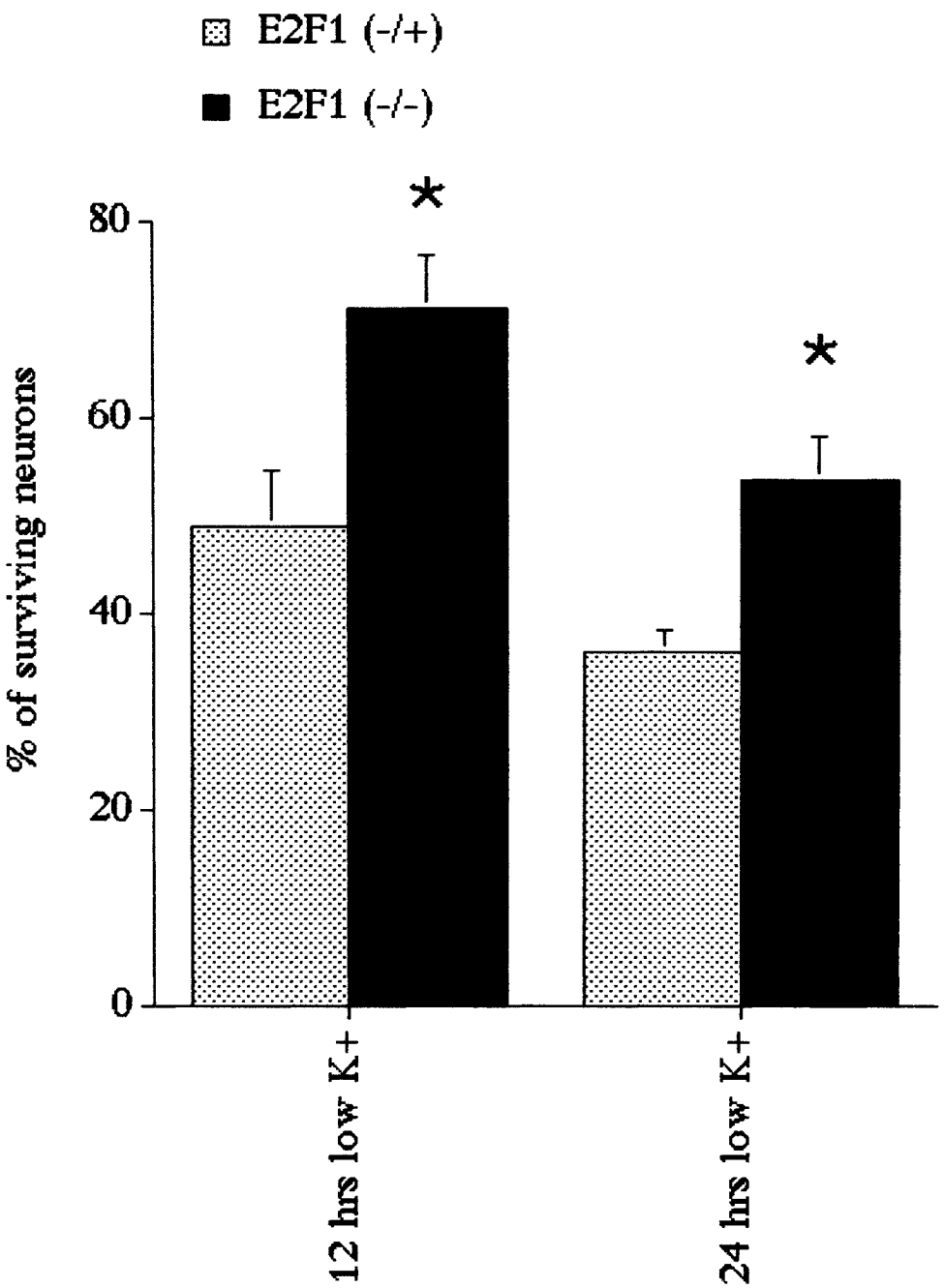
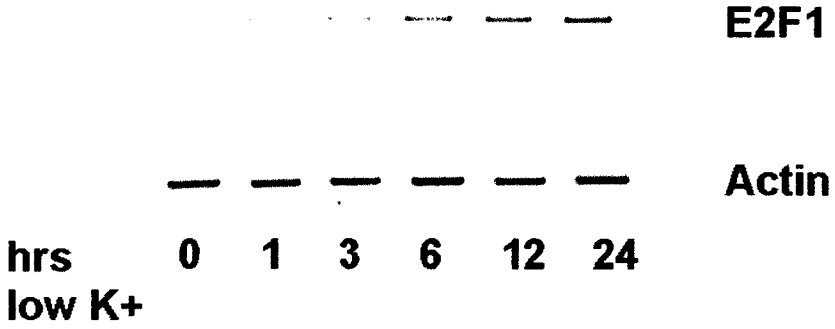

E2F1 (-/+)
E2F1 (-/-)
*
*
80
60
40
20
0
% of surviving neurons
12 hrs low K+
24 hrs low K+

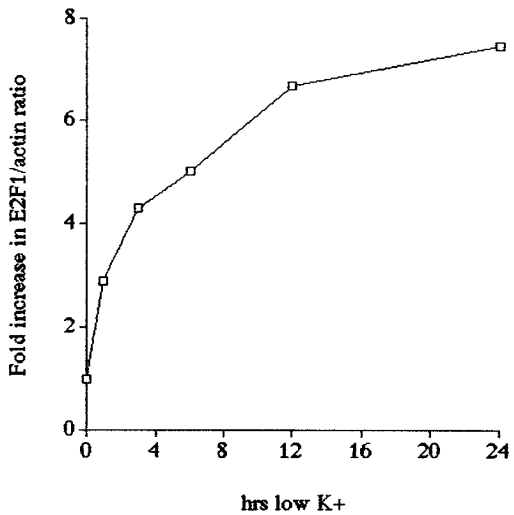
Figure 8: E2F-1 mRNA transcripts increase during K^+ deprivation.

(A) Representative time course of E2F-1 and β-actin transcripts during K^+ deprivation of CGNs. The image has been reversed to more clearly show the bands. (B) The densitometric ratio of E2F-1 to β-actin signal from the image in (A). (C) Combined data from 3 independent experiments at 0 and 6 hours following K^+ deprivation.

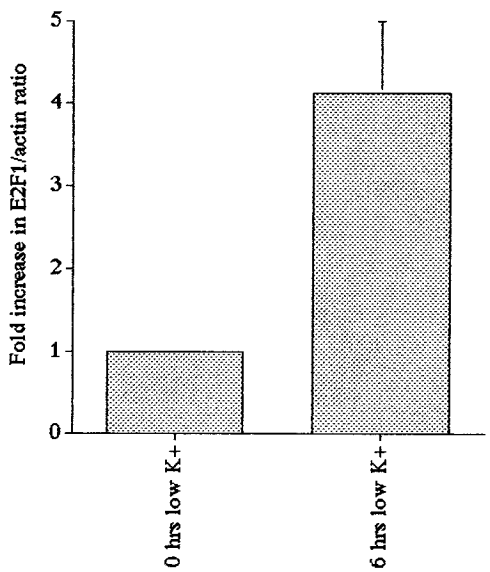
A


E2F1
Actin
hrs low K+
0
1
3
6
12
24

B


Fold increase in E2F1/actin ratio
0
2
4
6
8
0
4
8
12
16
20
24
hrs low K+

C

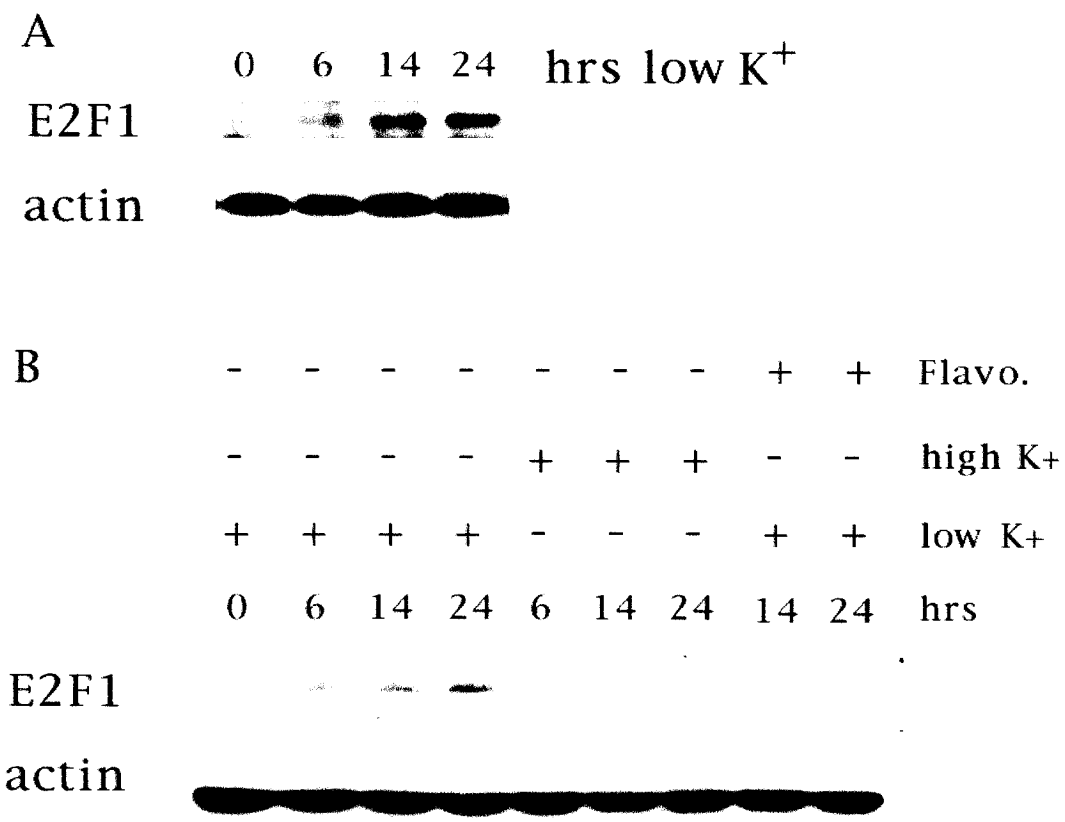

Fold increase in E2F1/actin ratio
0
1
2
3
4
5
0 hrs low K+
6 hrs low K+

Flavopiridol inhibits Cdk4/6 activity and Rb phosphorylation, and prevents death of CGNs evoked by low K^+ (Padmanabhan et al., 1999). Treatment with flavopiridol suppressed the increase in E2F-1 protein, suggesting that the Cdk4/6/Rb pathway is required for increased E2F-1 expression during K^+ deprivation (Fig 9B). An E2F-1 reactive band was not detected in E2F-1 deficient neurons deprived of K^+ (data not shown).

Taken together, our results indicate that E2F-1 levels increase and that this increase plays a modulatory role in K^+ deprivation induced death of CGNs. However, our data also indicate that factors other than E2F-1 must contribute to the death signal.

Figure 9: E2F-1 protein levels increase during K^+ deprivation of CGNs (A) and this increase is blocked by the CDK inhibitor flavopiridol (B).

Protein was harvested prior to K^+ deprivation (0 hours) and following 6, 14, and 24 hours of incubation in either 5mM K^+ media, 25mM K^+ media, or 5mM K^+ media plus flavopiridol (1μM). After probing with E2F-1 antibody the membrane was stripped and analyzed for β-actin.


A
0 6 14 24 hrs low K+
E2F1
actin
B
- - - - - - - + + Flavo.
- - - - + + + - - high K+
+ + + + - - - + + low K+
0 6 14 24 6 14 24 14 24 hrs
E2F1
actin

DISCUSSION

Previous results suggested that death of CGNs evoked by low K^+ is regulated by activation of cyclin dependent kinases (Padmanabhan et al., 1999). Accordingly, we examined the involvement of E2F-1, a transcription factor regulated by Rb and a potential effector of the CDK/Rb pathway. We investigated whether expression of E2F-1 was sufficient to evoke neuronal death and whether E2F-1 was required to promote CGN death evoked by low K^+.

E2F-1 expression evokes caspase activation and death in a bax-dependent and p53-independent manner

The ability of E2F-1 to induce apoptosis was previously reported in proliferating cell systems and was thought to be a protection mechanism against uncontrolled cell division (Fueyo et al., 1998; Pan et al., 1998). In support of this, E2F-1 knockout mice show increased tumorigenesis (Yamasaki et al., 1996). However, its ability to evoke neuronal death and the mechanism by which it may cause this death is not clear. We presently show that E2F-1 expression evokes CGN death in a manner independent of p53 but dependent on Bax.

In dividing cells, E2F-1 mediated death is reported to proceed through both p53-dependent (Qin et al., 1994; Wu and Levine, 1994; Kowalik et al., 1995) and independent (Hsieh et al., 1997; Phillips et al., 1997) processes. In the case of the former, E2F-1 is thought to signal via the tumor suppressor $p19^{ARF}$. E2F-1 is reported to directly activate transcription of $p19^{ARF}$ which binds to MDM2, a negative regulator of p53, with

consequent stabilization of p53 levels (Bates et al., 1998). Our results suggest that E2F-1 evoked death of CGNs is independent of p53. This is significant in the light of previous evidence demonstrating that apoptosis in the developing embryo due to Rb loss is dependent on both E2F-1 and p53 in the CNS (Field et al., 1996; Tsai et al., 1998). This indicates that neuronal death due to RB loss in the CNS proceeds through a different signaling mechanism than that of E2F-1 mediated overexpression alone.

Very little is known regarding the factors which mediate E2F-1 induced apoptosis in the absence of p53. Experiments using mutant forms of E2F-1 have shown that the transactivation domain of E2F-1 is dispensable for induction of p53 independent apoptosis in Saos-2 cells, whereas the DNA binding domain is required (Hsieh et al., 1997; Phillips et al., 1997). In addition, Phillips et al. have suggested that p53 independent E2F-1 mediated apoptosis involves the inhibition of antiapoptotic signals and may require signaling via death receptors (Phillips et al., 1999). Whether this is true in neurons remains to be determined. The recent identification of p63 and p73 (Kaelin, 1999) raises the possibility that E2F-1 may signal apoptosis in different cell types through multiple p53 family members.

E2F-1 expression results in caspase activation and death which is dependent upon the presence of Bax. We found that caspase activity, as indicated by DEVD-AFC cleavage, increases in response to E2F-1 expression in wild type CGNs but not Bax deficient CGNs. Most importantly, these Bax deficient CGNs are also resistant to the death induced by E2F-1. These results suggest an E2F-1 mediated death signaling pathway proceeding through Bax and subsequent caspase activity. Bax has been reported as an upstream activator of caspase activity in a number of cell death systems (Miller et

al., 1997b; Deckwerth et al., 1998; Cregan et al., 1999a). Bax activation of caspases is thought to occur via translocation to the mitochondrial membrane, consequent cytochrome-c release into the cytoplasm, and activation of the apaf1/caspase 9 containing apoptosome (Adams and Cory, 1998). Bax activity has also been shown to be required for other neuronal death paradigms, including K^+ deprivation of CGNs (Miller et al., 1997b; McGinnis et al., 1999). Accordingly, our results are consistent with the importance of Bax in apoptotic death of CGNs.

Role of E2F-1 in death of CGNs evoked by low K^+

We have shown that E2F-1 levels increase during death evoked by low K^+ and that this increase appears to be regulated by CDKs. Because E2F-1 is known to regulate its own transcription, this result is consistent with the notion of deregulated activation of E2F-1 complexes during death induced by low K^+; a concept suggested by previous reports demonstrating both Rb phosphorylation and loss in this same death paradigm (Padmanabhan et al., 1999). Our findings of significant neuroprotection as a result of E2F-1 deficiency are also consistent with the *in vivo* observations of others showing that E2F-1 deficiency is partially protective against death induced by Rb deficiency (Tsai et al., 1998) and ischemia (MacManus et al., 1999a). In addition, we have also recently shown that E2F-1 deficiency is protective against β-amyloid toxicity (Giovanni et al., 2000).

Our present findings extend these observations of E2F-1 involvement in neuronal death. However, our results indicating incomplete protection with E2F-1 deficiency suggest that other Rb interacting members may also be involved in the CDK mediated

death signal. In support of this, recent studies have raised the possibility that expression of E2F-2 and E2F-3 can also evoke death in proliferating cell systems (Dirks et al., 1998; Vigo et al., 1999). Accordingly, it is possible that functional redundancy amongst E2F members may control death of CGNs by low K^+. Other studies, however, have shown that E2F-1 is the only E2F member capable of evoking apoptosis in proliferating cells (DeGregori et al., 1997). In addition, although E2F-1 deficiency prolongs life of Rb deficient mice, it does not protect from all forms of apoptosis, suggesting that Rb functions through proteins other than E2F-1 (Tsai et al., 1998).

The involvement of numerous signaling pathways other than those directly related to the cell cycle have also been well established in K^+ deprivation induced death of CGNs. For example, activation of the c-Jun N-terminal kinase (Watson et al., 1998) and the Fas/FasL (Green and Reed, 1998) pathway have been demonstrated in CGNs exposed to low K^+ conditions. Other signals mediated through PI3 kinase (Miller et al., 1997a), and MAP kinase (Bonni et al., 1999) have been demonstrated to be neuroprotective in these cells. This involvement of multiple factors suggests that neurons simultaneously activate several biochemically distinct but functionally cooperative pathways which regulate the death response to low K^+. It will be important to relate these signaling events to obtain a more full understanding of how neuronal death is controlled.

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Chapter 3.

O'Hare, M.J. *et al.* (2005) Differential Roles of Nuclear and Cytoplasmic Cyclin-Dependent Kinase 5 in Apoptotic and Excitotoxic Neuronal Death. *J. Neurosci.* 25(39):8954-66

This work compares the activities of cdk5 in the cytoplasm vs. the nucleus. We find that nuclear cdk5, which is regulated by p25, is required for death induced by an excitotoxic stimulus but not for death induced DNA damage. Cdk5 also appears to play a pro-survival function within the cytoplasm, where it is regulated by p35. Inhibition of this cytoplasmic function of cdk5 can potentiate death induced by both excitotoxic and DNA damage.

All experiments were performed by M.J. O'Hare with the following exceptions: N. Kushwaha assisted in the production of NLS and NES constructs; Y. Zhang provided a number of cortical neuron cultures; S.M. Callaghan was responsible for viral construction. H. Aleyasin, R.S. Slack, and P.R. Albert provided valuable technical and theoretical insight as well as critical review of the manuscript. The manuscript was written by M.J. O'Hare. D.S. Park provided overall guidance and assisted in editing the manuscript.

Differential Roles of Nuclear and Cytoplasmic Cdk5 in Apoptotic and Excitotoxic Neuronal Death

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SUMMARY

Cyclin-dependent kinase 5 (cdk5) is a member of the cyclin-dependent kinase family whose activity is localized mainly to post-mitotic neurons due to the selective expression of its activating partners p35 and p39. Deregulation of cdk5, as a result of calpain cleavage of p35 to a smaller p25 form, has been suggested to be a central component of neuronal death underlying numerous neurodegenerative diseases. However, the relevance of cdk5 in apoptotic death that relies on the mitochondrial pathway is unknown. Furthermore, evidence that cdk5 can also promote neuronal survival has necessitated a more complex understanding of cdk5 in the control of neuronal fate. Here we explore each of these issues using apoptotic and excitotoxic death models. We find that apoptotic death induced by the DNA damaging agent camptothecin is associated with early transcription mediated loss of p35 and with late production of p25 that is dependent on Bax, Apaf1, and caspases. In contrast, during excitotoxic death induced by glutamate neurons rapidly produce p25 independent of the mitochondrial pathway. Analysis of the localization of p35 and p25 revealed that p35 is mainly cytoplasmic whereas p25 accumulates selectively in the nucleus. By targeting a dominant negative cdk5 to either the cytoplasm or nucleus we show that cdk5 has a death promoting activity within the nucleus, and that this activity is required in excitotoxic death, but not apoptotic death. Moreover, we also find that cdk5 contributes to pro-survival signaling selectively within the cytoplasm and manipulation of this signal can modify death induced by both excitotoxicity and DNA damage.

INTRODUCTION

Cyclin dependent kinases (CDKs) are a family of proline-directed serine/threonine kinases best known for their role in controlling proliferation. Once bound to an activating cyclin protein, CDKs control progression through the phases of the cell cycle by phosphorylation of specific substrates. For example, at the G1-S phase transition cyclinD/cdk4/6 and cyclinA/cdk2 complexes phosphorylate and inactivate the retinoblastoma protein (pRb), a major brake on the cell cycle (Johnson and Walker, 1999; Ekholm and Reed, 2000; Massague, 2004). Inactivation of pRb allows for E2F-mediated transcription of genes which promote S-phase (Dyson, 1998).

Some members of the CDK family, however, are involved in functions unrelated to the cell cycle. One such member, cdk5, is inactive in most tissues except for the CNS due to the neuron-specific expression of its non-cyclin activating partners p35 and p39. cdk5 is a multi-functional kinase required for a variety of normal neuronal functions including cortical development, axon migration, cell adhesion, and synaptic activity (Dhavan and Tsai, 2001).

Evidence suggests that DNA damage may be a central event in neurodegeneration. For instance, DNA strand breaks occur as an early event following reperfusion of ischemic brain (Tobita et al., 1995; Chen et al., 1997; Cui et al., 2000) and DNA lesions have been reported in Parkinson's and Alzheimer's diseases (Robison and Bradley, 1984; Alam et al., 1997; Gabbita et al., 1998; Jenner, 1998; Lovell and Markesbery, 2001). Furthermore, mutations in DNA repair genes have been linked to the development of neurodegenerative conditions in humans (Date et al., 2001; Moreira et al., 2001; Takashima et al., 2002), and an increase in DNA damage within the promoters

of genes involved with synaptic plasticity and neuronal survival may underlie the neuronal loss seen in the aged human brain (Lu et al., 2004). Neuronal death due to DNA damage can be modeled *in vitro* using camptothecin (CA), an inhibitor of topoisomerase 1 (Morris and Geller, 1996; Liu et al., 2000). Treatment of cultured cortical neurons with CA induces apoptotic death that is dependent on p53, Bax, and the mitochondrial death pathway (Xiang et al., 1998; Morris et al., 2001).

Interestingly, a wide range of evidence has accumulated implicating cell cycle CDKs as required signals in neuronal death induced by DNA damage. For example, CA induces cyclinD-associated kinase activity and leads to an increase in pRb phosphorylation (Park et al., 1998b; Park et al., 2000b). Inhibition of cell cycle CDKs by pharmacological means or by expression of dominant negative cdk4/6 prevents DNA damage induced death (Park et al., 1997b; Park et al., 1998b). Cell cycle CDKs have also been implicated in other models of neuronal death, including trophic factor withdrawal and β-amyloid induced death (Park et al., 1997a; Copani et al., 1999; Giovanni et al., 1999; Padmanabhan et al., 1999; Konishi et al., 2002).

More recently, cdk5 has also received considerable attention as a regulator of neuronal death. Evidence suggests that cdk5 becomes an inducer of death when its activator p35 is cleaved into a smaller p25 form by calpains (Patrick et al., 1999; Kusakawa et al., 2000; Lee et al., 2000). Accumulation of p25 and/or involvement of cdk5 has been observed in neurons treated with excitotoxins, β-amyloid, and oxidative stress (Alvarez et al., 1999; Lee et al., 2000; Gong et al., 2003), as well as in animal models of stroke (Wang et al., 2003), Parkinson's disease (Smith et al., 2003), and amyotrophic lateral sclerosis (Nguyen et al., 2001). Importantly, each of these models is

thought to involve Ca^{+2} signaling and do not have a strict reliance on the mitochondrial death pathway. Production of p25 and/or involvement of p25/cdk5 complexes in death models that are dependent upon the conserved apoptotic mitochondrial pathway, such as CA induced death, has yet to be carefully examined.

Although the evidence implicating cdk5 in neuronal death is compelling, some reports have provided contradictory evidence that cdk5 acts instead as a pro-survival signal (Cheung and Ip, 2004). For example, under certain conditions cdk5 has been shown to activate PI3K/Akt signaling (Li et al., 2003). Also, cdk5 can prevent apoptosis induced by UV irradiation through negative regulation of c-Jun-N-terminal kinase 3 (JNK3) (Li et al., 2002). Furthermore, cdk5 null mice, in addition to showing extensive abnormalities in cortical layering and cerebellar foliation, display degeneration of brain stem and spinal cord neurons (Ohshima et al., 1996).

Taken together, the above observations highlight two important questions with regards to the role cdk5 plays in neuronal death; 1) is cdk5, like cell cycle CDKs, important in a delayed model of neuronal apoptosis that has a strict dependence on the mitochondrial pathway, and 2) what factors determine whether cdk5 acts as a pro-survival or pro-death signal. To address these questions we examined p25 formation and the effect of cdk5 inhibition in apoptotic death induced by CA and in excitotoxic death induced by glutamate. We provide evidence that p35 can regulate a pro-survival activity of cdk5 within the cytoplasm whereas p25 regulates cdk5 pro-death activity within the nucleus. Moreover, although nuclear p25/cdk5 complexes accumulate in both excitotoxic and apoptotic death, they are functionally relevant only in the former, in which they are

produced early in the death process. In apoptotic death initiated by CA, p25 complexes are only produced as a late consequence of the mitochondrial death pathway.

MATERIALS AND METHODS

Primary Neuronal Cultures

For cortical neuron isolation brains from E15 CD1 or transgenic embryos were dissected as previously described (Xiang et al., 1996; Fortin et al., 2001) and neurons were maintained in Neurobasal media supplemented with B-27, N2, 0.5mM glutamine, and 0.05mg/mL penicillin-streptomycin (all from Gibco/Invitrogen). Cortical neurons were plated in Nunc 6-well dishes (2.5-3X10^6 cells/well) for Western blot, kinase assay, caspase assay, and RT-PCR, and in Costar 4-well dishes (3-5X10^5 cells/well) for immunocytochemistry and survival assay. Cerebellar granule neurons (CGNs) were cultured from CD1 or transgenic mice at post-natal day 7 or 8 as described previously (Cregan et al., 1999b). CGNs were maintained in EMEM media (Sigma) containing 10% dialyzed FBS, 25mM KCl, 0.1mg/m gentamycin, 2mM glutamine, and 25mM glucose. CGNs were plated in Costar 4-well dishes (3-5X10^5 cells/well in 750uL of media). All dishes were coated with poly-D-lysine (Sigma). AraC (10μM) was added to CGN cultures that were used for glutamate toxicity (Fig. 9, 10, and 11) but not to cultures used for camptothecin toxicity (Fig. 12).

DNA Damage and Survival Assays

All treatments with camptothecin (CA, Sigma) were at 10μM. Cortical neurons were treated with CA 3.5-4 days after plating and CGNs were treated 2 days after plating. For survival assays in Fig. 1C, Fig. 5, Fig 9C, and Fig. 12B, cell membranes were lysed and healthy nuclei were counted as previously described (Rukenstein et al., 1991; O'Hare et

al., 2000). The percentage of surviving neurons is expressed relative to untreated control wells. For survival of adenoviral infected cortical and CGN cultures exposed to CA (Fig. 8 and Fig. 12C) neurons were fixed and immunostained for MAP2 followed by nuclear staining with Hoescht. GFP expressing MAP2-positive cells were counted as either alive or dead according to the appearance of Hoescht staining. Live neurons displayed large intact nuclei while dead neurons displayed condensed and/or fragmented nuclei. Survival is expressed as the percentage of total cells that were classified as live.

Western Blot

Whole cell protein lysate was collected from 6-well plates (containing equal numbers of neurons) in solublization buffer (0.0625M Tris, 2.5 mM EDTA, 2.5 mM EGTA, 10% glycerol, 2% SDS, 0.001% bromophenol blue, and 5% β-mercaptoethanol). Equivalent volumes of lysate were separated on 10% or 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes according to standard procedure. After blocking membranes were probed with anti-p35 (C-19, SantaCruz sc-820, 1:2000), anti-cdk5 (C-8, SantaCruz, sc-173, 1:2000), anti-β-actin (Sigma, 1:3000), anti-pRb (BDPharMingen, 554136, 1:500), anti-cathepsinB (Biogenesis, 1910-8004, 1:3000), or anti-calpastatin (kind gift from Edon Melloni, University of Genoa, 1:1000). Prior to blocking, some membranes were stained with PonceauS (PS) to determine equivalent protein loading. Densitometry analysis was performed using ImageJ software (http://rsb.info.nih.gov/ij). For subcellular protein isolation (Fig. 6K), cells were collected and washed in 1X PBS. Pelleted cells were resuspened in buffer 1 (0.1X PBS pH 7.4, 0.4mM Na_2HPO_4, 0.15mM KH_2PO_4, 13.5mM NaCl, 0.25mM KCl, 0.5% Triton X-100, 2mM $MgCl_2$, and protease

inhibitor cocktail from Roche) which dissolves cell membranes. Nuclei were pelleted and the supernatant was harvested as the cytosolic fraction. Nuclei were washed 3 times with buffer 1 before lysis in solublization buffer (see above).

Kinase Assay

Neurons were collected and pelleted (at 4°C) in 1X PBS and then resuspended in IP Buffer (50mM Hepes pH 7.5, 150mM NaCl, 1mM EDTA, 2.5mM EGTA, 1mM DTT, 0.1% Tween-20, plus protease inhibitor cocktail from Roche). Lysates were incubated on ice for 10min, sonicated twice for 10 seconds at mid power, and then centrifuged at 10,000g for 6min. Supernatant was collected and protein concentrations were determined by Bradford assay. Equal amounts of protein were incubated with 1ug anti-cdk5 antibody (C-8, Santa Cruz) for 3hr and precipitated with Protein A-sepharose beads (Sigma). Immunoprecipitated complexes were incubated with 2μg Histone H1 and 5μCi P^{32}ATP and after 20min at 30°C the reaction was stopped with the addition of Western blot solublization buffer. Protein was separated by SDS-PAGE. The gel was stained with coommassie blue, dried, and then exposed to film.

Immunocytochemistry

At the indicated times neurons were fixed in 4% paraformaldehyde (containing 0.2% picric acid in 0.1M phosphate buffer pH 6.9) for 15min at RT. After washing, neurons were incubated with the primary antibodies anti-p35 (C-19 Santa Cruz, 1:500), anti-cdk5 (C-8 Santa Cruz, 1:500), or anti-MAP2 (HM2 Sigma, 1:500) overnight at 4°C. Secondary antibody to p35 and cdk5 was AlexaFluor 594 (Molecular Probes) and to

MAP2 was AlexaFluor 488 (Fig 6) or AlexaFluor 594 (Fig 7), all for 1hr at RT. Nuclei were stained with Hoescht 33258 (0.25μg/mL) for 20min at RT. Neurons were viewed using either a ZEISS Axiovert 100 or Axioskop2 fluorescence microscopes. Images were captured using a Sony 3CCD color digital video camera and Northern Eclipse ver 6.0 software. Final images were assembled using Adobe Photoshop ver 6.0.

Semiquantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufactures instructions. 50ng of RNA was used for RT-PCR using the SuperScript One-Step RT-PCR with Platinum *Taq* kit (Invitrogen). The following p35-specific primers were used at a final concentration of 500nM: upper-(5'-CTGCTGCGCTGCCTGGGTGAGTTT-3'), and lower-(5'-CAGGAAGGGCTTGAGCGGGTAGGA-3'). After DNase treatment cDNA synthesis was carried out at 45°C for 45mins. This was followed by 25 cycles of: 94°C for 30 sec, 67°C for 30 sec, 72°C for 1min. Preliminary experiments determined that 25 cycles fell within the linear range of amplification. These conditions generate a 297bp product. S12 RT-PCR was performed as described (Cregan et al., 2004b).

Caspase Activity Assay

Total cellular protein was harvested in caspase lysis buffer (1 mM KCl, 10 mM HEPES, pH 7.4, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml aprotinin, and 10% glycerol) as described (O'Hare et al., 2000). 7.5μg of protein was incubated with 15 μM DEVD-AFC in buffer as described (Stefanis et al., 1996). Fluorescence at 505nm was measured using a SLM 8000 fluorometer using an excitation wavelength of

400nm and slit length of 10.

Adenoviral Vectors

cDNA for dominant negative (D144N) or wild type versions of cdk5 were kind gifts from Dr. Li-Huei Tsai. The 3' end of D144N was ligated in frame to GFP containing either a nuclear localization (NLS from SV40 large T antigen, PKKKRKV (Kalderon et al., 1984; Groulx et al., 2000)) or nuclear exclusion sequence (NES from HIV Rev, LPPLERLTL (Fischer et al., 1995; Lee et al., 1999)) fused to the C-terminus. Also the 3' end of wild type cdk5 was ligated to GFP containing a NES. The resultant constructs (cdk5D144N-GFP-NES, cdk5D144N-GFP-NLS, and cdk5WT-GFP-NES, referred to as DN-NES, DN-NLS, and WT-NES, respectively) were inserted into pShuttle-CMV. All constructs were verified by sequencing. pShuttle constructs were processed using the AdEasy system as described (Sedarous et al., 2003) to yield final adenoviral products. Adenovirus expressing GFP-Calpastatin fusion protein was described previously (Sedarous et al., 2003). Virus titres were assessed by plaque assay. Multiplicity of infection equates to plaque forming units per cell.

Knockout Mice

All animal experimentation was approved by the University of Ottawa Animal Care Committee and conformed to guidelines set by the Canadian Council on Animal Care. Bax null mice were maintained on C57BL6 background and genotyped as reported (Knudson et al., 1995b). Apaf-1 deficient mice have been described (Cecconi et al., 1998) and were maintained on a C57BL6 genetic background. Genotyping of Apaf-1

mice was performed as reported (Fortin et al., 2001). Generation of p35 deficient animals, their characterization and genotyping, has been reported previously (Hallows et al., 2003).

Glutamate Toxicity

After 7-8 days in culture glutamate was added to CGNs at a final concentration of 20μM with or without 5μM MK801. Neurons were incubated at 37°C/5%$C0_2$ for 20 mins. Each well was then washed 2 times with 500uL of fresh media. After the final wash 600uL of 1:1 conditioned media to fresh media was added. Neurons were then returned to 37°C/5%$C0_2$ for the indicated times.

Materials

DEVD-AFC (N-acetyl-Asp-Glu-Val-Asp-AFC) was obtained from BIOMOL. DEVD-FMK (z-Asp-Glu-Val-Asp-FMK) and BAF (BOC-Asp-FMK) were obtained from Enzyme Systems Products (Livermore, CA). MDL28170 was obtained from Calbiochem (San Diego, CA). Camptothecin was obtained from Sigma (St. Louis, MO).

RESULTS

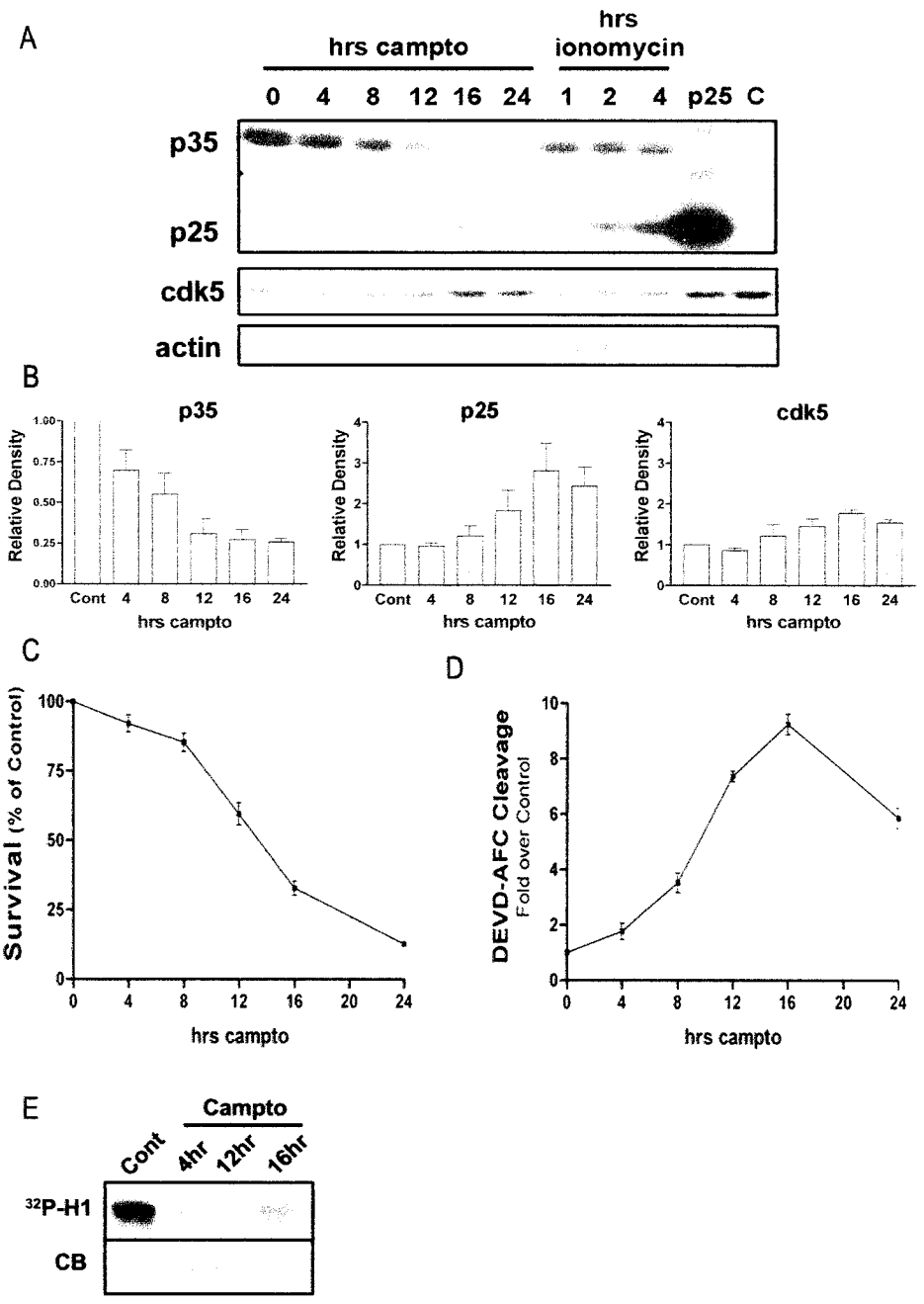
The significance of cdk5 signaling as regulated by p35 and p25 in different forms of neuronal death is not completely clear. Some reports have indicated that certain death models rely on a p25/cdk5 pathway, while others have suggested that cdk5 is instead a protective factor (Cheung and Ip, 2004). We propose that both roles are possible and that the exact function of cdk5 depends both on whether it is regulated by p35 or p25, and on the nature of the death stimulus. While clear evidence has implicated cdk5 signaling in oxidative and excitotoxic death, we first asked whether cdk5 is involved in a classic form of apoptotic death. As a model for such death, we first utilized embryonic cortical neurons treated with the DNA damaging agent camptothecin (CA). These neurons undergo apoptotic death dependent upon Bax and the mitochondrial pathway of caspase activation (Xiang et al., 1998; Keramaris et al., 2000; Morris et al., 2001).

p25 is produced in response to DNA damage

We began by examining whether p25 is produced in primary cortical neurons following treatment with CA. Immunoblot on total cell lysate using an antibody directed against the C-terminus of p35 (p35-C19, which recognizes both p35 and p25) reveals that p35 levels decrease and p25 is formed after DNA damage. Levels of p35 begin to decrease as early as 4h whereas p25 is detectable within ~12h and reaches maximal levels at 16h (Fig 1A and B). It is important to note that the level of basal p25 in untreated control neurons is somewhat variable between cultures. However, even in cultures with detectable basal levels of p25, there is a sharp increase in this protein between 12h and 16h. To ensure

Figure 1. p35 decreases early and p25 increases late in response to DNA damage.

A, representative Western blot showing relative p35, p25, and cdk5 levels from cortical neuron whole cell lysates following treatment with 10μM campto, 6μM ionomycin, or from HEK 293 cells transfected with pCMV-p25 (p25) or empty pCMV (C). Vehicle (DMSO) treatment did not alter p35, p25, or cdk5 levels. Probing for β-actin shows relative protein loading between lanes. B, Quantification of Western blots from 3 separate experiments is shown (Bars represent the mean ± SEM). Density values for each band were first normalized to actin and then to the zero time point. C, DEVD-AFC cleavage assay indicates caspase3-like activity increases within 8hr of campto treatment and peaks at 16hr. D, Survival assay showing that the majority of neuronal death occurs between 8hr and 16hr. Each data point represent the mean ± SEM from 3 experiments. E, In vitro kinase assay of immunoprecipitated cdk5 from 30μg of whole cell lysate using Histone-H1 as substrate.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32P-H1
CB

that the p25 band formed following DNA damage is the same as that reported by other groups under different death conditions, we have also examined protein from neurons treated with ionomycin, a Ca^{+2} ionophore which directly activates calpains, as well as protein from HEK293 cells transfected with pCMV-p25. The p25 band seen following DNA damage co-migrates with that produced by ionomycin and with p25 exogenously expressed from pCMV-p25 (Fig 1A). A second antibody directed against the N-terminus of p25 also confirmed that DNA damage induces an increase in p25 (data not shown). Interestingly, levels of cdk5 also increase following DNA damage at approximately the same time as p25 (Fig. 1A and B).

To determine the effect of DNA damage on cdk5 activity we performed *in vitro* kinase assay of immunoprecipitated cdk5 at various times following CA treatment. Cdk5 activity falls sharply within 4h (Fig. 1E). This is consistent with and parallels the loss of p35. Cdk5 activity rises again at 16 hrs, coinciding with the increase in p25 levels. This suggests that the early fall in cdk5 activity is in part due to loss of p35, while the late gain in activity may be due to p25 formation. However, other mechanisms of control may also be important, as suggested by the sharp decrease in cdk5 activity even in the presence of relatively abundant p35 (eg. at 4h and 8h).

Importantly, production of p25 following DNA damage occurs much later than signaling events that are known to be critical for the death process in this model. For example, increased cyclin-D-associated kinase activity (Park et al., 1998b), and upregulation of p53 and c-Jun (Morris et al., 2001; Ghahremani et al., 2002) protein are observed within 4h of CA treatment. Although a decrease in p35 levels occurs within this time frame, levels of p25 reach reliably detectable levels only at 12h. At this time

point caspases are activated 7 fold over control and survival has dropped to 50% (Fig. 1C and D). These findings present two important questions with regards to the regulation of p35 and p25 following DNA damage; 1) are p35 levels regulated independent from cleavage to p25, and 2) is p25 production a consequence or cause of late apoptotic processes?

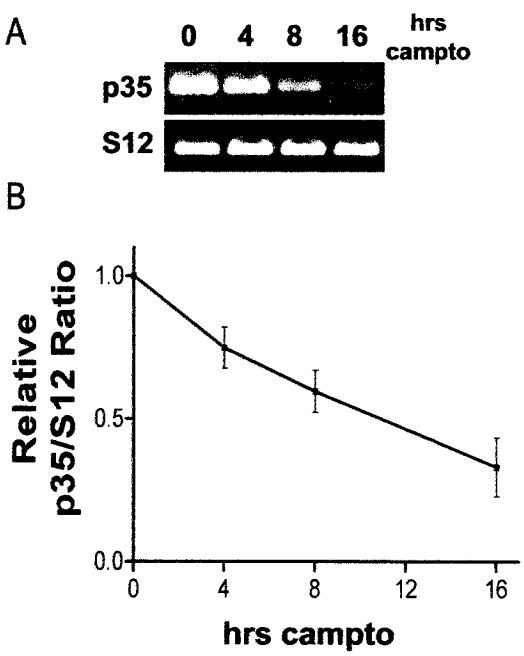
With regards to the first question, previous reports have shown that p35 levels can be regulated through proteosomal degradation (Patrick et al., 1998). However, proteosome inhibitors failed to block the early loss of p35 induced by DNA damage (data not shown). We therefore examined whether p35 was regulated at the transcriptional level. Semi-quantitative RT-PCR revealed that p35 mRNA levels decrease significantly between zero and 4h of DNA damage induction (Fig. 2), suggesting that downregulation of p35 transcription accounts for the early reduction in p35 protein, although this evidence is indirect. This result not only suggests that p35 protein levels are regulated independent from cleavage to p25, but also raises the question of whether early loss of p35 is relevant to death signaling. This possibility is more closely examined later.

p25 formation is regulated downstream of caspase activation

To address the question of whether p25 production following DNA damage is a cause or consequence of late apoptotic events we examined whether caspase activity was required for production of p25. Caspase activation in this model requires both Bax mediated cytochrome *c* release from the mitochondria and formation of the apoptosome complex consisting of cytochrome *c*, Apaf-1 and pro-caspase 9 (Xiang et al., 1998; Keramaris et al., 2000; Fortin et al., 2001). Co-treatment of CA with either of the caspase inhibitors

Figure 2. p35 mRNA decreases early in response to DNA damage.

A, RNA was extracted from cortical neurons at the indicated times following campto treatment and analyzed for p35 mRNA expression using semi-quantitative RT-PCR. S12 levels are shown as a control for equal input. B, p35 and S12 bands from 3 independent experiments were analyzed by densitometry using NIH Image v1.61. Data for p35 expression were normalized to S12 and expressed relative to the zero time point. Each point represents the mean $\pm$ SEM.


A
0
4
8
16
hrs campto
p35
S12
B
Relative p35/S12 Ratio
1.0
0.5
0.0
0
4
8
12
16
hrs campto

BAF or DEVD-FMK inhibited the production of p25 (Fig. 3A and B). Furthermore, neurons cultured from mice deficient for either Bax or Apaf-1 showed no production of p25 (Fig. 3C and D). These results show that p35 cleavage to p25 can be caspase-dependent. Interestingly, Bax deficient neurons displayed higher levels of p35 compared to heterozygous neurons at 16h (Fig. 3C). This relative increase in p35 level correlates with robust long lasting protection seen in Bax deficient neurons (Morris et al., 2001), which is in contrast to the more transient protection seen in Apaf-1 deficient neurons and neurons treated with caspase inhibitors (Cregan et al., 2002).

The dependency of p25 formation on caspases is not likely to be direct for 2 reasons; 1) p35 has no known caspase cleavage site within the region cleaved in the production of p25, and 2) purified p35 can not be cleaved to p25 by caspase-3, the predominant caspase in neurons (Lee et al., 2000). Therefore, calpains are still the most likely candidate responsible for p35 cleavage to p25 following DNA damage. To test if calpains are required for late production of p25 we co-treated neurons with CA and the calpain inhibitor MDL28170 (MDL). We found that calpain inhibition with MDL impeded the production of p25 (Fig. 4A). Similar results were found using additional inhibitors calpeptin and PD150606 (data not shown).

As we will show later, when p25 is produced in response to DNA damage, it accumulates selectively within the nucleus (see Fig. 6K). Associated with this accumulation is a switch in the profile of p35/p25 immunostaining from predominantly cytoplasmic, to a more diffuse staining in both the cytoplasm and nucleus (see Fig. 6A-I). We tested whether calpastatin, the endogenous inhibitor of calpains, could prevent this

Figure 3. p25 formation requires activation of the mitochondrial death pathway. Left panels display representative Western blots while centre and right panels display quantitative analysis of p35 and p25, respectively, from 3 or more separate experiments (Bars represent the mean ± SEM). Density values for each band were first normalized to loading control and then to the zero time point. A and B, cortical neurons were treated with campto alone or with campto plus 100μM BAF (A), or campto plus 50μM DEVD-FMK (B) for 8 and 16hrs. Total cellular protein was subjected to Western blot and analyzed for p35 and p25 levels. Caspase inhibition with BAF or DEVD-FMK prevents production of p25 following DNA damage. C and D, Neurons from individual Bax (C) or Apaf-1 (D) deficient embryos and from appropriate littermate controls were treated with campto and levels of p35 and p25 were analyzed by Western blot.

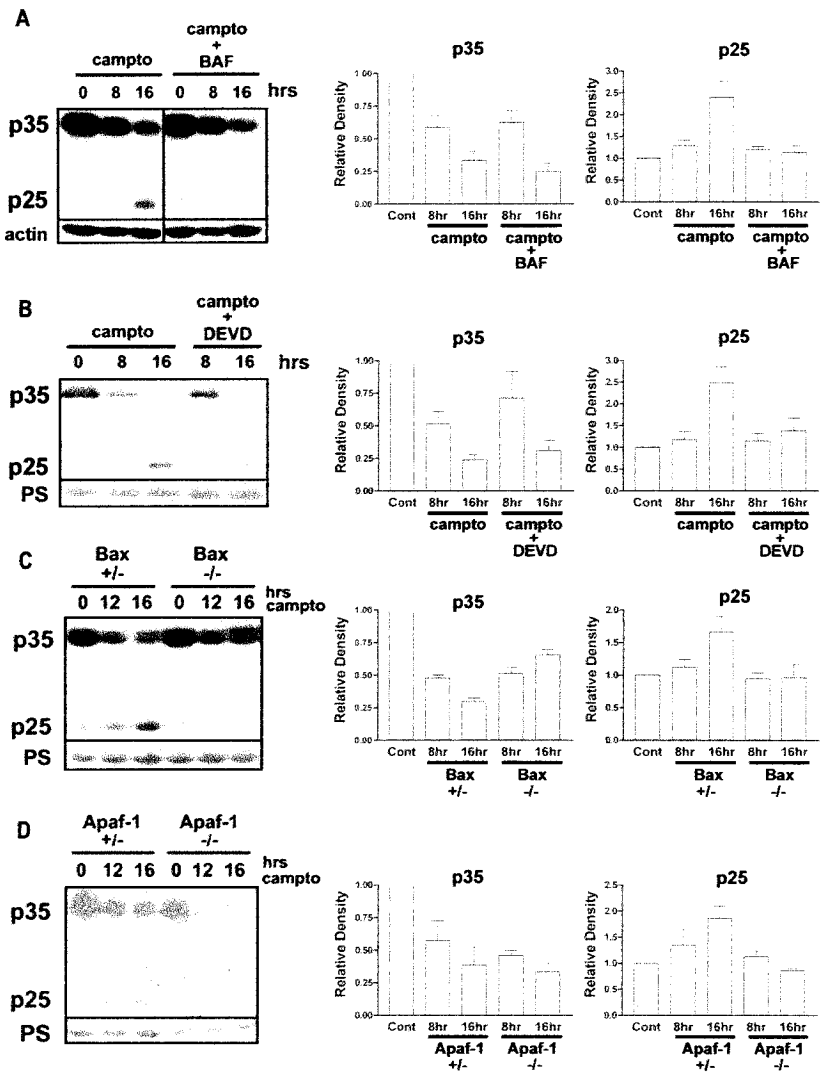
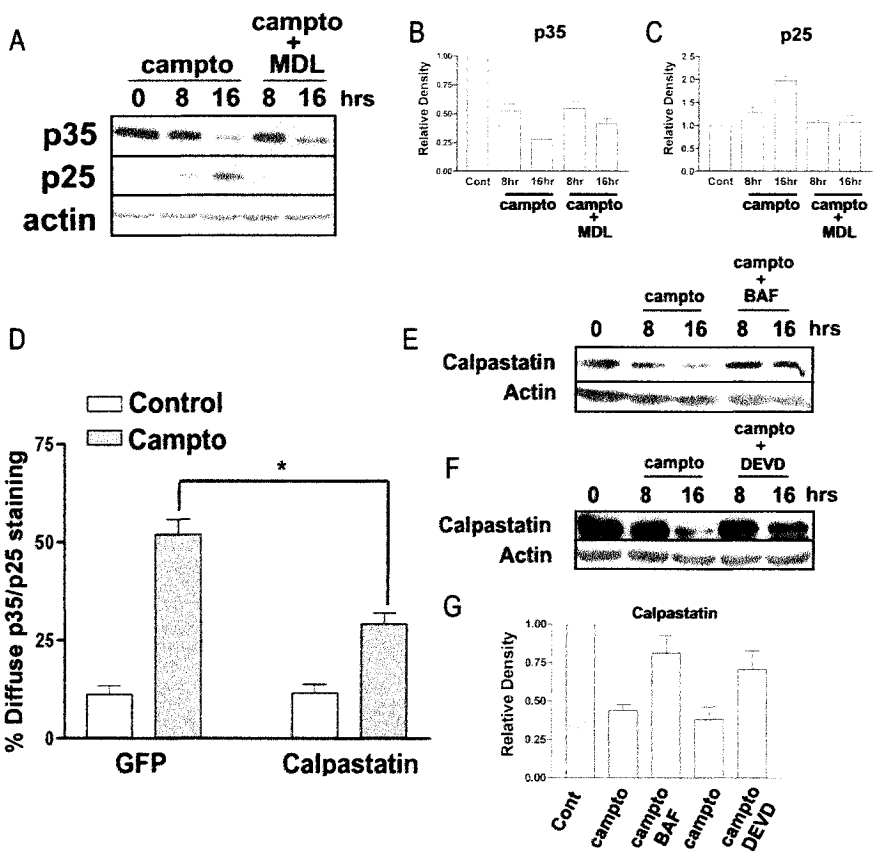

A
campto
campto + BAF
0 8 16 0 8 16 hrs
p35
p25
actin
p35
p25
Relative Density
Cont 8hr 16hr 8hr 16hr
campto
campto + BAF
B
campto
campto + DEVD
0 8 16 8 16 hrs
p35
p25
PS
p35
p25
Relative Density
Cont 8hr 16hr 8hr 16hr
campto
campto + DEVD
C
Bax +/-
Bax -/-
0 12 16 0 12 16 hrs campto
p35
p25
PS
p35
p25
Relative Density
Cont 8hr 16hr 8hr 16hr
Bax +/-
Bax -/-
D
Apaf-1 +/-
Apaf-1 -/-
0 12 16 0 12 16 hrs campto
p35
p25
PS
p35
p25
Relative Density
Cont 8hr 16hr 8hr 16hr
Apaf-1 +/-
Apaf-1 -/-

Figure 4. p25 formation requires calpain and caspase-mediated downregulation of calpastatin.

A, cortical neurons were treated with campto alone or campto plus the calpain inhibitor MDL28170 (75μM) for 8 and 16hrs. p35 and p25 levels were analyzed by Western blot. Quantification of relative p35 (B) and p25 (C) levels from 3 independent trials (Each bar represents the mean ± SEM). Density values for each band were first normalized to loading control and then to the control time point. D, 36hrs after plating cortical neurons were infected with either GFP-expressing or GFP-Calpastatin-expressing adenovirus using a multiplicity of infection of 75. 48hrs later neurons were treated with campto for 12hrs and then fixed and immuno stained using p35-C19 antibody. Nuclei were stained with Hoescht and overlap of p35-C19 staining and Hoescht was quantified. Each bar represents the mean ± SEM of data from 3 independent experiments. Significantly less GFP-Calpastatin expressing neurons treated with campto show overlap of p35-C19 and Hoescht compared to campto treated neurons expressing GFP alone (ANOVA, $p<0.001$, $n=3$). E and F, cortical neurons were treated with campto alone or campto plus 100μM BAF (E), or campto plus 50μM DEVD-FMK (F), for 8h and 16h. Total cellular protein was analyzed for calpastatin levels (~85kDa). G, Quantification of calpastatin levels at 16h from 3 independent experiments (mean ± SEM). Density values for each band were first normalized to loading control and then to the control time point.


A
campto
+
MDL
campto
0 8 16 8 16 hrs
p35
p25
actin
B
p35
Relative Density
Cont 8hr 16hr 8hr 16hr
campto
campto + MDL
C
p25
Relative Density
Cont 8hr 16hr 8hr 16hr
campto
campto + MDL
D
Control
Campto
% Diffuse p35/p25 staining
75
50
25
0
*
GFP
Calpastatin
E
campto
+
BAF
campto
0 8 16 8 16 hrs
Calpastatin
Actin
F
campto
+
DEVD
campto
0 8 16 8 16 hrs
Calpastatin
Actin
G
Calpastatin
Relative Density
1.00
0.75
0.50
0.25
0.00
Cont
campto
campto BAF
campto
campto DEVD

switch in localization of p35/p25 immunosignal. Cortical cultures infected with adenovirus expressing GFP-calpastatin showed significantly less neurons with diffuse p35/p25 staining following CA treatment compared to cultures expressing GFP alone (Fig. 4D). This ability of calpastatin to prevent the change in p35/p25 staining suggests that cleavage of p35 to p25 is mediated by calpains.

By what mechanism could both caspases and calpains be responsible for p35 cleavage to p25? Previous reports have shown that caspases can regulate calpain activity by cleaving and inactivating calpastatin (Porn-Ares et al., 1998; Wang et al., 1998; Kato et al., 2000). Similarly, we found that CA treatment induces a reduction in calpastatin protein levels and that this reduction is prevented by the caspase inhibitors BAF and DEVD-FMK (Fig. 4E-G). Together these observations are consistent with a model in which p35 cleavage by calpains occurs as a consequence of caspase degradation of calpastatin.

Addressing the functional importance of cdk5 in DNA damage

We have shown that the accumulation of p25 in neurons following DNA damage occurs downstream of caspase activation. Although this demonstrates that p35 cleavage to p25 is not a proximal event in this apoptotic model, it is still possible that the formation of p25 is essential for completion of the apoptotic program. As we have also shown, however, transcriptional mediated downregulation of p35 is a much earlier event, and this may represent a functionally important loss of cdk5 survival signaling.

To determine whether either of these possibilities is correct we treated neurons from p35 null mice (-/-), along with wild type (+/+) and heterozygous (+/-) littermate

controls, with CA and examined either for protection or sensitization. Untreated neurons from p35 -/- animals appeared similar to +/- or +/+ neurons and did not display any difference in basal survival (data not shown). Following CA treatment p35 -/- neurons did not show any difference in survival compared to +/+ or +/- littermates (Fig. 5).

The above result suggests that p25 is not required for neuronal death induced by DNA damage and that p35 is not involved in pro-survival cdk5 signaling in this model. Yet it is also possible that the homologous cdk5 activator p39 could be compensating in the absence of p35 (Tang et al., 1995). For this reason we sought to inhibit cdk5 activity more directly through expression of a dominant negative mutant of cdk5 (DN). However, this approach is also problematic because, if cdk5 regulates both pro-survival as well as pathogenic functions depending on whether it is regulated by p35 or p25, global inhibition would not allow the discrete effects of either to be observed. To avoid this problem we sought to inhibit pathogenic p25/cdk5 activity independent of p35/cdk5.

Some reports have shown that the localization of p25 differs markedly from that of p35, likely due to the loss of a myristoylation sequence at the N-terminus of p35 (Patrick et al., 1999; Kusakawa et al., 2000). This difference in localization could afford an opportunity to specifically inhibit p25/cdk5 through targeted expression of DN. Consequently, we investigated the subcellular localization of p35/p25, as well as cdk5, before and following DNA damage. Immunohistochemical staining using the p35 C-19 antibody, which does not distinguish between p35 and p25, revealed a change in signal localization after DNA damage (Fig. 6). Prior to CA treatment, when p35 is much more prevalent than p25, neuronal cell bodies show strong p35/p25 staining predominantly within the cytoplasm and relative absence of nuclear staining (Fig. 6A-D). After 16hrs of

Figure 5. p35 deficient cortical neurons are not resistant to DNA damage.

Cortical neurons were cultured from p35 null (-/-), heterozygous (+/-), or wild type (+/+) littermates and treated with campto for 12hrs. Neurons from the indicated number of mice with each genotype were cultured and tested individually. Cell membranes were lysed and intact healthy nuclei were counted according to the protocol outlined in Materials and Methods. Survival was expressed relative to untreated cells for each animal and then grouped according to genotype. Untreated neurons from p35 -/- embryos did not display any difference in survival compared to +/+ or +/- neurons. Each bar represents the mean $\pm$ SEM.

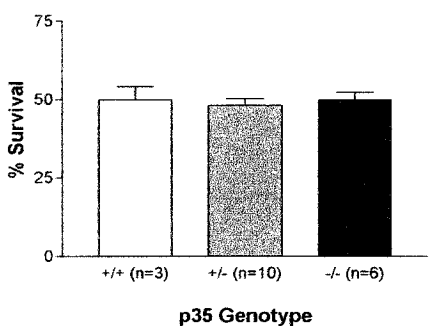
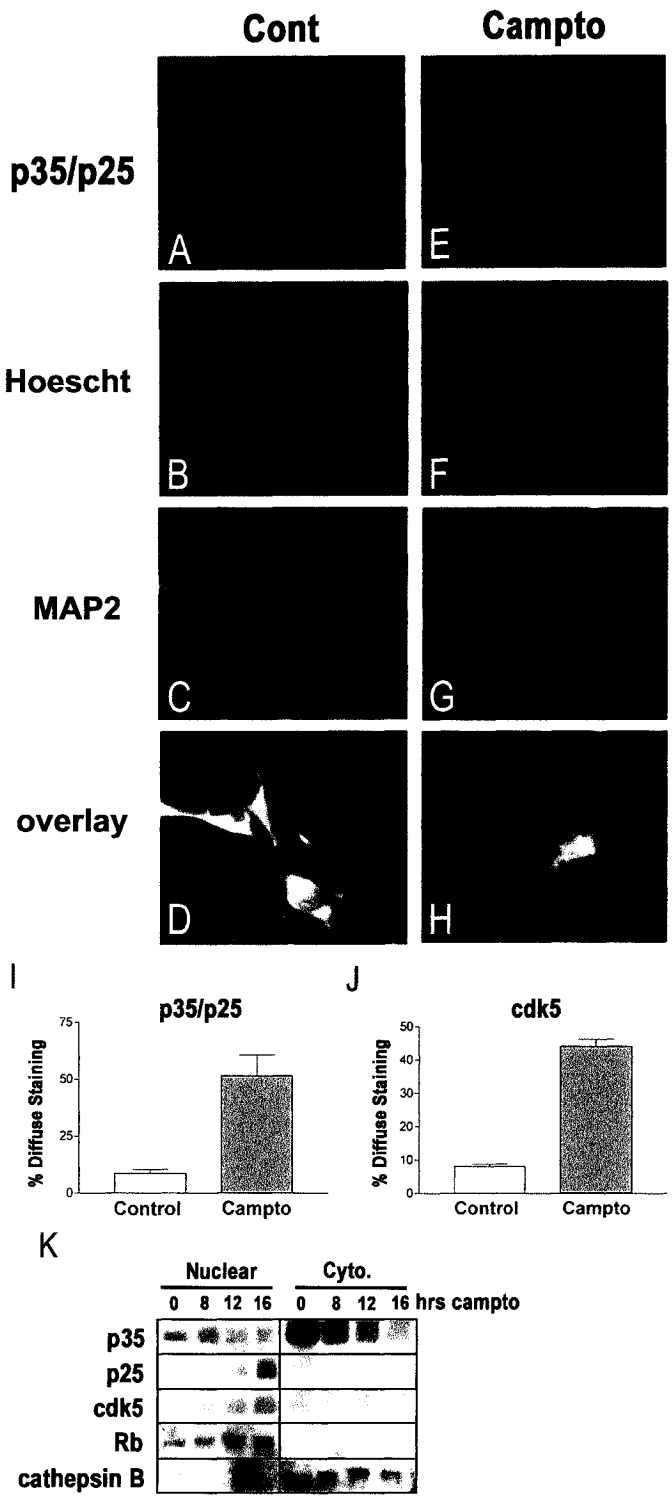

75
50
25
0
% Survival
+/+ (n=3)
+/- (n=10)
-/- (n=6)
p35 Genotype

Figure 6. p25 and Cdk5 accumulate selectively within the nucleus.

A-D, untreated neurons and neurons treated for 16hrs with campto (E-H) were fixed and immunostained for p35/p25 and MAP2. Nuclei were stained with Hoescht. Under control conditions (Cont) the p35/p25 signal predominates in the cytoplasm. Following campto treatment many neurons display more diffuse p35/p25 staining that overlaps with Hoescht. I, the percentage of p35-C19 stained neurons showing such a diffuse signal inclusive of the nucleus were quantified under control and campto treated conditions. J, Similar immunostaining for cdk5 also showed a predominantly cytoplasmic signal under control conditions and an increase in the percentage of neurons showing diffuse staining following campto treatment. Each bar represents the mean $\pm$ SEM of data from 3 independent experiments. K, protein from cytoplasmic and nuclear fractions was isolated as described in materials and methods. Western blot shows that p25 levels rise selectively within the nuclear fraction. cdk5 levels also rise only within the nuclear fraction. Purity of nuclear and cytoplasmic fractions was verified using pRb as a marker for nuclear protein and cathepsin-B as a marker for cytoplasmic protein.


Cont
Campto
p35/p25
Hoescht
MAP2
overlay
A
B
C
D
E
F
G
H
I
p35/p25
% Diffuse Staining
75
50
25
0
Control
Campto
J
cdk5
% Diffuse Staining
50
40
30
20
10
0
Control
Campto
K
Nuclear
Cyto.
0 8 12 16 0 8 12 16 hrs campto
p35
p25
cdk5
Rb
cathepsin B

CA treatment, when p25 levels are maximal, many neurons begin to show p35/p25 staining that is more diffuse throughout both the cytoplasm and nucleus (Fig. 6E-H). Only 10% of control neurons show this diffuse p35/p25 staining, whereas 51% of neurons treated with CA for 16hrs show such staining (Fig. 6I). Immunostaining for cdk5 was remarkably similar to that seen for p35. Quantification of cdk5 immunostaining revealed that only 9% of control, but 44% of CA treated neurons, displayed diffuse staining (Fig. 6J and data not shown). Together with our observation that total cellular p25 and cdk5 reach maximal levels 16hrs after CA treatment (Fig. 1), these results suggest the possibility that p25/cdk5 complexes accumulate within the nucleus.

Because immuno staining for p35/p25 does not distinguish between p35 and p25, we performed immunoblotting on subcellular protein fractions to more directly determine whether p25 is selectively accumulating in the nucleus. Our previous whole cell Western blots had shown that under control conditions p35 levels are much higher relative to p25, and that 16h following CA treatment p25 levels reach maximum. Subcellular fractionation reveals that the majority of p35 is cytoplasmic and that p25, once it accumulates, is present predominantly within the nucleus (Fig. 6K). Moreover, we found that cdk5 levels increase only within the nuclear compartment (Fig. 6K). Combined with the immunochemistry data above, these results reveal a dramatic downregulation of p35/cdk5 in the cytoplasm and an increased formation of p25/cdk5 in the nucleus.

This unique localization of p35 and p25 allowed us to devise a strategy to selectively inhibit cdk5 activity regulated by each. We constructed adenoviral vectors expressing DN-cdk5 C-terminal fused to GFP carrying either a nuclear localization

sequence (DN-NLS) or a nuclear exclusion sequence (DN-NES) (Fig. 7). Expression of DN-NLS was localized to the nucleus as judged by overlap with Hoescht and expression of DN-NES was localized to the cytoplasm as judged by non-overlap with Hoescht and overlap with microtubule associated protein 2 (MAP2) (Fig 7 A-D and E-H). In vitro kinase assays of cdk5 immunoprecipitated from neurons infected with these constructs confirmed that they effectively suppress cdk5 activity (data not shown).

Cortical neurons were infected 24h after plating with DN-NES, DN-NLS, or a control GFP expressing adenovirus and treated with CA 48h later. After CA treatment neurons were fixed and nuclei were stained with Hoescht. GFP-positive neurons were assayed for survival according to the appearance of Hoescht staining. Importantly, expression of DN-NLS did not provide any significant protection against DNA damage (Fig 8). Together with the observations that nuclear p25 formation occurs late following DNA damage, and that p35 null neurons are not protected, this suggests that the nuclear production of p25/cdk5 is not required for neuronal death induced by DNA damage.

Interestingly, expression of DN-NES caused a significant sensitization to DNA damage (Fig. 8). Sensitization due to cytoplasmic inhibition of cdk5 would be expected if p35/cdk5 were performing a pro-survival role within the cytoplasm. Our observations that p35 mRNA and protein levels decrease early following CA treatment are also consistent with a pro-survival role for cytoplasmic p35/cdk5. Together, these data suggest that in this apoptotic model of death, p25/cdk5 complexes increase and are present in the nucleus. However, this increase occurs too late to be of functional significance. In contrast, early loss of p35/cdk5 complexes in the cytoplasm may lead to sensitization.

Figure 7. Characterization of DN-NLS and DN–NES adenoviral constructs. Recombinant adenovirus expressing the fusion constructs DNcdk5-GFP-NLS or DNcdk5-GFP-NES were used to infect cortical neurons 24hrs after plating at a multiplicity of infection of 75. After 36hrs neurons were fixed and stained for MAP2 and Hoescht. DN-NLS overlaps strongly with Hoescht but not with MAP2 (A-D). DN-NES signal overlaps strongly with MAP2 but not with Hoescht (E-H).

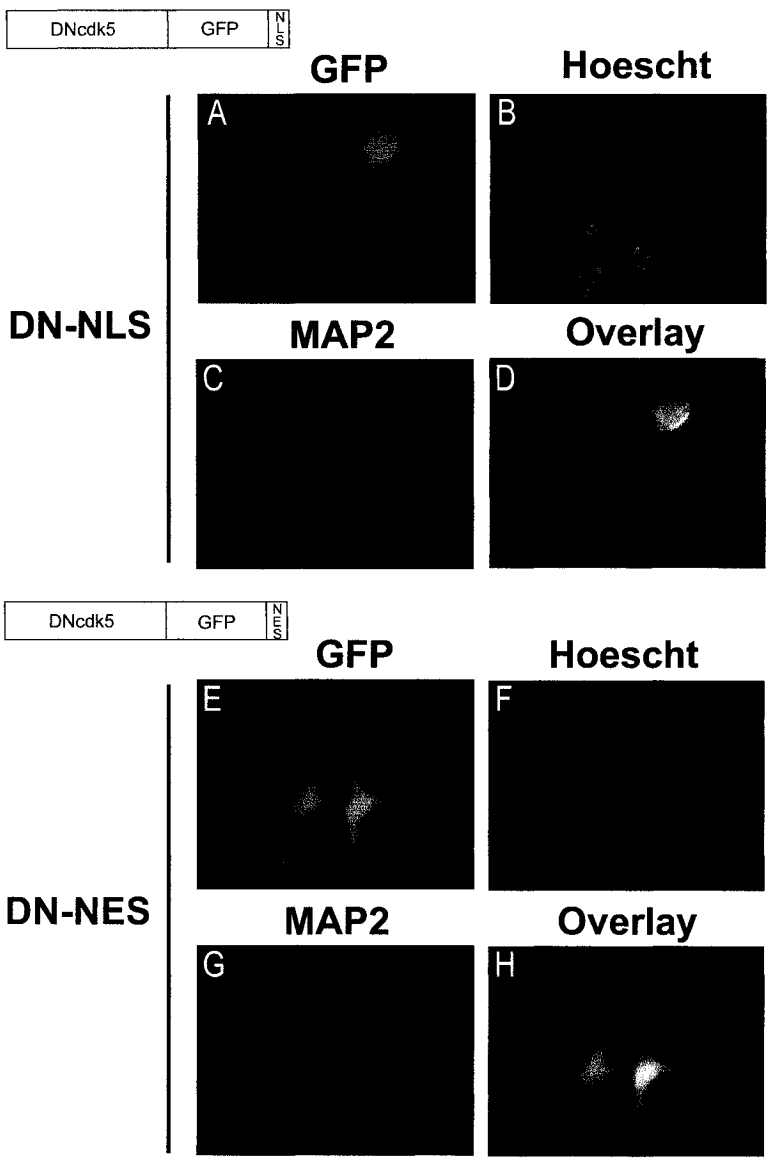
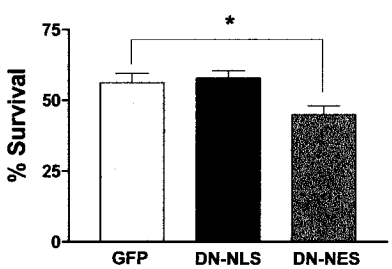

DNcdk5
GFP
NLS
GFP
Hoescht
A
B
DN-NLS
MAP2
Overlay
C
D
DNcdk5
GFP
NES
GFP
Hoescht
E
F
DN-NES
MAP2
Overlay
G
H

Figure 8. Nuclear inhibition of Cdk5 does not protect from DNA damage. But cytoplasmic inhibition of Cdk5 sensitizes to DNA damage.

Cortical neuron cultures plated 24-36hrs previous were infected with adenoviral constructs expressing GFP, DN-NLS, or DN-NES at a multiplicity of infection of 75. Two days later neurons were treated with campto for 14hrs. Cultures were immunostained for MAP2 and nuclei were labeled with Hoescht. GFP expressing MAP2-positive cells were classified as either dead or alive according to the appearance of Hoescht. The ratio of live to total cells counted was used as a measure of survival. Bars represent the mean $\pm$ SEM for data from 3 independent experiments each done in triplicate. * indicates significance (ANOVA, $p<0.05$). A minimum of 150 cells were counted per well (450 cells per experiment). Infected cultures not treated with campto showed low toxicity (>85% survival for all constructs).


*
75
50
25
0
% Survival
GFP
DN-NLS
DN-NES

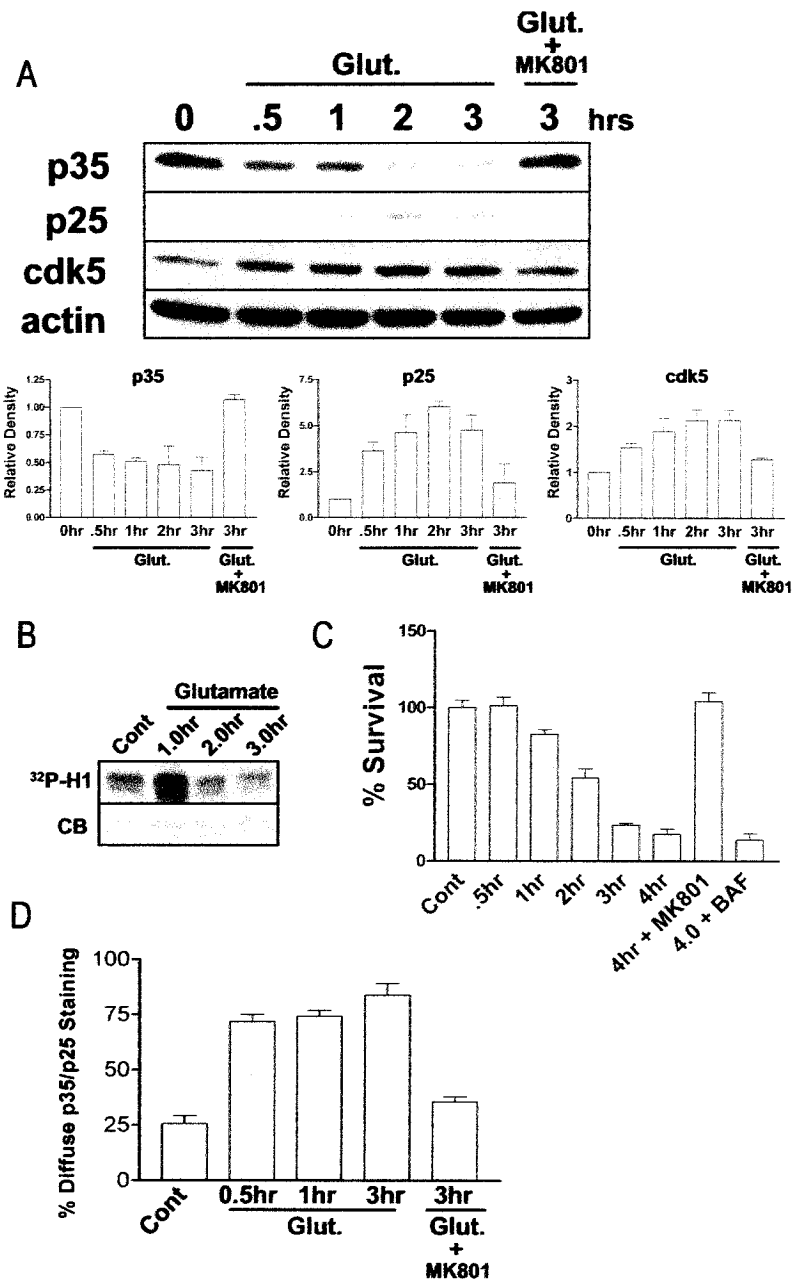
Nuclear and Cytoplasmic Roles for Cdk5 in Excitotoxicity

The relative contributions of p35-regulated versus p25-regulated cdk5 activity have not been addressed in non-apoptotic models of neuronal death in which cdk5 has been implicated, such as excitotoxic death. For this reason we examined the involvement of nuclear p25/cdk5 complexes and cytoplasmic p35/cdk5 complexes in excitotoxic death of cerebellar granule neurons (CGNs) exposed to glutamate. We found that CGNs treated with 20μM glutamate rapidly cleave p35 to p25 (Fig. 9A). Levels of p25 increase within 30min and reach maximal within 2h. Levels of cdk5 also increase within 30min of glutamate treatment (Fig. 9A). In vitro kinase assay of cdk5 activity shows an early increase in activity 1h following exposure (Fig. 9B). This increase is concurrent with the early rise in cdk5 and appearance of p25. However, the return of cdk5 activity to basal levels at 2h and 3h, even in the presence of increased p25 and cdk5, indicates that mechanisms in addition to protein levels contribute to cdk5 activity.

In contrast to what is seen following DNA damage induced death, cell survival following glutamate treatment begins to decline only after the appearance of p25. Reduced cell survival is detectable within 1h of glutamate exposure and death proceeds rapidly between 1h and 3h (Fig. 9C). Potent protection with MK801 indicates that death occurs in an NMDA-dependant manner (Fig. 9C). Cotreatment with BAF did not inhibit death, suggesting that caspases are not involved in this death model (Fig. 9C). Although production of p25 following glutamate has been reported previously (Lee et al., 2000), the localization of this endogenously produced p25 has not been addressed. We found that greater than 70% of CGNs treated with glutamate for 30min display diffuse p35/p25

Figure 9. p25 is produced early following glutamate exposure.

A, Primary cultures of CGNs were treated with 20μM glutamate with or without 5μM MK801 for the indicated times. Total cellular protein was collected and analyzed by Western blot for p35, p25, and cdk5 levels. Densitometry analysis of p35, p25, and cdk5 levels over two experiments is shown below. B, In vitro kinase assay of cdk5 activity from 30ug of whole cell lysate reveals a peak in activity at 1.0h. C, Neuronal survival was assessed at the indicated times using the technique described in Materials and Methods. Bars represent the mean ± SEM for data from 3 independent experiments. D, CGNs were fixed at the indicated times following glutamate treatment. Neurons were immunostained for p35/p25 as described in materials and methods and nuclei were stained with Hoescht. The percentage of neurons displaying overlap of p35/p25 signal with Hoescht was quantified as described for Fig. 6. Bars represent the mean ± SEM for data from 3 independent experiments.


A
Glut.
Glut. + MK801
0 .5 1 2 3 3 hrs
p35
p25
cdk5
actin
p35
p25
cdk5
Relative Density
0hr .5hr 1hr 2hr 3hr 3hr
Glut.
Glut. + MK801
B
Glutamate
Cont 1.0hr 2.0hr 3.0hr
32P-H1
CB
C
% Survival
Cont .5hr 1hr 2hr 3hr 4hr 4hr + MK801 4.0 + BAF
D
% Diffuse p35/p25 Staining
Cont 0.5hr 1hr 3hr 3hr
Glut.
Glut. + MK801

immunoreactivity in both cytoplasm and nucleus (Fig. 9D), compared to only ~25% of non-treated control cells. This finding is consistent with nuclear accumulation of p25.

We next tested whether p25 production during excitotoxicity was dependent on the mitochondrial death pathway, as it is following DNA damage. We found that p25 production in response to glutamate is not dependent upon the presence of Bax (Fig. 10A) and occurs in the absence of any increase in caspase activity (Fig. 10B). Together with the data presented in Fig. 3, these results imply distinct differences in the mechanisms controlling p35 cleavage to p25 in rapid excitotoxic death versus delayed apoptotic death.

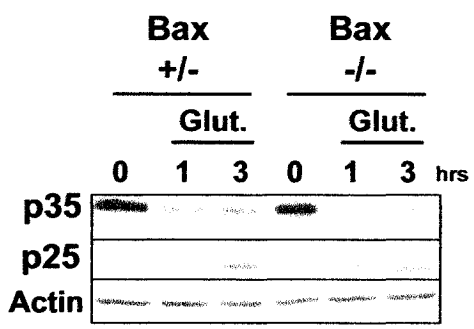
To determine whether the rapid caspase-independent increase in p25 is functionally required for glutamate induced death we tested the ability of DN-NLS to protect. Indeed, expression of DN-NLS is significantly protective following glutamate treatment (Fig. 11). This again contrasts with the DNA damage model, where the identical construct was ineffective in providing protection. These results are the first to suggest that inhibition of cdk5 exclusively within the nucleus is sufficient to prevent neuronal death.

Interestingly, although DN-NES expression did not sensitize to excitotoxicity as it did to DNA damage, we did find that wild type (WT) cdk5 expression selectively in the cytoplasm (WT-NES) provided protection (Fig. 11). Much like our finding that DN-NES sensitizes cortical neurons to DNA damage, this result again supports the idea that cdk5 performs a pro-survival role within the cytoplasm. However, because DN-NES did not sensitize to glutamate treatment within the time frame examined, it is unlikely that endogenous cytoplasmic cdk5 activity plays a functionally important role during

Figure 10. The mitochondrial pathway is not required for p25 production during excitotoxicity.

A, CGNs from Bax +/- and Bax -/- littermates were treated with glutamate for 1 and 3 hrs and total cellular protein was analyzed for p35 and p25 levels. B, Protein lysate from glutamate treated CGN cultures was analyzed for DEVD-AFC cleavage activity. Protein from neurons treated with low levels (5mM) of K^+ was included as a positive control for caspase activity. Each bar represents the mean $\pm$ SEM of data from three independent experiments.

A


Bax
+/-
Bax
-/-
Glut.
Glut.
0 1 3 0 1 3 hrs
p35
p25
Actin

B

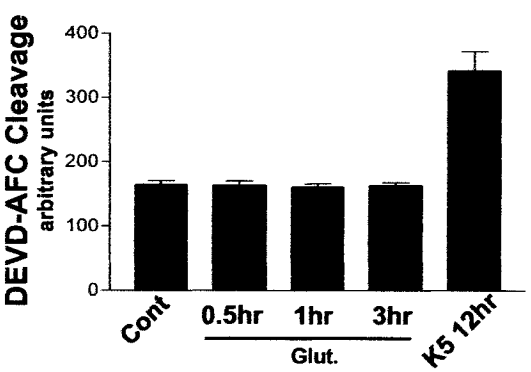
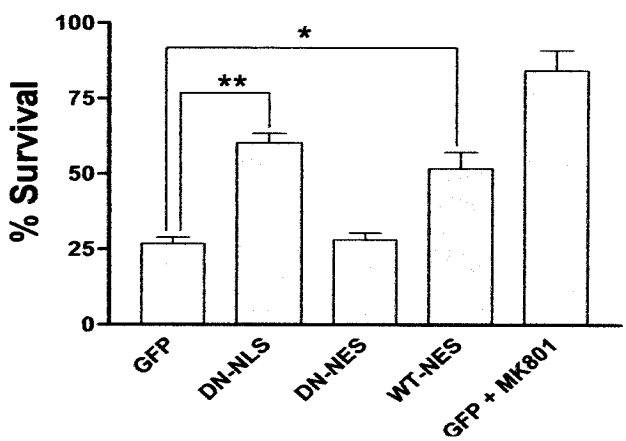

DEVD-AFC Cleavage
arbitrary units
400
300
200
100
0
Cont
0.5hr
1hr
3hr
Glut.
K5 12hr

Figure 11. Nuclear inhibition or cytoplasmic facilitation of cdk5 protects against excitotoxic death in CGNs.

After 5 days in culture CGNs were infected with the indicated adenoviral constructs at a multiplicity of infection of 20 and subjected to glutamate 2-3 days later. 3hrs following glutamate treatment cells were fixed and immunostained for MAP2. Nuclei were stained with Hoescht. The total number of GFP-positive live neurons (MAP2 positive cells) were counted in a circumscribed area. Survival is represented relative to non-glutamate treated cultures infected with the same construct. Adenoviral infections did not alter survival in non-glutamate treated cultures. Each bar represents mean $\pm$ SEM from 3 independent experiments. * and ** denote significance $p<0.001$ and $p<0.01$, respectively (ANOVA). Protection with MK801 verifies that death is NMDA dependent.

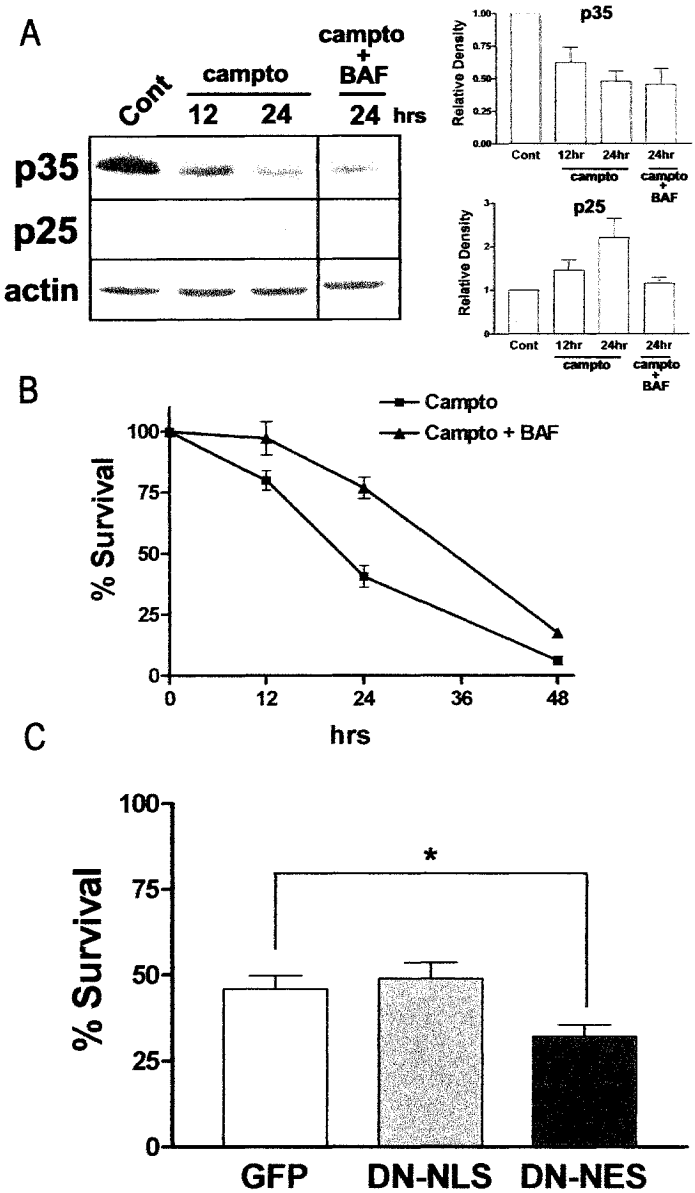

% Survival
100
75
50
25
0
*
**
GFP
DN-NLS
DN-NES
WT-NES
GFP + MK801

glutamate induced death. It is also possible that during glutamate induced death strong pro-death signals initiated by nuclear p25/cdk5 (and other death signals) overwhelm pro-survival signaling by cytoplasmic p35/cdk5. Importantly, the localization of DN-NLS and DN-NES in CGNs was similar to that observed in cortical neurons (Fig. 7), and the WT-NES construct also showed selective cytoplasmic localization (data not shown).

We next asked whether the differences we have observed in the contribution of cdk5 to apoptotic and excitotoxic death could be due simply to differences in the respective culture systems used. To address this question we subjected CGNs to DNA damage induced by CA. Similar to cortical neurons treated with CA, CGNs also upregulate p25 late in the death process (Fig. 12A). The kinetics of CA induced death in CGNs is slightly delayed compared to that in cortical neurons (Fig. 12B). However, the rise in p25 levels is also delayed, with the peak coming at 24h. At this time over 50% of neurons have died (Fig. 12B). Caspase inhibition by BAF significantly delays CA induced CGN death and also suppresses the production of p25 (Fig. 12A and B). The transient inhibition of death seen with BAF is similar to that described in cortical neurons (Cregan et al., 2002). Most importantly, the effect of expressing DN-NLS or DN-NES in CGNs exposed to DNA damage was analogous to that seen in cortical neurons. At 24h after CA treatment DN-NLS expression did not change survival with respect to GFP expression control, while DN-NES expression resulted in a small but significant reduction in survival (Fig. 12C). These results indicate that even within the same cell type (CGNs), cdk5 plays very distinct roles in delayed apoptotic death and rapid excitotoxic death. Furthermore, these results also suggest that the differences observed

Figure 12. DNA damage induced death of CGNs is associated with caspase-dependent p25 production and sensitization by DN-NES.

A, Western blot analysis of total cellular protein from CGNs treated 2 days after plating with campto alone or campto plus BAF (100μM) for the indicated times. Quantification of relative p35 and p25 levels is shown on the right. Density values for each band were normalized to actin and then to the control time point. B, CGNs treated with campto alone or campto plus BAF (100μM) were assayed for survival at the indicated times according to the protocol outlined in Materials and Methods. C, CGNs were infected at the time of plating with an MOI of 20. 48h after plating/infection neurons were treated with 10μM campto for the indicated times. Survival was assessed after 24h of campto treatment. GFP-positive neurons were classified as either dead or alive according to the appearance of Hoescht. The ratio of live to total cells counted was used as a measure of survival. Bars represent the mean ± SEM for data from 3 independent experiments each done in triplicate. * indicates significance (ANOVA, $p<0.05$). A minimum of 100 cells were counted per well. Infected cultures not treated with campto showed low toxicity (>85% survival for all constructs).


A
Cont
campto
12
24
campto + BAF
24 hrs
p35
p25
actin
p35
Relative Density
1.00
0.75
0.50
0.25
0.00
Cont
12hr
24hr
24hr
campto
campto + BAF
p25
Relative Density
3
2
1
0
Cont
12hr
24hr
24hr
campto
campto + BAF
B
Campto
Campto + BAF
% Survival
100
75
50
25
0
0
12
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36
48
hrs
C
% Survival
100
75
50
25
0
*
GFP
DN-NLS
DN-NES

between excitotoxic and apoptotic death are not an artifact of the different culture systems utilized.

In summary, we propose that in apoptotic death induced by DNA damage, downregulation of p35/cdk5 activity in the cytoplasm plays a modulatory role in survival while caspase-dependent nuclear p25 formation occurs temporally late and does not play a role. In contrast, in a glutamate model of death, rapid accumulation of nuclear p25 is sufficient and required to induce death while driving cytoplasmic cdk5 can be protective.

DISCUSSION

cdk5 in DNA damage signaling induced by CA

We had initially identified two questions of interest in regards to the role of cdk5 in neuronal death. The first was whether cdk5 was functionally important in a model of neuronal death that is dependent on the mitochondrial pathway. We raised this question because a number of previous reports have suggested cdk5 is a common regulator of neuronal death in multiple types of neurodegenerative conditions, yet few reports have examined the role of cdk5 in core apoptotic signaling. We examined this question using a model in which neuronal death occurs as a result of DNA damage.

A number of signaling pathways have been found to be required for neuronal death induced by DNA damage. For example, neurons lacking p53, a major response protein to DNA damage in mammalian cells, are robustly protected against a variety of DNA damage inducers including CA (Xiang et al., 1998; Morris et al., 2001). In response to DNA damage in neurons, p53 acts as the main upstream activator of the mitochondrial death pathway (Cregan et al., 1999b; Fortin et al., 2001; Morris et al., 2001). Activation of cell cycle CDKs and phosphorylation of pRb are also known to be critical for induction of the mitochondrial pathway and death in this model (Park et al., 1997b; Park et al., 2000b; Morris et al., 2001). The mitochondrial pathway involves cytochrome *c* release from the mitochondria induced by BH3-only members of the Bcl-2 family such as Bax, followed by formation of the apoptosome complex (Apaf-1, cytoC, dATP, and procaspase 9) and activation of caspases (Green and Kroemer, 2004). We provide several lines of evidence that p25 production following DNA damage occurs downstream of this pathway. Firstly, levels of p25 accumulate relatively late in the death

process, only after caspase activity has begun to increase. Second, caspase inhibitors prevent p25 production. Third, Bax or Apaf-1 deficient neurons do not display any increase in p25. Together these observations indicate that p35 cleavage to p25 can occur as a late, caspase-dependent apoptotic event. This finding is consistent with results from others showing that staurosporine induced apoptotic death of cortical neurons is associated with a late increase in p25 production (Kusakawa et al., 2000). Combined with our observations that neither p35 deficiency nor selective inhibition of p25/cdk5 activity (using DN-NLS) afforded protection against DNA damage, we conclude that the late appearance of p25 is a result of the death process rather than a required component. This result indicates that the presence of p25 in neurodegenerative or neurotoxic conditions, particularly those that involve delayed apoptotic signaling, does not necessarily point toward a direct requirement for cdk5 in the death process.

Despite the late caspase-dependent appearance of p25 we do not believe that caspases directly mediate p35 cleavage. Calpains are the only proteases shown to be capable of cleaving p35 in the region necessary to produce p25 (Kusakawa et al., 2000; Lee et al., 2000). However, multiple reports have shown that caspases can regulate calpain activity by cleaving and inactivating calpastatin, an endogenous calpain inhibitory protein (Porn-Ares et al., 1998; Wang et al., 1998; Kato et al., 2000). Our results show that in response to DNA damage calpastatin levels decrease in a caspase-dependent manner, and calpain inhibition (either with MDL or with overexpression of calpastatin) prevents both p25 production and appearance of nuclear p35/p25 immuno signal. These results are consistent with a model in which calpain mediated cleavage of p35 to p25 occurs as a result of caspase degradation of calpastatin.

Our previous results had shown that calpains were activated early following DNA damage and that this activation was required for the upregulation of p53 (Sedarous et al., 2003). An important question then is why do we not also observe an early cleavage of p35 to p25? Calpains are a large family of distinct but homologous proteases, some with tissue specific expression, some with ubiquitous expression, and each with a unique Ca^{+2} concentration requirement (Goll et al., 2003). Some reports have also identified nuclear specific calpains and/or nuclear transport of select calpains (Mellgren et al., 1993; Mellgren and Lu, 1994; Ma et al., 2001; Gil-Parrado et al., 2003). This variety and specificity in localization may explain why calpain regulation of p53, a predominantly nuclear protein, is distinct from that of p35, a cytoplasmic/cell membrane protein. Further studies are required to determine whether these two proteins are associated with distinct pools of calpain that are activated at different stages following DNA damage.

p35/cdk5 promotes survival within the cytoplasm while p25/cdk5 promotes death within the nucleus.

Our second objective was to resolve contradictory reports in the literature which suggest that cdk5 can act as both a pro-survival and a pro-death signal. Most findings have agreed that cdk5 pro-death signaling is regulated by p25 (Patrick et al., 1999; Kusakawa et al., 2000; Lee et al., 2000; Cheung and Ip, 2004). By default, therefore, pro-survival signaling seems likely to be regulated by p35. However, the relative contributions of p25/cdk5 and p35/cdk5 to death/survival in single models of neuronal death have not been addressed. One reason for this has been the inability to study cdk5 function regulated by p35 independent from that regulated by p25. Traditional means of

cdk5 inhibition (pharmacological, molecular, and genetic) do not distinguish between p35/cdk5 and p25/cdk5. Our finding that p35 is localized predominantly to the cytoplasm while p25 is found mainly within the nucleus allowed us to target inhibition of either p35/cdk5 or p25/cdk5 by localizing a dominant negative mutant of cdk5 to the cytoplasm or nucleus, respectively. Using this approach in both DNA damage and glutamate excitotoxicity, we find evidence supporting a model in which pro-survival signaling by cdk5 occurs mainly within the cytoplasm while pro-death cdk5 signaling is contained to the nucleus.

Our evidence that p35 regulates a pro-survival cdk5 function within the cytoplasm is threefold. Firstly, neurons actively downregulate p35 mRNA and protein in the early phase following DNA damage. This downregulation may represent a damping of pro-survival cdk5 signaling. Consistent with this is our observation that cytoplasmic cdk5 inhibition sensitizes neurons to DNA damage induced death. Thirdly, cytoplasmic expression of wild type cdk5 provides protection against glutamate toxicity. Furthermore, we have observed that CGNs undergoing death induced by low levels of K^+ also downregulate p35 protein, without production of p25 (data not shown). These findings are consistent with the reported findings that p35 protein decreases without p25 production under a variety of neurotoxic conditions including treatment with cyclosporine A, etoposide, okadaic acid, and 4-hydroxynonenal (Kerokoski et al., 2001).

What is the nature of this potential pro-survival cytoplasmic signal activated by p35/cdk5? Previous reports have shown that cdk5 can phosphorylate and inactivate the c-Jun N-terminal kinase 3 (JNK3) and that this property of cdk5 can inhibit neuronal death induced by UV irradiation (Li et al., 2002). Also, our previous work has identified

JNK phosphorylation of cJun as a required event in neuronal death induced by CA (Ghahremani et al., 2002). Together these results suggest the possibility that p35/cdk5 promotes survival in neurons exposed to CA by negatively regulating the JNK-cJun pathway. Further work will be needed to test this possibility.

We have also shown that, in the context of glutamate excitotoxicity, p25 promotes a pro-death cdk5 activity selectively within the nucleus. In support of this, we show first that p25 is formed rapidly and localizes almost exclusively to the nuclear compartment, and second that inhibition of cdk5 only within the nucleus is sufficient to inhibit glutamate induced death. Although we have not identified here the relevant nuclear cdk5 target(s), work from others has shown that in response to glutamate and oxidative stress, cdk5 phosphorylation of the MEF2 transcription factor is essential for death (Gong et al., 2003). This phosphorylation of MEF2 results in inactivation of its pro-survival activity.

In conclusion, our evidence points to a model by which the functional relevance of cdk5 to different neuronal death models depends upon at least two critical factors, localization and timing. Localization, which is controlled by binding either to p35 or p25, determines whether cdk5 suppresses or activates cell death mechanisms. Timing, in the production of p25, determines the importance of cdk5 in death. Late caspase dependent production of p25 can occur as a consequence of apoptotic death, but early non-caspase dependent p25 production plays a strong role in promoting death.

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Chapter 4. Summary and Discussion

The following discussion is divided into three main sections and is designed to supplement the discussions presented in Chapters 2 and 3. Since the publication of the data in Chapter 2, many reports from other groups have emerged which further support the hypothesis that E2F1 plays a critical role in promoting neuronal death. The first section below provides a summary and analysis of this data with an emphasis on insights into the mechanisms through which E2F1 induces death. The following section is an attempt to link the themes of Chapters 2 and 3 by providing an example of a situation in which E2F1 and cdk5 may cooperate to induce neuronal death. Also, recent evidence will be analyzed to suggest the possibility of a general linkage between cell cycle CDK pathways and cdk5. The final section will highlight the significance and impact of the preceding works on the field of neuronal death research, as well as the potential clinical implications of this work.

Neuronal Death Induced by E2F1

Since the publication of Chapter 2 other groups have confirmed that E2F1 expression causes neuronal death (Hou et al., 2000; Hou et al., 2001a; Liu and Greene, 2001). Increased expression of E2F1 protein and/or mRNA has been confirmed for the K+ deprivation model of CGN death (Trinh et al., 2001; Konishi and Bonni, 2003) and has also been observed during death induced by toxic levels of dopamine (Hou et al., 2001b), inhibition of histone deacetylases (Boutillier et al., 2003), oxygen-glucose deprivation (Gendron et al., 2001), and *in vivo* kainic acid treatment (Verdaguer et al., 2002). The central involvement of E2F1 in neuronal death processes is highlighted by the cases in which its inhibition prevents death. E2F1 downregulation or deletion has

since been shown to prevent death *in vitro* induced by β-amyloid (Giovanni et al., 2000), hypoxia (Gendron et al., 2001), staurosporine (Hou et al., 2000), and dopamine toxicity (Hou et al., 2001a). More importantly, E2F1 has also been directly implicated in *in vivo* neuronal death associated with stroke. E2F1 protein levels increase in neurons dying following a focal stroke treatment (Osuga et al., 2000), and E2F1 null mice show up to 77% reduction in infarct size compared to littermate controls and display significant improvement in motor function (MacManus et al., 1999a; MacManus et al., 2003). In combination with reports showing that CDK inhibition is an effective neuroprotectant in various stroke models (Osuga et al., 2000; Wang et al., 2002; Rashidian et al., 2005), this data indicates that elements of the G1 to S cell cycle control circuit may be important targets for therapeutic intervention in human cases of stroke.

Mechanisms of E2F1 Induced Death

In Chapter 2 I showed that E2F1-induced neuronal death was directly dependent on Bax, yet independent of p53, and was associated with caspase activation. Subsequent work has greatly expanded our understanding of E2F1 in neuronal death. Cdk1 is a target of E2F mediated transcription in proliferating cells. Konishi and Bonni found that cdk1 is also an essential target of E2F1 in neuronal death induced by low [K^+] (Konishi and Bonni, 2003). This study showed that E2F binding elements in the cdk1 promoter are essential for death, and that interruption of E2F1 signaling prevents low [K^+] induction of cdk1 and promotes survival (Konishi and Bonni, 2003). Inhibition of cdk1, by expression of a DNcdk1, is itself protective (Konishi et al., 2002). This suggested that cdk1 is an essential death target of E2F1. Although other work in proliferating cell

systems had correlated cdk1 induction with apoptosis, nothing was known about the mechanism by which it might mediate this effect. Konishi and Bonni have also addressed this question by showing that cdk1 can directly link cell cycle signaling to the intrinsic death pathway. They observed that Cdk1 can phosphorylate the BH3 only protein Bad on ser-128 (Konishi et al., 2002). In healthy cells growth factor induced phosphorylation of Bad on ser-136 prevents death by promoting the association of Bad with 14-3-3 proteins (Datta et al., 1997; Blume-Jensen et al., 1998; Bonni et al., 1999). Cdk1 mediated phosphorylation on ser-128 manages to override ser-136 phosphorylation, leading to an accumulation of Bad, release of cytochrome *c*, activation of caspases, and death (Konishi et al., 2002).

The above data provide valuable insight into the method by which E2F1 evokes neuronal death and at the same time provides a mechanistic link between cell cycle reactivation and activation of conserved death pathways. In fact, the link between E2F1 and the intrinsic death pathway is much more extensive. A number of elements of this pathway have been found to be direct targets of E2F1. For instance, Apaf-1, which is required for formation of the apoptosome and subsequent caspase activation, is upregulated in an E2F1-dependent manner following the loss of pRb (Moroni et al., 2001; Furukawa et al., 2002). E2F1 also directly controls the transcription of multiple BH3-only proteins including (Muller et al., 2001; Nahle et al., 2002; Cao et al., 2004; Fortin et al., 2004; Hershko and Ginsberg, 2004). Finally, E2F1 also mediates expression various caspases, including caspase 3, and 7 (Muller et al., 2001; Nahle et al., 2002). These data indicate that E2F1 has direct control over the expression of the major signaling elements of the mitochondrial death pathway. The data to date, therefore,

implicates E2F1 as a central convergence point between inappropriate cell cycle activation and initiation of apoptotic programs.

The findings that E2F1 overexpression can induce death seem to suggest that death occurs through direct gene activation. However, we must remember that two distinct mechanisms of gene induction at E2F target sites exist, activation and derepression. A mutant of E2F1 lacking the transactivation domain, and the pRb binding region within it, has been used to dissect the relative contribution of direct gene activation versus gene derepression. Overexpression studies confirmed that this mutant is still capable of potently inducing death (Hou et al., 2001a; Liu and Greene, 2001). This transactivation mutant E2F1 would be expected to act as a dominant negative against E2F-dependent transactivation, since it can displace endogenous E2F at target sites yet cannot promote transcription. Also, in being capable of binding DNA but being unable to bind pRb, it would be expected to displace endogenous inhibitory E2F/p130 complexes, and thereby function as an inducer of derepression. Taken in this way, these experiments indicate that death promoting targets of E2F1 are induced through derepression rather than activation. This model is supported by additional data from Liu and Greene. They found that overexpression of a 'decoy' oligonucleotide containing multiple E2F binding sites was also effective at promoting death (Liu and Greene, 2001). The authors interpret this as support for a derepression mechanism since the oligo would sequester free and complexed E2F away from endogenous E2F target sites. Interestingly, this result also suggests that neuronal survival depends upon active repression of E2F genes.

Potential Relationship Between E2F1 and Cdk5

We reported in Chapter 3 that cdk5 performs a pro-death signal when regulated by p25 within the nucleus. The nuclear targets of cdk5 that mediate this death signal are not known with certainty but could include the transcription factors MEF2 and p53, which others have identified as cdk5 substrates and are also well known to be involved in death and survival responses (Zhang et al., 2002; Gong et al., 2003). The possibility remains that cdk5 regulates the activity of additional nuclear factors. Is it possible that cdk5 exploits the death inducing potential of E2F1? To date this has not been directly explored. However, E2F1 does contain at least one consensus cdk5 phospohorylation site. The preferred cdk5 target sequence is T/SPXK/R, where X is a neutral or basic residue (Shetty et al., 1993; Veeranna et al., 1998). Human E2F1 contains the amino acid sequence SPGK starting at position 307. This sequence is conserved in mouse, rat, and worm and is distinct from sites known to be phosphorylated by cyclin A/cdk2 complexes. Other phosphorylations in this region of E2F1 have not been reported, making it difficult to predict what the effect of phosphate addition at this site would be. E2F2 and E2F3 do not contain a homologous cdk5 consensus site. Whether or not this site is in fact phosphorylated by cdk5 needs to be tested. It is interesting to note that a requirement for both E2F1 and cdk5 has been demonstrated in models of stroke (MacManus et al., 2003; Wang et al., 2003), and in β-amyloid toxicity of cultured cortical neurons (Alvarez et al., 1999; Giovanni et al., 2000). It will be important not only to establish whether cdk5 actively phosphorylates E2F1, but also whether cdk5-mediated death occurs in the absence of E2F1. A simple experiment could be to compare the toxicity of p25 in cultures of wild type and E2F1 null neurons.

Is it possible that there exists a more general relationship between cell cycle mediated pathways and cdk5 in the context of neuronal death than that discussed above? Although very little investigation has been performed along these lines, there is some evidence that deregulation of cdk5 can occur upstream of cell cycle reactivation. In a mouse model of ALS both cdk5 and cdk4 activities are upregulated and mislocalized in affected spinal motor neurons (Nguyen et al., 2003). Phospho-pRb is detectable and can be immunoprecipitated with cdk4 but not with cdk5, consistent with cyclin D/cdk4-mediated phosphorylation of pRb (Nguyen et al., 2003). The activation of cdk4 is alleviated by the expression of neurofilament H, a proposed phospohorylation sink for cdk5 (Nguyen et al., 2001). This suggests that deregulated cdk5 activity in this model precedes cdk4 deregulation.

Other evidence suggests that cdk5 may in fact act in a similar manner to cell cycle CDKs in the inactivation of pRb. In *in vitro* assays cdk5/p25 can efficiently bind and phosphorylate pRb (Lee et al., 1997). Cdk5 can also be coprecipitated with pRb from embryonic mouse brain (Lee et al., 1997). Also, inducible expression of p25 in a neuronal cell line is followed quickly by the phospohorylation of pRb (Hamdane et al., 2005). Other later events downstream of p25 induction include a decrease in expression of p27 and increases in the levels of cyclin A, cyclin B, and cdk1 (Hamdane et al., 2005). All of these are

Significance and Contribution to the Field

Prior to the initiation of the studies presented in Chapter 2 there was no direct information on the involvement of E2F1 in neuronal death. It was known that E2F1

expression could, under certain circumstances, induce apoptosis of proliferating cells, but this was thought to occur purely as a failsafe mechanism to eliminate cells exposed to inappropriate, and potentially oncogenic, growth signals. Given the post-mitotic and highly differentiated nature of neurons there was little reason to expect the need for a similar mechanism. Nevertheless, existing data clearly showed that cell cycle regulators upstream of E2F1 (eg. Cyclin D and cdk4/6) were activated and even required for some forms of neuronal death. This data was suggestive of a role for E2F1 in the death of neurons but left many important questions unanswered. Among these was whether or not E2F1 expression could induce apoptosis in a neuronal environment, and whether or not endogenous E2F1 was involved in the stimulus induced death of neurons. Our work answered both of these questions in the affirmative and also began to address the mechanism by which E2F1 induces death in neurons by showing that it was dependent upon Bax, independent of p53, and associated with caspase activation. The fact that a number of groups repeated the observation of E2F1-induced death (Azuma-Hara et al., 1999; Hou et al., 2001b; Hou et al., 2001a; Kobayashi et al., 2002; Konishi and Bonni, 2003), and that the means by which E2F1 induces neuronal death was further investigated in several excellent reports (Hou et al., 2001a; Liu and Greene, 2001; Konishi and Bonni, 2003; Liu et al., 2004; Biswas et al., 2005), points towards the importance of our work. Moreover, subsequent findings that E2F1 is essential for ischemia induced neuronal death (Gendron et al., 2001; MacManus et al., 2003) highlight the clinical relevance of our findings.

Unlike our work on E2F1, our studies of cdk5 were preceded by numerous reports showing that it could indeed signal neuronal death. In the case of cdk5, however, there

was a lack of clarity in the field as to the types of death in which it plays a role, and in fact some suggestions that it could signal survival. And so what was needed in the cdk5 field was rationalization. Because most previous work had implicated cdk5 in death models with a strong calcium-signaling component but with weak or unknown reliance on conserved death pathways, we set out to test whether cdk5 participated in a neuronal death model induced by DNA damage through the intrinsic pathway. Our findings were surprising and led to several novel findings that greatly increase the understanding of cdk5 in neuronal death. We found first that p35 cleavage to p25 could occur as a late consequence of death rather than an early prelude. Perhaps most importantly, we showed that p25 formation could in fact be dependent on the intrinsic death pathway. Previous observations of p25 production were often interpreted as strongly implicating cdk5 in the death process. Our finding that p25 can be produced simply as a result of caspase activity, and that cdk5 inhibition is not protective in this case, necessitates new interpretations. Findings of increased p25 in human neuropathologies and animal models of disease may simply reflect a late effect of classical apoptosis, rather than direct involvement of cdk5 in the pathology.

The most controversial aspect of cdk5 research has been the reports that it functions in a pro-survival role (Li et al., 2002; Li et al., 2003). No previous studies had attempted to rationalize these findings with the much more prevalent literature describing a pro-death function. This is an imperative task, particularly in light of the fact that therapeutic agents designed to inhibit pathogenic cdk5 activity could have the unintended effect of further compromising neuronal survival. Clearly, a more complete understanding of cdk5's function in both survival and death was required. Our work was

the first to address simultaneously the potential dual functionality of cdk5 in death/survival signaling. We showed for the first time that the death inducing function of cdk5 occurs selectively within the nucleus, where it is regulated by p25. In contrast, a clear survival activity was observed for cdk5 selectively within the cytoplasm, where it is regulated by p35. These results suggest that cdk5 acts as either a death-inhibiting or death-promoting factor based on its localization. Localization itself appears to be determined by binding to either p35 or p25. Therefore, whether or not cdk5 acts to promote or suppress death is dependent on events upstream of p35 cleavage to p25. These upstream events include the previously characterized calcium activation of calpains, which appears to act early in response to certain stimuli, such as excitotoxicity, to produce p25 and commit cdk5 to an essential death promoting role. Our data identify caspases as another upstream mediator of p35 cleavage. This pathway occurs during death mediated by the intrinsic pathway and may be an attempt to eliminate pro-survival signaling.

The main rationale for investigating the molecular mechanisms of neuronal death is to gain a better understanding of the causes of neurodegeneration and to identify rational targets for therapeutic intervention. The results presented in Chapter 2 of this thesis identified E2F1 as a target of interest, and generated interest from other researchers to pursue this possibility further. When considered alongside observations of ectopic expression of E2F1 and other cell cycle proteins in neurodegenerative disorders and animal models of disease, these results support the concept of targeting E2F. The results presented in Chapter 3 provide a new framework for the design of cdk5 inhibition

strategies. Localization of cdk5 inhibition to the nucleus may provide more effective downregulation of cdk5 death signals while at the same time avoiding the disruption of pro-survival signals.

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Appendix A. Additional Co-Author Publications

PUBLICATIONS

Zhang Y, O'Hare MJ, Callaghan SM, Slack RS, Park DS. (2005) The Chk1/Cdc25A Pathway as Activators of the Cell Cycle in Neuronal Death Induced by DNA damage. In Revision *J. Neurosci*

O'Hare MJ, Kushwaha N, Zhang Y, Aleyasin H, Slack RS, Vincent I, Albert PR, Callaghan SM, Park DS. (2005) Differential Roles of Nuclear and Cytoplasmic Cdk5 in Apoptotic and Excitotoxic Neuronal Death. *J. Neurosci.* 2005 Sep 28;25(39):8954-66.

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CONFERENCE PRESENTATIONS

Smith PD, O'Hare MJ, Mount MP, Callaghan S, Anisman H, Slack RS, Vincent I, Park DS. (2004) Calpain-mediated deregulation of the Cdk5 activator p35 is associated with dopaminergic cell loss *in vivo.* Society for Neuroscience

O'Hare MJ, Rashidian J, Slack RS, Park DS. (2003) The role of cyclin-dependent kinases in stroke models of neuronal injury. Society for Neuroscience

O'Hare MJ, Slack RS, Park DS. (2002) Alterations in protein levels, kinase activity, and subcellular localization of p35/Cdk5 in response to DNA damage. Society for Neuroscience

O'Hare MJ, Aleyasin H, Slack RS, Park DS. (2001) Role of $NF_{kappa}B$ in neuronal cell death following camptothecin induced DNA damage. Society for Neuroscience

Keramaris E, O'Hare MJ, Slack RS, Park DS. (2001) ATM and calpains mediate p53 induction/activation in neuronal death evoked by DNA damage. Society for Neuroscience

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Sincerely,

Michael O'Hare

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