Investigation of the role of cyclin dependent kinases in neuronal apoptosis

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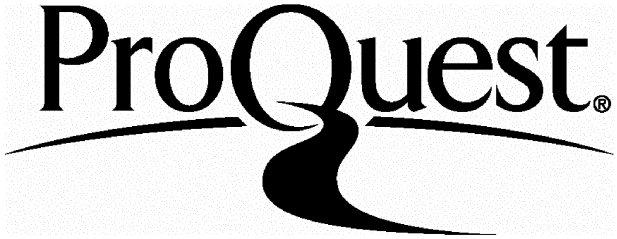

Abstract

Apoptosis and cell proliferation are fundamental processes that occur in all mammalian cells and there is evidence that there may be cross-talk between the two processes. Previous work has shown that activation of Cdk2 kinase is essential for apoptosis in resting thymocytes. The aim of this thesis was to determine whether cyclin dependent kinases (Cdks) can also regulate cell death in neuronal PC12 cells and sympathetic neurons.

In the presence of nerve growth factor (NGF) PC12 cells stop dividing and acquire a neuronal phenotype. The removal of NGF causes these differentiated cells to undergo apoptosis. An increase in Cdk2 kinase activity and cyclin E–associated kinase activity was observed following NGF withdrawal in PC12 cells. However, the early activation of Cdk2 can not be attributed to cyclin E. In contrast, Cdk5, Cdc2, Cdk4 or cyclin A-associated kinase activity did not change. Roscovitine, a pharmacological Cdk inhibitor that specifically inhibits Cdk2, Cdk5 and Cdc2, was able to block this increase in Cdk2 kinase activity and protected PC12 cells from NGF withdrawal-induced apoptosis. Therefore, the activation of Cdk2 by an unknown regulator and cyclin E appears to be required for apoptosis in PC12 cells.

Developing sympathetic neurons also die by apoptosis when deprived of NGF. An increase in Cdk2, Cdk5 and Cdc2 kinase activity was observed following NGF withdrawal. Cyclin A and cyclin E-associated kinase activity, and Cdk4 and cyclin D1-associated kinase activity did not change. Roscovitine was able to protect sympathetic neurons from NGF withdrawal-induced apoptosis. HD and NG-75, which are more potent pharmacological Cdk inhibitors, were also able to protect sympathetic neurons from apoptosis. Thus, Cdk2, Cdc2 and Cdk5 are activated during apoptosis in sympathetic neurons, and Cdk activation is upstream of mitochondrial cytochrome c release.

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My thesis is dedicated to my family, my mother and father, my sisters Gail and Margaret, my brothers Seán, Barry, Paul and Brian, my nieces Shannon, Daria, and RóiseMae, and my godsons Cormac and Conor.

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Abbreviations

AD	Alzheimer's disease
ASK	apoptosis signal-regulating kinase
ATP	adenosine 5′-triphosphate
BrdU	5-bromo-2′-deoxyuridine
CDC	cell division cycle
Cdk	cyclin depedent kinase
CKI	cyclin dependent kinase inhibitor
DN	dominant negative
ERK	extracellular signal-regulated kinase
HD	hymenialdisine
IC_{50}	inhibitory concentration of compound evoking 50% maximal effect
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
NGF	nerve growth factor
PCNA	proliferating cell nuclear antigen
SAPK	stress-activated kinase
SCG	superior cervical ganglia
TNFα	tumour necrosis factor α
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling

Chapter 1: Introduction

1.1 Apoptosis

1.1.1 History and definition of apoptosis

The phenomenon of physiological cell death has been discovered independently several times over the past 150 years (Kerr *et al.*, 1972; Sulston and Horvitz, 1977). The first account of naturally occurring developmental cell death was from Carl Vogt's study of dying notochordal and cartilaginous cells during amphibian metamorphosis in 1842. The next milestone was August Weissmann's detailed description in 1864 of massive cell death in pupating diptera. Spontaneous cell death as a physiological event was discussed as soon as tissue stains became available. "Chromatolysis" was the name Flemming gave to the formation of pyknotic chromatin as well as his observation of what now are called apoptotic bodies loose in the cavity of ovarian follicles in 1885. These morphological changes were also observed in other tissues such as breast cancer by Ströbe in 1892, so that by 1914 there was enough data available for Ludwig Gräper to publish a paper entitled "A new prospective on the physiological "switching-off" of cells". The early German literature, unfortunately, was inaccessible to the embryologists, such as Ernest, Fell and Glücksmann in the period between the two wars. It was not until 1951 when the importance of cell death during development was stated by Glücksmann (Clarke and Oppenheim, 1995). It was Kerr in 1971 who noted that cells in mildly hypoxic livers showed different characteristics to the previously reported structural changes associated with cell death. The cells had shrunken cell bodies, loss of contact with their neighbours, condensed chromatin, and were efficiently phagocytosed and degraded (Kerr, 1971). To stress the potential importance of this form of cell death as a reciprocal process to mitosis in regulating cell populations, the term "apoptosis" was invented, derived from the Greek meaning the naturally-occurring falling of leaves from trees, or petals from flowers (Kerr *et al.*, 1972).

It was only after the discovery that cell death in multicellular organisms is subject to genetic control and that abnormalities in cell death regulation can cause

diseases that the impact of physiological cell death on cell production and homeostasis was widely recognised (Ellis and Horvitz, 1986; Vaux *et al.*, 1988). Recent advances in apoptosis research include the discovery of death receptors and their ligands, definition of the distinct apoptosis signaling pathways, and classification of the components of the shared apoptotic effector machinery and their biochemical functions (these will be discussed in more detail below).

Historically, apoptosis was defined on a morphological basis to distinguish it from another type of cell death, necrosis (Kroemer *et al.*, 1995). Cells undergoing necrosis swell and their chromatin appears as fluorescent masses that eventually disappear to leave nuclear ghosts. DNA is non-specifically degraded and characteristically appears as a smear when size fractionated on an agarose gel. At the molecular level the process of apoptosis can be divided into three parts: initiation, execution, and termination of apoptosis. Apoptosis is initiated by a variety of extracellular and intracellular signals including cross-linking of death receptors (e.g. Fas and TNF receptors), ultraviolet (UV) and ionising radiation, anticancer drugs, growth factor deprivation, and overexpression of certain oncogenes or tumour suppressor genes. During the execution phase, chromatin condenses and forms aggregates at the periphery of the nucleus, which, in turn, becomes convoluted, whilst the nucleolus becomes enlarged and granular in appearance. DNA is cleaved, by different activated endonucleases, between histone octamers to generate a nucleosomal ladder of fragments. Cells shrink, adherent cells round up, dilation of the endoplasmic reticulum and blebbing of the plasma membrane occurs. In the termination phase, cells disintegrate into multiple membrane-enclosed vesicles, some of which contain nuclear fragments, that are phagocytosed without eliciting an inflammatory response (Wyllie, 1997).

Techniques have been developed to measure the molecular changes that occur during apoptosis, e.g. internucleosomal DNA cleavage and randomisation of the distribution of phosphatidyl serine (PS) between the inner and outer plasma membrane. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) at DNA breaks, and staining of cell surface-exposed PS with annexin V are a few of the standard tools used for measuring apoptosis (Gavrieli *et al.*, 1992).

1.1.2 Programmed cell death in the nematode worm

The nematode *Caenorhabditis elegans* has been a good model organism for studying programmed cell death at the cellular, genetic, and molecular levels. Each worm is born with 1090 cells, 131 of which die during development which leaves a final total of 959 in the adult. Initially *ced-3* and *ced-4* (*ce*ll *d*eath abnormal genes) were identified as being necessary for cell death. When these genes are inactivated by mutation none of the 131 normal cell deaths occurs (Ellis and Horvitz, 1986). On the other hand, *Ced-9* inhibits cell death because mutations that inactivate this gene lead to widespread and lethal embryonic cell death (Hengartner *et al.*, 1992). Finally, the *egl-1* gene (for *egg l*aying abnormal) was discovered because gain-of-function mutations in this gene resulted in the death of the two hermaphrodite-specific neurons that are required for egg laying. By contrast, a loss-of-function mutation prevented most if not all somatic programmed cell death (Conradt and Horvitz, 1998).

CED-3 is a cysteine protease that cleaves its substrate proteins after specific aspartic acid residues, and is a member of the caspase (**c**ysteine **asp**artyl prote**ase**) family (Alnemri *et al.*, 1996). CED-3 is synthesised as an inactive zymogen, which is activated through self-cleavage (Thornberry and Lazebnik, 1998). CED-4 is an adaptor protein that interacts with CED-3 and CED-9 (Chinnaiyan *et al.*, 1997). CED-9 and EGL-1 are members of the same family (the Bcl-2 family) but CED-9 is anti-apoptotic whereas EGL-1 is pro-apoptotic. When bound to CED-3, CED-4 promotes CED-3 activation, whereas CED-9 binds to CED-4 and prevents CED-3 activation (Chinnaiyan *et al.*, 1997). Under normal conditions, CED-9 is complexed with CED-4, thereby preventing activation of CED-3 and apoptosis. During apoptosis, EGL-1 binds to CED-9 which frees CED-4 which can then activate CED-3 and cause apoptosis.

In vertebrates entire gene families that resemble *C. elegans* cell death genes have evolved. The mammalian homologues of CED-3 are caspases (Thornberry and Lazebnik, 1998). The Apaf-1 (apoptotic protease activating factor) family are the mammalian CED-4 homologues (Zou *et al.*, 1997). The Bcl-2 family proteins are the mammalian homologues of CED-9 and EGL-1 (Yang and Korsmeyer, 1996).

1.1.3 Caspases

Caspases are a family of aspartate-specific cysteine proteases that are necessary for execution of apoptosis. Fourteen caspases have been identified in mammals so far. Caspases are produced as inactive zymogens that contain three domains: an NH_2-terminal pro-domain, a large subunit (~20 kDa), and a small subunit (~10 kDa). They are activated by proteolytic cleavage at conserved aspartate (Asp) residues. Because caspases cleave their substrates at Asp residues and are themselves activated by proteolytic processing at Asp residues, these proteases can collaborate in proteolytic cascades where caspases activate themselves and each other (Thornberry and Lazebnik, 1998).

Caspases are usually regarded as either upstream 'initiator' caspases (e.g. caspases-8, -9, and –10), or downstream 'effector' caspases (e.g. caspases-3, -6, -7, and –14). The zymogen forms of upstream initiator caspases have long N-terminal pro-domains that are important in protein-protein interactions, by associating with proteins that trigger caspase activation. These long pro-domains can be further classified into two types: the pro-domains of caspase-2, and -9 are termed caspase recruitment domains (CARD), whereas the pro-domains of procaspase-8 and –10 contain two tandem repeats of a motif known as the death effector domain (DED). Downstream effector caspases contain short N-terminal pro-domains and are largely dependent on upstream caspases for their proteolytic processing and activation (Thornberry and Lazebnik, 1998).

The principal mechanism for activating the caspase cascade involves the 'induced proximity or oligomerisation model' and illustrates how upstream initiator caspases are convened into action. This model is based on the observations that procaspases possess low but detectable activity and when brought into close contact through protein interactions the caspases become active. It has also been shown that procaspases that are overexpressed in cells and artificially cross-linked become active (Salvesen and Dixit, 1999).

Two major pathways that use the induced-proximity mechanism to activate caspases have been elucidated. One of these pathways is the tumour necrosis factor (TNF) receptor pathway (see death receptor pathway section). Binding of ligand to these receptors results in the recruitment of several intracellular proteins to the cytosolic

domains of the receptors, including procaspase-8, forming a death inducing signaling complex (DISC) that triggers activation of caspase-8 and the apoptosis signal is initiated (Medema *et al.*, 1997). Activation of the CARD containing procaspase-9 is mediated through interaction with cytochrome c and Apaf-1, which also contains a CARD, and causes the assembly of a multi-protein caspase-activating complex known as the 'apoptosome' (see mitochondria section). This oligomerisation of procaspase-9 in the apoptosome results in its autoproteolytic cleavage to generate active caspase-9, which is then capable of activating downstream caspases (Srinivasula *et al.*, 1998).

Activation of downstream effector caspases leads to proteolysis that is directly responsible for the characteristic morphological changes associated with apoptosis and ultimately cell death. Caspase-3, when activated is able to cleave the cytosolic inhibitor of apoptosis protein (IAP) allowing the release of active effector caspases (Slee *et al.*, 2001). Caspase-3 also cleaves Bcl-2 and Bcl-x_L which abolishes the anti-apoptotic capacity of these proteins and releases pro-apoptotic C-terminal fragments (Cheng *et al.*, 1997). Caspase-8 cleaves Bid, a pro-apoptotic Bcl-2 family member, generating truncated Bid that induces mitochondrial cytochrome c release (Li *et al.*, 1998; Luo *et al.*, 1998) (see Death receptor pathway section). Caspase-3 cleaves ICAD/DFF45, an inhibitor of the nuclease responsible for DNA fragmentation, CAD (caspase-activated deoxyribonuclease), allowing CAD to translocate to the nucleus and cut DNA (Liu *et al.*, 1997; Enari *et al.*, 1998). Caspase-3 is also responsible for cleaving and activating gelsolin, a protein that regulates actin dynamics and assists both cytoplasmic and nuclear apoptosis, including DNA fragmentation (Kothakota *et al.*, 1997). Caspase-6 cleaves lamins, which are structural proteins of the nuclear envelope, and may be partly responsible for the nuclear changes observed during apoptosis (Takahashi *et al.*, 1996). Cleavage of β-catenin has been implicated in promoting cellular packaging and phagocytosis (Brancolini *et al.*, 1997; Wen *et al.*, 1997). Poly (ADP-ribose) polymerase (PARP), is a key DNA double-strand break repair enzyme, cleavage of which may facilitate DNA degradation, a major characteristic of apoptosis (Casciola-Rosen *et al.*, 1996).

There have been reports of caspase-independent cell deaths. For instance, overexpression of Bax kills cells even in the presence of the broad-spectrum caspase

inhibitor zVAD-fmk. One of the functions of Bax is insertion into the mitochondrial membrane and induction of cytochrome-c release. Caspase inhibition may transiently protect cells from this cytochrome-c release however the energy depletion and build up of reactive oxygen species (ROS) will ultimately lead to apoptosis. Bax may also cause the release of endonuclease G from the mitochondria, this caspase-independent endonuclease may also contribute to the demise of the cell (Kaufmann and Hengartner, 2001). Recent findings show that a serine protease called Omi/HtrA2 is released from the mitochondria during apoptosis and inhibits the function of IAPs (see IAP section) by direct binding in a similar way to Smac (see mitochondria section). Moreover, extramitochondrially targeted overexpression of Omi induces atypical cell death, which is caspase-independent (Suzuki *et al.*, 2001).

1.1.4 The Bcl-2 protein family

The Bcl-2 family are a growing family of proteins that regulate apoptosis (Adams and Cory, 1998; Chao and Korsmeyer, 1998). This family can be divided into anti-apoptotic (Bcl-2, Bcl-x_L, Bcl-w, Bfl-1, Brag-1, Mcl-1, A1, E1B19K, LMW5-HL, and EBV BHRF1), and proapoptotic (Bax, Bak, Bcl-x_S, Bad, Bid, Bik, Blk, Hrk, Bim, and Bok) proteins. All members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4). Most anti-apoptotic members contain at least BH1 and BH2 domains along with the carboxy-terminal hydrophobic transmembrane region which are all important for functional activity. Bax, Bak and Bok contain BH1, BH2, and the BH3 domains, however Bik, Blk, Hrk, Bad, Bim, and Bid only possess the BH3 domain. The BH3 domain is essential for cytotoxicity activity in the pro-apoptotic family members. These conserved (BH1-4) domains mediate homo- and heterotypic dimer formation among Bcl-2 family members suggesting that these molecules function at least in part through protein-protein interactions (Adams and Cory, 1998; Chao and Korsmeyer, 1998).

Bcl-2 was the first member of this family to be identified due to its overexpression in follicular B cell lymphomas (Tsujimoto *et al.*, 1985). The overexpression of Bcl-2 prolonged cell survival when pro-B-cell lines and promyeloid cell lines where deprived of IL-3 (Chao and Korsmeyer, 1998). Bcl-2 was also able to

protect T cells against a variety of apoptotic signals, including glucocorticoids, γ-irradiation, phorbol esters, and ionomycin. Overexpression of Bcl-2 targeted to B cells or T cells in transgenic mice caused follicular hyperplasia and T cell lymphomas respectively.

Bcl-x_L is the closest mammalian relative of Bcl-2 and it too acts as a death inhibitor (Zornig *et al.*, 2001). Transgenic deregulation of Bcl-x_L in the lymphoid lineage has similar consequences to those of Bcl-2 overexpression. The similar properties of Bcl-2 and Bcl-x_L and their capacities to heterodimerise with proapoptotic Bcl-2 family members like Bak and Bax suggest that Bcl-x_L/Bcl-2 operate in a common pathway to inhibit apoptosis. Their different patterns of expression however indicate that their physiological roles are distinct, e.g. Bcl-x_L-deficient mice, unlike those lacking Bcl-2, exhibit embryonic lethality at around day E13 which is associated with massive apoptosis in the brain and hematopoietic tissues (Zornig *et al.*, 2001).

Bcl-2-associated protein X (Bax) was the first pro-apoptotic protein isolated by co-immunoprecipitation with Bcl-2 (Oltvai *et al.*, 1993). Observations that overexpression of the anti-apoptotic proteins, Bcl-2 and Bcl-x_L can inhibit the pro-apoptotic activity of Bax presented a model whereby the balance between the pro- and anti-apoptotic proteins control the fate of the cells (Korsmeyer *et al.*, 1993).

The BH3-only domain Bcl-2 family members, e.g. Bad, Bid, Bim, EGL-1, are able to bind to the pro-survival Bcl-2 family members and inhibit their function. Bad was originally identified by yeast 2-hybrid screening and shown to bind to Bcl-2/Bcl-x_L and promote apoptosis. Bad does not possess a C-terminal hydrophobic transmembrane region and is therefore probably not a membrane-associated protein. Its localisation appears to be actively controlled by its association/dissociation with other Bcl-2 proteins. Regulation of Bad function is associated with phosphorylation. In response to growth factors Bad is phosphorylated at Ser-112, Ser-136, and Ser-155, it is then sequestered by the cytosolic 14-3-3 protein and functionally inactivated (Antonsson, 2001). Bid, another BH3-only molecule, is regulated by proteolytic cleavage. Bid is cleaved by caspase-8 and the truncated form (tBid) translocates to the mitochondria where it activates Bax and Bak (Antonsson, 2001).

As is the case for the *C. elegans* CED genes there is biochemical evidence to suggest that the anti-apoptotic proteins of the Bcl-2 family, may function by directly inhibiting the ability of pro-apoptotic Bcl-2 family members, to activate caspases. Bcl-2/Bcl-x_L may inhibit activation of the initiator caspase-9 by Apaf-1. In this model the role of the pro-apoptotic Bcl-2 proteins would be to displace Bcl-2/Bcl-x_L from the Apaf-1/cytochrome-c/procaspase-9 complex and thereby trigger caspase-9 autoactivation (Chinnaiyan, 1999) (see section 1.1.6).

The possibility that Bcl-2 family proteins may act as membrane channels came from the observation that the 3-D structure of Bcl-x_L resembles the pore-forming domains of diptheria toxin and bacterial colicins (Muchmore *et al.*, 1996). Using lipid bilayers and liposomes both pro- and anti-apoptotic proteins were shown to possess channel-forming activity (Minn *et al.*, 1997), although at present there is no evidence to suggest *in vivo* ion channel formation. Bax has been shown to interact with the voltage dependent anion channel (VDAC) and the adenine nucleotide translocator (ANT) and cause cytochrome-c release (see section 1.1.6). It has also been suggested that after oligomerisation Bax is capable of forming pores that allow the release of cytochrome-c. This cytochrome-c release can be blocked by Bcl-2 and Bcl-x_L (Antonsson, 2001).

1.1.5 Inhibitors of Apoptosis (IAPs)

Members of the IAP family of proteins were originally described as viral products used by baculoviruses to inhibit insect cell apoptosis after viral infection (Crook *et al.*, 1993). Since then multiple human members of this family, including cIAP1, cIAP2, XIAP, NAIP, survivin, and livin have been identified (Miller, 1999). IAP's anti-apoptotic activity has been ascribed to their ability to bind and inhibit active caspases-3, -7, and –9. XIAP, cIAP1, and cIAP2 all block caspase-8 and caspase-9-initiated apoptotic events *in vitro* as well as directly inhibiting the activities of purified caspase-3, and -7 at high concentrations (Deveraux *et al.*, 1997; Roy *et al.*, 1997). A common feature of the anti-apoptotic IAPs is the presence of multiple baculovirus inhibitor repeat (BIR) domains, which chelate zinc to form a finger-like structure. Structural analysis of the binding between IAP's and caspases shows that BIR domains

bind to the caspase surfaces in such a way as to block caspase active sites (Chai *et al.*, 2001; Huang *et al.*, 2001).

It is important to note, however, that not all BIR-containing proteins are inhibitors of apoptosis. For example, survivin has been shown to play an important role in regulating mitosis rather than apoptosis (discussed in more detail below).

1.1.6 Role of mitochondria in apoptosis

The crucial role of mitochondria in mammalian cell apoptosis came to light when biochemical studies identified several mitochondrial proteins that are able to directly activate apoptotic pathways (Wang, 2001). Under normal circumstances, these proteins are present in the intermembrane space of mitochondria. In response to various apoptotic stimuli they are released into the cytosol and/or nucleus and promote apoptosis by activating caspases and nucleases or by blocking cytosolic inhibitors of apoptosis.

Cytochrome c, a component of the mitochondrial electron transfer chain initiates proteolytic caspase activation when released from the mitochondria during apoptosis (Liu *et al.*, 1996; Li *et al.*, 1997). In the cytosol, cytochrome c binds to Apaf-1, a cytosolic protein which contains a caspase recruitment domain (CARD), a nucleotide-binding domain, and multiple WD-40 repeats (Zou *et al.*, 1997). Binding of cytochrome c to Apaf-1, increases Apaf-1's affinity for dATP/ATP by 10-fold. The binding of dATP/ATP to the Apaf-1/cytochrome c complex results in its oligomerisation and the formation of the apoptosome, a multimeric Apaf-1/cytochrome c complex (Zou *et al.*, 1999). In the apoptosome the CARD domains of Apaf-1 become exposed and recruit multiple procaspase-9 molecules to the complex, which facilitates their autoactivation. Caspase-9 is then able to cleave and activate downstream caspases, which subsequently cleave many important intracellular substrates, leading to the characteristic morphological changes of apoptosis (see section 1.1.3). Loss of Apaf-1, caspase-9, or caspase-3 inhibits most of the neuronal apoptosis which occurs during normal development. The majority of caspase-9 knockout mice die perinatally with a greatly enlarged and malformed cerebrum caused by reduced apoptosis during brain development (Kuida *et al.*, 1998). Apaf-1 deficient mice display reduced apoptosis in the brain and noticeable craniofacial abnormalities with hyperproliferation of neuronal cells

(Yoshida *et al.*, 1998). Brain development in caspase-3 deficient mice is exceptionally affected, resulting in a variety of hyperplasias and disorganised cell deployment (Kuida *et al.*, 1996).

Smac/Diablo is another mitochondrial protein which is released into the cytosol during apoptosis (Du *et al.*, 2000; Verhagen *et al.*, 2000). Smac contains a mitochondrial targeting sequence at its N-terminus that is lost upon mitochondria entry. Removal of this sequence generates a new N-terminus in the Smac protein, Ala-Val-Prol-Ile (AVPI), which is able to bind the BIR (baculovirus IAP [inhibitor of apoptosis] repeat) domain of IAPs (see section 1.1.5). The BIR domain is also responsible for binding caspase-9 resulting in the inhibition of caspase-9 activity, which is alleviated when Smac competes off caspase-9 (Srinivasula *et al.*, 2000).

Apoptosis inducing factor (AIF) is a flavoprotein that resides in the mitochondrial intermembrane space. Upon induction of apoptosis, AIF translocates from the mitochondria to the nucleus and causes chromatin condensation and DNA fragmentation (Susin *et al.*, 1999).

The release of cytochrome c and other apoptogenic proteins from the mitochondria is known to be regulated by the Bcl-2 family of proteins. The pro-apoptotic members promote the release of apoptogenic factors whereas the anti-apoptotic members prevent this (see section 1.1.4).

Mitochondria are the bioenergetic and metabolic centres of eukaryotic cells. During apoptosis, mitochondria sustain specific damages that result in the loss of their function. Release of cytochrome c, a key component of the electron transfer chain, can potentially halt electron transfer resulting in the loss of mitochondrial membrane potential ($\Delta\psi_m$) and ATP synthesis. γ-irradiation induces apoptosis in thymocytes and disruption in the electron transport chain. Ligation of Fas also leads to a disruption in cytochrome c function in electron transport (Wang, 2001). A variety of stimuli that cause apoptosis lead to a loss in $\Delta\psi_m$, indicating the opening of a large conductance channel known as the mitochondrial permeability transition (PT) pore (Green and Reed, 1998). This consists of inner mitochondrial membrane proteins such as the adenine nucleotide translocator (ANT), and outer membrane proteins, such as the voltage dependent anion channel (VDAC). Opening of the non-selective ANT in the inner

membrane allows an equilibration of ions within the matrix and intermembrane space of the mitochondria, thus diffusing the H^+ gradient across the inner membrane and uncoupling the respiratory chains. PT pore opening also results in volume dysregulation. Water and solutes enter the matrix, causing matrix swelling and outer membrane disruptions, leading to the release of cytochrome c and other proteins.

Inhibitors of PT pore opening e.g. cyclosporins via cyclophilin D, appear to block apoptosis in some systems. It has been suggested that Bax causes a change in VDAC permeability to allow proteins such as cytochrome c to pass through (see section 1.1.4). Similarly, it has been reported that Bax and ANT may also form some kind of pore. The mitochondrial PT pore appears to be instrumental in controlling apoptosis. However, some studies have provided evidence that cytochrome c release and caspase activation is upstream of $\Delta\psi_m$ loss (Bossy-Wetzel *et al.*, 1998). The release of cytochrome c before, or in the absence of, a loss in $\Delta\psi_m$ in some cells suggests that different regulatory events control permeability of the inner and outer mitochondrial membranes.

1.1.7 Death receptor pathways

An additional signalling mechanism that actively directs cells to die by apoptosis has evolved in mammals and is particularly prevalent in the immune system. Death receptors are cell surface receptors that transmit apoptosis signals initiated by specific death ligands and play a pivotal role in this process.

Death receptors are a subfamily of the TNF/NGF receptor superfamily, which are characterised by similar cysteine-rich domains (CRDs) in their amino-terminal region. The death receptors have an intracellular death domain (DD), which is essential for inducing apoptosis. Members of the death receptor family include TNF-R1 (also called p55 or CD120a), Fas (CD95/APO-1), death receptor 3 (DR3; also called APO-3, LARD, TRAMP, WSL-1), DR4 (TRAIL-R1, APO-2), DR5 (TRAIL-R2, KILER, TRICK2) and DR6. Closely related to the death receptors are the decoy receptors, which function as inhibitors of this apoptotic signaling pathway. This subgroup includes decoy receptor (DcR) 1 (also called TRAIL-R3, LIT, TRID), DcR2 (TRAIL-R4. TRUNDD), osteoprotegerin (OPG) and DcR-3 (TR6) (Ashkenazi and Dixit, 1999).

The death receptors are activated by a group of structurally related ligands that belong to the TNF ligand family. These ligands include TNF, Fas ligand (FasL/CD95L), Apo2 ligand (Apo2L, also called TRAIL), Apo3L (TWEAK) and lymphotoxin-α (LT-α). FasL binds to Fas; TNF and LT-α bind to TNFR-1; Apo3L binds to DR3; and Apo2L binds to DR4 and DR5 (Ashkenazi and Dixit, 1998). Most of these ligands are synthesised as membrane-anchored trimers, and it seems that extensive receptor cross-linking is required for signalling (Schneider *et al.*, 1998). It has recently been reported that there is an extracellular region of TNF-R1 and Fas that mediates receptor self-association in the absence of ligands (Chan, 2000). TNF and FasL have been reported to be functional in soluble form (Krammer, 1999).

Of all the death receptor-ligand pairs, the best characterised and most widely studied is that of Fas-FasL (Figure 1.1). Ligation of Fas with FasL triggers the association of Fas monomers into trimeric Fas-complexes, which leads to the clustering of the receptor's DDs. Oligomerisation of Fas leads to recruitment of the serine phosphorylated Fas-adapter protein, FADD (Fas-associated death domain; also called Mort-1) (Martin *et al.*, 1998; Scaffidi *et al.*, 1999). Fas/FADD interaction is co-ordinated via conserved DDs found in both proteins. FADD is critical for Fas-mediated apoptosis, because expression of a dominant negative mutant of FADD completely abolishes Fas-induced apoptosis (Wajant *et al.*, 1998). At its N-terminus, FADD possesses a death effector domain (DED) that binds to a comparable domain in the N-terminus of procaspase-8 (Boldin *et al.*, 1996; Muzio *et al.*, 1996). The recruitment of procaspase-8 by FADD to the activated Fas receptor generates a death-inducing signalling complex (DISC) (Kischkel *et al.*, 1995). Procaspase-8 is autoproteolytically cleaved at the DISC (Medema *et al.*, 1997). Caspase-8 then activates downstream effector caspases including caspase-3, -6, -7, and –9, which commit the cell to apoptosis (see section 1.1.3).

Caspase-8 can also cleave the pro-apoptotic Bcl-2 homologue, Bid, forming an active truncated Bid (tBid) fragment that oligomerises with Bax and Bak in the outer mitochondrial membrane to initiate the mitochondrial death sequence. tBid induces release of cytochrome c from the mitochondria, and cytochrome c binds Apaf-1, the mammalian homologue of CED-3, thereby initiating apoptosis (see section 1.1.6).

FLIP (FLICE-inhibitory protein) has been shown to block Fas-mediated apoptosis. FLIP is another molecule with a DED, and is thought to compete with procaspase-8 at the DISC thereby inhibiting activation of caspase-8 (Sharma *et al.*, 2000).

Fas-mediated apoptosis may also be activated by the recruitment of molecules other than FADD. For example, RIP (receptor-interacting protein), and RAIDD (RIP-associated ICH/CED-3 homologous protein with a DD), and procaspase-2 is an alternative Fas-induced apoptotic signalling pathway (Sharma *et al.*, 2000).

p75, the low affinity neurotrophin receptor also has a recognised death domain, which has highly significant homology to the DDs of Fas and TNF receptors. NGF, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 can bind to p75 with approximately equivalent affinities and therefore p75 was originally reported to function as an accessory receptor modulating the survival signals through the Trk A receptor (Rodriguez-Tébar *et al.*, 1990, 1992; Ip *et al.*, 1993). However, evidence indicates that p75 signals on its own and in certain cellular contexts this signalling leads to apoptosis (Rabizadeh *et al.*, 1993). p75 has been shown to induce apoptosis of cultured neonatal sympathetic neurons, motor neurons, sensory neurons, oligodendrocytes, and in neurons when Trk activation is reduced or absent (Mamidipudi and Wooten, 2002). The apoptotic signalling mechanism of p75 is thought to be different to the Fas-induced pathway as the DD of p75 does not self-associate or bind to the death molecules such as FADD, TRADD, or RIP. (Nichols *et al.*, 1998). In addition the cytoplasmic domain of p75 and not the DD is required and sufficient to induce death signals (Kong *et al.*, 1999). One recently elucidated signalling mechanism of p75 involves JNK-p53-Bax, which are activated in sympathetic neurons by p75 activation (Aloyz *et al.*, 1998).

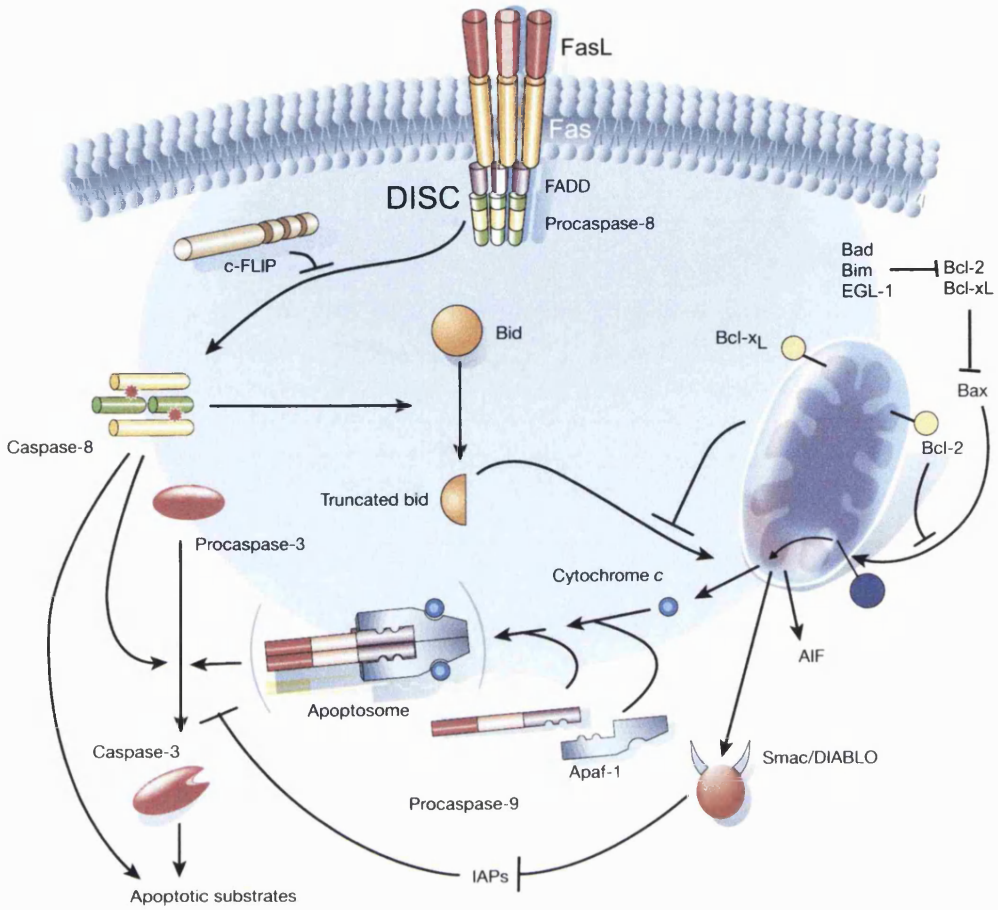


Figure 1.1 Two major apoptotic pathways in mammalian cells.

The death receptor pathway is triggered by members of the death receptor family (e.g. TNF and Fas receptors). Binding of FasL to the Fas receptor induces receptor clustering and formation of a death-inducing signalling complex (DISC). The adaptor molecule FADD, recruits multiple procaspase-8 molecules, resulting in caspase-8 activation through induced proximity. Caspase-8 activation can be blocked by recruitment of c-FLIP. The mitochondrial pathway is triggered by release of cytochrome c and induced by various pore-forming pro-apoptotic Bcl-2 family proteins such as Bax. In the cytosol, cytochrome c binds and activates Apaf-1, allowing it to bind and activate procaspase-9. This pathway is suppressed by anti-apoptotic Bcl-2 family proteins, which prevent cytochrome c release. The anti-apoptotic Bcl-2 family proteins, in turn, are suppressed by BH3-only proteins such as Bad. Active caspase-9 and caspase-8 cleave and activate effector caspases, e.g. caspase-3, which then cleave apoptotic substrates. Active caspases can be directly inhibited by IAP-family proteins, which themselves are antagonised by Smac/Diablo, which is released from the mitochondria. Cross-talk between the death receptor and mitochondrial pathways is provided by Bid. Caspase-8-mediated cleavage of Bid greatly increases the pro-apoptotic activity, and results in its translocation to the mitochondria, where it promotes cytochrome c exit. Figure adapted from Hengartner, 2000.

1.1.8 Physiological apoptosis

Apoptosis plays an important role in many physiological processes, especially in development and in the immune system (Krammer, 1999; Vaux and Korsmeyer, 1999). The best studied model of physiological cell death in development is the nematode *C. elegans* (see section 1.1.2). During metamorphosis of *Drosophila,* larval organs such as the salivary glands and gut undergo apoptosis. Mutations of the *rpr, hid,* and *grim* genes prevent apoptosis and cause severe developmental defects and malformations in the fly (White *et al.*, 1994).

Physiological apoptosis is critical for the normal development and function of multicellular organisms. It is involved in the formation of tubes, the sculpting of digits, the reconstruction of bone, and involution of mammary glands (Vaux and Korsmeyer, 1999). During the development of the vertebrate nervous system, both neurons and oligodendrocytes are produced in excess and approximately half are eliminated by apoptosis (Oppenheim, 1991). In the immune system, apoptosis occurs during T and B lymphocyte selection (Green and Scott, 1994) and cytolytic T-cell-mediated killing (Matiba *et al.*, 1997). Apoptosis also plays an important role in the removal of excess T- and B-cells thereby maintaining cellular homeostasis during immune responses (Scott *et al.*, 1996). In addition, mammalian cells can initiate apoptosis if the cell is unable to repair defects such as DNA damage (Clarke *et al.*, 1993).

1.1.9 Pathological apoptosis

There is substantial evidence to suggest that alterations in control of cell death and survival contribute to the pathogenesis of many human diseases. Generally, there are diseases with too little apoptosis, for example cancer, autoimmune disorders, and viral infections, or with too much apoptosis, such as AIDS, neurodegenerative disorders, toxin-induced liver disease, and some autoimmune diseases.

It has become clear that as well as deregulated growth, inhibition of apoptosis plays a pivotal role in tumorigenesis. The proto-oncogene *bcl-2*, an inhibitor of apoptosis, was first discovered as the target gene present at the translocation breakpoint in the tumour cells of approximately 80% of patients with human follicular B-cell lymphoma (Strasser *et al.*, 1997). The p53 tumour suppressor gene is functionally

inactivated in 70% of human tumours (Evan *et al.*, 1995). Frameshift mutations in the pro-apoptotic *bax* gene have been detected in human colon carcinomas (Rampino *et al.*, 1997). Relapse in childhood acute lymphoblastic leukaemia (ALL) is associated with a decrease in the Bax/Bcl-2 ratio, and with loss of spontaneous caspase-3 processing *in vivo* (Prokop *et al.*, 2000). In human non-Hodgkins lymphoma 11% of cases studied showed Fas mutations indicating a link between Fas receptor mutation, cancer, and autoimmunity (Gronbaek *et al.*, 1998). Missense mutations in the death domain of the Fas receptor were detected in 11% of gastric cancer cases (Park *et al.*, 2001). Certain tumours may also escape Fas ligand-dependent immune-cytotoxicity attack by expressing a decoy receptor e.g. DcR3, that Fas ligand binds to and thus loses its killing ability (Pitti *et al.*, 1998). The DcR3 gene is amplified and expressed in about 50% of primary lung and colon tumours studied (Ohshima *et al.*, 2000). The anti-apoptotic protein c-FLIP inhibits death receptor-induced apoptosis and has been reported to be over-expressed in human melanomas (Medema *et al.*, 1999). Caspase 8, an activator of apoptosis, is frequently inactivated in neuroblastomas, (a childhood tumour of the peripheral nervous system) with MycN amplification (Teitz *et al.*, 2000). The inhibitor of apoptosis (IAP) family member survivin, is undetectable in terminally differentiated adult tissues, but is highly expressed in most human cancers of lung, colon, pancreas, prostate, and breast (Ambrosini *et al.*, 1997). Survivin is also found in approximately 50% of high-grade non-Hodgkins lymphomas (Ambrosini *et al.*, 1997) and high survivin expression is associated with poor prognosis in human neuroblastomas (Islam *et al.*, 2000).

The autoimmune lymphoproliferative syndrome (ALPS) is characterised by mutations in Fas, FasL, and caspase-10, which impair deletion of T lymphocytes by apoptosis (Jackson *et al.*, 1999). Apoptosis is used as a defence mechanism against viruses and other intracellular pathogens. Expression of adenovirus protein E1A promotes viral replication but also promotes host cell apoptosis via p53. The adenovirus proteins E1B55kD, which directly interferes with p53 function, and E1B19kD, a Bcl-2 homologue, inhibit this apoptotic pathway (Debbas and White, 1993).

T cells from HIV-infected patients exhibit both increased Fas receptor expression and enhanced susceptibility to Fas-mediated death (Debatin *et al.*, 1994; Katsikis *et al.*,

1995). Fas ligand is elevated in the peripheral blood mononuclear cells from HIV-infected patients (Mitra *et al.*, 1996) and the plasma level of soluble Fas L is increased in HIV-positive patients (Hosaka *et al.*, 1998). The β-amyloid protein, which builds up in the brain during Alzheimer's disease, causes cultured neurons to die by apoptosis (Cotman and Anderson, 1995). Apoptotic cell death has been found in neurons and glial cells in Alzheimer's disease. Taken together these results indicate that abnormalities in apoptosis can play a major role in disease processes.

1.2 Cell Cycle

1.2.1 History and definition of the cell cycle

The cell cycle is a series of events through which all cells must traverse in order to divide (Sherr, 1996). The fundamental task of the cell cycle is to ensure that DNA is faithfully replicated once during S phase and that identical chromosomal copies are distributed equally to two daughter cells during M phase. The fission yeast *Schizosaccharomyces pombe* has been used for many years as an excellent model system for investigation of the fundamental regulation and control mechanisms of the eukaryotic cell cycle. *S. pombe* is an ideal system for genetically investigating the cell cycle because it is easy to isolate mutants altered in cell cycle progression which define CDC (cell division cycle) genes. As in the higher eukaryotes, the fission yeast cell cycle can be divided into discrete phases. G_0 is the quiescent state in which cells are metabolically active but do not undergo cell division. Upon stimulation with growth factors, cells leave G_0 and enter the first gap phase called G_1, during which they prepare to replicate their DNA (see Figure 1.2). It is during the G_1 phase that the cell integrates mitogenic and growth inhibitory signals and makes the decision to proceed, pause or exit the cell cycle. At a point late in G_1, cells make an irreversible commitment to divide; this is called the restriction point (START in yeast) (Hartwell *et al.*, 1974; Pardee, 1974; Nurse, 1975). Beyond the restriction point, cells enter S-phase, where DNA synthesis occurs. Following DNA replication, cells enter a second gap phase called G_2, during which the cell prepares for the process of division. During this phase, cells survey the newly synthesised DNA for errors and verify that DNA replication has proceeded to completion. Cell division, or mitosis, ultimately occurs during M-phase, the phase in which the replicated chromosomes are segregated into separate nuclei and cytokinesis occurs to form two daughter cells. The checkpoints of the cell cycle ensure that critical events in a particular phase are completed before the next phase can be initiated, thereby preventing the formation of abnormal cells. Thus it is the cell cycle checkpoints that serve as molecular switches at which cells must determine whether to complete cell

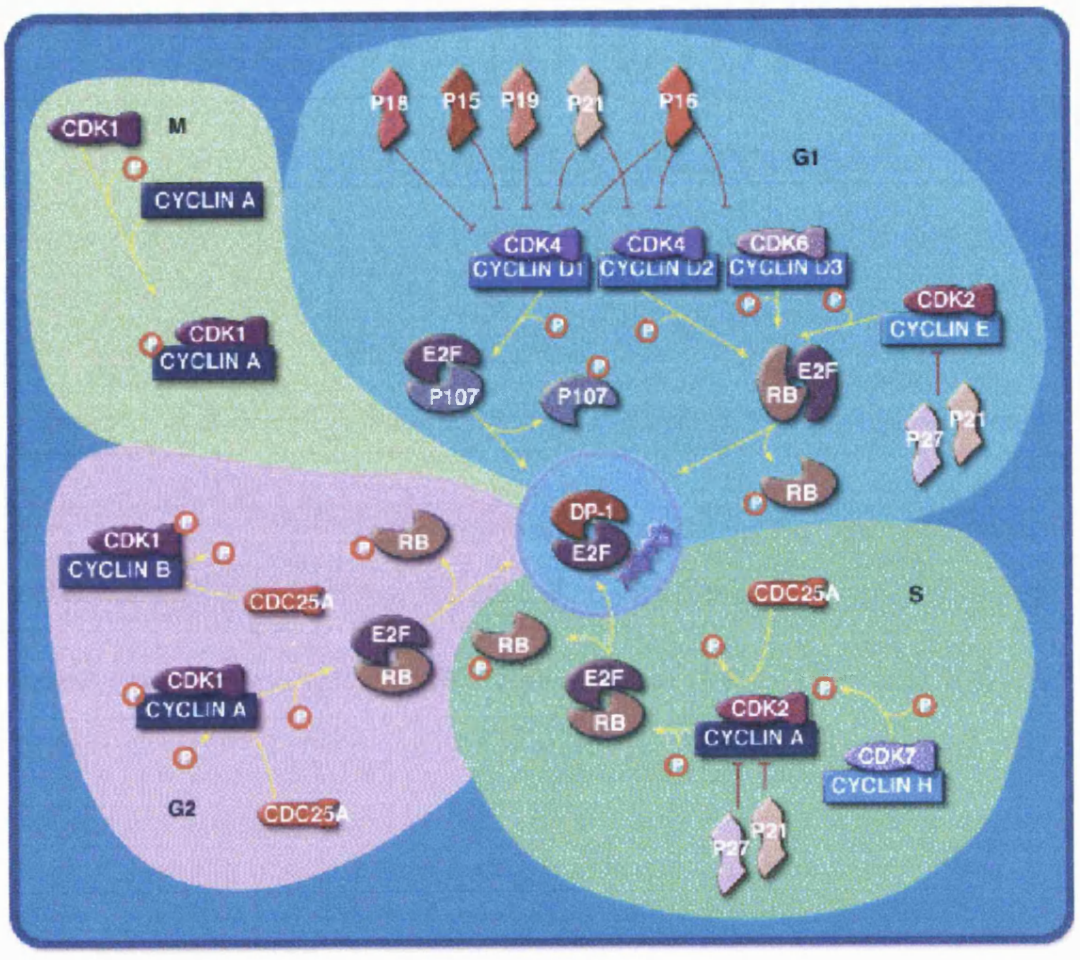


Figure 1.2 Schematic diagram of the cell cycle

The cell cycle is divided into different stages: 1) G_1 phase where cells prepare to synthesise DNA, 2) S phase where DNA synthesis occurs, 3) G_2 phase when cells prepare for mitosis and 4) M phase where mitosis occurs. The cell cycle is regulated by the interplay of many molecules. Key among these are the cyclins, which are expressed and then, degraded in a concerted fashion to drive the stages of the cell cycle (discussed in more detail in the text). Cyclins bind to Cdks to form activated kinases that phosphorylate proteins required for cell cycle progression. (diagram from www.biocarta.com)

division, arrest growth to repair cellular damage, or undergo apoptosis if the damage is too severe to be repaired or if the cell is incapable of repairing the DNA (Lundberg and Weinberg, 1999).

In fission yeast, Cdc2 is the only Cdk directly involved in cell cycle regulation (Moser and Russell, 2000). During the cell cycle Cdc2 levels remain constant whereas cyclin levels oscillate. Cdc2 activity is regulated through its association with four different cyclins, Cig1, Cig2, Puc1, Cdc13. Accumulation of the Cdc2 inhibitor, Rum1 ensures that Cdc2 activity is kept low throughout G_1 phase. Late in G_1 phase, active Cig1- and Puc1-Cdc2 complexes are formed and these phosphorylate Rum1, which targets it for degradation. In the absence Rum1, Cdc2-Cig2 activity increases and induces entry into S phase. Cdc13 levels rise during S phase and Cdc13 associates with Cdc2 however, due to the inhibitory phosphorylation of this complex mediated by Mik1 and Wee1 it remains inactive. Activation of the Cdc2-Cdc13 complex occurs late in G_2 when the phosphatase dephosphorylates the complex thereby driving cells into M phase. Cdc2 activity declines after cells exit mitosis due to degradation of Cdc13 and accumulation of Rum1.

Much that has been ascertained from the fission yeast *S.pombe* can also be applied to Metazoa including human cells. For example the human *cdc2* gene can completely substitute for the fission yeast *cdc2* gene because the two genes are so similar (Lee and Nurse, 1987). This indicates that basic aspects of cell cycle control are highly conserved from yeast to humans and by studying these controls in more convenient model systems it is possible to extrapolate to analogous controls in human cells.

1.2.2 Cyclin dependent kinases (Cdks) and Cdk activation

In higher eukaryotes, the orderly progression of the cell cycle is driven by sequential activation of cyclin dependent kinases (Cdks) to pass the checkpoints (Pardee, 1974; Pardee, 1989; Murray, 1994), primarily during the G_1/S and G_2/M transitions of the cell cycle. Cdks allow progression through the different phases of the cell cycle by phosphorylating critical serines and threonines on their target substrates. This family of kinases requires association with a cyclin regulator subunit for activity (Solomon *et al.*,

1990). Different Cdk/cyclin complexes are active during each phase of the cell cycle. To date, at least nine Cdks and more than twelve different cyclin members have been described. Cdks are closely related in size (35-40 kD), and sequence (40-75% identity among Cdk1 through to Cdk7) (Meyerson *et al.*, 1992). The structures of Cdks consist of a small N-terminal lobe, and a larger C-terminal lobe, with an ATP binding site in the cleft between the two lobes (De Bondt *et al.*, 1993) (Figure 1.3). Regions conserved between Cdks include the glycine-rich loop of the ATP binding site and the highly conserved PSTAIRE motif. The typical Cdk catalytic subunit contains a 300 amino acid catalytic core that is completely inactive when monomeric and unphosphorylated (De Bondt *et al.*, 1993) (Figure 1.3). In its inactive form the core of the protein substrate binding cleft is almost completely blocked by a large loop (the 'T-loop', containing the Thr160 phosphorylation site). This loop has several residues that block access to the γ phosphate of ATP, as well as acting as an auto-inhibitor of substrate binding. Thr14 and Tyr15 are phospho-acceptor sites within the glycine-rich loop that serves as a phosphate anchor in ATP binding. Phosphorylation at one or both of these sites inhibits kinase activity by interfering with substrate binding, or by indirectly affecting ATP orientation by changing the conformation of the glycine-rich loop. Wee1 and Myt1 have been identified as the kinases responsible for the phosphorylation of these inhibitory sites on Cdk2 and Cdc2 (Parker and Piwnica-Worms, 1992; Mueller *et al.*, 1995). The inhibitory phosphorylations of Cdks are removed by the action of the Cdc25 family of protein phosphatases (Sebastian *et al.*, 1993). Interestingly, the Cdc25 proteins are themselves substrates of Cdk/cyclins, and their phosphorylation stimulates the phosphatase activity (Hoffmann *et al.*, 1994). The extent of inhibitory phosphorylation is regulated by the opposing activities of Wee1 and Myt1 kinases and Cdc25 phosphatases (De Bondt *et al.*, 1993).

The mechanism of Cdk activation has been revealed by the structure of Cdk-cyclin complexes (Figure 1.3). The association of a Cdk with its corresponding cyclin produces a partially active complex (Jeffrey *et al.*, 1995), full activity being achieved after phosphorylation of the Cdk on a conserved threonine residue (Thr 172 in Cdk4/6, Thr 160 in Cdk2, and Thr 161 in Cdc2) (Gu and Du, 1992; Gu *et al.*, 1992; Solomon *et al.*, 1992)

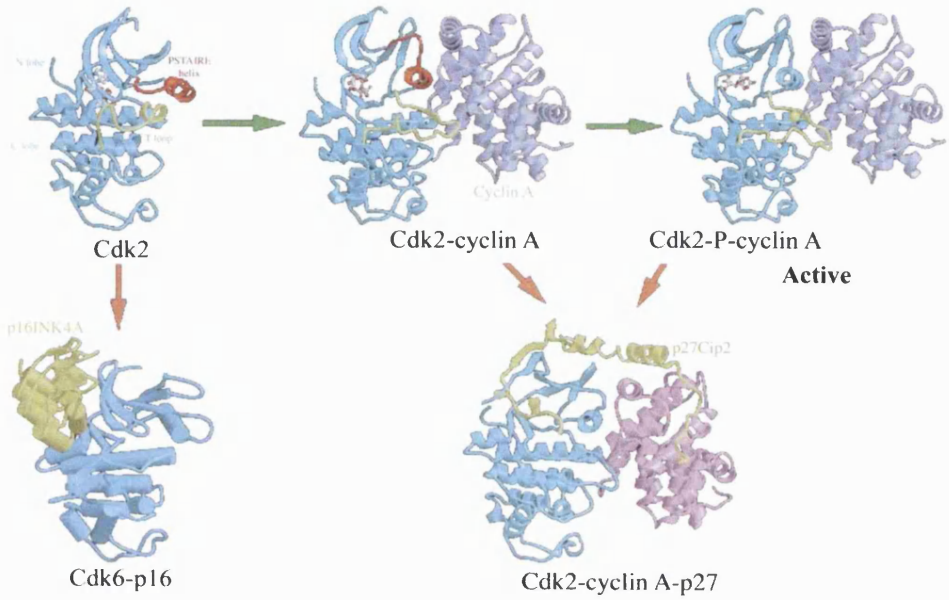


Figure 1.3 Cyclin dependent kinase regulation.

Cdk structures corresponding to non-activated (monomeric Cdk2), partially active (Cdk2-cyclin A complex), inhibited (p27-Cdk2-cyclin A complex), and non-activable (p16-Cdk6 complex) states. In the monomeric Cdk2 and cyclin A-bound Cdk2 structures, the PSTAIRE helix is in red and the T loop in green. In the phosphorylated Cdk2-cyclin A complex, the phosphate group is indicated by a green sphere. ATP shown in ball-and-stick representation. Figure adapted from Pavletich 1999.

Cdk activating kinase (CAK), the kinase responsible for phosphorylating the conserved threonine, is itself a complex of a Cdk subunit, Cdk7, and a cyclin-like subunit, cyclin H. CAK activity appears to remain constant during the cell cycle, but is induced in some cell types during the transition from G_0 into S (Kaldis, 1999).

Cyclins vary in relative molecular mass from 30-45 kD and share a homologous region of about 100 amino acids (30-50% identity among cyclins A, B, D1, and E) termed the cyclin box (Kobayashi *et al.*, 1992). Cyclin A binds to one side of the catalytic cleft, interacting with both lobes of Cdk2 to form a large, continuous protein-protein interface (Jeffrey *et al.*, 1995). The cyclin box (residues 209-310), is the key element at the cyclin-Cdk2 interface, forming the binding site for the PSTAIRE helix and making contact with the T-loop of Cdk2 (Jeffrey *et al.*, 1995). The exact sequence in the PSTAIRE region is specific to the cyclin preference of Cdks, and mutations there eliminate cyclin binding (Kobayashi *et al.*, 1992). Following cyclin A binding, extensive hydrogen bonding induces a conformational change in the T-loop immediately preceding the PSTAIRE helix. This is associated with a translation of the helix into the catalytic cleft towards ATP. The significance of this movement is that it brings the side chain of Glu 51, which belongs to a triad of catalytic site residues conserved in all eukaryotic kinases, into the catalytic site. This triad (Lys 33, Glu 51, and Asp 145) is involved in ATP catalysis. This movement also directs the T-loop away from the entrance of the catalytic cleft and exposes the Thr 160 hydroxyl group so it can be phosphorylated by CAK (Jeffrey *et al.*, 1995) (Figure 1.3). Substrates containing the ZRXL motif (where Z and X are usually basic) bind to a hydrophobic patch on the surface of cyclin A, which contains a MRAIL sequence conserved among a number of cyclins (Schulman *et al.*, 1998).

Critical Cdk/cyclin complexes for cell cycle function are Cdk4/cyclinD, Cdk2/cyclin E, Cdk2/cyclin A, and Cdc2/cyclin B. The kinase activity of these complexes is dependent on the presence of the cyclins, whose abundance varies substantially during the cell cycle.

1.2.3 G_1/S phase progression

During the G_1 phase, cells respond to extracellular signals by either advancing toward another division or withdrawing from the cell cycle into a resting state (G_0) (Pardee, 1989; Sherr, 1994). The D-type cyclins are the first to be induced as cells enter the cell cycle. Synthesis of D-type cyclins in early G_1 phase is under growth factor control, and the cyclin D promoter contains several regulatory elements that respond to growth factor signalling (Hinz *et al.*, 1999). The activation of cyclin D1 gene transcription is dependent on the activation of the Ras-Raf-MAPK pathway (Albanese *et al.*, 1995; Lavoie *et al.*, 1996). Cdk4 and Cdk6 are the major catalytic partners of D-type cyclins, associating with them and forming active complexes that have a distinct substrate preference for the retinoblastoma protein (pRb) (Ewen *et al.*, 1993) (Figure 1.2).

The pRb plays a critical role in regulating G_1 progression and is a key component of the molecular network controlling the restriction point. Hypophosphorylated pRb and the other Rb-like proteins p107 and p130, block cell cycle progression by binding to E2F transcription factors and repressing the E2F-responsive genes that contribute to S phase entry (Weintraub *et al.*, 1992; Zhu *et al.*, 1993; Vairo *et al.*, 1995). Phosphorylation of pRb by D-type cyclin kinases leads to the release of bound E2F proteins and allows them to act as activators of transcription (Ewen *et al.*, 1993) (Figure 1.2). The E2F transcription factor consists of a heterodimer between E2F and DP family proteins (Helin *et al.*, 1993) (Figure 1.2). Six members of the E2F family (E2F-1 to E2F-6) and three members of the DP family (DP1, 2, and 3) have been identified to date. E2F factors activate the transcription of genes involved in the cell cycle and DNA synthesis, including cyclin E and A, Cdc2, B-myb, dihydrofolate reductase, thymidine kinase, and DNA polymerase α (Helin, 1998).

Through the activation of E2F, cyclin E is the next cyclin to be expressed in mid to late G1 phase (Geng *et al.*, 1996) (Figure 1.2). Cyclin E associates with Cdk2, and this kinase complex is required for cells to make the transition from G_1 into S phase (Ohtsubo *et al.*, 1995). Cdk2/cyclin E phosphorylates the phosphatase Cdc25A at the G_1/S transition which is required for further Cdk2/cyclin E activation, thus creating a positive feedback loop (Hoffmann *et al.*, 1994). The Cdk2/cyclin E complex drives

another positive feedback loop through further phosphorylation of Rb and release of additional E2F activity (Johnson *et al.*, 1994). This feedback loop is further enhanced by the increased expression of the *E2F-1, E2F-2,* and *E2F-3* genes which are themselves targets of E2F transactivation (Wu *et al.*, 2001). As well as the irreversible commitment to enter S phase, Rb inactivation shifts from being mitogen-dependent (cyclin D-driven) to mitogen-independent (cyclin E-driven). Cyclin D1 degradation occurs after phosphorylation of Thr 286 by glycogen synthase kinase 3β (GSK-3β) which targets the cyclin to the proteosome (Diehl *et al.*, 1998). Degradation of cyclin D1 can occur when it is complexed with Cdk4/6, or present as a free molecule (Diehl *et al.*, 1997; Germain *et al.*, 2000). Cdk2/cyclin E has been shown to phosphorylate S phase-specific substrates, for example NPAT (nuclear protein in the ataxia telangiectasia region), which is involved in the activation of histone gene transcription (Zhao *et al.*, 2000a). Like many other Cdk/cyclin complexes, Cdk2/cyclin E phosphorylates histone H1, and this activity may be important for the chromatin rearrangement required during the replication of the genome (Herrera *et al.*, 1996). Cdk2/cyclin E also phosphorylates the Cdk inhibitor p27 (see section 1.2.5) and cyclin E, which promotes the degradation of both of these proteins (Won and Reed, 1996; Vlach *et al.*, 1997).

Cyclin A, which is also regulated in part by E2F (Schulze *et al.*, 1995), is expressed soon after cyclin E at the G_1/S boundary and also forms complexes with Cdk2 and, to a lesser extent, with Cdc2 (Tsai *et al.*, 1991) (Figure 1.2). Cdk2/cyclin A activity is required for S phase transition and control of DNA replication (Tsai *et al.*, 1993a). Cdc25A is active from the G_1/S boundary to mitosis, and is responsible for the activation of Cdk2/cyclin A (Hoffmann *et al.*, 1994) (Figure 1.2). A known substrate for Cdk2/cyclin A is Cdc6, phosphorylation of which leads to its export from the nucleus. Cdc6 is required for the initiation of DNA replication, phosphorylation by Cdk2/cyclin A has been implicated in preventing re-replication of DNA. HIRA, the human homologue of the yeast repressors of histone gene transcription Hir1p and Hir2p, is another Cdk2/cyclin A substrate. Phosphorylation of HIRA abolishes its repressor activity and hence increases histone transcription. Cdk2/cyclin A also phosphorylates Skp2 and Cdc20, two components of proteolytic pathways involved in cell cycle progression (Obaya and Sedivy, 2002).

1.2.4 G_2/M phase regulation

After the completion of DNA replication, cells enter the G_2 phase of the cell cycle with a 4N DNA content and begin preparations for mitosis and cytokinesis. Cdc2 regulates the passage of cells through these later phases of the cell cycle in association with both cyclin A and cyclin B (Pines and Hunter, 1989; Nurse, 1990) (Figure 1.2). Cdc2 requires cyclin binding and phosphorylation of Thr 161 for complete activation (Lorca *et al.*, 1992). Cdc2 activity can be negatively regulated by phosphorylation on Thr 14 and Tyr 15 (Norbury *et al.*, 1991). This negative control mechanism provides a checkpoint control pathway whereby a halt in the cell cycle occurs if DNA replication is incomplete, DNA is damaged, or spindle assembly has not occurred correctly. Cdc25B dephosphorylates Cdc2/cyclin B1 in the cytoplasm prior to its transport to the nucleus (Norbury *et al.*, 1991). Cdc25C further regulates entry into mitosis by activating nuclear Cdc2/cyclin B1 (Hoffmann *et al.*, 1993).

Targets of Cdc2/cyclin B1 include structural proteins involved in the initiation of mitotic events, and regulatory proteins that are important in the control and timing of this process. Nuclear lamins, nucleolar proteins (nucleolin and NO38), microtubule-associated protein-4 (MAP-4), proteins of the nuclear pore complex, centrosomal proteins, and Eg5 (a kinesin-related motor) have all been described as Cdc2/cyclin B substrates (Obaya and Sedivy, 2002). Cdc2/cyclin B1 participates in the inhibition of transcription and translation that occurs in mitosis by phosphorylating the TFIIH subunit of RNA polymerase II and the ribosomal S6 protein kinase. Cyclin B1-associated kinase activity blocks DNA replication by phosphorylating minichromosome maintenance protein-4 (MCM4) and preventing its interaction with DNA. Cyclin B2 is thought to play a part in the segregation of organelles during cytokinesis by phosphorylating targets such as the matrix protein GM130 (Obaya and Sedivy, 2002)

For cells to exit mitosis, cyclin A and cyclin B must be degraded. The APC/cyclosome is activated by the phosphorylation of its Cdc20 subunit by Cdc2 and is involved in the eventual proteolytic degradation of cyclin B1 and cyclin A (Stewart *et al.*, 1994; Kotani *et al.*, 1999). After mitosis, cells again enter G_1 and, at the restriction point, must decide whether to proceed into another cell cycle.

1.2.5 Regulation of Cdk/cyclin complexes by Cdk inhibitors

An additional mechanism for regulating Cdk/cyclin activity is the interaction with Cdk inhibitors (CKIs). Two major classes of CKIs have been identified: the Ink4 family (p16, p15, p18, and p19) and the Cip/Kip family (p21, p27, and p57) (Sherr and Roberts, 1995; Sherr and Roberts, 1999) (Figure 1.2).

The Ink4 CKIs are composed of multiple ankyrin repeats and were initially found to bind monomeric Cdk4 or Cdk6 (Serrano *et al.*, 1993; Hannon and Beach, 1994) (Figure 1.2). The site on Cdk4 required for CKI binding overlaps with the cyclin binding site, and Ink4 CKIs have been shown to block the formation of Cdk4/cyclin D complexes (Coleman *et al.*, 1997). However, ternary Ink4/Cdk/cyclin D complexes have been detected when p15 is expressed at high levels (Reynisdottir and Massague, 1997). The signals that lead to the synthesis of Ink4 proteins remain poorly understood, however it is clear that p15 is induced by TGF-β and contributes to its ability to induce G_1 phase arrest (Hannon and Beach, 1994). p16 is upregulated during senescence (Alcorta *et al.*, 1996), whereas p18 and p19 are expressed during fetal development and may be involved in terminal differentiation (Zindy *et al.*, 1997). Overexpression of Ink4 proteins can arrest the cell cycle in the G_1 phase and this process is dependent on the status of Rb (Guan *et al.*, 1994). An obvious explanation for this block in the cell cycle would be that Rb phosphorylation is prevented by Ink4-mediated inhibition of Cdk4 kinase activity, thereby maintaining Rb in a hypophosphorylated state. However, cell cycle arrest caused by the expression of the Ink4 CKIs has been reported to require the presence of at least one Cip/Kip CKI to inhibit Cdk2 activity (McConnell *et al.*, 1999). The necessity to inhibit Cdk2 also illustrates why p16 can impose a sustained G1 arrest, whereas the overexpression of a constitutively active Rb cannot (Lukas *et al.*, 1999). Another function attributed to Cdk4-6/cyclin D complexes during the G1 phase of the cell cycle is the sequestration of p27 from Cdk2/cyclin E complexes, allowing Cdk2 to be activated. Cdk4/6/cyclin D complexes accumulate p27 as cells progress through G_1 (Ladha *et al.*, 1998; Perez-Roger *et al.*, 1999). Once Cdk2 is activated it phosphorylates p27 thereby triggering its degradation (Vlach *et al.*, 1997). Furthermore, in TGF-β-treated cells, accumulation of p15 leads to a reduction in the amount of Cdk4/cyclin D complexes, which frees p27, allowing it to interact with, and

inhibit, Cdk2 (Reynisdottir *et al.*, 1995). Inducible expression of p16 and displacement of cyclin D1 from Cdk4 and Cdk6 also drives p21 into complexes with Cdk2 (McConnell *et al.*, 1999).

The Cip/Kip family of CKIs is characterised by their binding to preformed Cdk/cyclin complexes (Harper *et al.*, 1993; Polyak *et al.*, 1994; Lee *et al.*, 1995) (Figure 1.2). In vitro, these CKIs can block the activity of all Cdk/cyclin complexes with different potencies (Sherr and Roberts, 1999). p21 is upregulated by p53 in response to DNA damage (el-Deiry *et al.*, 1993; el-Deiry *et al.*, 1994), and it is also upregulated during replicative senescence (Stein *et al.*, 1999). Proliferating cell nuclear antigen (PCNA) has been found in p21/cyclin/Cdk complexes. PCNA is an elongation factor for DNA polymerase δ, as well as a component of the DNA repair machinery (Kelman and Hurwitz, 1998). The binding of the p21 complex to PCNA inhibits the ability of PCNA to function in DNA replication but not DNA repair (Li *et al.*, 1994). p27 has been described as a Cdk2/cyclin E inhibitor responsible for the antiproliferative effects of transforming growth factor (TGF)-β (Polyak *et al.*, 1994). Similarly p57 appears to regulate Cdk2/cyclin complexes, although its expression pattern is more restricted (Lee *et al.*, 1995). p27 levels are high in quiescence and gradually decrease as cells progress through G_1 due to a reduction in transcription, and an increase in protein turnover (Hengst and Reed, 1996). Mitogenic signalling results in the rapid degradation of p27 at the end of G_1, indicating that p27 is an important regulator for S phase entry (Aktas *et al.*, 1997). Degradation of p27 by the ubiquitin/proteasome pathway is initiated by Cdk2/cyclin E phosphorylation of Thr 187 and is required for S phase entry (Pagano *et al.*, 1995; Vlach *et al.*, 1997).

1.2.6 Pharmacological Cdk inhibitors

Functional inhibition of Cdks can also be achieved with small molecule inhibitors. Deregulation of Cdks in a number of diseases (see section 1.2.7) has encouraged an active search for selective pharmacological inhibitors of Cdks. Over fifty inhibitors have been identified among which more than twenty have been co-crystallised with Cdk2. Although diverse in structure all inhibitors target the ATP-binding pocket of the catalytic site of the kinase and act as ATP competitive ligands (Meijer et al., 1997).

Flavopiridol is a flavone and is the most advanced Cdk inhibitor, currently in phase II clinical trials for refractory myeloma, advanced gastric carcinoma, and high-grade non-Hodgkin's and mantle cell lymphoma (Sedlacek, 2001). The compound is a nonselective kinase inhibitor showing *in vitro* activity against Cdk4, Cdc2, Cdk2, and PKC (Table 1.1)

Olomoucine, roscovitine, and NG-75 are members of the purine class of Cdk inhibitors (De Azevedo *et al.*, 1997; Meijer *et al.*, 1997; Chang *et al.*, 1999). Olomoucine was discovered as a lead compound through the screening of substituted purines. Although not as potent as flavopiridol, it is more specific for Cdk2, Cdc2, and Cdk5 and inactive with respect to Cdk4 and Cdk6 (Table 1.1). Roscovitine is a second-generation purine in which the 2- and 9-substituents are increased in size (Figure 1.4). This compound shows a 10-fold improved potency for Cdk2, Cdc2, and Cdk5 and maintains its selectivity with respect to a number of other kinases (Table 1.1). The Cdk2-roscovitine complex structure is almost identical to the Cdk2-ATP complex (Figure 1.4). Roscovitine binds in the ATP-binding pocket, with the purine ring of roscovitine occupying approximately the same region as the purine ring of ATP (De Azevedo *et al.*, 1997). However, the purine ring in the roscovitine complex has a different orientation than ATP with respect to the protein. In the roscovitine molecule the benzyl ring points towards the outside of the ATP-binding pocket and occupies a region not engaged by any parts of the ATP in the Cdk-ATP complex. Binding of a hydrophobic group in this region is important for increasing the specificity of the inhibitors for Cdk2. The association between roscovitine and Cdk2 is characterised by predominantly hydrophobic and van der Waal's interactions. The total contacts between roscovitine and Cdk2 are 53, 38% of these contacts are made by Ile 10, Leu 83, and Leu 134 (De Azevedo *et al.*, 1997).

Hymenialdisine (HD) belongs to a family of chemically and metabolically related, marine-sponge-derived natural products (which contain both bromopyrrole and guanidine groups) (Meijer *et al.*, 2000). HD has been found to inhibit Cdk2, Cdc2, and

	$IC_{50}(\mu M)$			
Enzyme	Flavopiridol	Olomoucine	Roscovitine	HD
Cdc2/cyclin B	0.03-0.4	7	0.65	0.022
Cdk2/cyclin A	0.1	7	0.7	0.07
Cdk2/cyclin E	0.1	7	0.7	0.04
Cdk4/cyclin D1	0.02-0.04	>1000	>100	0.6
Cdk5/p25 p35		3	0.16	0.028
Cdk6/cyclin D	0.06	>250	>100	0.7
Erk-1	16	50	34	0.47
Erk-2	6	40	14	2
c-src tyrosine kinase	34	>1000	250	7
c-abl tyrosine kinase		>100	>1000	4
Insulin receptor tyrosine kinase		400	70	75
Protein kinase A	122-145			
Protein kinase C*	6			
Protein kinase C α		>1000	>100	0.7
Protein kinase C β_1		>1000	>100	1.2
Protein kinase C β_2		>1000	>100	1.7
Protein kinase C γ		800	100	0.5
Protein kinase C δ		>1000	>1000	1.1
Protein kinase C ε		>1000	>100	6.5
Protein kinase C η		930	>100	2
Protein kinase C ζ		>1000	>1000	60
c-Jun amino-terminal kinase		200	50	8.5
cAMP-dependent protein kinase		>2000	>1000	8
cGMP-dependent protein kinase		>2000	>1000	1.7
GSK3-β	0.45			0.01
ASK-γ (plant GSK-3)		130	220	0.08
CK1				0.035
Ref.	Sedlacek,2001	Meijer *et al.*, 1997	Meijer *et al.*, 1997	Meijer *et al.*, 2000

Table 1.1 IC_{50} values for flavopiridol, olomoucine, roscovitine, and HD, added to various purified enzymes. (* Isoform not reported).

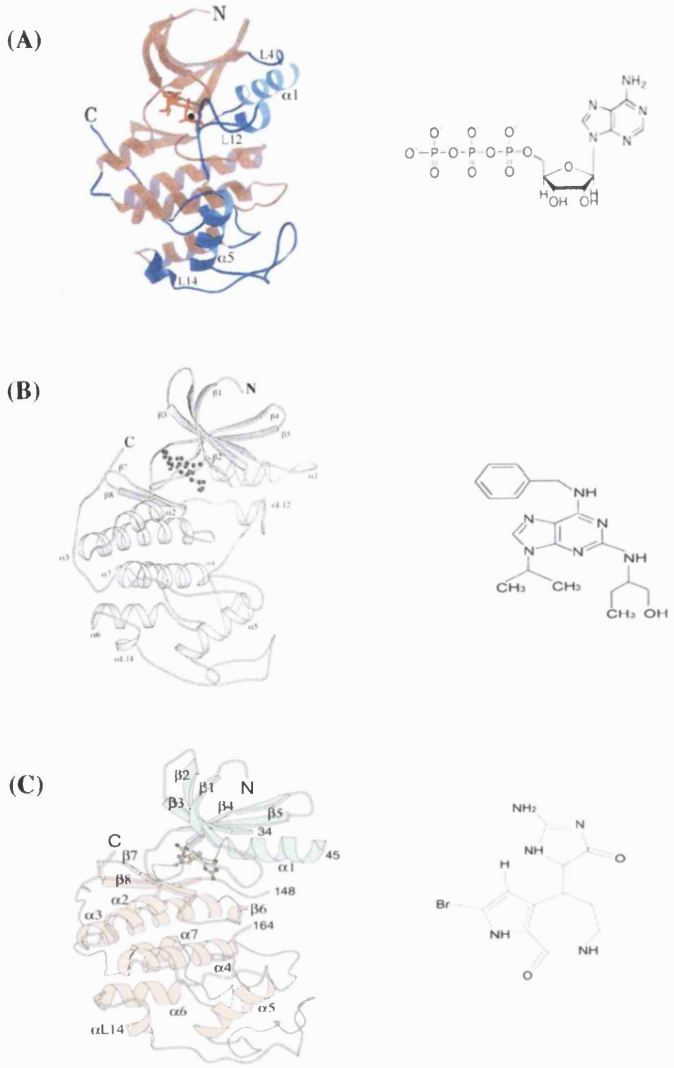


Figure 1.4 Structure of Cdk2 with (A) ATP, (B) roscovitine, and (C) HD, in the ATP-binding pocket between the smaller N-terminal domain and the larger C-terminal domain, and their respective chemical structures.

Secondary structural elements are indicated by arrows for β strands and coils for α helices. These complexes were adapted from De Bondt *et al.*, 1993; De Azevedo *et al.*, 1997; Meijer *et al.*, 2000.

Cdk5, and has limited action on Cdk4 and Cdk6 (Table 1.1). GSK-3β and CK1 kinases are also highly sensitive to HD. HD binds to the ATP-binding pocket and three hydrogen bonds link HD to the Glu 81 and Leu 83 residues of Cdk2 (Figure 1.4). The bromine atom of HD is bound to the ligand-binding pocket of Cdk2, which is occupied by the benzyl group of roscovitine.

1.2.7 Cell cycle control and disease

Cell cycle deregulation has important implications in relation to human disease. Inappropriate cell cycle progression is a critical feature of tumour cells, and a major characteristic of transformed cells is that they lack appropriate checkpoint controls. The Rb kinase pathway has been reported to be heavily mutated in human tumour cells, and it is estimated that potentially all human tumour cells contain mutations in this pathway (Sherr, 1994; Millard and Koff, 1998).

Cyclin D1 translocations are associated with certain B-cell lymphomas (Bodrug *et al.*, 1994) and cyclin D1 gene amplification occurs in a subset of breast, oesophageal, bladder, lung, and squamous cell carcinomas (Wang *et al.*, 1994a; Wang *et al.*, 1994b). Cyclin D2 and D3 have also been reported to be overexpressed in some tumours (Leach *et al.*, 1993). Mutant Cdk4 and Cdk6 proteins that are resistant to negative regulation by Ink4 inhibitors, and therefore retain kinase activity, have been isolated from human tumours (Leach *et al.*, 1993; Wolfel *et al.*, 1995). A familial melanoma syndrome is associated with an inactivating mutation in p16, and many sporadic tumours have inactivated p16. p15 deletions are found in many of the sporadic tumours with inactivated p16 and, specific deletions of p15 sequences have been found in a few cases of leukaemia and lymphomas (Lee and Yang, 2001). A p18 mutant unable to bind Cdk6 has been isolated from a breast cancer cell line. Cyclin E has been found to be amplified, overexpressed, or both in some breast, colon, and leukaemic cancers. Although very few alterations in p21 are found in human cancers, it is implicated in tumorigenesis through its regulation by the p53 tumour suppressor protein. Decreased expression of p27 is frequently observed in human cancers, including breast, prostate, gastric, lung, skin, colon, and ovarian cancers (Lee and Yang, 2001).

There is evidence to suggest that re-expression of various cell cycle regulators is associated with neuronal vulnerability in Alzheimer's disease (Raina *et al.*, 2000). Neurons in Alzheimer's disease have a phenotype that suggests that the

cells are attempting to cycle, rather than remain in a terminally differentiated non-cycling state. Cdk4 and p16 protein levels are both increased in the brains of cases of Alzheimer's disease compared with age-matched controls (McShea *et al.*, 1997). An increase in Cdc2 and cyclin B1 protein levels and Cdc2 kinase activity in degenerating neurons of Alzheimer's disease brain has been reported (Vincent *et al.*, 1997). Neuronal nuclear expression of PCNA was increased in Alzheimer's disease, as well in Down's syndrome patients and in those with Pick's disease (Nagy *et al.*, 1997).

1.3 The Cell Cycle and Apoptosis

Two of the most fundamental properties of a cell are the ability to divide and the ability to undergo apoptosis. The co-ordinated regulation of cell proliferation and apoptosis is crucial for the normal development of an organism and the maintenance of tissue homeostasis. An imbalance in either of these processes is associated with a wide variety of pathological conditions (see section 1.1.9 and 1.2.7). Excessive cell proliferation and compromised apoptosis contribute to hyperplasia and cancer. For instance, increased cell cycling due to overexpression of the c-Myc oncoprotein and reduced apoptosis due to overexpression of the Bcl-2 oncoprotein co-operate in lymphomagenesis (Strasser *et al.*, 1990). Many studies have provided evidence that cell cycle regulatory molecules influence apoptosis and, that conversely, molecules known to regulate apoptosis can impinge on cell cycle control.

1.3.1 Rb and E2F

The retinoblastoma (Rb) gene product functions as a tumour suppressor gene. Although the level of Rb protein remains relatively unchanged during the cell cycle, its state of phosphorylation does vary (see section 1.2.3). The best characterised target of Rb is the E2F transcription factor family. It has been shown that dephosphorylated Rb binds E2F-1 and inhibits its activity. When Rb is phosphorylated it no longer binds E2F-1 and free E2F-1 is involved in the transcriptional control of genes responsible for cell growth control.

Irradiation of an osteosarcoma cell line containing mutant Rb leads to apoptosis in a time and dose-dependent manner (Haas-Kogan *et al.*, 1995). Expression of wild type Rb protein blocked apoptosis whereas a mutant Rb, unable to bind E2F, did not. This suggests that the inhibitory effect of Rb on apoptosis is

through inhibition of E2F-mediated gene transcription. Further evidence relating inactivation of Rb function to apoptosis comes from studies of *Rb* -/- mice. These mice die during embryonic development after 13-15 days of gestation. Histological analysis of the embryos revealed widespread apoptosis in both the peripheral and central nervous systems (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Apoptosis is substantially elevated in the ocular lens of Rb deficient embryos but this apoptosis is inhibited in embryos deficient in both Rb and p53 (Morgenbesser *et al.*, 1994). Consistent with these findings was the observation that treatment of mouse embryonic fibroblasts (MEFs) with chemotherapeutic agents causes p53 accumulation regardless of Rb status (Almasan *et al.*, 1995). However, the induction of p53 leads to apoptosis in *Rb* -/- cells but only growth arrest in both *Rb* +/- and *Rb* +/+ cells. The loss of Rb function therefore triggers the p53 apoptotic pathway which may serve as an intrinsic protective mechanism for eliminating cells in which the Rb pathway is deregulated. A model has been elucidated to explain the link between Rb and p53 in regulating apoptosis. Loss of Rb allows accumulation of free E2F-1, E2F-1 can then induce mouse p19ARF (DeGregori *et al.*, 1997) or human p14ARF (Bates *et al.*, 1998) which then stabilises p53 levels by blocking Mdm-2-mediated degradation of p53 (Pomerantz *et al.*, 1998).

Mice lacking the Rb family member p130 show a strain-dependent phenotype ranging from embryonic lethality to being viable and fertile. In the C57/BL6 background, *p130* -/- mice reach adulthood and do not show increased tumour incidence. However, in a Balb/c background *p130* -/- mice die embryonically after 11-13 days of gestation showing elevated cellular proliferation and extensive apoptosis in various tissues (LeCouter *et al.*, 1998).

The E2F family of transcription factors, which contains six members, is a major cellular target of Rb. E2F binding sites are found in many of the promoters of genes whose expression is repressed by Rb and which are involved in cell cycle progression (see section 1.2.3). The interactions of E2Fs with Rb family members are regulated during the cell cycle by phosphorylation, cellular localisation and protein turnover. Among the E2F family, E2F-1 is unique in its ability to induce both proliferation and apoptosis.

As a consequence of its action on proliferation, E2F-1 can act as an oncogene. It co-operates with Ras in transformation assays and the transformed cells produce tumours in nude mice (Johnson *et al.*, 1994). E2F-1 expression alone is not

sufficient to induce tumours but it does lead to hyperplasia. Loss of p53 function or co-expression of activated Ras along with E2F-1 overexpression was required for tumour development (Pierce *et al.*, 1998a; Pierce *et al.*, 1998b). However, a central paradox in the action of E2F-1 is that although it can act as an oncogene it also has tumour suppressor effects. *E2F-1* -/- and heterozygous mice are predisposed to tumour formation (Yamasaki *et al.*, 1996). The anomalous nature of E2F-1 in tumourigenesis may be a consequence of its ability to regulate apoptosis.

Overexpression of E2F-1 can trigger apoptosis in fibroblasts (Shan and Lee, 1994). This effect has been reported to be p53-dependent (Qin *et al.*, 1994; Wu and Levine, 1994). E2F-1-mediated apoptosis is significantly reduced in transgenic keratinocytes in a *p53*-/- background (Pierce *et al.*, 1998). Furthermore, p53-dependent apoptosis in the CNS of mice is significantly reduced upon loss of functional E2F-1 (Pan *et al.*, 1998). As described above, the pathway of E2F-1-induced p53-dependent apoptosis has been outlined and shown to act via ARF and Mdm2.

Recent evidence suggests that E2F-3 may also play a role in the regulation of apoptosis. Analysis of *Rb* -/- *E2F-3* -/- embryos show that lack of E2F-3 suppresses both in inappropriate proliferation and the p53-dependent apoptosis arising in Rb null embryos. This suggests that E2F-3 may contribute to the apoptosis resulting from Rb loss. The authors suggested that lack of free E2F-1 and E2F-3 may lead to uncontrolled proliferation and if these levels rise above a certain threshold this results in apoptosis (Ziebold *et al.*, 2001).

E2F-1 can also induce apoptosis in a p53-independent manner. A substantial component of the p53-independent pathway of E2F-1-induced apoptosis is mediated through the induction of p73. E2F-1 regulates p73 levels directly through recognition and transactivation of the p73 promoter, as opposed to p53 on which it acts indirectly (Irwin *et al.*, 2000; Stiewe and Putzer, 2000). p73 contains an oligomerisation domain (OD) and data suggests that p73, like p53, functions as a homotetramer (Davison *et al.*, 1999). p73 with a mutant OD blocks p73-dependent transcriptional activation and apoptosis in tumour cells lacking p53 (Irwin *et al.*, 2000). This provides a mechanism whereby E2F-1 induces apoptosis in the absence of p53. Some of the target genes activated by p73 are also targets of p53 e.g. *mdm2, Gadd45*α (Stiewe and Putzer, 2000). It remains to be seen whether p73-dependent apoptosis involves the induction of the same set of genes as those activated in p53-

induced apoptosis. A clear biological distinction between p53 and p73 is seen in the case of T cell receptor activation-induced cell death (TCR AICD). TCR AICD is p53-independent but Lissy *et al* demonstrated that this process requires the induction of p73. They also showed that splenocytes from mice null for both E2F-1 and p73 do not undergo TCR AICD when compared to wild type cells (Lissy *et al.*, 2000).

1.3.2 The Bcl-2 family

The Bcl-2 family of proteins are the best studied components of the apoptosis regulatory machinery and in addition they have been shown to directly influence cell cycle progression. This relationship was primarily established in studies with T cells from transgenic mice. The *bax* transgene was overexpressed in the T cell compartment of mice and the number of cells in the various phases of the cell cycle determined. About twice as many thymocytes were in S phase in *bax* transgenic mice compared to non-transgenic littermates (Brady *et al.*, 1996). To determine whether this effect was cell autonomous a synchronised population of primary T cells was allowed to re-enter the cell cycle following IL-2 re-addition. The presence of the *bax* transgene accelerated the entry into S phase of cycling T cells whereas those from *bcl-2* transgenic mice showed delayed entry into S phase. Bcl-2 overexpression was shown to reduce the number of thymocytes in S phase (O'Reilly *et al.*, 1996), whereas *bcl-2* -/- mice have an increased number of thymocytes in S phase compared to wild type mice (Linette *et al.*, 1996). An interesting point regarding the action of Bax and Bcl-2 on cell cycle entry is their ability to influence the level of p27, which in turn regulates the activity of cyclin dependent kinases. Bax overexpression accelerates p27 degradation whereas Bcl-2 impairs it (Brady *et al.*, 1996; Linette *et al.*, 1996). This effect is not seen for other CKI's, such as p21 (Brady *et al.*, 1996) or p16 (Vairo *et al.*, 1996). As well as elevated p27 levels, delayed cell cycle entry in *bcl-2* transgenic cells is correlated with hypophosphorylated pRb (Mazel *et al.*, 1996) and increased levels of the pRb relative p130 (Lind *et al.*, 1999). However, the Bcl-2 mediated delay in cell cycle entry is independent of pRb (Vairo *et al.*, 2000) and p53 (O'Reilly *et al.*, 1996). In quiescent fibroblasts overexpression of Bcl-2 leads to elevated levels of p27 and p130. In addition, overexpression of Bcl-2 in *p130* -/- fibroblasts had no effect on cell cycle entry showing the requirement for p130 in this process (Vairo *et al.*, 2000). Bcl-2 retards E2F-1 accumulation. Furthermore E2F-4 augments, whereas E2F-1 overrides, the action of Bcl-2 on cell cycle entry. These

authors suggested that a possible explanation for the ability of p130 to restrain cell cycle progression may involve complex formation with E2F-4 which can repress expression of E2F-1.

It has been suggested that Bcl-2's ability to inhibit apoptosis and ability to retard cell cycle entry are genetically distinguishable. Mutation of a tyrosine residue in the N-terminal BH4 domain had no effect on Bcl-2's survival function but abrogated its ability to slow down cell cycle entry (Huang *et al.*, 1997). However, any mutation that abolishes the survival function of Bcl-2 also abolishes the effect of Bcl-2 on cell cycle entry suggesting an interdependence of the two processes (O'Reilly *et al.*, 1996). Overexpression of other members of the Bcl-2 family, Mcl-1 (Fujise *et al.*, 2000) and Bcl-x_L (O'Reilly *et al.*, 1996) can also retard cell cycle progression. In the case of Mcl-1, this effect has been attributed to its ability to bind to PCNA.

1.3.3 FADD

TNF receptor family members such as TNFR-1 and CD95 initiate signalling pathways which can trigger either cell proliferation or apoptosis (Ashkenazi and Dixit, 1998). The outcome depends on the status of the cell and the type of stimuli to which it is exposed. It was initially thought that following receptor ligation, signalling via the FADD adaptor protein led to apoptosis whereas signalling via TRADD and TRAF molecules led to cell proliferation or differentiation. However, studies using transgenic and knockout mice have shown that FADD's function is not so clear cut. *FADD* -/- die during embryonic development but chimaeric mice could be generated containing *FADD* -/- lymphocytes. The thymocytes of these mice decrease to an undetectable level with age suggesting FADD has important survival functions in these cells. Unexpectedly, activation-induced proliferation is impaired in *FADD* -/- peripheral T cells despite production of IL-2 again suggesting that of FADD is required for T cell proliferation (Zhang *et al.*, 1998). In transgenic mice expressing dominant–negative (DN) FADD, mitogen-induced proliferation of mature T cells is inhibited (Newton *et al.*, 1998; Zornig *et al.*, 1998). Experiments in both T cells and fibroblasts expressing DN FADD showed that the release of intracellular calcium is impaired upon activation of either cell type (Hueber *et al.*, 2000). In a form of TNF-induced apoptosis in NIH3T3 cells where death is preceded by G_2/M

accumulation, FADD conversely appears to have a survival effect (Luschen *et al.*, 2000).

1.3.4 IAPs/Survivin

Survivin is a mammalian protein that carries a motif typical of the IAP proteins (see section 1.1.5). Its expression is upregulated in many common cancers (Ambrosini *et al.*, 1997). In HeLa cells, survivin is expressed predominantly in the G_2/M phase of the cell cycle and its transcription is regulated in a cycle-dependent manner (Li *et al.*, 1998). The protein is degraded in a cell cycle-dependent (G_1 phase specific) manner mediated by the ubiquitin-proteasome pathway (Zhao *et al.*, 2000b). Survivin associates with microtubules of the mitotic spindle at the beginning of mitosis. Disruption of this association results in the loss of survivin's anti-apoptotic function and leads to increased caspase-3 activity. Treatment of HeLa cells with antisense survivin oligonucleotides and a DN survivin mutant leads to apoptosis and polyploidy (Li *et al.*, 1999; Chen *et al.*, 2000). Survivin was also shown to co-localise with caspase-3 and p21 within the centrosome. Furthermore the ability of survivin to control ploidy was shown to require p21. The authors suggest that this apoptosis pathway regulated by survivin could act as a general regulator of mitosis and that an essential requirement of this pathway is co-localisation of p21, caspase-3 and survivin to the centrosomes. Deletion of the mouse survivin gene by homologous recombination leads to embryonic death. *In vitro* studies from E2.5 and E5.5 show that *survivin* -/- embryos have altered microtubule organisation and form giant nuclei. This further implies a crucial role for survivin in mitosis (Uren *et al.*, 2000).

Yeasts also contain IAP proteins, but lack caspases. They are not believed to implement an apoptosis programme suggesting that these proteins have an alternative function (Fraser and James, 1998). Mutations of the single BIR-containing IAP proteins in *S. cerevisiae* (BIR1) and *S. pombe* (bir1) cause a pleiotropic cell division defect in yeast, with dysregulation of cellular ploidy and overall cell morphology (Uren *et al.*, 1999; Li *et al.*, 2000). Similarly, disruption of the *C. elegans* IAP genes, *bir-1* and *bir-2,* leads to incomplete cytokinesis and multinucleation in embryos further implying that the IAP proteins present in non-mammalian cells are required for cell division (Fraser *et al.*, 1999).

1.3.4 Cdks

The Cdks and their regulatory subunits, the cyclins, are key regulators of cell cycle progression (see section 1.1.2). Several lines of evidence suggest a requirement for Cdk-cyclin activity in apoptosis.

Apoptosis induced in S phase arrested cells by compounds such as caffeine and staurosporine as well as TNFα leads to activation of the cyclin A-dependent kinases, Cdk2 and Cdc2 (Meikrantz *et al.*, 1994). When deprived of serum, Rat1a fibroblasts constitutively overexpressing c-Myc show elevated cyclin A transcription but no change in cyclins B, C, D1 and E. Inducible expression of cyclin A in these cells correlated with apoptosis in a dose-dependent manner (Hoang *et al.*, 1994). These data suggest a role for cyclin A in promoting cell death under certain conditions.

In Jurkat cells, Cdc2 kinase activity was induced by Granzyme B and primarily associated with cyclin A (Shi *et al.*, 1996). A concomitant activation of Cdk2-cyclin A was also detected. Activation of Cdc2 kinase has been implicated in chemotherapeutic drug-induced apoptosis of breast cancer cells. Taxol activates Cdc2 kinase in these cells, leading to cell cycle arrest in G_2/M phase followed by apoptosis. Both a chemical inhibitor of Cdc2 kinase activity and a dominant-negative (DN) version of Cdc2 blocked this taxol-induced apoptosis (Yu *et al.*, 1998a).

A clear link has been established between Cdk2 kinase activation and thymocyte apoptosis. As apoptosis proceeds Cdk2 kinase activity increases. When this activity is blocked by pharmacological inhibitors the thymocytes are protected against dexamethasone and DNA damage-induced apoptosis (Gil-Gomez *et al.*, 1998; Hakem *et al.*, 1999). One study showed that the elevated Cdk2 kinase activity is not associated with either cyclin E or cyclin A and requires *de novo* synthesis of proteins for activation to occur. This suggests that Cdk2 may require a novel partner to regulate kinase activity during thymocyte apoptosis (Gil-Gomez *et al.*, 1998). Another study has shown that the Cdk2 activation is upstream of the alterations of mitochondrial permeability and caspase activation. However, they suggest that Cdk2 activation maybe associated with cyclin E and cyclin A (Hakem *et al.*, 1999). Cdc2 kinase is not activated during thymocyte apoptosis (Norbury *et al.*, 1994; Gil-Gomez *et al.*, 1998).

Upregulation of Cdk2 activity was also observed in human endothelial cells undergoing apoptosis after growth factor withdrawal. This activity was found to require cyclin A and is preceded by caspase-mediated cleavage of both p27 and p21 (Levkau *et al.*, 1998). This is at variance with the idea that the activation of Cdk2 is exclusively upstream of caspase cleavage. Comparable data was obtained with human hepatoma cells in which activation of caspase-3 was found to be upstream of Cdk2 activation (Jin *et al.*, 2000).

Further studies have examined whether caspases regulate Cdk2 activation. DN Cdk2 blocks the chromatin condensation that occurs when HeLa cells are exposed to TNFα or staurosporine. However, caspase activation is not blocked nor is the loss of mitochondrial membrane potential and membrane phospholipid asymmetry. These data suggest that caspase activity is necessary for Cdk2 activation, which in turn is required for apoptosis. However, these events can be dissociated since other caspase-associated events such as loss of mitochondrial membrane potential can proceed in the absence of Cdk2 activity (Harvey *et al.*, 2000).

Expression of DN mutants of either Cdc2, Cdk2 or Cdk3 in HeLa cells suppressed TNFα and staurosporine-induced apoptosis whereas DN Cdk5 did not (Meikrantz and Schlegel, 1996).

Clearly, Cdks have an important role in regulating certain apoptotic pathways. To understand the way in which activation of Cdks influence apoptosis it is important to identify the substrates that they phosphorylate. These might include proteins contributing to the morphological similarities between apoptotic and mitotic cells, such as proteins involved in cell rounding, nuclear membrane breakdown or chromatin condensation. Alternatively, Cdks may be activating apoptosis-specific substrates. If so, then it would be interesting to know how Cdk activity is targeted to these substrates only during apoptosis.

1.3.5 CKIs

It is becoming clear that in addition to its role in cell cycle progression (see section 1.2.5) p27 contributes to apoptosis. However, a complex picture is emerging with evidence for both pro- and anti- apoptotic effects.

p27 -/- mesangial cells and fibroblasts were found to be more susceptible to serum withdrawal-induced apoptosis than their wild type counterparts (Hiromura *et al.*, 1999). Further evidence for an anti-apoptotic role for p27 was obtained when p27

was overexpressed in human myeloid leukaemia cells. This delayed apoptosis in response to a variety of DNA damaging agents as well as cycloheximide and an agonistic Fas antibody (Eymin *et al.*, 1999a; Eymin *et al.*, 1999b). p27 is downregulated after a variety of apoptotic stimuli in lymphoid cells of both B and T cell origin. Depending on the apoptotic stimulus, lymphoid cells use at least two pathways to downregulate p27. One involves PARP cleavage, indicative of caspase-3 cleavage and is ZVAD-fmk and Boc-D-fmk sensitive. The other pathway is insensitive to these inhibitors and occurs in the absence of caspase-3 and caspase-8 (Frost and Sinclair, 2000). Decreased levels of p27 are associated with apoptosis in response to various stimuli in thymocytes and this can occur in the absence of protein synthesis (Gil-Gomez *et al.*, 1998). However, in complete contrast, several studies have shown that high level expression of p27 from an adenovirus vector has been shown to induce apoptosis in transformed epithelial cells and fibroblasts (Katayose *et al.*, 1997; Schreiber *et al.*, 1999).

p21 has also been implicated in cell death pathways. Initially, it was observed that p21 increases during DNA damage-induced cell death (el-Deiry *et al.*, 1994). Subsequently it was found that inducing p21 can decrease apoptosis in differentiating muscle cell lines (Wang and Walsh, 1996) and prostaglandin-treated colon carcinoma cell lines (Gorospe *et al.*, 1996). Reduction of p21 levels during differentiation of a neuroblastoma cell line increases apoptosis (Poluha *et al.*, 1996). Thus p21 seems to have an anti-apoptotic function.

Another interesting insight into p21's mode of action was gained from a study of the receptor tyrosine kinase ErB2. Overexpression of ErB2 in breast cancers leads to Taxol resistance. The overexpressed ErB2 causes upregulated transcription of p21 in breast cancer cell lines (Yu *et al.*, 1998a). The p21 associates with Cdc2 and inhibits the Taxol-mediated increase in Cdc-2 kinase activity. This seems to delay entry of the cell into G_2/M and also inhibits Taxol-induced apoptosis. ErB2 was unable to inhibit Taxol-induced apoptosis in breast cancer cells transfected with p21 antisense or in *p21* -/- fibroblasts, further suggesting that ErB2 blocks Taxol induced apoptosis via p21. p21 has been shown to play a role in the resistance to Fas-mediated apoptosis seen in the human hepatoma cell line Hep G2. p21 is thought to act by directly binding procaspase-3 and thereby preventing its activation. However, it remains to be seen if this can account for resistance to Fas-induced death in other cell types (Suzuki *et al.*, 1998).

Both p27 and p21 have been shown to have a role in apoptosis in endothelial cells after growth factor withdrawal. When these cells undergo apoptosis the p27 and p21 proteins are truncated due to a specific cleavage within the C-termini (Levkau *et al.*, 1998). This cleavage is mediated by caspase-3 and other DEVD-sensitive caspases. Truncated p21 leaves the nucleus having lost its nuclear localisation sequence. The levels of p21 and p27 associated with cyclin E and cyclin A–CDK2 complexes are greatly reduced in apoptotic endothelial cells. A mutant of p21 resistant to caspase cleavage partially suppressed apoptosis. The caspase-mediated cleavage of p27 and p21 regulates apoptosis of endothelial cells and this appears to involve activation of CDK2. Similar findings were obtained with human hepatoma cells undergoing apoptosis after treatment with the drug Ginsenoside Rh-2 (Jin *et al.*, 2000). Again p21 was shown to undergo caspase-3-mediated cleavage during apoptosis but p27 cleavage was not observed in this system.

1.4 Neuronal Apoptosis

1.4.1 Apoptosis in neural development and disease

Apoptosis occurs throughout the nervous system in neurons, glia, and neural progenitor cells. At least half of the original cell population is eliminated as a result of apoptosis in the developing nervous system (Oppenheim, 1991; Burek and Oppenheim, 1996). Apoptosis during neural development is required for the refinement of patterns of electrical synaptic connectivity (Burek and Oppenheim, 1996). It is believed that a neuron's chance of survival during development is dependent on the extent of its connections to a postsynaptic target, and competition with other neurons for target-derived neurotrophic factors (Cowan *et al.*, 1984).

If neurons die by apoptosis during development, could apoptotic pathways be activated in neurological diseases? Alzheimer's disease (AD) is the most common cause of dementia among the elderly. AD is the result of damage to selective neuronal circuits in the neocortex, hippocampus, and basal forebrain cholinergic system. The hallmarks of AD include senile plaques, consisting of extracellular depositions of β-amyloid peptide and neurofibrillary tangles, which are intracellular depositions of the protein tau, in a hyperphosphorylated and ubiquitinated state (Honig and Rosenberg, 2000). Four specific genes have been proven to be involved in the occurrence of this disease. The membrane spanning protein genes, β-amyloid

precursor protein (βAPP), presenilin-1 (PS1), and presenilin-2 (PS2), mutations of which are associated with familial AD. The fourth gene, apolipoprotein E attributes to the risk of developing late-onset AD. β-amyloid is neurotoxic to primary cells and overexpressing β-amyloid protein in transgenic mice causes neurodegeneration. β-amyloid protein has been shown to induce apoptosis in PC12 cells, neuroblastoma cells, and cortical and hippocampal neurons *in vitro*. The importance of apoptosis in AD pathogenesis is further supported by evidence of increased TUNEL staining and activated caspases in postmortem analysis of AD brains (Honig and Rosenberg, 2000).

Loss of motor neurons is a major characteristic of amyotrophic lateral sclerosis (ALS), a relatively rapid progressive disease in which spinal motor neurons and upper motor neuron pathways degenerate, leading to complete paralysis of all general somatic musculature (Honig and Rosenberg, 2000). Examination of ALS spinal cord has shown evidence of apoptosis by TUNEL staining, as well as decreased levels of Bcl-2 mRNA and increased Bax mRNA levels in spinal neurons. Expression of mutant SOD1 (Cu-Zn superoxide dismutase), which is detected in one third of ALS familial cases, induces apoptosis in neuronal cells. Mutant SOD1-induced apoptosis can be inhibited by Bcl-2, anti-oxidants, and caspase inhibitors. Furthermore, overexpression of Bcl-2 has been shown to prolong SOD1 mutant mice survival.

Stroke is a major cause of disability and death world-wide. Brain damage following stroke results from a reduced blood supply to brain cells which reduces their access to oxygen and glucose. Recent findings implicate apoptosis in neuronal degeneration in cell culture and animal models of ischaemic brain injury. In these systems, reactive oxygen species (ROS) production, perturbed calcium homeostasis, caspase activation, and mitochondrial dysfunction appear to be important mediators of neuronal apoptosis following ischaemic injury (Mattson, 2000).

There is evidence to suggest that apoptosis occurs in neurodegenerative diseases, and this implies that the apoptotic pathway may be an effective therapeutic target. However, it remains to be seen whether intervening in the apoptotic pathway, or completely blocking apoptosis, would have any significant impact on the signs or symptoms of these diseases.

1.4.2 PC12 cells as a model of apoptotic cell death

The PC12 cell line was cloned from a rat phaeochromocytoma (Greene and Tischler, 1976) and resembles adrenal chromaffin cells of neural crest origin. In culture, treatment of PC12 cells with NGF leads to a slowing or cessation of cell division and differentiation into sympathetic neuron-like cells with neurite outgrowth (Greene and Tischler, 1976; Ignatius *et al.*, 1985). Differentiated PC12 cells are dependent on NGF for survival. Differentiation of PC12 cells requires activity of the Ras/ERK pathway, whereas inhibition of this pathway had no effect on survival or proliferation. Instead, PI3 kinase signalling is necessary for PC12 survival (Klesse *et al.*, 1999).

The role of NGF as a differentiation factor is associated with its ability to regulate the cell cycle. NGF treatment of PC12 cells leads to a decrease in proliferation rates, a decrease in DNA synthesis, accumulation of the hypophosphorylated form of Rb, and G_0/G_1 phase arrest (Greene and Tischler, 1976; Gunning *et al.*, 1981; Ignatius *et al.*, 1985; Rudkin *et al.*, 1989; Yan and Ziff, 1995). This suggests that the development of NGF-mediated neuronal characteristics in PC12 cells is linked to cell cycle arrest in G_1 phase (Rudkin *et al.*, 1989; van Grunsven *et al.*, 1996b). However, Ignatius *et al* (1985) and Buchkovich and Ziff (1994) both report that NGF slows DNA synthesis but does not stop it and that mitotic activity can persist in morphologically differentiated PC12 cells.

NGF induces changes in the cell cycle regulatory machinery which may account for the withdrawal from the cell cycle of NGF-responsive PC12 cells during differentiation. NGF-induced differentiation of PC12 cells results in a reduction in levels of Cdk2, Cdc2, Cdk4, Cdk6, cyclin A, cyclin B, and the S phase marker PCNA, as well as the level of phosphorylated Rb (Buchkovich and Ziff, 1994; Dobashi *et al.*, 1995; Yan and Ziff, 1995; van Grunsven *et al.*, 1996a; van Grunsven *et al.*, 1996b). These decreases coincide with a decrease in the enzymatic activity of cyclin A-Cdc2, cyclin B-Cdc2, cyclin E-Cdk2, and cyclin A-Cdk2 (Buchkovich and Ziff, 1994). Constitutive overexpression of Cdk2 inhibits neuronal differentiation of PC12 cells (Dobashi *et al.*, 1995), and simultaneous suppression of Cdk2 and Cdc2 activities induces neuronal differentiation of PC12 cells in the absence of NGF (Dobashi *et al.*, 2000). This indicates that downregulation of Cdk2 and Cdc2 activity is an important event for the neuronal differentiation of PC12 cells. This correlates

with *in vivo* observations whereby a decline in the expression of Cdc2 was observed during terminal differentiation of neurons in the rat cerebral cortex, cerebellum, hippocampus, and olfactory bulb (Hayes *et al.*, 1991; Okano *et al.*, 1993).

There is an increase in the level of cyclin D1 protein when PC12 cells are treated with NGF, which is characteristic of cells in G_1 (Dobashi *et al.*, 1995; Yan and Ziff, 1995; van Grunsven *et al.*, 1996b). However, due to an increase in the levels of the Cdk inhibitor p21 complexed to cyclin D1/Cdk kinase complexes, there was no increase in cyclin D1-associated kinase activity after NGF treatment. Inducible expression of p21 in PC12 cells led to complete withdrawal from the cell cycle and accelerated NGF-induced differentiation (Erhardt and Pittman, 1998a; Erhardt and Pittman, 1998b). Ectopic p21 expression led to accumulation of cyclin D and cyclin E, and a decrease in cyclin A, cyclin B, Cdc2, and Cdk4, as well as a decrease in DNA synthesis. These results imply that the mechanism by which NGF induces the many cellular changes associated with growth arrest during differentiation is through p21 induction.

The nuclear localisation of E2F-4 and its association with the Rb family member p130 increased during neuronal differentiation and forced expression of E2F-4 enhanced the rate of PC12 differentiation by NGF. Moreover, the down-regulation of E2F-4 expression inhibited NGF-induced neurite outgrowth. E2F-4 may be an important transcriptional regulator of growth arrest or differentiation (Persengiev *et al.*, 1999).

Cdk5 protein levels increase during NGF-induced differentiation of PC12 cells (van Grunsven *et al.*, 1996a) and Cdk5 activity is only detectable in post-mitotic neurons of the CNS (Tsai *et al.*, 1993b). It has been shown that two members of the intermediate filament family, which are major cytoskeletal components of axons, are substrates of Cdk5. Cdk5 kinase complexes can also phosphorylate microtubule-associated proteins, such as tau and MAP2, which are important regulators of neuronal polarity and microtubule dynamics (Lew *et al.*, 1995). p35 is a brain-specific Cdk5-associated protein that binds and activates Cdk5 and does not activate other members of the Cdk family (Tsai *et al.*, 1994). Cdk5/p35 kinase activity is essential for neurite outgrowth in cortical neurons because overexpression of dominant negative mutants of Cdk5 or antisense p35 reduced neurite length while coexpression of Cdk5 and p35 induced longer neurites (Nikolic *et al.*, 1996). These results suggest a role for the Cdk5/p35 kinase in neurogenesis.

It is important to understand the mechanisms by which cell cycle controls are regulated during PC12 differentiation as there is increasing evidence to suggest that reactivation of cell cycle regulators in differentiated PC12 cells leads to apoptosis (see below).

In addition to promoting the differentiation and cell cycle arrest of PC12 cells, NGF serves as a critical survival factor because differentiated PC12 cells die when deprived of NGF. Following NGF withdrawal, PC12 cells exhibit cell and chromatin condensation and active membrane blebbing which are major characteristics of apoptosis, and there is a decrease in metabolic activity that precedes a decline in cell number (Mesner *et al.*, 1992). Apoptosis of neuronal PC12 cells induced by NGF withdrawal requires new transcription and translation, which reflects a requirement for novel gene products to effect cell death (Batistatou and Greene, 1991; Mesner *et al.*, 1992).

Broad-spectrum inhibitors of caspases are able to inhibit the death of PC12 cells deprived of NGF. However, caspase-1 specific inhibitors were much less effective. Caspase-3 activity is observed in PC12 cells deprived of NGF and inhibition of this activity by a caspase-3 specific inhibitor partially blocks apoptosis (Haviv *et al.*, 1997). Other investigators showed that caspase-3 activity is not required for NGF withdrawal-induced apoptosis of PC12 cells (Stefanis *et al.*, 1998), whereas caspase-2 activation has a required role in neuronal apoptosis (Troy *et al.*, 1997).

In PC12 cells, phosphatidylserine (PS) redistributes from the inside to the outside of the plasma membrane, during NGF withdrawal-induced apoptosis (Rimon *et al.*, 1997), as has been observed in other cell types.

1.4.3 Sympathetic neurons as a model of apoptotic cell death

Sympathetic neurons from rat superior cervical ganglia (SCG) provide the most extensively characterised model for *in vitro* studies of the molecular mechanisms of developmental neuronal cell death. *In vivo*, essentially all SCG neurons are post-mitotic at the time of birth, and approximately 30% die by apoptosis between days 3 and 7 of postnatal development (Wright *et al.*, 1983). This developmental cell death can be reproduced *in vitro*. NGF-dependent sympathetic neurons can be isolated from the SCG of embryonic day 21 or postnatal day one rats and cultured in the presence of NGF for 5-7 days. Removal of NGF during this time

results in apoptotic cell death in most of the neurons by 48 hours (Martin *et al.*, 1988). Advantages to this model are that a relatively homogenous population of neurons is obtained that undergo apoptosis in a synchronous and reproducible manner. Furthermore, cell death in culture is typical of the physiological growth factor withdrawal-induced neuronal death that occurs *in vivo*. One of the disadvantages of this model is that it is difficult to obtain large numbers of sympathetic neurons for biochemical and molecular analysis.

An early event, which occurs in sympathetic neurons at three hours after NGF withdrawal, is an increase in the level of reactive oxygen species (ROS), which return to basal levels by eight hours (Greenlund *et al.*, 1995a). Preventing this increase in ROS by expression of SOD1 or inhibition of NADPH oxidase, promotes survival of NGF-deprived sympathetic neurons (Greenlund *et al.*, 1995a; Tammariello *et al.*, 2000). When deprived of NGF for 12 hours in culture, sympathetic neurons begin to exhibit morphological changes: the neurites begin to degenerate, the plasma membrane loses its smooth appearance, and the cell body begins to atrophy (Martin *et al.*, 1988; Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994). DNA fragmentation is detected after 18 hours of NGF deprivation (Edwards *et al.*, 1991). The nuclei begin to shrink and condense by 18-24 hours (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994).

Several observations confirm the importance of caspases as mediators of apoptosis in sympathetic neurons (see section 1.1.3). Viral proteins such as p35 and crmA, which are naturally occurring caspase inhibitors, protect against NGF withdrawal-induced apoptosis (Gagliardini *et al.*, 1994; Martinou *et al.*, 1995). The caspase inhibitor BOC-aspartyl(Ome)-fluoromethylketone (BAF) completely blocks the death of sympathetic neurons *in vitro* (Deshmukh *et al.*, 1996), while the caspase-3 specific inhibitor, acetyl-Asp-Glu-Ala-Asp-aldehyde (Ac-DEVD-CHO) inhibited death when microinjected into NGF-deprived neurons (McCarthy *et al.*, 1997). Conversely, caspase-3 activity was not detected during sympathetic neuron death whereas caspase-2 activity was (Deshmukh *et al.*, 1996), and antisense RNA targeted to caspase-2 protected against neuronal death (Troy *et al.*, 1997). However, neurons cultured from caspase-2 deficient mice are still sensitive to NGF deprivation (Bergeron *et al.*, 1998), perhaps as a result of functional redundancy during development. More intriguingly, neurons from caspase-9 knockout mice show

reduced cell death after NGF withdrawal, indicating that the apoptosome complex plays a role in the death of these neurons (see section 1.1.6) (Deshmukh *et al.*, 2000).

Cytochrome c has been shown to be an important mediator of apoptosis in sympathetic neurons. Microinjection of neurons with an antibody against cytochrome c promoted survival in the absence of NGF (Neame *et al.*, 1998). NGF deprivation induced the loss of cytochrome c from the mitochondria in sympathetic neurons. This event was dependent on protein synthesis and Bax function but occurred upstream to caspase activation (Deshmukh and Johnson, 1998). Surprisingly, microinjection of neurons with cytochrome c did not alter the rate of apoptosis in the presence or absence of NGF (Deshmukh and Johnson, 1998; Neame *et al.*, 1998). However, when neurons were deprived of NGF, microinjection of cytochrome c was able to induce caspase-dependent apoptosis in the presence of cycloheximide (Deshmukh and Johnson, 1998). From these results it has been suggested that NGF deprivation induced the translocation of cytochrome c and another step that is independent of protein synthesis and termed "competence-to-die".

Another major apoptotic regulator, Bcl-2, has been shown to play a role in sympathetic neuron apoptosis. Neurons microinjected with Bcl-2 expression vectors show increased survival rates in response to NGF withdrawal (Garcia *et al.*, 1992). Furthermore, neurons from *bcl-2* transgenic mice are protected from naturally-occurring developmental apoptosis (Martinou *et al.*, 1994). However, *bcl-2* -/- mice show no decrease in the number of sympathetic neurons at birth, but do have 58% fewer sympathetic neurons at postnatal day 44 compared to wildtype controls. Neurons cultured from these mice undergo a faster rate of apoptosis compared to controls (Greenlund *et al.*, 1995b). Sympathetic neurons derived from *bax* -/- mice do not undergo apoptosis after NGF withdrawal, and the mice have a three-fold increase in the number of sympathetic neurons per ganglion (Deckwerth *et al.*, 1996). The BH3-only Bcl-2 family members, Dp5/Hrk and Bim, are induced after NGF withdrawal in sympathetic neurons and during the hyperpolarization of cerebellar granule neurons (Imaizumi *et al.*, 1997; Harris and Johnson, 2001; Putcha *et al.*, 2001; Whitfield *et al.*, 2001). Overexpression of Bim_{EL} in sympathetic neurons induces cytochrome c redistribution and apoptosis in the presence of NGF, and neurons injected with Bim antisense oligonucleotides or isolated from *bim* -/- knockout mice die more slowly after NGF withdrawal (Putcha *et al.*, 2001; Whitfield

et al., 2001). However, loss of bim was not as effective as inactivation of bax, perhaps because dp5 can compensate for bim.

The death of sympathetic neurons after NGF withdrawal requires *de novo* RNA and protein synthesis (Martin *et al.*, 1988; Edwards *et al.*, 1991). These observations suggest that neuronal apoptosis is an active process involving the synthesis of proteins which ultimately bring about physiologically appropriate cell death. This has led to a search for genes and proteins that are upregulated following NGF withdrawal, since these may be important in mediating apoptotic cell death. For instance, the level of *c-jun, cyclin D1, dp5* and *bim* mRNA increases during apoptosis caused by NGF-withdrawal in cultured sympathetic neurons (Estus *et al.*, 1994; Freeman *et al.*, 1994; Ham *et al.*, 1995; Imaizumi *et al.*, 1997; Putcha *et al.*, 2001; Whitfield *et al.*, 2001).

The cell death programme initiated by NGF withdrawal can be stopped if neurons are refed with NGF prior to an irreversible "commitment point". The commitment point has been defined as the time after NGF withdrawal (about 22 hours) at which half the neurons can no longer be rescued (Edwards *et al.*, 1991; Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994).

The c-Jun N-terminal kinases (JNK), also called stress-activated protein kinases (SAPK), belong to the stress-activated group of mitogen-activated protein (MAP) kinases that are involved in the stress response and cell death. Jun kinases can stimulate the transcriptional activity of c-Jun by phosphorylating serines 63 and 73 in the transactivation domain and by stimulating *c-jun* gene expression. After NGF withdrawal, JNK activity, c-Jun levels, and c-Jun N-terminal phosphorylation increase in sympathetic neurons (Ham *et al.*, 1995; Virdee *et al.*, 1997 Eilers *et al.*, 1998). Expression of c-Jun protein and apoptosis are blocked by DN c-Jun (Ham *et al.*, 1995; Eilers *et al.*, 1998), and NGF withdrawal-induced death is also blocked by microinjection of an antibody specific for c-Jun (Estus *et al.*, 1994). These results suggest that c-Jun activates target genes that promote apoptosis, as well as the *c-jun* gene itself. Direct inhibition of JNK activity also promotes the survival of neurons after NGF withdrawal (Eilers *et al.*, 1998; Harding *et al.*, 2001). Expression of activated or DN mutants of JNK kinase kinases, such as apoptosis signal-regulating kinase (ASK) 1, and of Rho family GTPases, such as Cdc42, indicate that these proteins are crucial components of the upstream signalling pathway that triggers JNK activation in sympathetic neurons (Bazenet *et al.*, 1998; Kanamoto *et al.*, 2000).

1.4.4 The role of cell cycle regulators in neuronal apoptosis

It is important to distinguish between the Cdk activity associated with cell death and that associated with cell cycle progression in a normal dividing cell. Therefore, biochemical studies to determine the mechanism of Cdk-driven apoptosis would be best performed in non-dividing cells to minimise the interference from cell cycle-related Cdk activity. Post-mitotic neurons represent an ideal model in which to examine this proposition because they are not in the cell cycle but readily undergo apoptosis. Accumulating evidence indicates that cell cycle components are involved in neuronal death.

Freeman *et al,* (1994) carried out the first study investigating the expression of cell cycle genes in post-mitotic neurons undergoing apoptosis following NGF withdrawal. RT-PCR analysis showed a general reduction in the level of mRNAs for cell cycle genes except for cyclin D1, which was induced (Freeman *et al.*, 1994). Subsequent work on a neuroblastoma cell line undergoing apoptosis following serum withdrawal showed that in this system there was an increase in cyclin D1-dependent kinase activity. This was attributed to an increase in cyclin D1 protein levels and an increase in Cdk4-cyclin D1 complex formation (Kranenburg *et al.*, 1996). Induction of both cyclin D1 and Cdk4 proteins was also detected in differentiated PC12 cells undergoing apoptosis (Davis *et al.*, 1997). An increase in Cdc2 and PCNA protein levels was also observed in this study along with an increase in histone H1 kinase activity that was attributed to Cdc2 kinase. Cyclin B mRNA and protein levels increased in PC12 cells after NGF withdrawal. This coincided with an increase in cyclin B-associated kinase activity (Gao and Zelenka, 1995).

Several agents that inhibit cell cycle progression promote the survival of trophic factor-deprived sympathetic neurons and/or neuronally differentiated PC12 cells. The G_1/S phase blockers mimosine, ciclopirox, and deferoxamine are effective in preventing NGF withdrawal-induced apoptosis of post-mitotic PC12 cells and sympathetic neurons (Farinelli and Greene, 1996). The pharmacological Cdk inhibitors, flavopiridol and olomoucine, have been shown to inhibit NGF withdrawal-induced apoptosis of both neuronally differentiated PC12 cells and sympathetic neurons (Park *et al.*, 1996) and caspase activation (Stefanis *et al.*, 1996). These authors went on to observe that overexpression of the Cdk inhibitors p16, p21 and p27 in sympathetic neurons blocks apoptosis. In addition, expression of DN

forms of Cdk4 or Cdk6, but not Cdk2 or Cdk3, protects NGF-deprived sympathetic neurons from apoptosis (Park *et al.*, 1997a). This implies a role for certain Cdks in neuronal apoptosis. It is interesting to note that although olomoucine is an effective inhibitor of neuronal apoptosis it is not a potent inhibitor of Cdk4 or Cdk6 kinase activity (Meijer *et al.*, 1997; see Table 1.1). Apoptosis induced by withdrawal of neurotrophic support from CNS (rat retinal) and PNS (chick sympathetic, sensory, and ciliary) neurons were equally rescued by olomoucine and roscovitine (Maas *et al.*, 1998). In this study the survival-promoting activity of olomoucine correlated with its invitro IC_{50} for c-Jun amino-terminal kinase-1 and its potency to repress c-Jun induction in live PC12 cells. Roscovitine was more potent in rescuing neurons than in inhibiting Jun kinase. It was therefore concluded that the anti-apoptotic action of roscovitine might be due to inhibition of additional kinases.

Neuronal apoptosis induced by DNA damaging agents also appears to involve signaling pathways that normally control the cell cycle. Pharmacological G_1/S blockers and Cdk inhibitors inhibit death of neuronal PC12 cells, sympathetic neurons, and cortical neurons evoked by UV irradiation, camptothecin, and AraC (the S phase inhibitor cytosine arabinoside) (Park *et al.*, 1997b; Park *et al.*, 1998b; Park *et al.*, 2000a). During camptothecin-induced apoptosis of cortical neurons there is an increase in cyclin D1-associated kinase activity and expression of DN Cdk4/6 protects against apoptosis (Park *et al.*, 1998a). A model has been proposed in which camptothecin treatment of cortical neurons leads to Cdk activation, which lies upstream of cytochrome c release and caspase activation (Stefanis *et al.*, 1999). Overexpression of the Cdk inhibitors p16, p21, and p27, as well as DN Cdk4/Cdk6 can protect sympathetic neurons against UV irradiation- and AraC-induced death (Park *et al.*, 1998a). These studies indicate that Cdk4/6 activity plays a role in DNA damage-induced neuronal apoptosis. However these investigations failed to show that there was an increase in Cdk4 or Cdk6 kinase activity during DNA damage-induced apoptosis, or that DN Cdk4/6 mutants specifically block Cdk4/6 activity. Moreover, olomoucine specifically inhibits Cdk2, Cdc2, and Cdk5 kinase activity and is not an effective inhibitor of Cdk4 or Cdk6 activity.

Flavopiridol and DN Cdk4/Cdk6 protect cortical neurons from β-amyloid-induced apoptosis. In addition, the substrate of Cdk4/6, pRB/p107, is phosphorylated during β-amyloid-evoked neuronal death (Giovanni *et al.*, 1999). Several groups

have reported abnormal upregulation of a variety of cell cycle proteins in the brains from Alzheimer's disease patients (Vincent *et al.*, 1997; Busser *et al.*, 1998). Induction of cell cycle proteins has also been observed when post-mitotic neurons are exposed to oxidative stress. An increase in cyclin B expression occurs during dopamine- and peroxide-induced apoptosis in chick post-mitotic sympathetic neurons. Both apoptosis and cyclin B induction can be blocked by antioxidant treatment, suggesting a functional role for cyclin B induction during neuronal apoptosis. An increase in Cdk5 protein was also observed (Shirvan *et al.*, 1998). Although Cdk5 has not been shown to be required to regulate cell cycle there is some evidence to suggest it may be involved in apoptosis during development. Cdk5 expression is correlated with apoptosis in various developing tissues (Zhang *et al.*, 1997) as well as the expression of its regulatory subunit p35 (Ahuja *et al.*, 1997). Cdk5 kinase activity has been reported to increase in the rat brain following ischaemia (Green *et al.*, 1997). Increased expression of both Cdk5 and p35 occurs in the rat substantia nigra during apoptosis (Henchcliffe and Burke, 1997; Neystat *et al.*, 2001). During neuronal PC12 apoptosis, increased phosphorylation of the Cdk5 substrate, tau, has been observed (Davis and Johnson, 1999).

1.5 Aim

Cell cycle regulators have been implicated in the programmed cell death of sympathetic neurons and PC12 cells after NGF withdrawal, as well as in several other models of apoptosis. In this study, the expression and activity of Cdks, cyclins and Cdk inhibitors, in PC12 cells and sympathetic neurons, will be examined during NGF withdrawal-induced death to determine the role of Cdks in neuronal apoptosis. In addition, the pharmacological Cdk inhibitors, roscovitine, NG-75 and HD, which specifically block Cdk2, Cdc2 and Cdk5 activity, will be used to determine whether these kinases are required for NGF withdrawal-induced death. The effect of specifically blocking Cdk2 expression and activity in sympathetic neurons, with Cdk2 antisense oligonucleotides, will also be assessed.

The release of cytochrome c from the mitochondria is an important event in sympathetic neuron apoptosis. Therefore, if Cdks play a role in neuronal apoptosis, it will be necessary to determine whether Cdk activation is upstream or downstream of the release of mitochondrial cytochrome c.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals and equipment

The following is a list of suppliers of reagents and equipment.

Agar Scientific

Number 5 Dumont forceps

Amersham Pharmacia Biotech

ECL chemiluminescence reagent, Hybond-C extra ECL nitrocellulose membrane, GammaBind G Sepharose, [γ-^{32}P] ATP

Biognostik

rat specific Cdk2 antisense (Cdk2AS) phosphorothioate oligonucleotide #21592 (sequence not disclosed by company), CG-matched randomised-sequence (NS) phosphorothioate oligonucleotide

Bio-Rad Laboratories

Gel dryer model 583, Mini-Protean 3 electrophoresis cell, Protean II xi electrophoresis cell, Mini Trans-Blot electrophoretic transfer cell

Boehringer Mannheim/Roche

TUNEL assay kit, phenyl methyl sulphonyl fluoride (PMSF), Histone H1, cell proliferation ELISA, BrdU kit

Calbiochem

Hoechst 33342 dye

Citifluor

Citifluor AF1 glycerol/PBS solution

Dow Chemical Company

Saran wrap

Gibco BRL Life Technologies

L-glutamine, penicillin/streptomycin, L15 (Leibovitz) medium, RPMI 1640 medium, Dulbecco's Modified Eagle's medium (DMEM)

John Weiss

Dissection scissors and forceps

Kodak

Scientific imaging film Biomax MS

Merck Ltd. BDH

Glycine, methanol, sodium chloride, Tris(hydroxymethyl)methylamine, glacial acetic acid, type 1 coverslips (circular, 13 mm diameter), microscope slides, Nonidet P40 (NP40)

Molecular Probes

Live / dead viability / cytotoxicity assay kit

PAA Laboratories

Foetal calf serum (FCS), horse serum (HS).

Promega

2.5S nerve growth factor (NGF), CellTiter 96® AQueous One Solution Cell Proliferation assay

Santa Cruz

retinoblastoma protein (pRb) tagged with glutathione S transferase (GST)

Sigma-Aldrich Chemical Company

Ammonium persulphate (APS), bovine serum albumin (BSA), bromophenol blue, calcium chloride ($CaCl_2$), Coomassie Brilliant Blue R-250, collagen (Rat tail, type VII, acid soluble), dimethyl sulfoxide (DMSO), 1,4 – dithiothreitol (DTT),

Dulbecco's Modified Eagle's Medium (DMEM), ethylenediamine tetra-acetic acid (EDTA), FCS (for sympathetic neurons), 5-fluoro-2-deoxyuridine, goat serum, glycerol, β-mercaptoethanol, magnesium chloride ($MgCl_2$), paraformaldehyde, phosphate buffered saline (PBS) without calcium chloride and magnesium chloride, PBS with calcium chloride and magnesium chloride, poly-L-lysine, sodium dodecyl sulphate (SDS), N, N, N′, N′ - tetramethylethylene diamine (TEMED), Triton X-100, trypsin (T-4665), trypsin / EDTA, trypan blue solution (0.4%), Tween 20, uridine, laminin, sodium fluoride, sodium pyrophosphate, β-glycerophosphate, sodium orthovanadate, aprotinin, leupeptin, antipain, soyabean trypsin inhibitor, adenosine 5′ - triphosphate (ATP)

Worthington Biochemicals

Collagenase type II

2.1.2 Antibodies

Amersham Pharmacia Biotech

Donkey anti-rabbit immunoglobulin, conjugated to horseradish peroxidase

Sheep anti-mouse immunoglobulin, conjugated to horseradish peroxidase

Donkey anti-sheep/goat immunoglobulin, biotinylated

Donkey anti-rabbit immunoglobulin, biotinylated

Goat anti-mouse immunoglobulin, biotinylated

Streptavidin immunoglobulin conjugated to horseradish peroxidase

Boehringer Mannheim/Roche

anti-NGF monoclonal antibody

Jackson ImmunoResearch

Donkey anti-mouse immunoglobulin, conjugated to fluoroscein (FITC)

Oncogene Research Products

Mouse monoclonal anti-PCNA (Ab-1) antibody

PharMingen

Mouse monoclonal anti-cytochrome c antibody	

Santa Cruz

Rabbit polyclonal anti-Cdk2 (M2) antibody	sc-163
Cdk2 blocking peptide, human aa 232-298	sc-163P
Rabbit polyclonal anti-Cdk5 (C-8) antibody	sc-173
Cdk5 blocking peptide, human aa 284-291	sc-173P
Rabbit polyclonal anti-Cdc2 p34 (17) antibody	sc-54
Cdc2 blocking peptide, human aa 224-230	sc-54P
Rabbit polyclonal anti-Cdk4 (C-22) antibody	sc-260
Cdk4 blocking peptide, mouse aa 282-290	sc-260P
Rabbit polyclonal anti-cyclin D1 (C-20) antibody	sc-717
Cyclin D1 blocking peptide, mouse aa 276-295	sc-717P
Rabbit polyclonal anti-cyclin A (C-19) antibody	sc-596
Cyclin A blocking peptide, mouse aa 405-423	sc-596P
Rabbit polyclonal anti-cyclin E (M-20) antibody	sc-481
Cyclin E blocking peptide, rat aa 378-396	sc-481P
Rabbit polyclonal anti-p21 (C-19) antibody	sc-397
Rabbit polyclonal anti-p27 (C-19) antibody	sc-528

2.1.3 Stock Solutions

All solutions were made up in MilliQ deionised water unless specified otherwise.

Western blot transfer buffer	192 mM glycine
	25 mM Tris base
	20% methanol
SDS running buffer	250 mM glycine
	25 mM Tris base
	0.1% w/v SDS
Laemmli buffer A	125 mM Tris-Cl pH 6.8

	4% SDS 20% glycerol 10% β-mercaptoethanol 0.02% bromophenol blue
Laemmli buffer B	33 mM Tris-Cl pH 6.8 3.3% SDS 30% glycerol 17% β-mercaptoethanol 0.02% bromophenol blue
TBS-T	20 mM Tris-Cl pH 7.5 500 mM NaCl 0.1% v/v Tween-20
Lysis buffer	50 mM Tris-Cl pH 7.5 150 mM NaCl 20 mM EDTA 0.5% NP40
Kinase assay buffer	50 mM Tris-Cl pH 7.5 10 mM $MgCl_2$
Coomassie Brilliant Blue Solution	50% methanol 10% acetic acid 0.25% Coomassie
Destaining solution	10% methanol 5% acetic acid
4% paraformaldehyde	4% paraformaldehyde in PBS
Propidium Iodide (PI) solution	50 μg/ml PI

	0.1% sodium citrate 0.1% Triton X-100
L15+ medium	L15 (Leibovitz) 0.1% FCS 100 units/ml penicillin 100 μg/ml streptomycin
Trypsin solution	0.025% trypsin (T-4665) in PBS without calcium chloride and magnesium chloride
Collagenase solution	0.2% collagenase in PBS with calcium chloride and magnesium chloride
SCG medium	DMEM (with 4.5 g/l glucose, 0.11 g/l pyruvate) 10% FCS (batch tested) 100 units/ml penicillin 100 μg/ml streptomycin 2 mM L-glutamine 20 μM uridine 20 μM fluorodeoxyuridine 50 ng/ml NGF
PC12 medium	DMEM 10% FCS 5% HS 100 units/ml penicillin 100 μg/ml streptomycin 2 mM L-glutamine

PC6-3 medium	RPMI 1640 medium
	10% FCS
	5% HS
	100 units/ml penicillin
	100 μg/ml streptomycin
	2 mM L-glutamine

2.2 Methods

2.2.1 Tissue culture

2.2.1.1 PC12 cell culture

Undifferentiated PC12 cells were grown in DMEM containing 10% foetal calf serum (FCS), 5% horse serum (HS), L-glutamine (2 mM), 100 units/ml penicillin and 100 μg/ml streptomycin. PC6-3 cells, a PC12 subclone which were selected for their increased dependency on NGF for survival (Pittman *et al.*, 1993), were grown in RPMI 1640 containing 5% FCS, 10% HS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Both cell lines were grown on collagen-coated 10 cm culture dishes, in a humidified atmosphere containing 5% CO_2 at 37°C. Collagen was dissolved in 0.1% glacial acetic acid and diluted to 125 μg/ml in 30% ethanol. Plates were coated overnight, washed with PBS and sterilised by UV treatment. PC12 and PC6-3 cells were subcultured every 4 days by rinsing the cells with PBS and incubating for 2-3 minutes with 1 x trypsin. When the cells had detached PC12/PC6-3 medium was added to inactivate the trypsin and cells were diluted 1:3 in PC12/PC6-3 medium and plated in collagen-coated plates.

For long term storage in liquid nitrogen, after inactivating the trypsin, cells were pelleted by centrifugation at 1200 rpm for 5 minutes. Cells were then resuspended in PC12/PC6-3 medium containing 50% FCS and 20% DMSO and 1 ml aliquots were frozen overnight at -70°C and then transferred to liquid nitrogen. Frozen cells were thawed by incubation at 37°C until a small ice crystal remained and cells were then pipetted into 30 ml of PC12/PC6-3 medium to dilute the DMSO.

Cells were pelleted by centrifugation and resuspended in 10 ml of fresh PC12/PC6-3 medium and plated in 10 cm collagen-coated plates.

To differentiate the cells and obtain a sympathetic neuron-like phenotype, PC12 cells were plated at a density of 1 x 10^6 per plate in 10 ml of DMEM containing 1% HS, L-glutamine (2 mM), 100 units/ml penicillin and 100 μg/ml streptomycin plus 100 ng/ml NGF. PC6-3 cells were differentiated with RPMI 1640 containing 2% HS, 1% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 100 ng/ml NGF. Cells were differentiated for 7-8 days and the medium was changed every 2 days. Following differentiation, cells were deprived of NGF and incubated for various amounts of time in media anti-NGF antibody at 100 ng/ml.

2.2.1.2 Method for coating glass coverslips with poly-L-lysine and laminin

Coverslips were baked in glass petri dishes at 140°C for 4 hours. Poly-L-lysine was dissolved in sterile, tissue culture quality water at 1 mg/ml. The baked coverslips were placed in a 10 cm tissue culture dish and covered with poly-L-lysine solution. The sealed dish was then placed on a shaker and gently rotated for 24 hours at room temperature. After coating, the poly-L-lysine solution was removed and the coverslips were washed with tissue culture quality water overnight for three days, and the water was changed each day. The coverslips were dried by standing them around the edge of a dry 10 cm tissue culture dish inside a Class II tissue culture hood overnight. Poly-L-lysine-coated coverslips were coated with laminin on the day of a sympathetic neuron prep. Poly-L-lysine-coated coverslips were placed in the centre of 3.5 cm tissue culture dishes and 60 μl of 20 μg/ml laminin solution was pipetted onto each coverslip so as to cover three quarters of the whole surface. The coverslips were left at room temperature for two hours. Just before plating the cells, the laminin solution was aspirated off and the coverslips were washed with 60 μl of SCG medium.

2.2.1.3 Sympathetic neuron culture

Superior cervical ganglia (SCG) were removed from one-day-old Sprague Dawley rats obtained from the Biological Services Unit, University College London. The ganglia were desheathed using Dumont number 5 forceps and dissociated by incubation in 5 ml of 0.025% trypsin in PBS for 30 minutes at 37°C, followed by 5 ml of 0.2% collagenase in PBS (containing Mg^{2+} and Ca^{2+}) for 30 minutes at 37°C.

The enzymatic reaction was stopped by the addition of 10 ml of SCG medium. The medium was removed but not discarded, 1 ml of fresh medium was added and the ganglia were dissociated by trituration through the tip of a Gilson P1000. The cell suspension was spun for 10 minutes at 1000 rpm and the cell pellet was resuspended in 1 ml of medium by gentle trituration. 9 ml of SCG medium was then added and the SCG neuron population was enriched by pre-plating for three hours in a 10 cm tissue culture dish, to allow non-neuronal cells to attach. After pre-plating, the neurons were gently rinsed off, centrifuged at 1000 rpm for 10 minutes and resuspended in 400 μl of SCG medium. The number of cells was determined by mixing 20 μl of cell suspension with 20 μl of 0.4% trypan blue solution and counting in a 0.2 mm deep, modified Fuchs-Rosenthal haemocytometer. Cells were plated onto poly-L-lysine/laminin-coated 13 mm glass coverslips, placed in a 3.5 cm tissue culture dishes, at 5,000 - 8,000 cells per coverslip in a volume of 50 μl. Cells were cultured at 37°C in 10% v/v CO_2 for 7 days. Fresh medium was added after 1, 2, 4, and 6 days in culture. To reduce the number of non-neuronal cells the concentration of uridine and fluorodeoxyuridine, which have anti-mitotic properties, was increased to 40 μM on day 1 and 2. For NGF withdrawal experiments, the cells were washed once with SCG medium lacking NGF, and then refed with SCG medium without NGF, containing 100 ng/ml of anti-NGF antibody.

2.2.1.4 Preparation of Cdk inhibitors

Roscovitine, HD, and NG-75 were a kind gift from Dr Laurent Meijer. Roscovitine stock solution was prepared at 60 mM in DMSO and stored at -20°C. Concentrations of 10, 25 and 50 μM roscovitine were made in SCG medium or PC12 medium and used in cell viability and TUNEL experiments. 10 mM stocks of HD and NG-75 were made in DMSO, and concentrations of 10 and 25 μM were prepared in SCG medium.

Cdk2 antisense (Cdk2AS) and control (NS) oligonucleotides were prepared at 100 μM in Tris-EDTA (TE) – buffer. 40 μl of Cdk2AS or NS oligonucleotides was added directly into SCG or PC12 medium to make a 2 μM solution. Sympathetic neurons were treated with 2 μM Cdk2AS or 2 μM NS for 24 hours before NGF withdrawal. Neurons were then deprived of NGF for 48 hours in the presence of 2 μM Cdk2AS or 2 μM NS and cell viability determined using the live/dead

viability/cytotoxicity assay (see below). PC12 cells were treated with 2 μM Cdk2 AS or 2 μM NS for 24 hours. Following 24 hours, fresh medium was added and cells were treated with 2 μM Cdk2AS or 2 μM NS for a further 48 hours. Cells were harvested and the effect of Cdk2AS on Cdk2 protein levels and kinase activity was determined using immunoblot and kinase assay analysis respectively (see below).

2.2.1.5 Live/dead viability/cytotoxicity assay

The percentage of viable sympathetic neurons in a culture was determined using the live/dead viability/cytotoxicity assay kit from Molecular Probes. The principle of this assay is that viable cells convert calcein AM to calcein, which fluoresces green, and exclude ethidium homodimer. Apoptotic cells lose membrane integrity and take up ethidium homodimer, which binds to DNA, and hence have red nuclei.

A 10 X stock solution of 5 μM calcein AM and 10 μM ethidium homodimer was made in DMEM. 0.22 ml of this stock solution was added to cells in 2 ml of medium, and incubated at 37°C in 10% CO_2 for 30 minutes. Cells were examined on an inverted fluorescence microscope on 20 X magnification. The number of live cells and the number of dead cells in 10 fields was determined.

% of viable cells = no. of live cells ÷ (no. of live cells + no. of dead cells) x 100

2.2.1.6 Measurement of apoptosis by propidium iodide staining and flow cytometry

Propidium iodide staining of nuclei was used to attempt to quantify apoptosis of PC12 cells after NGF withdrawal. The amount of PI bound correlates with the content of DNA within a given cell. (Nicoletti *et al.,* 1991). PI intercalates into the major groove of double-stranded DNA and produces a highly fluorescent adduct that can be excited at 488 nm with a broad emission centred around 600 nm. Once cells are stained, they are analysed on a flow cytometer. The relative content of DNA indicates the distribution of a population of cells throughout the cell cycle. Cells in the G_0/G_1 phases of the cell cycle are diploid, or have a 2n amount of DNA. Cells in G_2/M phases have a DNA content of 4n, while S phase cells have a DNA content greater than 2n and less than 4n. Apoptotic nuclei have fragmented DNA which is represented as a sub G_1 peak in a DNA histogram.

1 x 10^6 PC12 cells were plated in collagen-coated 10 cm dishes and differentiated for 7 days in medium containing 100 ng/ml NGF or cultured in PC12 medium. After differentiation cells were incubated in medium containing fresh NGF or 100 ng/ml anti-NGF antibody for 48 hours. Analysis of the cell cycle status of proliferating, differentiated and differentiated PC12 cells deprived of NGF for 48 hours was determined. Cells were washed once with PBS and pelleted by centrifugation at 1200 rpm for 5 minutes. Cell pellets were resuspended in 300 µl of PI solution (PI 50 µg/ml in 0.1% sodium citrate plus 0.1% Triton X-100). The amount of fluorescence emitted by each sample was measured using a flow cytometer.

2.2.1.7 CellTiter 96® AQueous One Solution Cell Proliferation assay

The CellTiter 96® AQueous One Solution Cell Proliferation assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The CellTiter 96® AQueous One Solution Reagent contains a novel tetrazolium compound [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulphate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. The MTS tetrazolium compound is bioreduced by dehydrogenase enzymes in metabolically active cells into a coloured formazan product that is soluble in tissue culture medium.

PC12 cells were plated at 2.5 x 10^3 cells/well in collagen-coated 96 well plates and differentiated for 7 days in the presence of 100 ng/ml NGF. After differentiation cells were cultured in medium containing 100 ng/ml anti-NGF antibody for 0, 24, 48, 72, 96, or 120 hours. 20 µl of CellTiter 96® AQueous One Solution Reagent was added to each well of the samples containing 100 µl of culture medium. The plate was then incubated for 3 hours at 37°C in a humified, 5% CO_2 atmosphere. The absorbance was recorded at 490 nm using a 96 well plate reader.

2.2.1.8 Cell proliferation assay, 5-bromo-2'-deoxyuridine (BrdU) incorporation

The amount of proliferation in dying PC12 cells was measured using the cell proliferation ELISA, BrdU kit from Boehringer Mannheim/Roche. PC12 cells were plated in a collagen-coated 96-well plate at 2.5 x 10^3/well and differentiated for 7

days in medium containing NGF. After differentiation cells were incubated in medium containing anti-NGF antibody for 0, 24, 48 and 72 hours and cell proliferation was determined.

Cells were labelled with 10 μM BrdU for 5 hours at 37°C. During this labelling period BrdU is incorporated in place of thymidine into the DNA of proliferating cells. The labelling medium was removed and cells were fixed, and DNA denatured by adding FixDenat solution (available from kit) for 30 minutes at room temperature. Cells were then incubated with a monoclonal BrdU antibody conjugated with peroxidase for 2 hours. Cells were washed three times with PBS. The substrate solution, containing tetramethylbenzidine (TMB), was then added to the cells for 30 minutes. The reaction product was quantified by measuring the absorbance at 490 nm using a microplate reader. The developed colour and hence the absorbance values correlate to the amount of DNA synthesis and thereby to the number of proliferating cells.

2.2.2 Protein Analysis

2.2.2.1 Preparation of protein extracts from cells

PC12 cells were treated with 1 x trypsin/EDTA for 5 minutes at 37°C, and then harvested and pelleted by centrifugation at 1200 rpm for 5 minutes at 4°C. SCG neurons were removed from the coverslips by rinsing with medium (any contaminating fibroblasts and Schwann cells remained behind) and pelleted by centrifuging at 1000 rpm for 10 minutes at 4°C. The cell pellets were washed once in ice-cold PBS and stored at -70°C until required. The frozen cell pellets were resuspended in lysis buffer (see section 2.1.3) by pipetting and incubated on ice for 30 minutes. After centrifugation at 1200 rpm for 30 minutes at 4°C, the supernatant was transferred to a new microfuge tube.

2.2.2.2 Protein concentration assay

The protein concentration in cell extracts was determined by the method of Bradford (Bradford, 1976), using a BioRad assay kit, with BSA as the standard. Protein concentrations were measured at OD_{595} using a BioRad model 550 microplate reader.

2.2.2.3 SDS-Polyacrylamide gel electrophoresis of proteins

One dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (Laemmli, 1970). Gels were run using the BioRad Mini-Protean 3 system. Resolving gels were made of 10%, 12% or 14% acrylamide:bis-acrylamide [30:0.8] in 0.375 M Tris-Cl, pH 8.8, 0.1% SDS, 0.05% N, N, N′, N′ - tetramethylethylene diamine (TEMED) and 0.05% ammonium persulphate (APS). The resolving gel was poured to fill approximately 75% of the space between two glass plates separated by 0.75 mm spacers and overlaid with isopropanol until polymerised. The stacking gel consisted of 4% acrylamide:bis-acrylamide [30:0.8], 0.126 M Tris-Cl, pH 6.8, 0.1% SDS, 0.1% TEMED and 0.05% APS and was poured on top of the resolving gel. SDS running buffer (1x) was added to the gel tank and the wells in the gel were flushed using a syringe and 16 G needle. Samples were mixed with Laemmli loading buffer (A or B, see section 2.1.3), heated to 100°C for 5 minutes, and then loaded on to the gel. The gel was electrophoresed at 80 V until the bromophenol blue dye had run off the bottom of the gel.

2.2.2.4 Immunoblotting of proteins

Following electrophoresis, proteins were transferred to Hybond ECL nitrocellulose membrane by electroblotting using the BioRad Mini Trans-Blot system. Prior to transfer the gel and the membrane were equilibrated with transfer buffer for 10 minutes. The gel was placed onto the membrane and sandwiched between three pieces of Whatmann 3MM paper on either side. To remove air bubbles the sandwich was carefully rolled with a pipette and placed into the transfer apparatus containing 1 x transfer buffer. Protein gels were left to transfer for 1-2 hours at 4°C with a constant voltage of 90 V. After transfer, protein loading and transfer was checked by staining the membranes in 0.2% ponceau-S solution, and then washing in TBS-T.

To detect immobilised proteins, membranes were blocked overnight at 4°C in blocking solution (TBS-T containing 5% non-fat milk powder). Membranes were incubated for two hours at room temperature with the primary antibody diluted in TBS-T (usually between 1:500-1:2000). Membranes were washed three times for 10 minutes each in TBS-T. Membranes were then incubated with a biotinylated secondary antibody diluted in blocking solution (1:2000) for one hour at room temperature. Membranes were washed three times for 10 minutes or more in TBS-T

and then incubated with streptavidin-HRP in TBS-T for 30 minutes. Following three washes in TBS-T the membranes were treated with ECL reagent for one minute and exposed to Kodak film.

2.2.3 Kinase assay

2.2.3.1 Immunoprecipitation of kinases

Immunoprecipitation of Cdk2, Cdk5, p35-, cyclin A- and cyclin E-associated kinases was performed as follows. Frozen PC12 cell pellets (2 x 10^5 cells) and SCG neurons (4 x 10^4 cells) were resuspended in ice-cold lysis buffer containing 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml antipain, 20 μg/ml soyabean trypsin inhibitor (ALAS), 100 μg/ml phenyl methyl sulphonyl fluoride (PMSF) and 1 mM DTT. To immunoprecipitate Cdc2, Cdk4 and cyclin D1-associated kinases frozen cell pellets were resuspended in ice-cold SDS lysis buffer containing ALAS, 100 μg/ml PMSF, 1 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 0.1 mM sodium orthovanadate and 1 mM DTT. Samples were incubated on ice for 30 minutes and then spun at 13,000 rpm for 30 minutes at 4°C. Following centrifugation the supernatant was transferred to a new tube. Protein concentrations were determined as described in section 2.2.2.2. Equal amounts of protein from cell lysates were incubated with 0.4 μg/ml antibody, 20 μl of protein G sepharose beads in lysis buffer (containing protease inhibitors as described above and 1 mM DTT), and rotated at 4°C for 2-3 hours or overnight. 10 μl of blocking peptide was added to the negative controls.

2.2.3.2 Histone H1 kinase reaction

Following immunoprecipitation, beads were pelleted by pulse centrifugation at 13,000 rpm for 10 seconds, washed three times in 400 μl of lysis buffer containing 1 mM DTT and once in 500 μl of kinase buffer containing 1 mM DTT. Beads were resuspended in kinase buffer containing 2 μg of Histone H1, 20 μM ATP, 10 μCi of [γ-^{32}P] ATP and 1 mM DTT and incubated at 30°C for 30 minutes. Samples were mixed with Laemmli loading buffer (A or B), heated to 100°C for 5 minutes, and then loaded onto a 12% SDS-polyacrylamide gel. PC12 cell samples were run overnight at 40 V using the Protean II xi electrophoresis cell, whereas SCG samples were run for 1.5 – 2 hours at 80 V using the Mini-Protean 3 electrophoresis cell.

Electrophoresis was stopped when the bromophenol blue had run off the end of the gel. Gels were dried for 2 hours at 80°C and exposed in a phosphorimager cassette.

2.2.3.3 Rb-GST kinase reaction

Following immunoprecipitation, beads were pelleted by pulse centrifugation at 13,000 rpm for 10 seconds, washed three times in 400 μl of lysis buffer containing 1 mM DTT and once in 500 μl of kinase buffer containing 1 mM DTT. Beads were resuspended in kinase buffer containing 0.5 μg of GST-Rb, 100 μM ATP, 10 μCi of [γ-^{32}P] ATP and 1 mM DTT and incubated at 30°C for 30 minutes. Samples were mixed with Laemmli loading buffer (A or B), heated to 100°C for 5 minutes, and then loaded on to a 12% SDS-polyacrylamide gel as described above in section 2.2.3.2.

2.2.4 Immunocytochemistry

2.2.4.1 TUNEL and bisbenzimide (Hoechst 33342) staining

Neurons were washed in PBS, fixed in 4 % paraformaldehyde for 30 minutes at room temperature and then permeabilised with 0.5 % Triton X-100 in PBS for 5 minutes at room temperature. Following permeabilisation, neurons were washed and then incubated with terminal deoxynucleotide transferase (TdT) and fluorescein-conjugated dUTP at 37°C for 60 minutes in a wet box in the dark. After washing, cells were treated with Hoechst dye at 10 μg/ml in water for 5-10 minutes at room temperature. The cells were then washed twice in distilled water before mounting in Citifluor on glass slides. The coverslips were sealed to the slide by using clear nail varnish.

2.2.4.2 Cytochrome c immunocytochemistry

Neurons were washed in 10 mM glycine in PBS, fixed in 4 % paraformaldehyde for 30 minutes at room temperature and then washed in 10 mM glycine. Cells were permeabilised and non-specific binding was blocked by incubation in 50% goat serum, 0.5% Triton X-100, 0.5% BSA in PBS for 30 minutes at room temperature. Cells were incubated with anti-cytochrome c monoclonal antibody diluted 1:100 in blocking/permeabilization solution for 4 hours at room temperature. The cells were washed in PBS before incubation with the fluorescein-

conjugated anti-mouse IgG secondary antibody diluted 1:100 in blocking/permeabilization solution for 60 minutes at room temperature in the dark. After washing in PBS, nuclei were detected by staining with Hoechst 33342 dye (10 μg/ml) for 5 minutes. The cells were then washed twice in distilled water, left to dry before mounting in Citifluor on glass slides. The coverslips were sealed to the slide by using clear nail varnish.

Chapter 3 Expression and function of cell cycle regulators during NGF-induced differentiation and NGF withdrawal-induced apoptosis of PC12 cells

3.1 Introduction

Apoptosis occurs extensively during the development of the nervous system and may contribute to numerous pathological conditions, including stroke, spinal cord injury, and certain neurodegenerative diseases (Oppenheim, 1991; Linnik *et al.*, 1993; Choi, 1996; Stefanis *et al.*, 1997; see section 1.4.1). Neuronal survival depends on the presence of neurotrophic factors, such as NGF, which are produced by target cells, and neurons that do not obtain an adequate supply of survival factors undergo apoptosis (Korsching, 1993). The death of vertebrate neurons deprived of survival factors requires *de novo* RNA and protein synthesis and appears to result from a specific cascade of molecular and cellular events (Martin *et al.*, 1988; Ellis *et al.*, 1991; Oppenheim, 1991).

A study that looked at gene expression during the NGF withdrawal-induced death of developing sympathetic neurons showed a reduction in the level of expression of cell cycle-related genes, with the exception of cyclin D1, which was induced (Freeman *et al.*, 1994). Later work showed that there was an increase in cyclin D1-dependent kinase activity in neuroblastoma cells undergoing apoptosis (Kranenburg *et al.*, 1996). An increase in both cyclin D1 and Cdk4 proteins was also observed in differentiated PC12 cells undergoing apoptosis (Davis *et al.*, 1997). The pharmacological Cdk inhibitors flavopiridol and olomoucine, inhibit the NGF withdrawal-induced death of both neuronally differentiated PC12 cells and sympathetic neurons (Park *et al.*, 1996). Roscovitine, another pharmacological Cdk inhibitor, has been shown to block primary neuronal apoptosis induced by withdrawal of neurotrophic factors (Maas *et al.*, 1998). Roscovitine is not a potent inhibitor of Cdk4 activity, and this would suggest that Cdks, other than Cdk4, may be involved in neuronal apoptosis.

PC12 cells are a useful model system for investigating the role of cyclin dependent kinases in neuronal apoptosis. When grown in serum-containing medium, PC12 cells divide and resemble precursors of adrenal chromaffin cells and

sympathetic neurons. This is advantageous as large quantities of cells can be obtained to carry out biochemical and molecular analysis. When PC12 cells are treated with NGF they stop dividing and differentiate into sympathetic neuron-like cells with neurites (Greene and Tischler, 1976; Ignatius *et al.*, 1985). Fully differentiated PC12 cells undergo transcription-dependent cell death after the removal of NGF (Batistatou and Greene, 1991).

Cell cycle arrest is essential for the differentiation and survival of neurons. Terminally differentiated neurons are arrested in a post-mitotic state and the expression of several key regulators of the cell cycle is downregulated in these cells (Hayes *et al.*, 1991; Okano *et al.*, 1993; Freeman *et al.*, 1994; Tang *et al.*, 1998). Treatment of PC12 cells with NGF leads to a decrease in proliferation rate, a decrease in DNA synthesis, and the accumulation of the hypophosphorylated form of the retinoblastoma protein (Greene and Tischler, 1976; Gunning *et al.*, 1981; Ignatius *et al.*, 1985). It has been reported that in many PC12 sublines, not all of the cells become dependent on NGF for survival, and following the removal of NGF, some of these cells begin proliferating in serum-containing medium (Pittman *et al.*, 1993). However, most of the differentiated cells die after the removal of NGF. PC6-3 cells, a subline of PC12, are highly dependent on NGF for survival and it has been reported that 90% of these cells undergo transcription-dependent cell death following removal of NGF (Pittman *et al.*, 1993).

Since I was interested in determining the role of cell cycle regulators in neuronal apoptosis it was crucial that the PC12 cells that I used would fully differentiate in the presence of NGF and were no longer cycling. It was important to establish that this was the case so that any changes in cell cycle regulators observed would be due to apoptosis and not a cell cycle effect. I therefore investigated the pattern of expression of various cell cycle regulators, and Cdk kinase activity, during the differentiation of PC12 cells and PC6-3 cells following NGF treatment, to determine which of these clones fully exited the cell cycle. After determining this, I chose to use PC12 cells to look at how cell cycle-related proteins were regulated in differentiated cells deprived of NGF.

3.2 Results

3.2.1 Cyclin dependent kinase, cyclin and Cdk inhibitor levels are differentially regulated by NGF during PC12 and PC6-3 cell differentiation

When PC12 cells are treated with NGF they stop dividing and differentiate into sympathetic neuron-like cells with long neurites. NGF causes a decrease in the proliferation rate of PC12 cells, reduces DNA synthesis and causes arrest in the G1 phase of the cell cycle (Greene and Tischler, 1976; Gunning *et al.*, 1981; Ignatius *et al.*, 1985; Rudkin *et al.*, 1989; Buchkovich and Ziff, 1994). Figure 3.1 shows the morphology of PC12 cells that were cultured in the presence of 100 ng/ml of NGF for 0, 3, and 7 days. A few short neurites are visible after 3 days of NGF treatment, and by 7 days most of the cells have fully extended neurites. To determine the effects of NGF on cell cycle regulatory proteins, the expression of these molecules was examined in NGF-treated and untreated PC12 and PC6-3 cells. Protein extracts were made from cells that had been treated with NGF for various times and from cells that had not been exposed to NGF and equal amounts of protein were resolved using 12% SDS polyacrylamide gels. Immunoblotting using antibodies specific for each cell cycle regulator was performed. The Cdk2, Cdk5, Cdc2, and Cdk4 antibodies used in these studies were highly specific, binding to proteins of the expected size with little or no background binding. Representative blots in Figure 3.2A, show there was a dramatic decrease in the level of the Cdk2 and Cdc2 proteins in NGF-treated PC12 cells, and a slight decrease in the level of the Cdk4 and Cdk6 proteins in the NGF-treated cells compared to the untreated cells. In contrast, Cdk5 protein levels increased upon NGF treatment. Figure 3.2B shows the percentage decrease of Cdk2 and Cdc2 expression to 36.7 ± 5.2% and 21.3 ± 6.8% respectively (mean ± SEM, n=3), in PC12 cells after 7 days differentiation with NGF compared to untreated cells, and that this decrease is significant $p < 0.05$. Cdk5 expression increased 1.67 ± 0.21 fold (n=2) in PC12 cells after NGF-induced differentiation (see Figure 3.2B). This confirms previously reported results that showed a decrease in Cdk2, Cdc2, Cdk4 and Cdk6, and an increase in Cdk5, protein levels in PC12 cells after NGF treatment (Buchkovich and Ziff, 1994; Dobashi *et al.*, 1995; Yan and Ziff, 1995; van Grunsven *et al.*, 1996a). Figure 3.3 shows similar data for PC6-3 cells. There was little or no change in Cdk2 and Cdk5 protein levels. This is consistent with a study

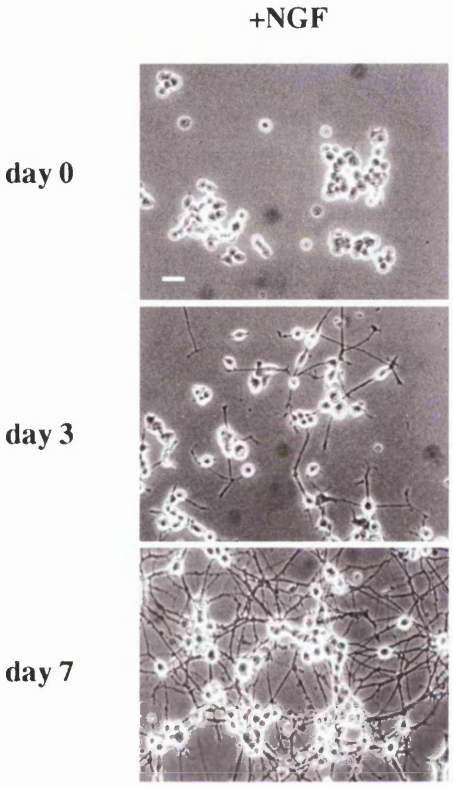


Figure 3.1 Treatment of PC12 cells with NGF causes differentiation into neuron-like cells with neurite outgrowth

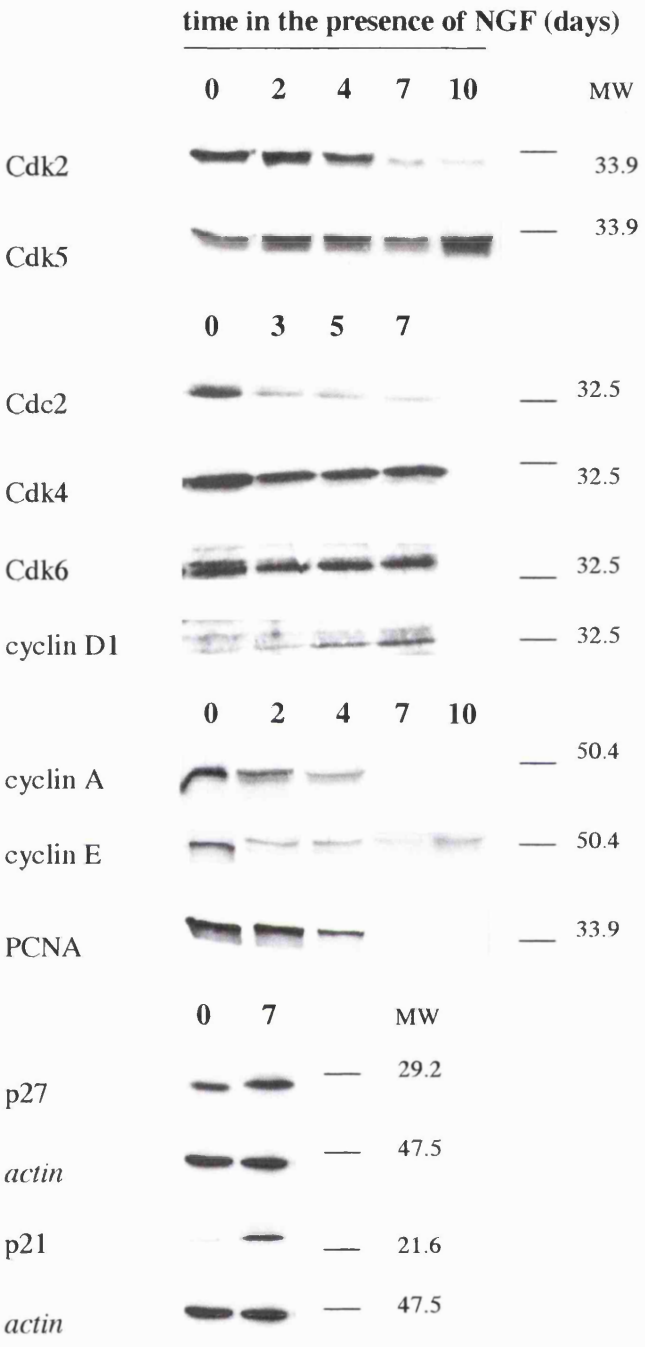
PC12 cells were grown in medium containing 100 ng/ml NGF for 0, 3, and 7 days. Phase-contrast photomicrographs were taken at these time points and neurite outgrowth was assessed. Bar, 20 μm.

Figure 3.2 Pattern of expression of Cdks, cyclins, and Cdk inhibitor proteins during the NGF-induced differentiation of PC12 cells.

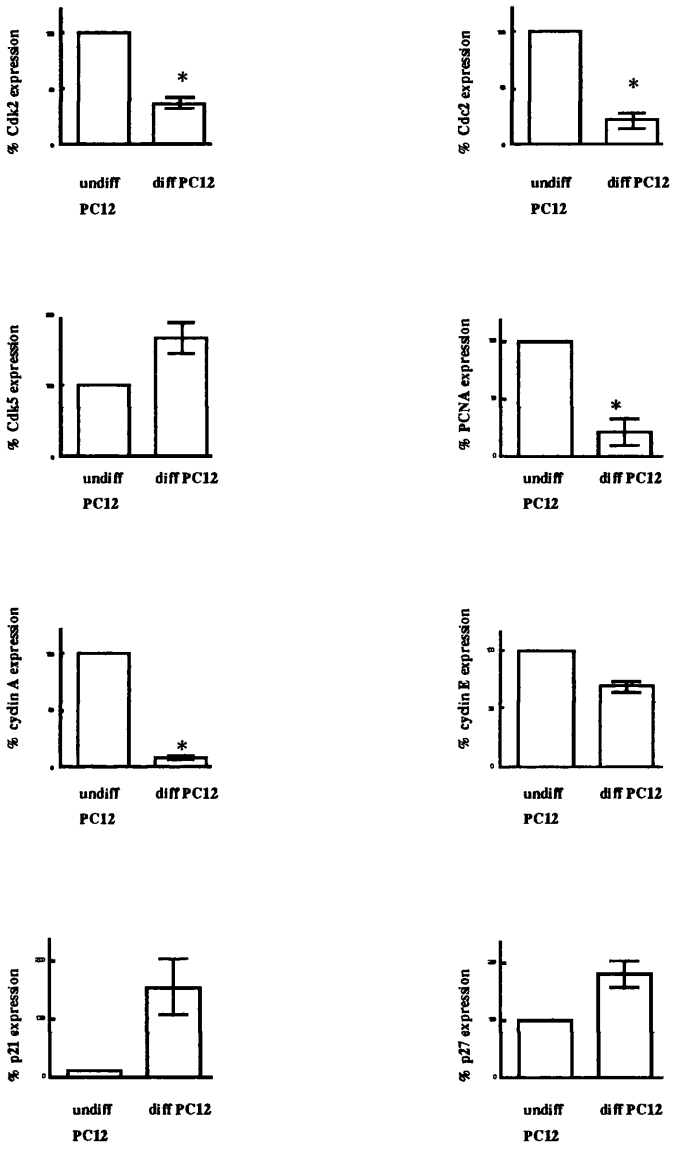
A) PC12 cells were treated with NGF and at 0, 2, 3, 4, 5, 7, or 10 days cell extracts were prepared and 30 μg of each extract was separated on 12% SDS polyacrylamide gels. Immunoblotting was performed using antibodies specific for Cdk2, Cdc2, Cdk5, Cdk4, Cdk6, cyclin A, cyclin E, cyclin D1, PCNA, p21, and p27. The positions and sizes in kilodaltons of molecular weight markers that were run in parallel are shown on the right. Representative immunoblots from independent extracts is shown.

B) Immunoblots were scanned on a densitometer and the relative amount of protein in extracts from cells cultured in the absence (undiff PC12) or presence (diff PC12) of NGF was determined. The data shown represents the mean ± range/SEM of data from 2-3 separate experiments and are expressed relative to the initial amount of protein in undifferentiated PC12 cells. Cdk2, Cdc2, cyclin A, cyclin E, PCNA, and p27 n=3, Cdk5 and p21 n=2. * Significantly different from undifferentiated PC12 cells ($p<0.05$).

(A)


time in the presence of NGF (days)
0 2 4 7 10
MW
Cdk2
33.9
Cdk5
33.9
0 3 5 7
Cdc2
32.5
Cdk4
32.5
Cdk6
32.5
cyclin D1
32.5
0 2 4 7 10
cyclin A
50.4
cyclin E
50.4
PCNA
33.9
0 7
MW
p27
29.2
actin
47.5
p21
21.6
actin
47.5

(B)


% Cdk2 expression
*
undiff PC12
diff PC12
% Cdc2 expression
*
undiff PC12
diff PC12
% Cdk5 expression
undiff PC12
diff PC12
% PCNA expression
*
undiff PC12
diff PC12
% cyclin A expression
*
undiff PC12
diff PC12
% cyclin E expression
undiff PC12
diff PC12
% p21 expression
undiff PC12
diff PC12
% p27 expression
undiff PC12
diff PC12

by Yan and Ziff who reported that there is no change in Cdk5 protein level in PC12 cells following NGF treatment, but in contradiction with other studies that describe an increase in Cdk5 protein levels during NGF-induced PC12 differentiation (van Grunsven *et al.*, 1996a).

The formation of active Cdk complexes requires the binding of cyclins. It was therefore important to determine the effects of NGF on the expression of the cyclin proteins during PC12 and PC6-3 differentiation. The antibodies used to detect cyclin A, cyclin E, and cyclin D1 in these studies did bind to proteins of the expected size, however non-specific binding did occur. The PCNA antibody however was highly specific. As shown in Figure 3.2A, cyclin A and cyclin E protein levels gradually decreased after NGF. Figure 3.2B shows that the 14.4 ± 3.6 fold decrease (n=3) in cyclin A activity in PC12 cells after 7 days differentiation in the presence of NGF is significant ($p < 0.05$). In contrast, cyclin D1 protein levels increased after the addition of NGF. NGF treatment led to a dramatic decrease in proliferating cell nuclear antigen (PCNA), which is a molecular marker for cells in S phase (see Figure 3.2A). Figure 3.2B shows that this 78.3 ± 11.6% decrease in PCNA expression during NGF-induced differentiation is significant ($p < 0.05$). These results agree with previous work which showed that a decrease in cyclin A and increase in cyclin D1 protein levels occurs during PC12 differentiation (Dobashi *et al.*, 1995; Yan and Ziff, 1995; van Grunsven *et al.*, 1996b). A decrease in cyclin A and cyclin E protein levels was also observed during PC6-3 differentiation as seen in Figure 3.3. PCNA protein levels, however, remained constant.

Cdks are positively regulated by cyclins and negatively regulated by cyclin dependent kinase inhibitors. p21 and p27 are well known Cdk inhibitors that negatively regulate cyclin D, E and A-dependent kinases. To determine whether p21 and p27 expression changed during PC12 differentiation, p21 and p27 levels were determined in the absence and presence of NGF. p21 and p27 antibodies did bind to proteins of the expected size however, non-specific binding did occur. Figure 3.2A and B show that p21 and p27 protein levels increased, 15.5 ± 4.5 fold (n=2) and 1.8 ± 0.25 fold (n=3) respectively, in PC12 cells after 7 days of NGF treatment. An increase in p21 protein levels during PC12 differentiation has been previously reported (Dobashi *et al.*, 1995; Yan and Ziff, 1995; van Grunsven *et al.*, 1996b). Similarly, Figure 3.3 shows that treatment of PC6-3 cells with NGF causes an increase in p21 protein levels.

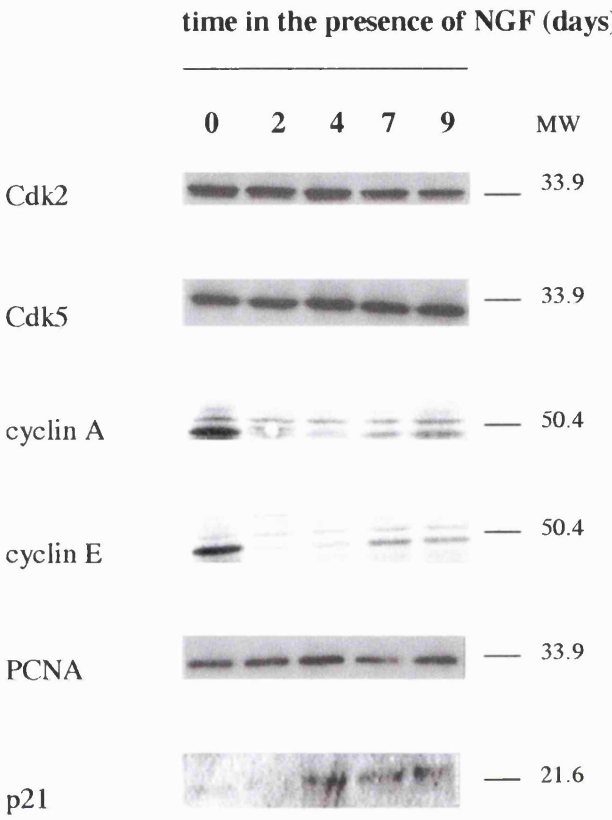


Figure 3.3 Pattern of expression of cell cycle proteins during the NGF-induced differentiation of PC6-3 cells.

PC6-3 cells were treated with NGF for 0, 2, 4, 7, and 9 days. Cell extracts were prepared and 30 μg of each extract was separated on 12% SDS-polyacrylamide gels. Immunoblotting was performed using antibodies specific for Cdk2, Cdk5, cyclin A, cyclin E, PCNA, and p21. The positions and size in kilodaltons of molecular weight markers that were run in parallel are shown on the right. Immunoblots represent independent extracts from the same differentiation experiment.

These results demonstrate that expression of cell cycle regulators are differentially regulated during the NGF-induced differentiation of PC12 and PC6-3 cells. The expression of cyclin A and cyclin E are reduced during differentiation of both PC12 and PC6-3 cells. However, PCNA, Cdk2, and Cdc2, which are crucial for cell proliferation, are dramatically reduced only in PC12 cells during differentiation.

3.2.2 Cdk and Cdk-associated kinase activity are regulated during the differentiation of PC12 cells.

The decrease in the level of the Cdk2, Cdc2, cyclin A, and cyclin E proteins during the NGF-induced differentiation of PC12 cells suggested that the kinase activity relating to these kinase complexes would also decrease. Cdk kinase activity was analysed in extracts prepared from PC12 or PC6-3 cells cultured in the absence or presence of NGF for 7 days. Cell lysates were incubated with antibodies to Cdk2, Cdc2, cyclin A, or cyclin E proteins and immunoprecipitation performed. The kinase activity in immune complexes was detected by using histone H1 as a substrate. Each experiment was performed 2-3 times; similar results were obtained in each experiment and representative data are shown.

Figure 3.4A and B show that there was a 78.6 ± 1.4% (n=2) decrease in Cdk2 kinase activity in PC12 cells after 7 days treatment with NGF, and a 53.2 ± 17.2% (n=2) decrease in Cdc2 kinase activity. Cyclin A-associated and cyclin E-associated kinase activities decreased to 24 ± 10.4% and 57.3 ± 30.1% of control cells respectively. Figure 3.5 shows that there was a 1.7 fold (n=1) decrease in Cdk2 kinase activity in NGF-treated PC6-3 cells compared to the untreated control. Cyclin A and cyclin E-associated kinase activities decreased 4.6 fold and 2 fold (n=1) respectively in PC6-3 cells after NGF treatment. These results agree with previous studies that also showed a decrease in both Cdk2 and Cdc2 kinase activity during the NGF-induced differentiation of PC12 cells (Buchkovich and Ziff, 1994; Dobashi *et al.*, 1995; Yan and Ziff, 1995).

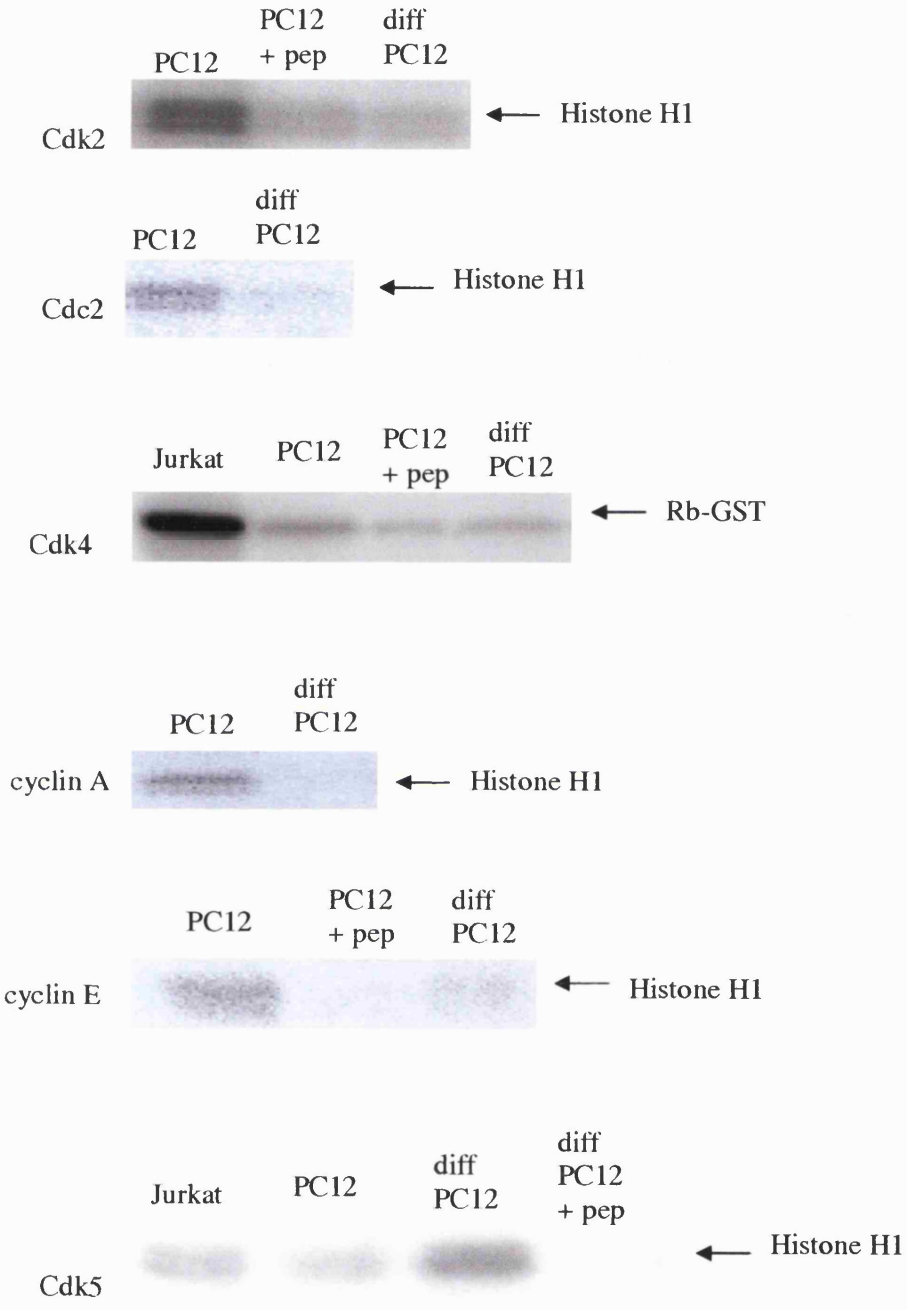
To measure Cdk4 kinase activity, Cdk4 was immunoprecipitated as described above but the kinase activity in immune complexes was detected by using GST-Rb as a substrate. As shown in Figure 3.4, Cdk4 kinase activity decreased 2.2-fold in PC12 cells following NGF treatment for 7 days. This confirms a previous report that Cdk4 kinase activity decreases in PC12 cells after NGF treatment (Yan and Ziff, 1995).

Figure 3.4 Cdk2, Cdc2, Cdk4, cyclin A-associated, and cyclin E-associated kinase activity decrease during the differentiation of PC12 cells, whereas Cdk5 kinase activity increases.

(A) Kinase assays were performed with independent extracts prepared from proliferating PC12 cells and differentiated PC12 cells that had been treated with NGF for 7 days (diff PC12). 30 μg of protein extract was used per immunoprecipitation. Blocking peptide (+pep) was added to positive controls to show that the kinase activity was specific to that kinase. Kinase activity in immune complexes was measured using histone H1 as a substrate, except in the case of Cdk4 for which Rb-GST was used. Reaction products were separated on 12% SDS polyacrylamide gels. After electrophoresis, gels were dried and substrate phosphorylation was detected by phosphorimaging.

(B) Relative kinase activity was determined by scanning phosphorimages on a densitometer. The level of kinase activity in undifferentiated PC12 cells was set at 100%. The results shown are the average of two independent experiments. Error bars indicate range.

(A)


PC12
PC12 + pep
diff PC12
Cdk2
Histone H1
PC12
diff PC12
Cdc2
Histone H1
Jurkat
PC12
PC12 + pep
diff PC12
Cdk4
Rb-GST
PC12
diff PC12
cyclin A
Histone H1
PC12
PC12 + pep
diff PC12
cyclin E
Histone H1
Jurkat
PC12
diff PC12
diff PC12 + pep
Cdk5
Histone H1

(B)

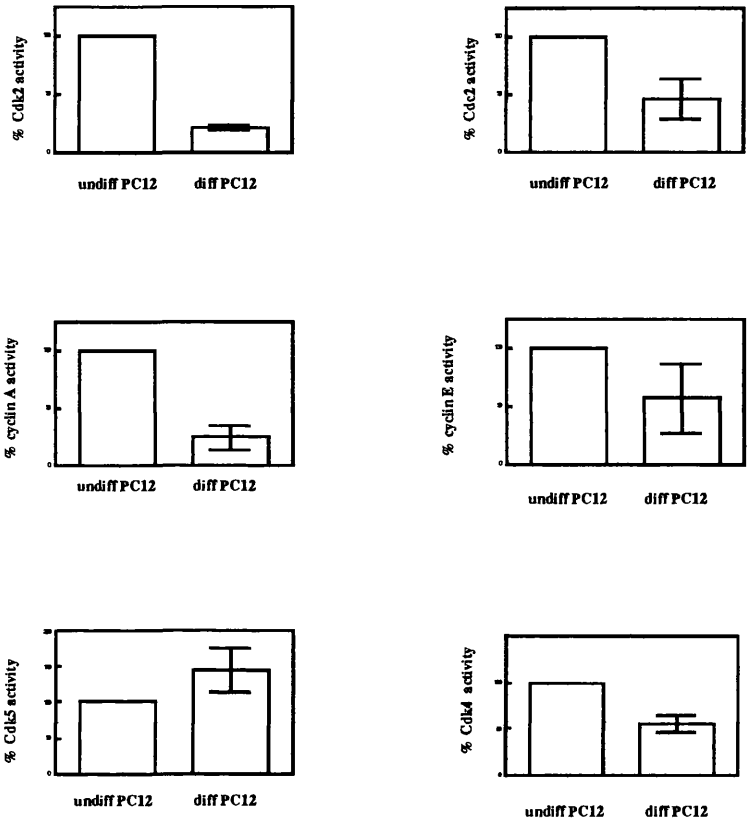
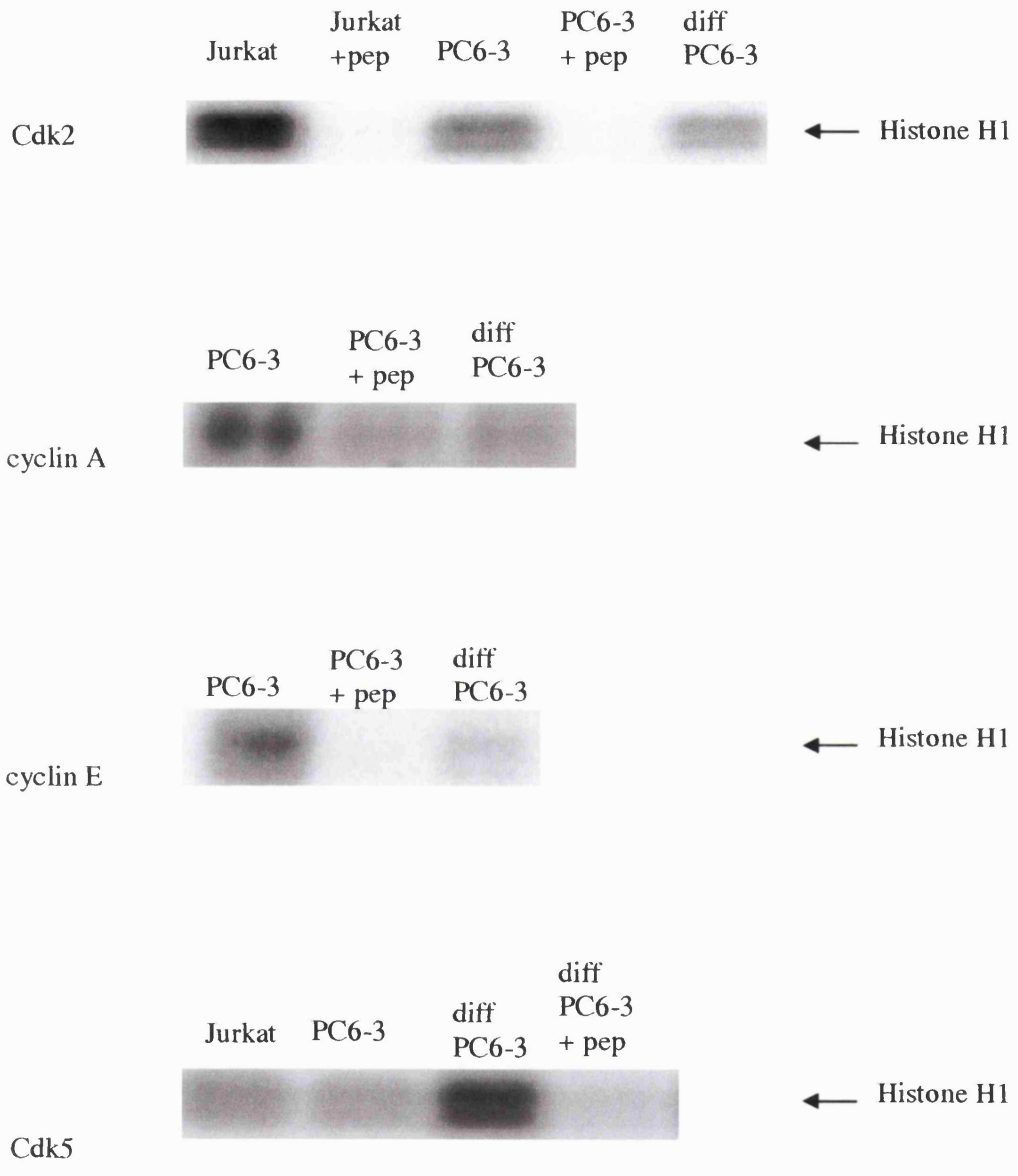

% Cdk2 activity
undiff PC12
diff PC12
% Cdc2 activity
undiff PC12
diff PC12
% cyclin A activity
undiff PC12
diff PC12
% cyclin E activity
undiff PC12
diff PC12
% Cdk5 activity
undiff PC12
diff PC12
% Cdk4 activity
undiff PC12
diff PC12

Figure 3.5 Cdk2, cyclin A-associated, and cyclin E-associated kinase activities decrease during NGF-induced differentiation of PC6-3 cells, whereas Cdk5 kinase activity increases.

Cell extracts were made from PC6-3 cells and differentiated PC6-3 cells that had been treated with NGF for 7 days (diff PC6-3). 30 μg of protein extract was used per immunoprecipitation. Blocking peptide (+pep) was added to positive controls (Jurkat cell extracts) to show that the kinase activity was specific to that kinase. Kinase activity in immune complexes was measured using histone H1 as a substrate. Reaction products were separated on 12% polyacrylamide gels. Histone H1 phosphorylation was detected by phosphorimaging. Phosphoimages represent kinase activity in independent extracts from the same experiment.


Jurkat
Jurkat +pep
PC6-3
PC6-3 + pep
diff PC6-3
Cdk2
Histone H1
PC6-3
PC6-3 + pep
diff PC6-3
cyclin A
Histone H1
PC6-3
PC6-3 + pep
diff PC6-3
cyclin E
Histone H1
Jurkat
PC6-3
diff PC6-3
diff PC6-3 + pep
Cdk5
Histone H1

Cdk5 protein levels increased slightly during PC12 differentiation. Cdk5 kinase activity was assessed as described above using histone H1 as the substrate. Figure 3.4A and B show that Cdk5 kinase activity increased 1.5 ± 0.3 fold (n=2) in PC12 cells after treatment with NGF for 7 days. Cdk5 kinase activity also increased in PC6-3 cells after NGF treatment (2.2-fold) as seen in Figure 3.5.

A blocking peptide to the various proteins, i.e. Cdk2, Cdk4, cyclin E, cyclin A, and Cdk5, was added to the in vitro kinase reaction of the positive controls (e.g. proliferating PC12 cells for Cdk2, Cdk4, cyclin E kinase activity, and differentiated PC12 cells for Cdk5 kinase activity). This showed that the kinase activity being measured was specific to the particular antibody being used to immunoprecipitate the protein. In the presence of a blocking peptide to a specific protein, the kinase activity associated with that protein will be inhibited in the kinase reaction (see Figure 3.4A and 3.5). Another control that could have been carried out would involve adding a different blocking peptide to the protein used in order to show that this does not block kinase activity. The amount of background phosphorylation could have been determined by carrying out a kinase reaction with no extract present. It is also important to quantify the amount of protein that was immunoprecipitated. This would ensure that the change in kinase activity observed is not due to different amounts of proteins being immunoprecipitated.

These results indicate that as well as regulating the expression of cell cycle regulators in PC12 and PC6-3 cells, NGF also regulates their activity.

3.3.3 Analysis of cell cycle-related protein expression during the NGF withdrawal-induced apoptosis of differentiated PC12 cells.

PC12 cells have proved to be a useful model system for studying neuronal cell death. NGF withdrawal causes neuronally differentiated PC12 cells to undergo apoptosis (Batistatou and Greene, 1991). Although I studied the differentiation of both PC12 and PC6-3 cells, I chose to focus on PC12 cells for NGF withdrawal experiments because my results suggest that PC12 cells exit the cell cycle more consistently when compared to PC6-3 cells. Following NGF differentiation of PC6-3 cells, Cdk2 and PCNA levels remained constant which could suggest a certain population of these cells are still in S phase. If this was the case any changes in Cdk expression or activity observed during apoptosis would be difficult to interpret as it could be due to a cell cycle effect rather than apoptosis. To determine the effects of

NGF withdrawal on the expression of cell cycle regulatory proteins, PC12 cells were differentiated for 7 days in the presence of NGF. After differentiation, NGF was removed and replaced with medium containing anti-NGF antibody. Protein extracts were prepared at various times after NGF withdrawal, notably 0, 24, 48, and 72 hours. Equal amounts of each extract were then resolved using 12% SDS polyacrylamide gels. Immunoblot analysis using antibodies specific for each cell cycle regulator was performed. Figure 3.6A shows the pattern of expression of cell cycle proteins during the NGF withdrawal-induced death of PC12 cells. Cdk2, Cdc2, and PCNA protein levels increased after NGF withdrawal, whereas Cdk4 and Cdk5 protein levels remained constant. Cdk2 expression increased 1.5 ± 0.1 fold (n=2), 48 hours after NGF withdrawal, and Cdc2 increased 1.8 ± 0.3 fold (n=2), as shown in Figure 3.6B. PCNA expression increased 1.95 ± 0.35 fold (n=2), 48 hours after NGF withdrawal compared to NGF treated cells (see Figure 3.6B). Cdk5 expression did not change after NGF withdrawal in two independent experiments (see Figure 3.6B). It has been previously reported that Cdc2 and PCNA protein levels increase in apoptotic PC12 cells, however, it has also been reported that there is an increase in Cdk4 protein levels and no change in Cdk2 levels (Davis *et al.*, 1997).

Since cyclins are important regulators of Cdks it was important to look at the expression of these proteins during the apoptosis of differentiated PC12 cells. Figure 3.6A shows that cyclin A, cyclin E, and cyclin D1 protein levels remained constant during the NGF withdrawal-induced death of PC12 cells.

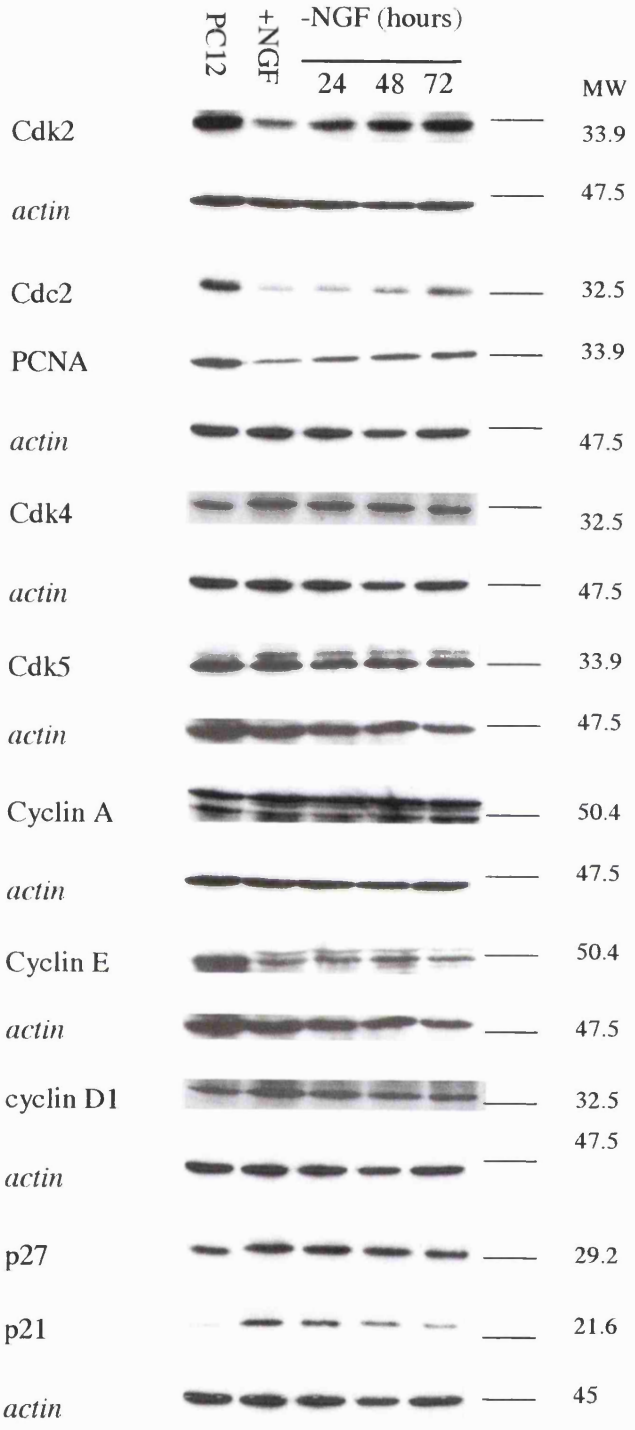
Cdk inhibitors negatively regulate Cdk activity so I also looked at the expression of these proteins during NGF withdrawal-induced apoptosis. Figure 3.6A shows that p27 levels remained constant during apoptosis of differentiated PC12 cells. However, p21 protein levels gradually decreased following NGF withdrawal. Figure 3.6B shows there is a 2.5 ± 0.2 fold (n=2) decrease in p21 expression following NGF withdrawal from differentiated PC12 cells.

Figure 3.6 Pattern of expression of cell cycle proteins during NGF withdrawal-induced death of PC12 cells.

(A) PC12 cells were differentiated with NGF for 7 days (+NGF). After differentiation, the medium was removed and replaced with medium lacking NGF that contained anti-NGF antibody. Cell extracts were made at 24, 48, and 72 hours after NGF withdrawal and 30 μg of protein extract was separated on 12% SDS polyacrylamide gels. Immunoblotting was performed using antibodies specific for Cdk2, Cdc2, Cdk4, Cdk5, cyclin A, cyclin E, cyclin D1, PCNA, p21, and p27. The membranes were reprobed with an actin antibody to control for any differences in loading. The positions and sizes in kilodaltons of molecular weight markers that were run in parallel are shown on the right. Immunoblots for Cdk2 and cyclin E, Cdc2 and PCNA, Cdk4 and cyclin D1, and p21 and p27 are from the same extracts.

(B) Densitometrical analyses of cell cycle regulator proteins. Values were calculated from two independent experiments and were normalised to those at time 0. Results were shown as mean ± range.

(A)


PC12
+NGF
-NGF (hours)
24
48
72
MW
Cdk2
33.9
actin
47.5
Cdc2
32.5
PCNA
33.9
actin
47.5
Cdk4
32.5
actin
47.5
Cdk5
33.9
actin
47.5
Cyclin A
50.4
actin
47.5
Cyclin E
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cyclin D1
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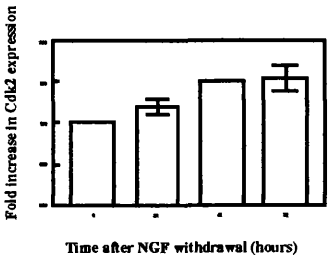
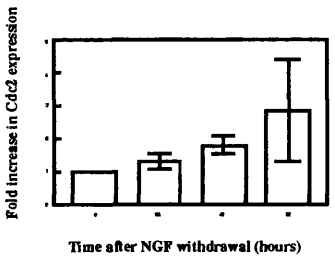
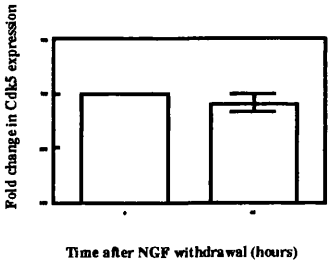
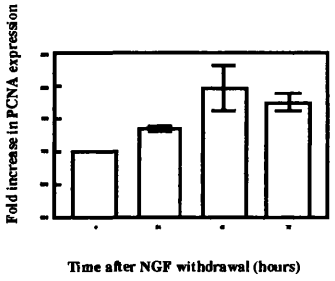
p27
29.2
p21
21.6
actin
45

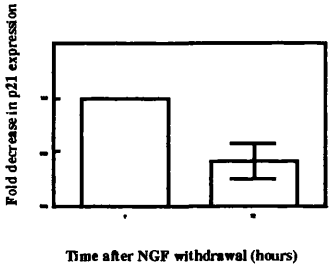
(B)


Fold increase in Cdk2 expression
Time after NGF withdrawal (hours)


Fold increase in Cdc2 expression
Time after NGF withdrawal (hours)


Fold change in Cdk5 expression
Time after NGF withdrawal (hours)

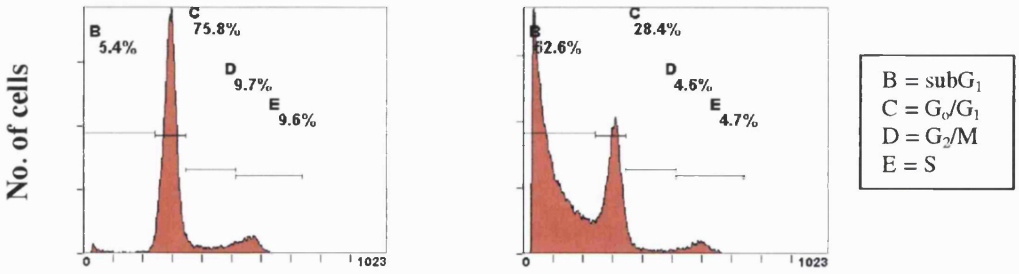

Fold increase in PCNA expression
Time after NGF withdrawal (hours)


Fold decrease in p21 expression
Time after NGF withdrawal (hours)

The level of cell death following NGF deprivation from differentiated PC12 cells at these time points was also determined. In the first instance, PI staining of DNA and assessing the sub-diploid population of cells from a cell cycle profile was used to quantitate apoptosis by flow cytometry. Figure 3.7A shows the DNA profile of proliferating PC12 cells, which represents a typical cell cycle profile of cycling cells (Nicoletti *et al.*, 1991). The DNA profile of PC12 cells changed dramatically after NGF-induced differentiation as seen in Figure 3.7B. The sub-diploid population of cells increased from 7.8 ± 1.0% (mean ± SEM, n=4) to 60.4 ± 2.8% (see Figure 3.7C). This gives the impression that there is an increase in the amount of apoptotic cells after NGF-induced differentiation. However, this is not the case as this sub-diploid population is due to debris, probably arising from fragmented neurites that occur after lysing the cells in hypotonic buffer (see Material and Methods section 2.2.1.6). Figure 3.7D shows the DNA profile of PC12 cells deprived of NGF for 48 hours. Since the sub-diploid background is very high (Figure 3.7B), there is no increase in this population after NGF withdrawal. Therefore, the effect of roscovitine on NGF withdrawal-induced apoptosis could not be determined using this technique.

Secondly, the Cell Proliferation MTS assay (Materials and Methods section 2.2.1.7), was used to determine the viability of differentiated PC12 cells after NGF withdrawal. PC12 cells were plated and differentiated for 7 days in collagen-coated 96 well plates in NGF containing medium. Cells were then deprived of NGF for various amounts of time before determining viability by incubating cells with the MTS tetrazolium compound and measuring absorbance at 490 nm. It was important to optimise the density of PC12 cells that would differentiate in a collagen-coated 96 well plate and give rise to a cell death curve after NGF withdrawal. The optimal density of cells that differentiated in the presence of NGF and produced a cell death curve after NGF withdrawal, were 2.5 x 10^3 cells/well. However, Figure 3.8A shows data from 3 separate experiments, whereby cells were plated at 2.5 x 10^3/well, and indicates a great variability between individual experiments. This variability could arise from dissimilar levels of differentiation between individual experiments, so that when NGF is removed varying proportions of the cells have become dependent on NGF for survival. Due to this variation this assay was considered unsuitable for measuring loss of cell viability in PC12 cells after NGF withdrawal.

(A) PC12 (B) diff PC12

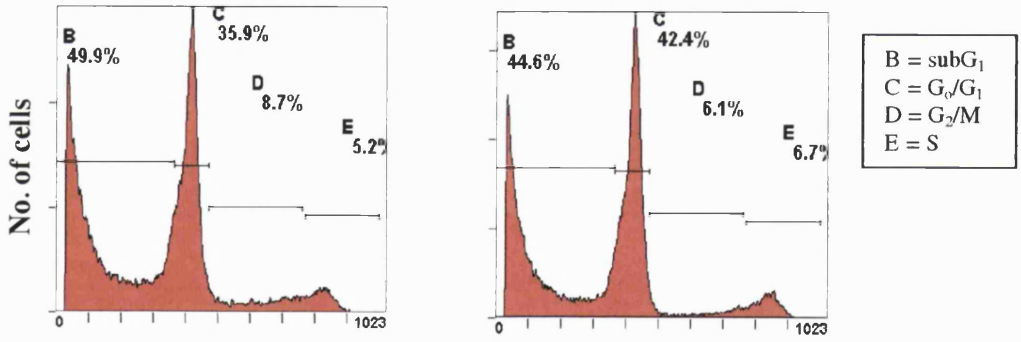


DNA content

(C)

	% of cells			
	$subG_1$	G_0/G_1	G_2/M	S
PC12	7.8 ± 1.0	75.2 ± 0.8	9.4 ± 1.4	8.0 ± 0.6
Diff PC12	60.4 ±2.8	28.3 ±1.4	7.2 ± 1.5	4.3 ± 0.4

(D) diff PC12 –NGF 48 hrs (E) diff PC12 –NGF 48 hrs + roscovitine 25 μM

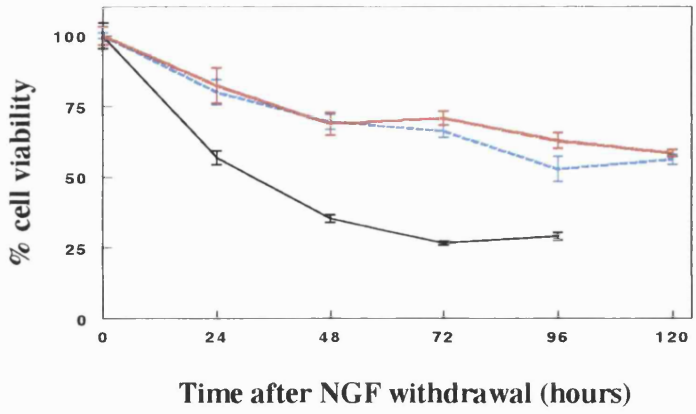


DNA content

Figure 3.7 Effect of NGF on cell cycle profile of PC12 cells

PC12 cells were differentiated in 100 ng/ml of NGF containing medium for 7 days. Cells were processed for cell cycle analysis with PI as described in Materials and Methods section 2.2.1.6.

(A)



(B)

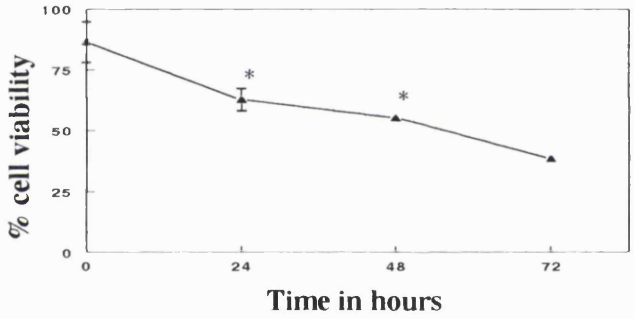


Figure 3.8 Viability of differentiated PC12 cells decrease after NGF withdrawal.

A) PC12 cells were differentiated for 7 days in collagen-treated 96 well plates in the presence of medium containing 100 ng/ml of NGF. Cells were then deprived of NGF for various amounts of times and cell viability was determined using the MTS viability assay (see section 2.2.1.7).

B) Cell viability was also determined using the the live/dead viability/cytotoxicity assay kit (see section 2.2.1.5). * Significant difference compared to cells grown in the presence of NGF $p < 0.05$.

The viability of PC12 cells cultured in the presence and absence of NGF was also determined using the live/dead viability/cytotoxicity assay kit (see section 2.2.1.5). Figure 3.8B shows that there is a significant difference in cell viability between cells grown in the absence of NGF for 24 and 48 hours, compared to cells grown in the presence of NGF. The viability of cells decrease from 86.6 ± 8.0% (mean ± SEM, n=3) to 62.8 ± 4.7% 24 hours after NGF withdrawal, and 55.2 ± 1.2% of cells are viable by 48 hours after NGF withdrawal. The increase in Cdk2, Cdc2, and PCNA levels observed correlates with an increase in NGF withdrawal-induced death. This assay was also used to assess the effect of roscovitine on NGF withdrawal-induced death of PC12 cells

In summary, these results show that Cdk2, Cdc2, and PCNA protein levels increase during the NGF withdrawal-induced death of differentiated PC12 cells whereas p21 levels decline.

3.3.4 Cdk2 kinase activity increases after NGF withdrawal in differentiated PC12 cells

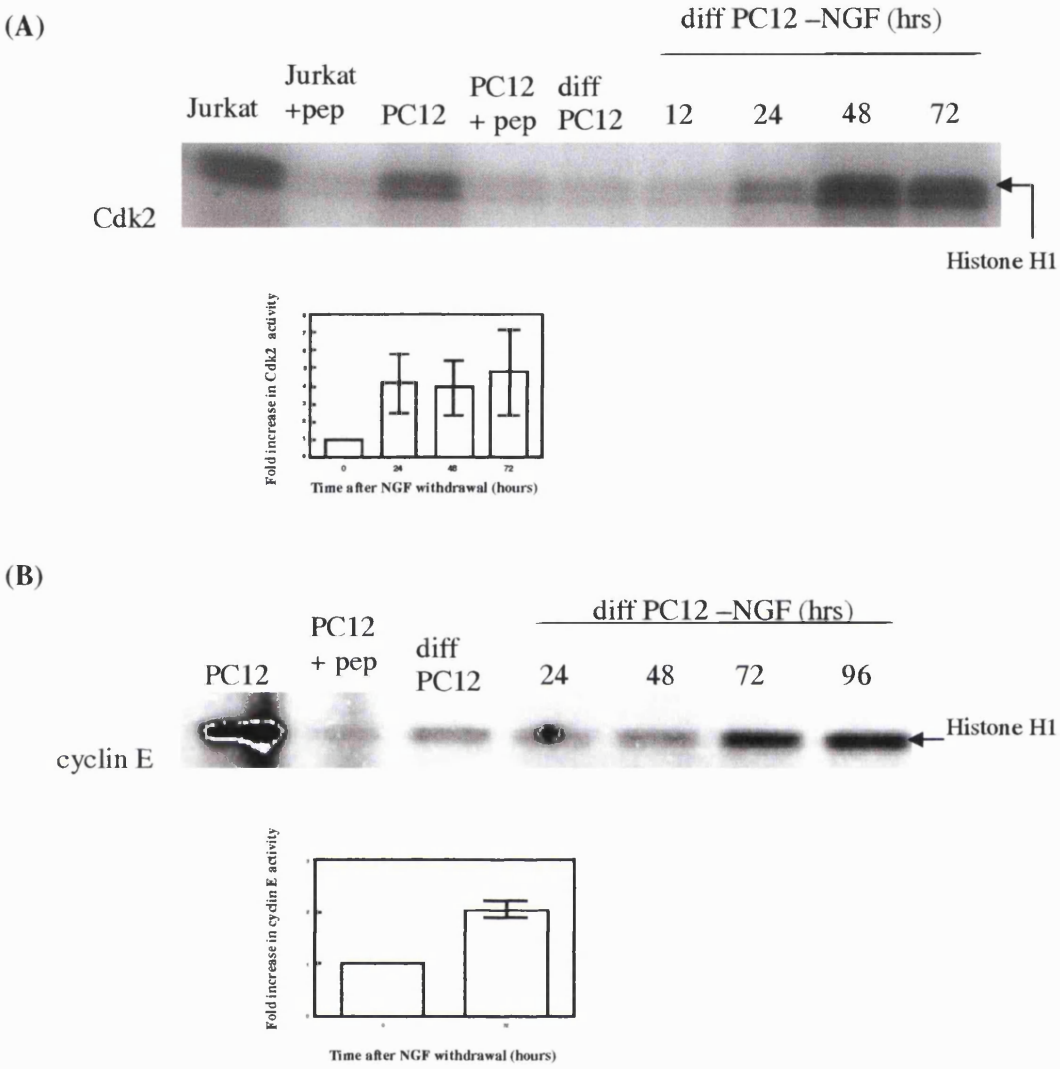
The increase in Cdk2 and Cdc2 protein levels that occurs during NGF withdrawal-induced apoptosis suggests that the kinase activity of these proteins may also increase during apoptosis. Cdk kinase activity was analysed in extracts prepared from differentiated PC12 cells cultured in the presence of NGF and at various timepoints after NGF withdrawal. Equal amounts of cell lysate were incubated with antibodies to Cdk2, Cdc2, Cdk5, Cdk4, cyclin A, or cyclin E proteins and immunoprecipitation carried out. The kinase activities of immune complexes were detected by using histone H1 or Rb-GST as a substrate. These experiments were performed 2-3 times and representative gels are shown.

Figure 3.9A shows that during the NGF withdrawal-induced apoptosis of differentiated PC12 cells, Cdk2 kinase activity increases. A 4.15 ± 1.65 (mean ± SEM, n=3) fold increase in Cdk2 kinase activity is observed at 24 hours after NGF withdrawal and a 3.9 ± 1.5 fold (n=3) increase after 48 hours. The Cdk2 kinase activity at 72 hours after NGF withdrawal is 4.75 ± 2.45 fold (n=3) higher than in the presence of NGF. As shown in Figure 3.9B cyclin-E associated kinase activity also increases during NGF withdrawal-induced apoptosis of PC12 cells.

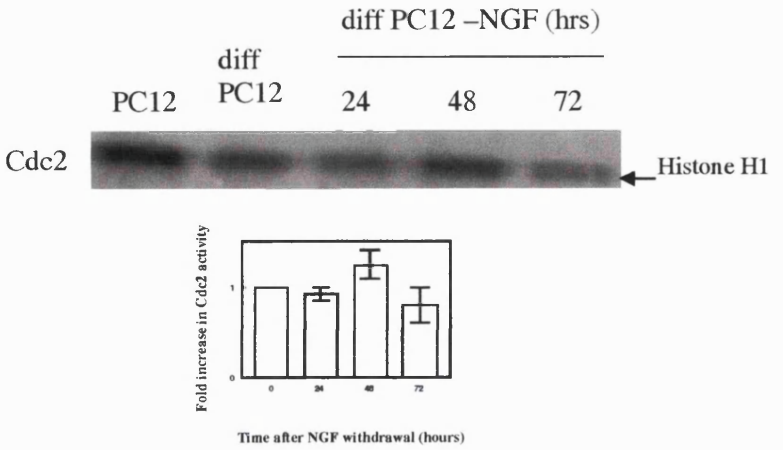
Figure 3.9 Cdk2 and cyclin E-associated kinase activity increases during NGF withdrawal-induced apoptosis of PC12 cells whereas cyclin A-associated, Cdk5, Cdc2, and Cdk4 kinase activity remain constant.

PC12 cells were differentiated in medium containing NGF for 7 days (diff PC12). After differentiation, the NGF-containing medium was removed and replaced with medium containing anti-NGF antibody. Cell extracts were made at 12, 24, 48, 72, or 96 hours after NGF withdrawal. 30 μg of protein from independent extracts was used per immunoprecipitation and blocking peptide (+pep) was added to positive controls to show that the kinase activity was specific to that kinase. The kinase activity in immune complexes was measured using histone H1 or Rb-GST as the substrate. Cycling Jurkat cells were used as a positive control in some experiments.

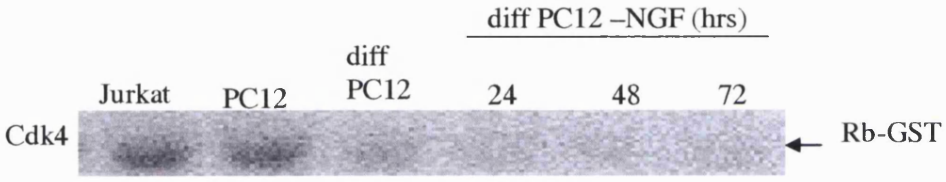
Relative kinase activity was determined by densitometry analyses. Each point is the mean ± range/SEM of data from 2-3 separate experiments and was normalised to those at time 0. Cdk2 kinase activity (n=3), Cdk5, Cdc2, and cyclin E-associated kinase activity (n=2).


(A)
diff PC12 –NGF (hrs)
Jurkat
Jurkat +pep
PC12
PC12 + pep
diff PC12
12
24
48
72
Cdk2
Histone H1
Fold increase in Cdk2 activity
Time after NGF withdrawal (hours)
(B)
diff PC12 –NGF (hrs)
PC12
PC12 + pep
diff PC12
24
48
72
96
cyclin E
Histone H1
Fold increase in cyclin E activity
Time after NGF withdrawal (hours)
(C)
diff PC12 –NGF (hrs)
PC12
diff PC12
24
48
72
cyclin A
Histone H1

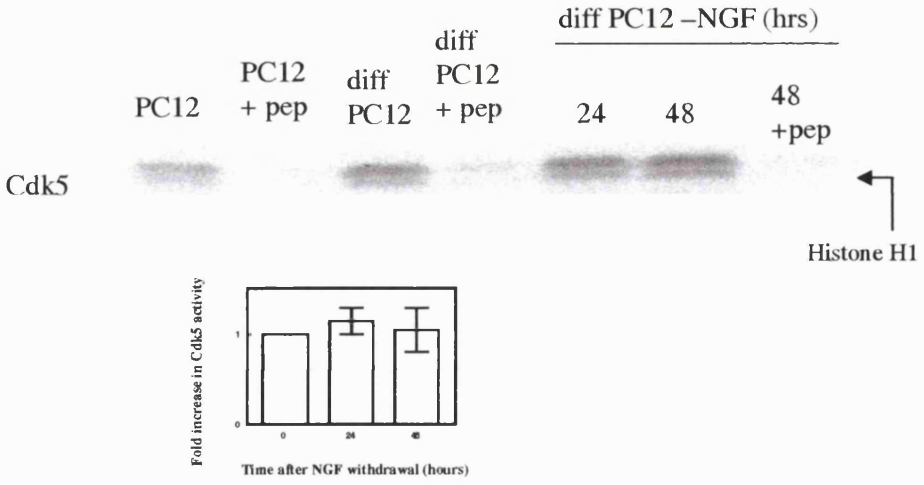
(D)


diff PC12 –NGF (hrs)
diff PC12
PC12
24
48
72
Cdc2
Histone H1
Fold increase in Cdc2 activity
Time after NGF withdrawal (hours)

(E)


diff PC12 –NGF (hrs)
diff PC12
Jurkat
PC12
24
48
72
Cdk4
Rb-GST

(F)


diff PC12 –NGF (hrs)
diff PC12 + pep
PC12
PC12 + pep
diff PC12
24
48
48 +pep
Cdk5
Histone H1
Fold increase in Cdk5 activity
Time after NGF withdrawal (hours)

At 72 hours after NGF withdrawal, cyclin E-associated kinase activity is 2.05 ± 0.15 (n=2) fold higher compared to that seen with differentiated PC12 cells cultured in the presence of NGF. Cyclin A-associated (Figure 3.9C), Cdc2 (Figure 3.9D), Cdk4 (Figure 3.9E), and Cdk5 (Figure 3.9F) kinase activities remain constant during differentiated PC12 cell apoptosis. The average activity of Cdc2 and Cdk5, from two individual experiments, after NGF withdrawal from PC12 cells is shown in Figure 3.9D and 3.9F respectively.

3.3.5 Effect of roscovitine, a pharmacological Cdk inhibitor, on Cdk activity and NGF withdrawal-induced death of PC12 cells.

Roscovitine is a small molecule Cdk inhibitor that specifically inhibits Cdk2, Cdc2, and Cdk5. Cdk2 and Cdc2 protein levels and Cdk2 kinase activity increase during NGF withdrawal-induced apoptosis of PC12 cells. Therefore it was important to determine whether roscovitine could inhibit these kinases in PC12 cells and if so, whether it had any effect on cell death. PC12 cells were differentiated with NGF for 7 days. After differentiation, the culture medium was replaced with medium lacking NGF containing anti-NGF antibody, or anti-NGF antibody and 10 or 25 μM roscovitine, for 24, 48, 72, and 96 hours. Protein extracts were prepared and equal amounts of protein were resolved using 12% SDS polyacrylamide gels. Immunoblot analysis using antibodies for Cdk2, Cdc2, and PCNA was performed. Cell viability was determined at 48 hours after NGF withdrawal in the presence and absence of roscovitine using the live/dead viability /cytotoxicity assay kit as described in the Materials and Methods chapter.

25 μM roscovitine inhibited the increase in Cdk2, Cdc2, and PCNA protein levels observed during the NGF withdrawal-induced death of PC12 cells (Figure 3.10A). Figure 3.10B shows that the increase in Cdk2 kinase activity during PC12 apoptosis was also blocked by 25 μM roscovitine.

Figure 3.10C shows that at 48 hours after NGF withdrawal roscovitine clearly protects differentiated PC12 cells against NGF withdrawal-induced death. The difference in cell death between roscovitine-treated and untreated PC12 cells is significant as determined by Student's t-test: for 10 μM roscovitine $p = 0.009$; for 25 μM roscovitine $p = 0.001$.

Figure 3.10 Roscovitine, a pharmacological Cdk inhibitor, can block NGF withdrawal-induced apoptosis of PC12 cells, and the increase in Cdk2, Cdc2, and PCNA protein levels and Cdk2 kinase activity.

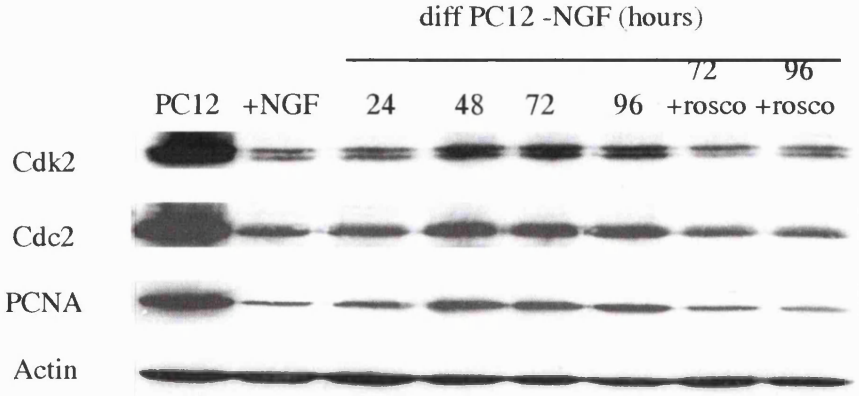
PC12 cells were differentiated in medium containing NGF for 7 days (+NGF). After differentiation, the NGF-containing medium was removed and replaced with medium containing anti-NGF antibody, or anti-NGF antibody and roscovitine at 25 μM (unless specified otherwise).

(A) Cell extracts were prepared at 0, 24, 48, and 72 hours after NGF withdrawal and 30 μg of protein was separated on 12% SDS polyacrylamide gels. Immunoblotting was performed using antibodies specific for Cdk2, Cdc2, and PCNA. The membranes were reprobed with an anti-actin antibody to control for differences in protein loading.

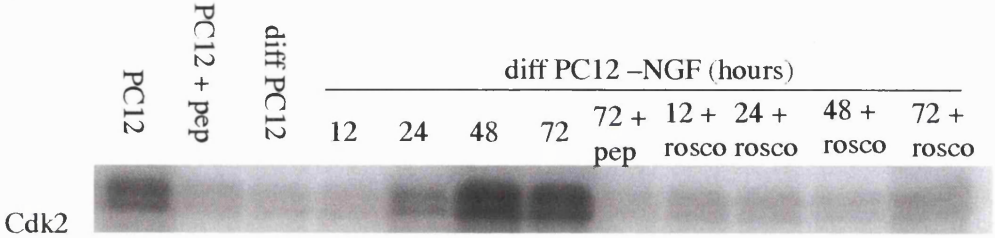
(B) Cell extracts were prepared at 0, 12, 24, 48, and 72 hours after NGF withdrawal. Approximately 30 μg of protein was used per immunoprecipitation and blocking peptide (+pep) was added to positive controls to show that the kinase activity was specific to that kinase. The kinase activity of immune complexes was measured using histone H1 as the substrate.

(C) Cell viability was determined at 0 and 48 hours after NGF withdrawal in the absence or presence of roscovitine using the Live/dead cell viability/cytotoxicity kit. The values for 10 and 25 μM roscovitine were compared to the -NGF value in a t-test. In both cases the increase in the % of viable cells was significant: * $p = 0.009$; # $p = 0.001$.

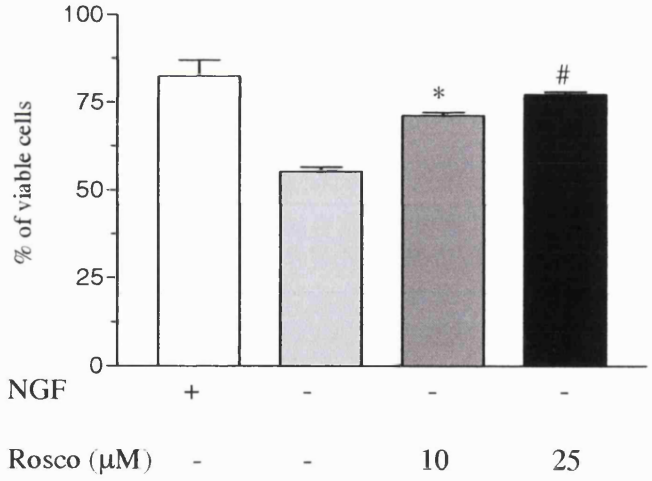
(A)


diff PC12 -NGF (hours)
PC12
+NGF
24
48
72
96
72 +rosco
96 +rosco
Cdk2
Cdc2
PCNA
Actin

(B)


PC12
PC12 + pep
diff PC12
diff PC12 –NGF (hours)
12
24
48
72
72 + pep
12 + rosco
24 + rosco
48 + rosco
72 + rosco
Cdk2

(C)


100
75
50
25
0
% of viable cells
*
#
NGF
+
-
-
-
Rosco (μM)
-
-
10
25

3.3.6 PC12 cells do not re-enter the cell cycle after NGF withdrawal.

PCNA is an S phase marker, so the increase in PCNA protein levels observed during NGF withdrawal-induced apoptosis of PC12 cells suggests that cells may be re-entering the cell cycle. To determine whether this was the case or not, 10^3 or 10^5, PC12 cells were differentiated for 7 days in collagen-coated 96 well microtitre plates. Cells were refed with medium containing fresh NGF, or medium lacking NGF supplemented with anti-NGF antibody for 24, 48, or 72 hours. Cells were then labelled with BrdU, fixed, incubated with a monoclonal BrdU antibody conjugated to peroxidase, washed and then incubated with the substrate tetramethylbenzidine. Absorbance was measured at 490 nm using a microplate reader.

Figure 3.11 shows that there is no significant increase in BrdU incorporation in PC12 cells during NGF withdrawal-induced apoptosis. This confirms that the increase in PCNA levels after NGF withdrawal is an apoptotic effect and not associated with a progression of the PC12 cells into S phase.

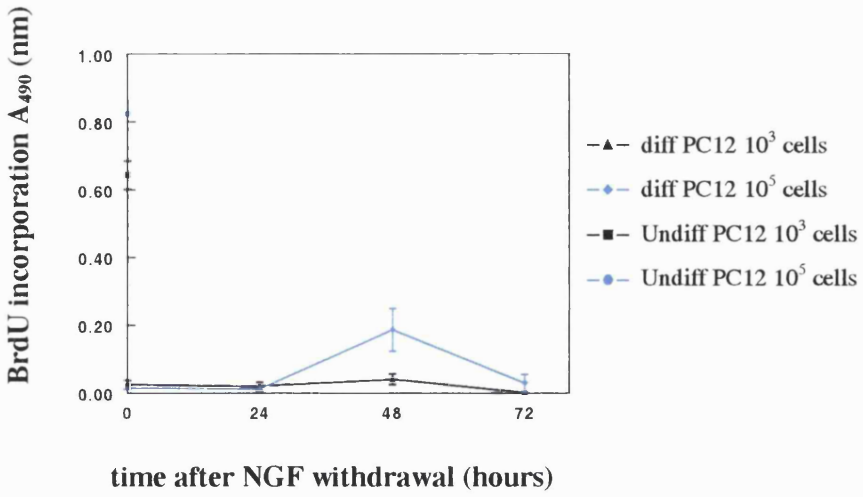


Figure 3.11 There is no change in DNA synthesis during NGF withdrawal-induced apoptosis of PC12 cells.

PC12 cells were differentiated for 7 days in medium containing 100 ng/ml of NGF. After differentiation, NGF was removed and DNA synthesis during NGF withdrawal-induced apoptosis was measured using BrdU incorporation as described in the Materials and Methods section 2.2.1.8. BrdU incorporation was also measured in proliferating PC12 cells (undiff PC12)

3.3 Discussion

The results described in this chapter confirm that the PC12 lines that I used differentiate into neuron-like cells in the presence of NGF, and analysis of cell cycle-associated protein expression and activity suggests that the cells exit from the cell cycle. During the NGF withdrawal-induced apoptosis of PC12 cells Cdk2, Cdc2, and PCNA protein levels increased whereas p21 protein levels decreased. The increase in Cdk2 protein levels correlates with an increase in Cdk2 kinase activity during NGF withdrawal-induced death. Roscovitine, a Cdk inhibitor, blocks the increases in Cdk2, Cdc2, and PCNA protein levels and Cdk2 kinase activity and slows down NGF withdrawal-induced death.

Several cell cycle-associated proteins have been directly implicated in the terminal differentiation process including the retinoblastoma protein (pRb) and the cyclin dependent kinase inhibitor p21 (Yan and Ziff, 1995; Erhardt and Pittman, 1998a). Moreover, it has been shown that these proteins have dual regulatory effects on cell cycle progression and cell differentiation that occur by distinct mechanisms (Di Cunto *et al.*, 1998; Novitch *et al.*, 1999). In order to investigate the role of Cdks in the NGF withdrawal-induced apoptosis of PC12 cells it was important to confirm that the cells exit the cell cycle when differentiated. Since the differentiated PC12 cells I used are out of the cell cycle any changes in cell cycle regulator expression or activity observed during NGF withdrawal-induced death, are not due to a cell cycle effect.

During PC12 cell differentiation there is a slight decrease in the levels of Cdk4 and Cdk6, G1 phase regulators of the cell cycle. Although there is an increase in cyclin D1 protein levels during PC12 differentiation there is a decrease in Cdk4 kinase activity. This may be explained by the increase in the level of the Cdk inhibitors, p21 and p27, which could inhibit Cdk4 kinase activity and cause the cells to stay in G1. A decrease in Cdk2, Cdc2, cyclin A, and cyclin E protein expression during NGF-induced differentiation of PC12 cells, correlates with a decrease in their respective kinase activities. The decrease in Cdk2 and Cdc2 protein levels and kinase activity during NGF-induced differentiation of PC12 cells has been shown previously (Buchkovich and Ziff, 1994; Dobashi *et al.*, 1995; Yan and Ziff, 1995). These results are consistent with the cells exiting from the cell cycle because Cdk2-cyclin E and Cdk2-cyclin A complexes are required for S phase progression, whereas

Cdc2-cyclin A is required for M phase progression (Sherr, 1994). The increase in p21 and p27 levels may also be responsible for the decrease in Cdk2 and Cdc2 kinase activity observed during differentiation. The decrease in the level of PCNA, which is an S phase marker, is consistent with previous studies (Yan and Ziff, 1995) and further suggests that the cells have exited from the cell cycle. In the case of PC6-3 cell differentiation there is also a decrease in cyclin A, and cyclin E protein levels as well as their particular kinase activities. There is no change in Cdk2 expression, however there is a decrease in Cdk2 activity, which is probably due to the decrease in cyclin A and cyclin E levels. Similarly p21 protein levels increase during differentiation of PC6-3 cells. However, PCNA protein levels do not decrease during the NGF-induced differentiation of PC6-3 cells, which might mean that there are cells in S phase of the cell cycle. In both of the PC12 clones that I studied there is an increase in Cdk5 protein levels during differentiation that corresponds with an increase in Cdk5 kinase activity. It has been reported that expression of Cdk5 increases progressively in the nervous system as increasing numbers of cells exit the proliferative cycle (Tsai *et al.*, 1993b). The increase in Cdk5 protein levels and kinase activity suggests that Cdk5 might play a functional role in the process of PC12 differentiation.

These results suggest that the PC12 cells that I have used fully differentiate and exit the cell cycle following treatment with NGF and therefore serve as a useful tool to look at the role of cell cycle regulators during NGF withdrawal-induced apoptosis. NGF appears to be preventing G_1 progression due to the decrease in Cdk4 kinase activity and the increase in p21 and p27 levels, whereas the increase in cyclin D1 levels is indicative of a G_0/G_1 phase arrest. NGF is also preventing the transition from G_1 into S phase by reducing the expression of Cdk2 and cyclin E. DNA synthesis is being affected by NGF as there is a reduction in Cdk2 and cyclin A levels, as well as PCNA. The reduction in Cdc2 suggests another function of NGF is to block mitosis. During NGF-induced differentiation of PC6-3 cells the levels of Cdk2 and PCNA remain fairly constant. This would suggest that a proportion of cells may be in S phase of the cell cycle and therefore still synthesising DNA. In order to be sure of this it would be necessary to measure the amount of BrdU incorporation during NGF-induced differentiation of PC6-3 cells, as well as looking at additional S phase markers.

Previous studies have provided evidence that cell cycle proteins, including Cdks and cyclins, may be involved in the apoptotic process (O'Connor *et al.*, 2000). For example, it has been reported that Cdk2 is a key regulator of thymocyte apoptosis (Gil-Gomez *et al.*, 1998). Cdk2 kinase activity significantly increases during thymocyte apoptosis and this increase in kinase activity in not associated with cyclin A or cyclin E. In addition, it has also been shown that the pharmacological Cdk inhibitors, flavopiridol and olomoucine can inhibit NGF withdrawal-induced apoptosis of PC12 cells.

The results described in this chapter show that Cdk2, Cdc2, and PCNA protein levels increase during the NGF withdrawal-induced apoptosis of PC12 cells. The increase in Cdk2 protein levels (1.5 ± 0.0 fold, at 48 hours) correlates with a more dramatic increase in Cdk2 kinase activity (3.9 ± 1.5 fold, at 48 hours). The increase in Cdk2 activity can not be completely contributed to the increase in Cdk2 protein as activation of Cdk proteins requires the binding of specific cyclins. Cyclin A or E usually bind to and activate Cdk2. Cyclin A and cyclin E protein levels remain constant during the NGF withdrawal-induced apoptosis of PC12 cells. Cyclin E-associated kinase activity increases 2.05 ± 0.15 fold, during PC12 cell apoptosis, but its time course of induction is 48 hours later than that of Cdk2 activity. The increase in Cdk2 kinase activity coincides with a decrease in the protein levels of the Cdk inhibitor, p21. Although Cdc2 protein levels increase during NGF withdrawal-induced apoptosis (1.8 ± 0.3, at 48 hours), Cdc2 kinase activity stays the same (1.25 ± 0.15, at 48 hours). This could be due to inhibition of kinase activity by the presence of the Cdk inhibitor p27, or due to a lack of the positive regulators cyclin A or cyclin B. PCNA protein levels also increase during NGF withdrawal-induced apoptosis of PC12 cells. PCNA is required for DNA replication. It encircles double stranded DNA in a homotrimeric form and functions as a sliding clamp required for procession of DNA polymerase δ and ε (Kelman and Hurwitz, 1998). Binding of p21 to PCNA suppresses PCNA-dependent DNA replication (Li *et al.*, 1994). The increase in PCNA protein levels and the decrease in p21 levels during NGF withdrawal-induced apoptosis of PC12 cells suggests that there maybe an increase in DNA synthesis and cells may be re-entering the cell cycle. However this does not appear to be the case as there is no significant increase in DNA synthesis following NGF withdrawal.

Roscovitine, a pharmacological Cdk inhibitor selective for Cdk2, Cdc2, and Cdk5, was able to protect against NGF withdrawal-induced apoptosis. The increase in Cdk2, Cdc2, and PCNA protein levels, as well as the increase in Cdk2 kinase activity, was inhibited by roscovitine. Roscovitine functions as a Cdk inhibitor by competing with ATP for binding to active Cdk/cyclin complexes. This property of roscovitine can explain how the increase in Cdk2 activity is blocked in PC12 cells after NGF withdrawal but it does not explain how roscovitine can prevent the increase in Cdk2, Cdc2, and PCNA protein levels. By blocking Cdk2 activity roscovitine may be preventing the phosphorylation of targets required to increase Cdk2, Cdc2, and PCNA protein levels e.g. transcription factors. A common putative transcription factor binding site found in the upstream region of Cdk2, Cdc2, and PCNA genes includes the C-myb binding site (Ferber *et al.*, 1991; Ku *et al.*, 1993; Shiffman *et al.*, 1996). C-myb has been shown to regulate the mRNA levels of PCNA (Travali *et al.*, 1991), as well as transactivate Cdc2 expression (Ku *et al.*, 1993). Induction of C- and B-myb proteins occur in cortical neurons, sympathetic neurons, and neuronal PC12 cells in response to DNA damage or NGF withdrawal, and overexpression of these proteins causes neuronal apoptosis (Liu and Greene, 2001). The mechanism by which mybs induce apoptosis is unclear, however as these proteins are transcription factors it seems likely that may regulate proapoptotic genes. It has been shown that Cdk2/cyclin A phosphorylates B-myb at sites that enhance its transactivation properties (Saville and Watson, 1998). Another putative transcription factor binding site identified in the upstream region of the human Cdk2 gene is Sp1 (Shiffman *et al.*, 1996). Cdk/cyclin A catalyses the phosphorylation and modulates the transcriptional activity of Sp1 (Haidweger *et al.*, 2001). Phosphorylation of Sp1 is required for increased FasL transcription during apoptosis of smooth muscle cells (Kavurma *et al.*, 2001). To link these findings with the results discussed in this chapter, an attractive suggestion would be that the increase in Cdk2 activity after NGF withdrawal results in the phosphorylation of Sp1 and/or myb proteins, that are responsible for the transactivation of Cdk2, Cdc2, and PCNA. This would suggest a positive feedback loop whereby Cdk2 activation results in an increase in Cdk2 protein, further enhancing Cdk activity and thereby increasing substrate phosphorylation.

Another possible explanation to why roscovitine prevents the increase in Cdk2, Cdc2, and PCNA protein levels following NGF withdrawal is that roscovitine

may be inhibiting protein synthesis. Inhibition of protein synthesis promotes survival of neuronally differentiated PC12 cells and sympathetic neurons deprived of NGF (Martin *et al.*, 1988). To determine whether roscovitine had an effect on protein synthesis the amount of leucine incorporation after NGF withdrawal in the absence and presence of roscovitine could have been measured. It has been reported that at least 80% inhibition of protein synthesis is required to protect sympathetic neurons from NGF withdrawal (Martin *et al.*, 1992). 1 μM flavopiridol and 200 μM olomoucine inhibited leucine incorporation in cultures of differentiated PC12 cells or sympathetic neurons by 25-30%. It was therefore concluded that it was unlikely that the mechanism by which flavopiridol and olomoucine rescue postmitotic neurons and PC12 cells is by inhibition of protein synthesis (Park *et al.*, 1996). It would also have been interesting to determine whether the increased expression of Cdk2, Cdc2, and PCNA was blocked by protein synthesis inhibitors e.g. cycloheximide. Roscovitine may also be blocking JNK activity which is required for NGF withdrawal-induced apoptosis of PC12 cells (Eilers *et al.*, 1998). To determine if roscovitine was blocking JNK activity in this system it would have been interesting to look at JNK activity in the absence and presence of roscovitine after NGF withdrawal. Roscovitine has been reported to be more potent at rescuing neurons from neurotrophic withdrawal-induced death than in inhibiting JNK activity (Maas *et al.*, 1998).

The results described in this chapter suggest that Cdk2 has a functional role in the NGF withdrawal-induced death of PC12 cells because Cdk2 kinase activity increases after NGF-withdrawal, and because inhibition of this activity using roscovitine blocks cell death.

Chapter 4 Expression and function of cell cycle regulators during NGF withdrawal-induced apoptosis of sympathetic neurons

4.1 Introduction

To further investigate the role of cell cycle regulators in neuronal apoptosis I chose to study developing sympathetic neurons cultured *in vitro*, a model of developmental neuronal cell death. Sympathetic neurons from rat superior cervical ganglia provide the most extensively characterised model for apoptosis in the nervous system. Sympathetic neurons depend on NGF for their survival both *in vivo* and *in vitro*. In the rat, essentially all SCG neurons are post-mitotic at the time of birth, and approximately 30% of them die between postnatal days 3 and 7 (Hendry, 1977; Wright *et al.*, 1983). *In vitro*, the removal of NGF from sympathetic neuron cultures results in nearly complete death by 48-72 hours (Martin *et al.*, 1988; Martin *et al.*, 1992) and DNA fragmentation, chromatin condensation, and membrane blebbing typical of apoptosis occur in the dying neurons (Edwards and Tolkovsky, 1994). Inhibitors of transcription or protein synthesis can protect sympathetic neurons against NGF withdrawal-induced death, suggesting that in this system new gene expression is required for death to occur (Martin *et al.*, 1988).

Studies investigating the mechanism by which neurons die when deprived of neurotrophic support have revealed the involvement of several regulatory components, for example, caspases (Gagliardini *et al.*, 1994; Troy *et al.*, 1997), pro- and antiapoptotic members of the bcl-2 family (Garcia *et al.*, 1992; Greenlund *et al.*, 1995b; Deckwerth *et al.*, 1996), and a requirement for the transcription of specific genes including *c-jun* (Estus *et al.*, 1994; Ham *et al.*, 1995). Cell cycle regulatory proteins have also been implicated in neuronal cell death. In sympathetic neurons, trophic factor withdrawal-induced death is associated with an increase in the level of cyclin D1 mRNA (Freeman *et al.*, 1994), and overexpression of cyclin D1 in neuroblastoma cells induces apoptosis (Kranenburg *et al.*, 1996). Moreover, chemical inhibitors of Cdks and DN forms of Cdk4 or Cdk6 promote survival of NGF-deprived sympathetic neurons (Park *et al.*, 1997a). Although these observations point to Cdk4 and Cdk6 having a functional role in neuronal cell death, the Cdk

inhibitor olomoucine is not an effective inhibitor of these kinases but does inhibit neuronal apoptosis. This therefore suggests that other Cdks may be involved.

In many cell types undergoing apoptosis, cytochrome c is released from the mitochondrial intermembrane space into the cytosol, where it interacts with Apaf-1 to trigger ATP-dependent autocatalytic processing of procaspase-9 (Li *et al.*, 1997). Caspase-9 then activates caspase-3 and other caspases. Microinjection of cytochrome c into the cytosol of living cells induces apoptosis (Li *et al.*, 1997). During sympathetic neuronal death, the release of cytochrome c from the mitochondria occurs before caspase activation and requires protein synthesis-dependent events (Deshmukh and Johnson, 1998). It has been shown that microinjection of an anti-cytochrome c antibody protects sympathetic neurons from apoptosis induced by NGF withdrawal (Neame *et al.*, 1998).

In this chapter I investigated whether roscovitine and other pharmacological inhibitors could protect sympathetic neurons from apoptosis, and whether roscovitine could prevent release of mitochondrial cytochrome c. I then went on to look at the expression and activity of various cell cycle regulators in sympathetic neurons following NGF withdrawal.

4.2 Results

4.2.1 Pharmacological Cdk inhibitors protect sympathetic neurons from NGF withdrawal-induced apoptosis

Since it is difficult to carry out biochemical analysis using primary sympathetic neurons, due to limitations in cell numbers, effects of the pharmacological Cdk inhibitors roscovitine, HD, and NG-75 on sympathetic neuron apoptosis were initially determined. Roscovitine, is a purine analogue and has been reported to specifically inhibit Cdk2, Cdc2, and Cdk5 kinase activity (Sielecki *et al.*, 2000). Two newly developed Cdk inhibitors, NG-75 (Chang *et al.*, 1999) and HD (Meijer *et al.*, 2000), are more potent Cdk inhibitors and like roscovitine also specifically inhibit Cdk2, Cdc2, and Cdk5 kinase activity (see Table 1.1). The viability of NGF-deprived neurons cultured with or without the various Cdk inhibitors, or in fresh NGF-containing medium, was determined after 48 hrs using the Live/dead viability/cytotoxicity assay kit. In parallel, TUNEL and Hoechst staining of neurons deprived of NGF in the presence or absence of 25 μM roscovitine, or cultured in fresh NGF-containing medium for 24 hours, was performed.

Figure 4.1 shows some typical fluorescence pictures of neurons incubated with calcein AM and ethidium homodimer 1, which were used to determine cell viability. In the presence of NGF, sympathetic neurons are viable and convert calcein AM to calcein, which fluoresces green. Neurons look healthy and their cell bodies are of similar size and have a smooth green appearance. Few cells fluoresce red. This indicates that cells are able to exclude ethidium homodimer 1. In contrast, neurons deprived of NGF are unable to exclude ethidium homodimer 1 because they have lost membrane integrity and as a result a larger percentage of cells fluoresce red. In addition, these neurons are reduced in size (atrophied) and the green fluorescence is very uneven. Neurons deprived of NGF but incubated with 25 μM roscovitine have a similar appearance to neurons cultured in the presence of NGF. Cell bodies are of similar size, have a smooth green appearance, are largely viable and few cells have red fluorescence. These findings are represented graphically in Figure 4.2.

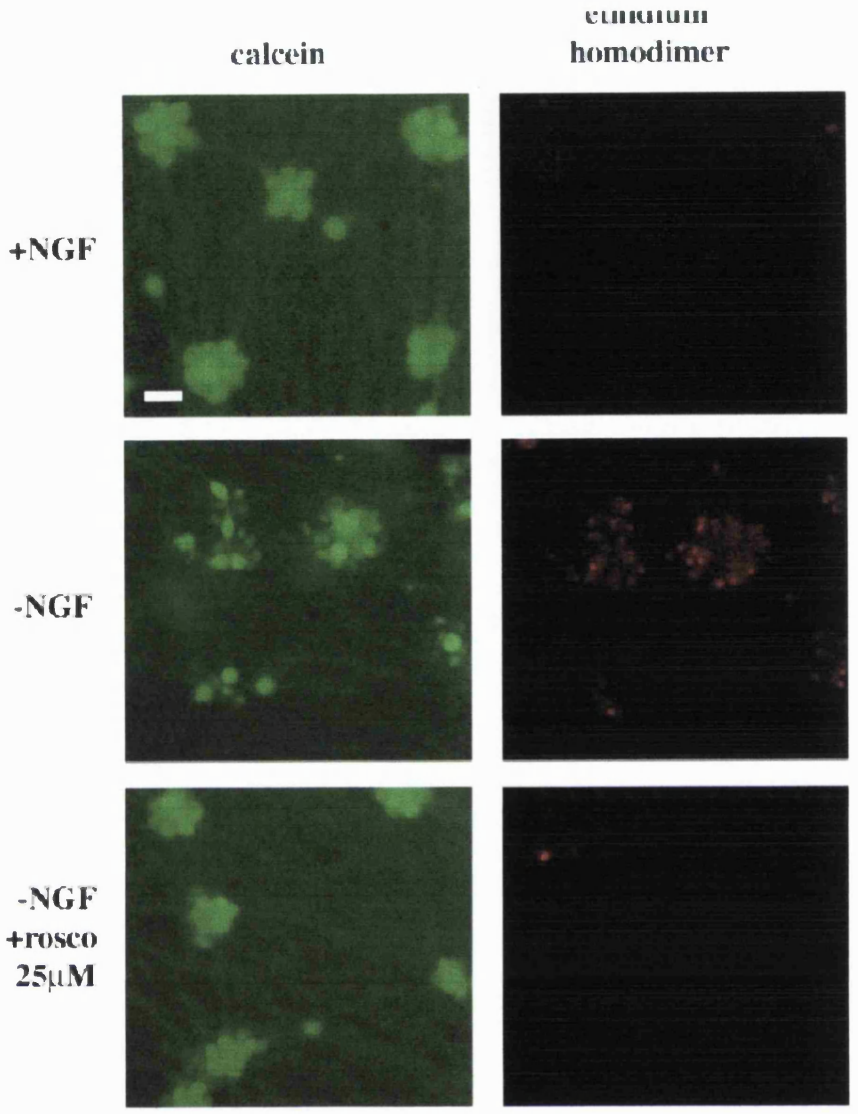


Figure 4.1 The Cdk inhibitor roscovitine protects sympathetic neurons against NGF withdrawal-induced apoptosis. Sympathetic neurons were isolated from neonatal rats and cultured for 7 days in the presence of NGF. The cells were then refed with medium lacking NGF, which had been supplemented with neutralising anti-NGF antibody (-NGF), with (-NGF+rosco25) or without 25 μM roscovitine, or with fresh NGF-containing medium (+NGF). 48 hours later, cell viability was determined using the live/dead viability/cytotoxicity assay kit. In the presence of NGF, the cells had a smooth green appearance and few cells were red. After NGF withdrawal, the green fluorescence became very uneven and a greater proportion of cell fluorsce red. Bar, 25 μm.

Figure 4.2A shows that 25 μM roscovitine was able to significantly protect sympathetic neurons from NGF withdrawal-induced death, whereas 10 μM roscovitine was not. At 48 hours after NGF withdrawal, 46.2 ± 6.6% (mean ± SEM from 3 experiments) of the neurons were alive, whereas 78 ± 8.2% survival was observed for neurons cultured in 25 μM roscovitine. 50 μM roscovitine also provided significant protection from NGF deprivation as seen in Figure 4.2B. After 48 hours in the absence of NGF, 54.6 ± 3.6% of neurons were viable, this increased to 87 ± 1% for cells cultured in medium containing 25 and 50 μM roscovitine.

Figure 4.2C shows that 10 μM HD and 10 μM NG-75, which are more potent Cdk inhibitors, are able to significantly protect sympathetic neurons from NGF withdrawal-induced death. After NGF withdrawal, 53 ± 5.2% of neurons were viable, whereas 80.7 ± 4.2% cell viability was obtained when cells are cultured in 10 μM HD, and 84.6 ± 1.4% cell viability in the presence of 10 μM NG-75. 25 μM HD and 25 μM NG-75 were also able to significantly protect sympathetic neurons from apoptosis causing 81.9 ± 4.4% and 80.1 ± 3.2% survival respectively. Maximal protection against NGF withdrawal-induced death was obtained with 25 μM roscovitine, and 10 μM HD and 10 μM NG-75. This data is consistent with the fact that HD and NG-75 are more potent Cdk inhibitors than roscovitine.

The fragmentation of chromosomal DNA into oligonucleosomal-sized fragments is a characteristic of apoptosis. Figure 4.3 shows that 25 μM roscovitine was able to significantly inhibit NGF withdrawal-induced DNA fragmentation in sympathetic neurons ($p < 0.05$, Student's t-Test).

In summary, these results show that roscovitine, HD, and NG-75 protect sympathetic neurons from NGF withdrawal-induced apoptosis, and this therefore suggests that Cdk2, Cdc2, and/or Cdk5 kinase activity is required for the NGF withdrawal-induced death of sympathetic neurons.

Figure 4.2 The pharmacological Cdk inhibitors, roscovitine, HD, and NG-75, protect sympathetic neurons from NGF withdrawal-induced apoptosis.

Sympathetic neurons were isolated from neonatal rats and cultured in the presence of NGF for 7 days. The cells were then refed with fresh NGF-containing medium, or with medium lacking NGF, which had been supplemented with neutralising anti-NGF antibody, + or –, and:

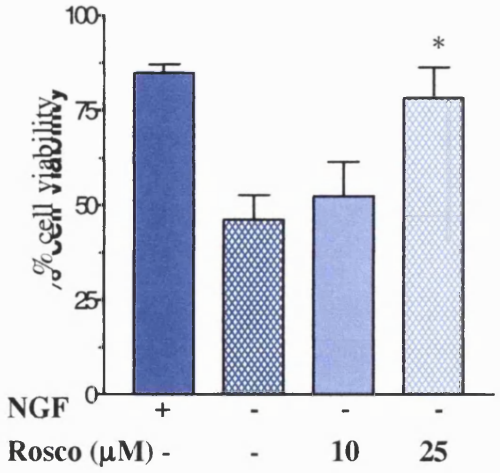
C) 10 μM roscovitine, 25 μM roscovitine,

D) 50 μM roscovitine,

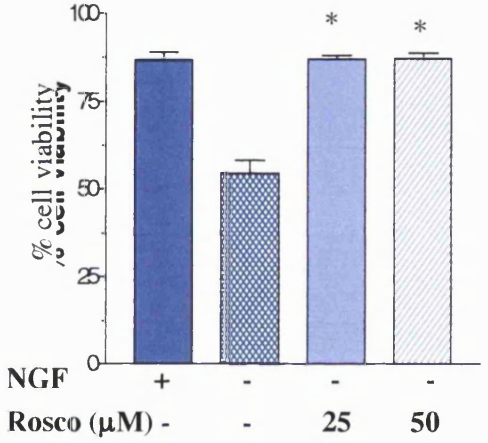
E) 10 μM HD, 25 μM HD, 10 μM NG-75, 25 μM NG-75.

48 hours later, cell viability was determined using the live/dead viability/cytotoxicity assay kit as described in Material and Methods. Student's t-tests were performed comparing the viability of cells cultured in anti-NGF antibody with cells cultured in medium containing the various Cdk inhibitors. * $p < 0.05$.

(A)


100
75
50
25
0
% cell viability
*
NGF + - - -
Rosco (μM) - - 10 25

(B)


100
75
50
25
0
% cell viability
*
*
NGF + - - -
Rosco (μM) - - 25 50

(C)

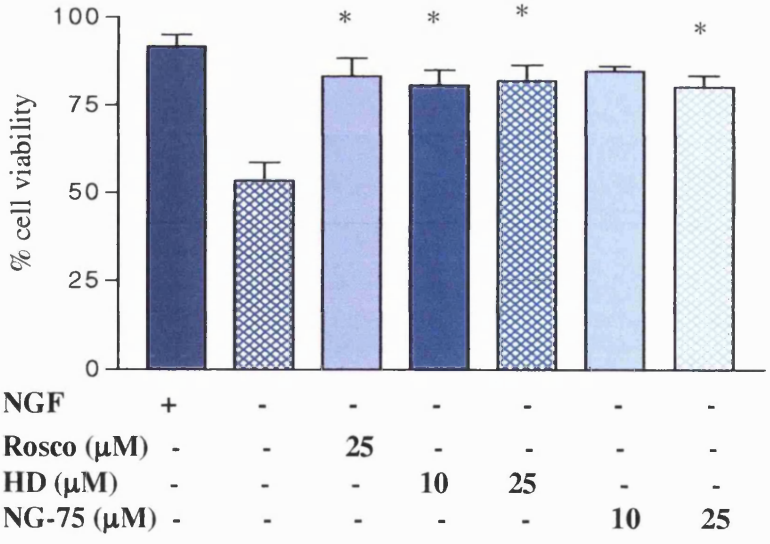
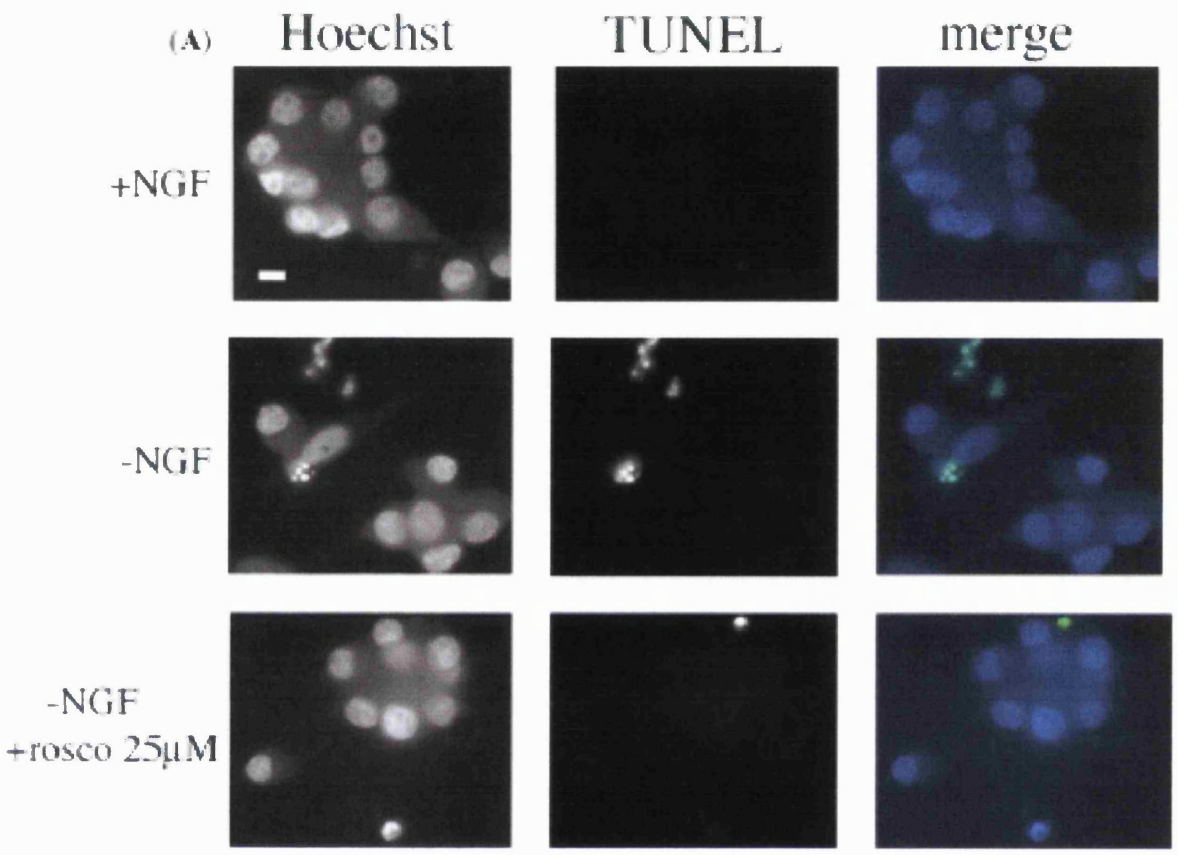
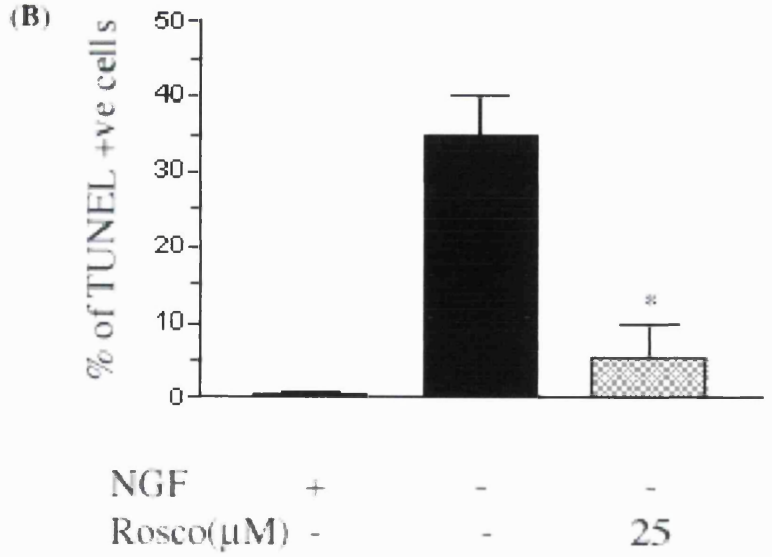

100
75
50
25
0
% cell viability
*
*
*
*
NGF + - - - - - -
Rosco (μM) - - 25 - - - -
HD (μM) - - - 10 25 - -
NG-75 (μM) - - - - - 10 25

Figure 4.3 Roscovitine inhibits NGF withdrawal-induced DNA fragmentation in sympathetic neurons.

(A) Sympathetic neurons were cultured on glass coverslips in the presence of NGF for 7 days and then refed with fresh medium containing NGF (+NGF), or lacking NGF (-NGF), or lacking NGF and containing 25 μM roscovitine (-NGF+rosco25). 24 hours later, the cells were fixed and TUNEL analysis and staining with Hoechst dye was performed as described in Materials and Methods (Chapter 2). In the presence of NGF, the nuclei stain evenly with Hoechst dye and a very low percentage are TUNEL positive. After NGF withdrawal, Hoechst staining reveals numerous condensed, distorted or fragmented nuclei, many of which are TUNEL positive. Roscovitine reduces the proportion of TUNEL positive cells caused by NGF withdrawal. Bar, 10 μm.

(B) Graphical representation of 3 experiments showing the mean % of TUNEL positive cells ± SEM. * $p < 0.05$ as determined by the Student's t-test.


(A)
Hoechst
TUNEL
merge
+NGF
-NGF
-NGF
+rosco 25μM


(B)
% of TUNEL +ve cells
50
40
30
20
10
0
*
NGF + - -
Rosco(μM) - - 25

4.2.2 Roscovitine prevents the release of mitochondrial cytochrome c in sympathetic neurons deprived of NGF.

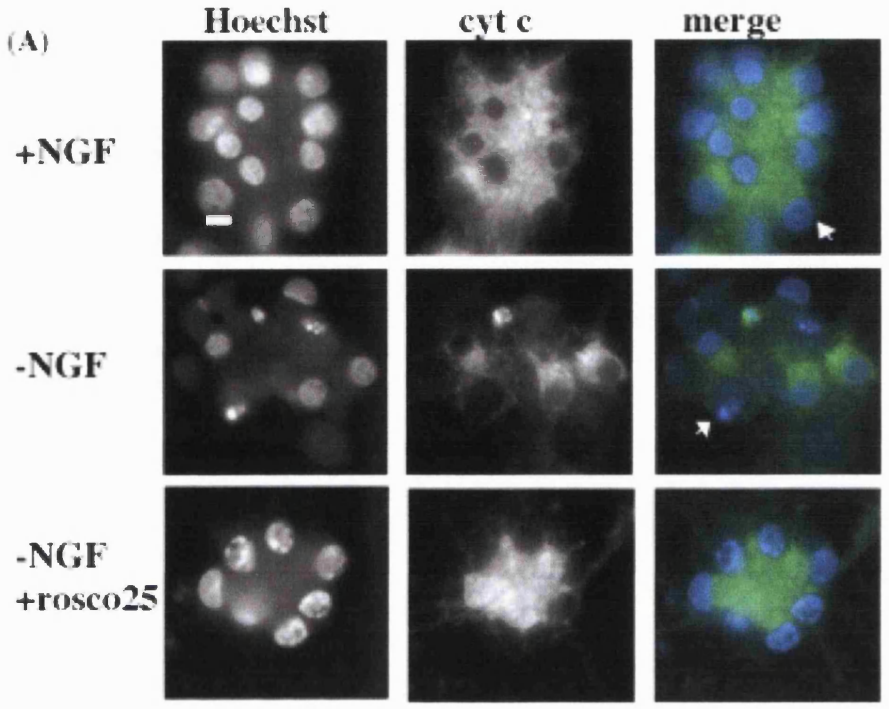
Release of cytochrome c from the mitochondria intermembrane space occurs during sympathetic neuronal death (Deshmukh and Johnson, 1998; Neame *et al.*, 1998), so it was important to determine if the effect of roscovitine was upstream or downstream of cytochrome c release.

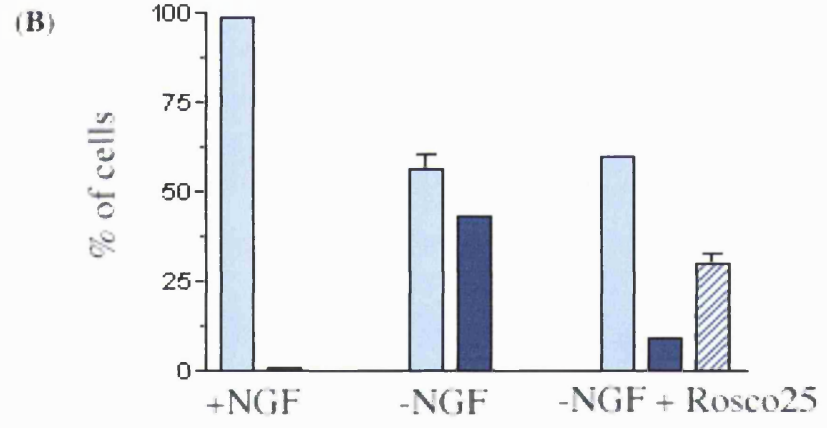
Figure 4.4A shows some typical immunofluorescence pictures of neurons stained with anti-cytochrome c antibody and Hoechst 33342 dye. The number of cells with normal or pyknotic nuclei, and with normal (punctate) or redistributed (diffuse) cytochrome c was determined. In the presence of NGF, cytochrome c immunoreactivity is excluded from the nuclear space and has a punctate pattern and the neurons have normal nuclei. In the absence of NGF, cytochrome c has a fainter, diffuse staining pattern that occurs throughout the whole cell and the neurons have pyknotic nuclei. In neurons cultured with 25 μm roscovitine in the absence of NGF, cytochrome c staining is punctate and excluded from the nuclear space, however, more of the nuclei are pyknotic compared to the control (+NGF). Figure 4.4B represents the average result from two experiments. In the presence of NGF ~ 100% of cells had normal nuclei and punctate cytochrome c staining. After removal NGF for 48 hours, ~ 45% of the cells had pyknotic nuclei and diffuse cytochrome c staining. 25 μM roscovitine significantly reduced the percentage of cells with diffuse cytochrome c, but the cells still had pyknotic nuclei, although the nuclear changes were not as pronounced as those observed in cells deprived of NGF. These results suggest that roscovitine inhibits cytochrome c release but some of the nuclear changes typical of NGF withdrawal-induced death can still occur. This is discussed in more detail later in the chapter.

Figure 4.4 Roscovitine prevents the release of cytochrome c from the mitochondria of sympathetic neurons deprived of NGF.

(A) Sympathetic neurons were cultured in the presence of NGF for 7 days and then refed with fresh medium containing NGF (+NGF), or lacking NGF (-NGF), or lacking NGF and supplemented with 25 μM roscovitine (-NGF+rosco25). After 48 hours, the cells were fixed and incubated with anti-cytochrome c antibody and stained with Hoechst dye. In the presence of NGF, cytochrome c immunoreactivity is excluded from the nuclear space and has a punctate pattern (see white arrow in the merged image). In the absence of NGF, there is a fainter, more diffuse staining pattern that occurs throughout the whole cell (arrow indicates a representative cell). Neurons incubated with 25 μM roscovitine have a similar cytochrome c staining pattern to cells cultured in the presence of NGF.

(B) The number of cells with normal or pyknotic nuclei, and with normal or diffuse cytochrome c was scored. The average of the results from two experiments is shown with the range. Bar, 10 μm.





normal nuclei
normal cytochrome c

pyknotic nuclei
diffuse cytochrome c

pyknotic nuclei
normal cytochrome c

4.2.3 Cdk2 antisense oligonucleotides protect sympathetic neurons from NGF withdrawal-induced apoptosis.

To determine whether levels of Cdk2 protein determine the susceptibility to apoptosis, the effect of Cdk2 antisense oligonucleotides on the survival of sympathetic neurons deprived of NGF was examined. Sympathetic neurons were pulsed with medium containing 2 μM Cdk2 antisense oligonucleotides (Cdk2AS), 2 μM randomised-sequence oligonucleotides (NS), or fresh NGF for 24 hours. Cells were then refed with medium containing either fresh NGF or anti-NGF antibody, or anti-NGF antibody and 25 μM roscovitine, or anti-NGF antibody and 2 μM Cdk2AS, or anti-NGF antibody and 2 μM NS. After 48 hours, cell viability was determined using the live/dead assay.

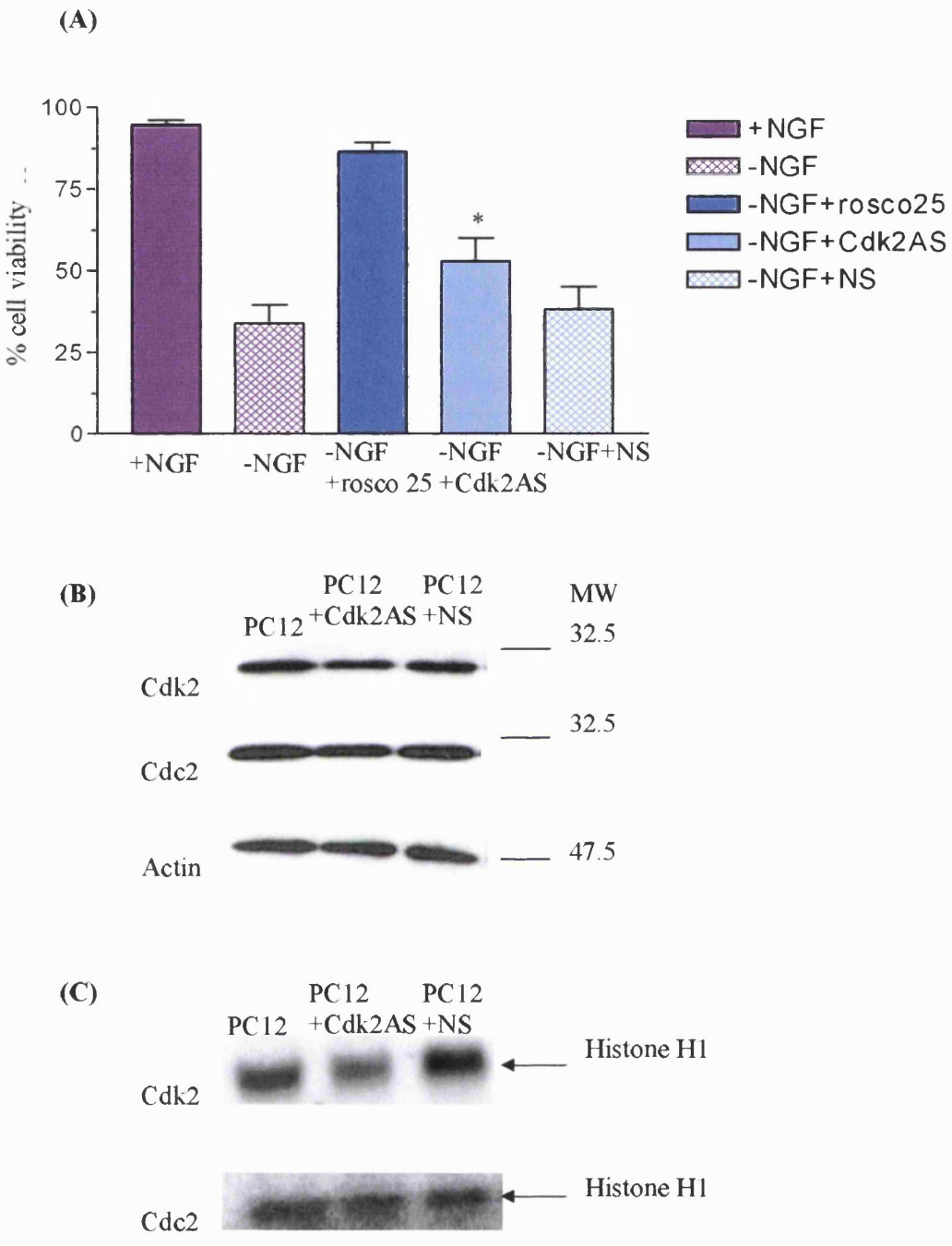
As shown in Figure 4.5A fewer neurons survived when cultured in medium containing anti-NGF antibody for 48 hours (33.9 ± 5.8%, mean ± SEM from 4 experiments) when compared to cells grown in NGF-containing medium (94.8 ± 1.4%). Treatment of cells with random sequence control oligonucleotides at 2 μM in the absence of NGF had no significant effect on neuronal survival (38.2 ± 6.9%) as seen in Figure 4.5A. However, cultures treated with a Cdk2 antisense oligonucleotide (2 μM) were significantly protected from NGF withdrawal-induced apoptosis, with 52.9 ± 6.9% of neuron survival ($p < 0.05$, antisense vs, random sequence, Student's t-Test).

It was essential to establish whether the survival effect obtained with the Cdk2 antisense oligonucleotide in sympathetic neurons was due to the specific inhibition of Cdk2 protein levels. To carry out this type of biochemical analysis using sympathetic neurons would require a lot of cells and Cdk2 antisense oligonucleotide. I therefore decided to determine if the Cdk2 antisense oligonucleotide specifically reduced Cdk2 protein levels in PC12 cells. PC12 cells were pulsed in medium containing 2 μM Cdk2 antisense oligonucleotide, or 2 μM random sequence control oligonucleotide, or fresh medium for 24 hours. Cells were then refed with the above and incubated for a further 48 hours. Cell extracts were made and equal amounts of protein were separated on 12% SDS polyacrylamide gels. Immunoblotting analysis using antibodies for Cdk2 and Cdc2 was performed. Equal amounts of each extract were immunoprecipitated with antibodies to Cdk2 and

Figure 4.5 A Cdk2 antisense oligonucleotide enhances neuronal viability after NGF withdrawal, and specifically reduces Cdk2 protein levels and kinase activity in PC12 cells.

(A) Sympathetic neurons were maintained *in vitro* for 7 days. Cells were cultured in medium containing fresh NGF with or without 2 μM Cdk2 antisense oligonucleotide (Cdk2AS), or 2 μM randomised-sequence oligonucleotide (NS) for 24 hours. Cells were then refed with medium containing NGF (+NGF), or anti-NGF antibody (-NGF), or anti-NGF antibody and 2 μM Cdk2AS (-NGF+Cdk2AS), or anti-NGF antibody and 2 μM NS (-NGF+NS). 48 hours later, cell viability was determined using the live/dead viability/cytotoxicity assay kit. The results shown are the average for 4 experiments ± SEM. Student's t-test was performed comparing the viability of cells cultured in anti-NGF antibody (-NGF) with cells cultured in medium containing anti-NGF antibody and Cdk2AS (-NGF+Cdk2AS). * $p < 0.05$.

Proliferating PC12 cells were incubated with medium only (PC12), medium containing 2 μM Cdk2AS (PC12+Cdk2AS), or medium containing 2 μM NS (PC12+NS) for 24 hours. Cells were then refed and cultured for a further 48 hours in the medium described above. **(B) Effect of Cdk2 antisense oligonucleotide on Cdk2 protein level.** Protein extracts were made and equal amounts of protein were separated on 12% SDS polyacrylamide gels. Immunoblotting was performed using antibodies specific for Cdk2, Cdc2, and actin. The positions and sizes in kilodaltons of molecular weight markers are shown on the right. **(C) Effect of Cdk2 antisense oligonucleotide on Cdk2 kinase activity.** 30 μg of protein extract was used per immunoprecipitation and kinase activity in the immune complexes was measured using histone H1 as a substrate. Reaction products were separated on 12% SDS polyacrylamide gels. Histone H1 phosphorylation was detected by phosphorimaging.


(A)
100
75
50
25
0
% cell viability
*
+NGF
-NGF
-NGF+rosco25
-NGF+Cdk2AS
-NGF+NS
+NGF
-NGF
-NGF +rosco 25
-NGF +Cdk2AS
-NGF+NS
(B)
PC12
PC12 +Cdk2AS
PC12 +NS
MW
32.5
Cdk2
32.5
Cdc2
Actin
47.5
(C)
PC12
PC12 +Cdk2AS
PC12 +NS
Histone H1
Cdk2
Histone H1
Cdc2

Cdc2 proteins and the kinase activity in immune complexes was detected using histone H1 as a substrate.

Figure 4.5B shows that 2 μM antisense Cdk2 oligonucleotide is able to reduce Cdk2 protein levels in proliferating PC12 cells by 18%. This decrease in Cdk2 protein levels correlates with a reduction of 22% in Cdk2 kinase activity in PC12 cells cultured with Cdk2 antisense oligonucleotide as seen in Figure 4.5C. Cdc2 protein levels appear to decrease slightly, however Cdc2 kinase activity does not change when proliferating PC12 cells are treated with Cdk2 antisense oligonucleotide (see Figure 4.5B and C). In retrospect, it would have been possible to confirm that Cdk2 antisense was specifically blocking Cdk2 in sympathetic neurons. The amount of Cdk2 activity in sympathetic neurons cultured in the presence of NGF, in the absence of NGF for 4 hours with or without 2 μM Cdk2 antisense oligonucleotide could have been determined by pooling 5 coverslips per 3.5 cm dish, with each coverslip containing 8,000 neurons. This would therefore result in a reduction in the use of animal resources as well as being more cost effective.

This data suggests that the Cdk2 antisense oligonucleotide reduces NGF withdrawal-induced apoptosis by specifically reducing Cdk2 protein levels and Cdk2 kinase activity.

4.2.4 Cdk2 kinase activity increases in sympathetic neurons following NGF withdrawal.

The observation that Cdk2 antisense oligonucleotides reduce NGF withdrawal-induced death suggests that Cdk2 might have a functional role in sympathetic neuron apoptosis. It was therefore necessary to look at Cdk2 kinase activity and protein levels in sympathetic neurons after NGF withdrawal. Cell extracts were made from sympathetic neurons at various times after NGF deprivation, notably 0, 2, 4, and 6 hours. Equal amounts of protein were immunoprecipitated with a Cdk2 antibody and the kinase activity of complexes was detected by using histone H1 as a substrate. Alternatively, equal amounts of each extract were resolved on a 12% SDS polyacrylamide gel and immunoblotting performed using a Cdk2 antibody.

Initially, Cdk2 kinase activity and protein levels in sympathetic neurons were determined at 24 and 48 hours after NGF withdrawal and no change was observed (data not shown). It has been reported that when sympathetic neurons are deprived of

NGF there is a 2-fold increase in c-Jun N-terminal kinase activity by 4 hours (Eilers *et al.*, 1998). It therefore seemed reasonable to look at Cdk2 kinase activity and protein levels at earlier timepoints after NGF withdrawal. Figure 4.6A shows that there is a gradual increase in Cdk2 kinase activity over 6 hours after NGF withdrawal. Figure 4.6B shows that there was a 1.7 ± 0.1 fold (mean ± SEM from 3 experiments) increase in Cdk2 kinase activity at 4 hours after NGF withdrawal compared to neurons cultured in medium containing NGF. This increase in Cdk2 kinase activity was significant as determined by Student's t-test, $p < 0.05$. It is important to note that control cells (+NGF) received fresh NGF-containing medium 4 hours before being harvested. This rules out the possibility that the increase in Cdk2 kinase activity was due to the addition of fresh serum. No reproducible change in Cdk2 protein level was observed during the same timecourse of NGF withdrawal (Figure 4.6C).

These results show that Cdk2 kinase activity increases in sympathetic neurons following NGF withdrawal. This increase in Cdk2 kinase activity does not coincide with an increase in Cdk2 protein levels indicating that the increase in kinase activity is due to an increase in the specific activity of Cdk2 kinase rather that a simple increase in the level of protein.

Figure 4.6 Cdk2 kinase activity increases during the NGF withdrawal-induced apoptosis of sympathetic neurons.

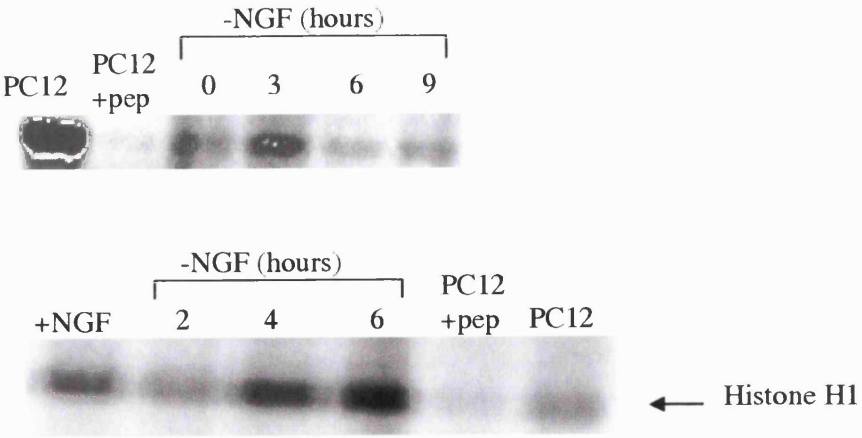
Sympathetic neurons were cultured in the presence of NGF for 7 days. The cells were then refed with medium lacking NGF, which had been supplemented with anti-NGF antibody (-NGF) for 0, 2, 4, or 6 hours, or with fresh NGF-containing medium (+NGF) and cell extracts were prepared. Half of the extract was used in a Cdk2 kinase reaction, and the other half was used to measure Cdk2 protein levels by SDS-PAGE analysis.

(A) Equal amounts of protein were used per immunoprecipitation and kinase activity in immune complexes was measured using histone H1 as a substrate. Reaction products were separated on 12% SDS polyacrylamide gels and substrate phosphorylation was detected by phosphorimaging.

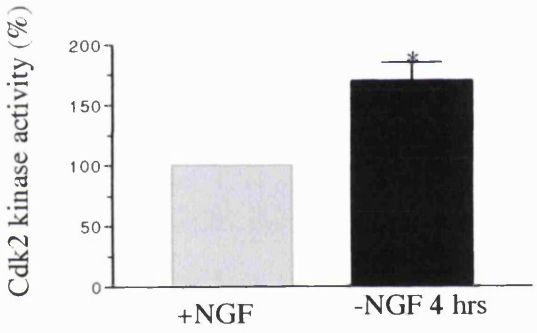
(B) Relative Cdk2 kinase activity was determined using the ImageMaster TotalLab software. The level of Cdk2 kinase activity in cells cultured in NGF (+NGF) was set at 100%. The results shown are the average of three independent experiments. Error bars indicate SEM. Student's t-test was performed to compare differences in kinase activity between cells cultured in medium containing NGF or cells cultured in medium lacking NGF for 4 hours. * $p < 0.05$.

(C) Equal amounts of protein were separated on 12% SDS polyacrylamide gels and immunoblotting was performed using an antibody specific for Cdk2. The membrane was reprobed with an actin antibody to control for any differences in loading. The positions and sizes in kilodaltons of molecular weight markers that were run in parallel are shown on the right. Immunoblots were scanned on a densitometer and the relative amount of Cdk2 protein in extracts from cells cultured in NGF (+NGF), or in the absence of NGF for 4 hours (-NGF 4hrs) was determined. The data shown represents 2 independent experiments ± range.

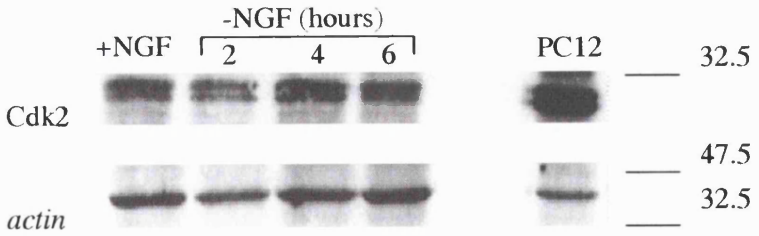
(A) Cdk2 kinase assay

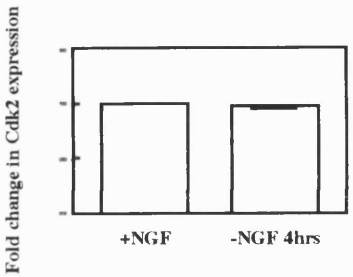


(B)



(C) Immunoblots





4.2.5 There is a transient increase in Cdc2 kinase activity when sympathetic neurons are deprived of NGF.

As described above, the pharmacological Cdk inhibitors, roscovitine, HD, and NG-75, protect sympathetic neurons from NGF withdrawal-induced apoptosis. As well as inhibiting Cdk2 kinase activity, these compounds also inhibit Cdc2 and Cdk5 kinase activity. It was therefore necessary to look at the activity of these kinases in sympathetic neurons following NGF withdrawal. Cdc2 kinase activity was analysed in extracts prepared from sympathetic neurons deprived of NGF for 2, 4, and 6 hours, and neurons refed with NGF-containing medium for 4 hours. Equal amounts of cell lysate were immunoprecipitated with Cdc2 antibody and the kinase activity in immune complexes was measured using histone H1 as a substrate. Equal amounts of protein were also separated on a 12% SDS polyacrylamide gel and immunoblotting was performed using a Cdc2 antibody.

Figure 4.7A shows that Cdc2 kinase activity increased in sympathetic neurons at 4 hours after NGF withdrawal but had then returned to basal levels by 6 hours. Figure 4.7B represents the average of 3 experiments and shows that Cdc2 kinase activity was 1.6 ± 0.05 fold higher at 4 hours after NGF withdrawal compared to cells cultured in medium containing NGF. This increase in Cdc2 kinase activity is significant compared to control cells (+NGF) as determined by Student's t-test, $p < 0.05$. Figure 4.7C shows there is no change in Cdc2 protein levels during the same timecourse of NGF withdrawal.

This data shows that Cdc2 kinase activity transiently increases in sympathetic neurons following NGF withdrawal and that this increase does not correlate with an increase in Cdc2 protein levels.

Figure 4.7 Cdc2 kinase activity increases in sympathetic neurons after NGF withdrawal whereas Cdc2 protein levels remain constant

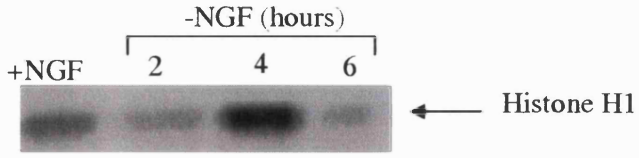
Sympathetic neurons were refed with medium containing anti-NGF antibody for 2, 4, or 6 hours, or with fresh NGF-containing medium and cell extracts were prepared. The cell extract was divided in two, one half was used to measure Cdc2 kinase activity and the other half was used in SDS-PAGE analysis to determine the amount of Cdc2 protein.

(A) Equal amounts of protein were used per immunoprecipitation and kinase activity in immune complexes was measured using histone H1 as a substrate. Reaction products were separated on 12% SDS polyacrylamide gels and histone phosphorylation was detected by phosphorimaging.

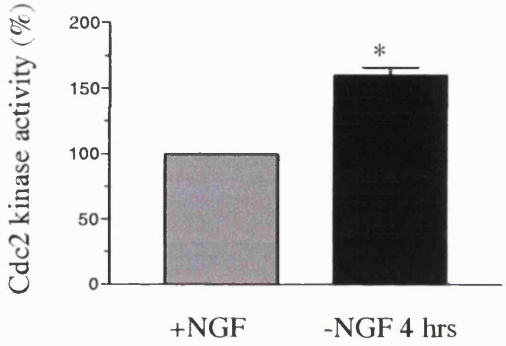
(B) Relative Cdc2 kinase activity was determined using the ImageMaster TotalLab software. The level of Cdc2 kinase activity in NGF-treated cells (+NGF) was set at 100%. The results shown are the average of three independent experiments. Error bars indicate SEM. A Student's t-Test was performed to determine whether there was a significant difference in kinase activity between cells cultured in the presence of NGF and cells deprived of NGF for 4 hours. * $p < 0.05$.

(C) Equal amounts of protein were separated on 12% SDS polyacrylamide gels and immunoblotting was performed using an antibody specific for Cdc2. The membrane was reprobed with an actin antibody to control for any differences in loading. The positions and sizes in kilodaltons of molecular weight markers that were run in parallel are shown on the right.

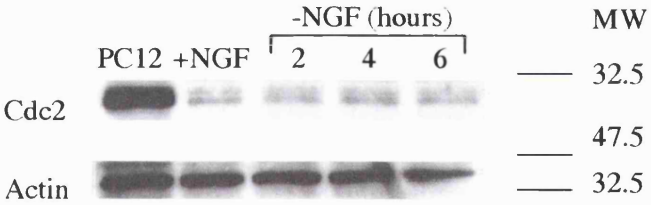
(A) Cdc2 kinase assay



(B)



(C) Immunoblot



4.2.6 Cdk5 kinase activity increases in sympathetic neurons after NGF withdrawal

An increase in Cdk5 expression and Cdk5 kinase activity in both adult and embryonic tissues undergoing cell death has been reported (Zhang *et al.*, 1997). Roscovitine, NG-75, and HD are all able to inhibit Cdk5 kinase activity. The level of Cdk5 activity in sympathetic neurons at different times after NGF withdrawal was therefore determined. Cell extracts were prepared from sympathetic neurons deprived of NGF for 2, 4, and 6 hours, and cells that had been refed with fresh NGF-containing medium for 4 hours. Equal amounts of protein were immunoprecipitated with Cdk5 antibody and kinase activity in immune complexes was measured using histone H1 as a substrate. Equal amounts of protein were separated on a 12% SDS polyacrylamide gel and immunoblotting was performed using a Cdk5 antibody.

Figure 4.8A shows that Cdk5 kinase activity began to increase 3 hours after NGF withdrawal and that this increase in kinase activity was maintained at 9 hours after NGF withdrawal. Figure 4.8B shows that there was a significant increase (1.8 ± 0.2 fold) in Cdk5 kinase activity at 4 hours after NGF withdrawal compared to cells cultured in NGF-containing medium (Student's t-test, $p < 0.05$). Cdk5 protein levels remained constant during the same timecourse of NGF withdrawal as seen in Figure 4.8C.

These findings show that Cdk5 kinase activity increases in sympathetic neurons after NGF withdrawal, whereas Cdk5 protein levels remain constant. This means that there is an increase in the specific activity of endogenous Cdk5 in sympathetic neurons following NGF withdrawal.

Figure 4.8 Cdk5 kinase activity increases after NGF withdrawal in sympathetic neurons but, Cdk5 protein levels remain constant.

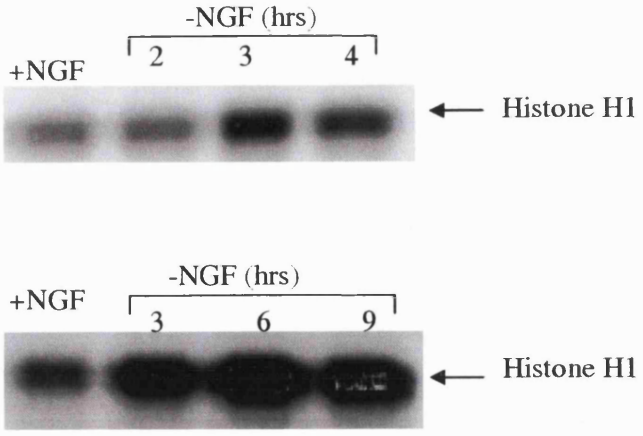
Sympathetic neurons were cultured for 7 days in medium containing NGF. Cells were then deprived of NGF for 0, 2, 3, 4, 6 or 9 hours and cell extracts were made. Half of the cell extract was used in a Cdk5 kinase reaction and the other half was used in SDS-PAGE analysis to determine the amount of Cdk5 protein.

(A) Equal amounts of protein were used per immunoprecipitation and kinase activity in immune complexes was measured using histone H1 as a substrate. Reaction products were separated on 12% SDS polyacrylamide gels and histone phosphorylation was detected by phosphorimaging.

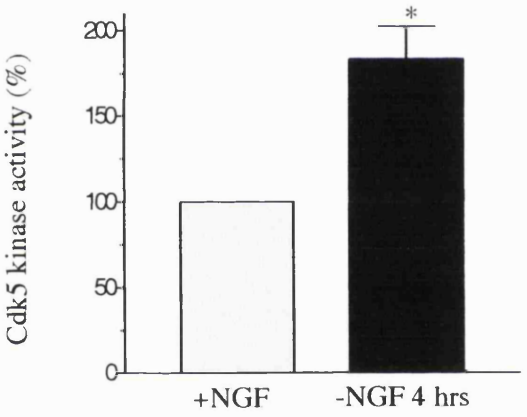
(B) Relative Cdk5 kinase activity was determined using the ImageMaster TotalLab software. The level of Cdk5 kinase activity in NGF-treated cells (+NGF) was set at 100%. The results shown are the average of three independent experiments. Error bars indicate SEM. A Student's t-test was performed to determine if there was a significant difference in kinase activity between cells cultured in medium containing NGF and cells deprived of NGF for 4 hours. * $p < 0.05$.

(C) Equal amounts of protein were separated on a 12% SDS polyacrylamide gel and immunoblotting was performed using an antibody specific for Cdk5. The positions and sizes of molecular weight markers are shown on the right.

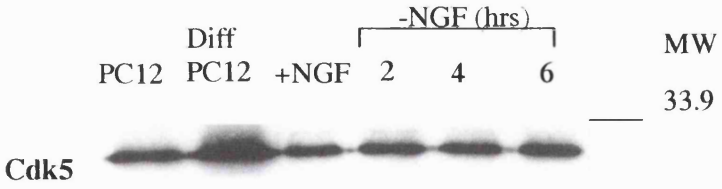
(A) **Cdk5 kinase assay**



(B)



(C) **Immunoblot**



4.2.7 There is no change in cyclin A or cyclin E-associated kinase activity in sympathetic neurons after NGF withdrawal.

Cyclin A and cyclin E are important regulators of Cdk2. Since Cdk2 is activated in sympathetic neurons following NGF withdrawal it was important to look at cyclin A and cyclin E-associated kinase activity under the same conditions. Cyclin A and cyclin E-associated kinase activity was measured in extracts prepared from sympathetic neurons cultured in the presence of NGF and at various times after NGF withdrawal. In parallel, equal amounts of protein were resolved on 12% SDS polyacrylamide gels and immunoblotting performed using antibodies specific for cyclin A and cyclin E.

Figure 4.9A shows that cyclin A and cyclin E-associated kinase activity both remain constant following NGF withdrawal. There is also no change in cyclin A or cyclin E protein levels in sympathetic neurons after the removal of NGF, as seen in Figure 4.9B.

These results indicate that cyclin A or cyclin E-associated kinase activity do not change following NGF withdrawal and this implies that the increase in Cdk2 kinase activity in sympathetic neurons after NGF withdrawal is not associated with cyclin A or cyclin E.

4.2.8 p35-associated and cyclin D1-associated kinase activity remains constant in sympathetic neurons following NGF withdrawal.

p35 is a neural-specific regulatory subunit for Cdk5 (Tsai *et al.*, 1994). Since Cdk5 kinase activity increases in sympathetic neurons after NGF withdrawal, it was important to establish whether an increase in the level of p35 was associated with this increase in Cdk5 activity. An increase in cyclin D1 RNA expression in sympathetic neurons following NGF withdrawal was previously reported (Freeman *et al.*, 1994), so cyclin D1-associated kinase activity following NGF withdrawal was also assessed. p35 and cyclin D1-associated kinase activities were measured in extracts prepared from sympathetic neurons deprived of NGF for 0, 2, 4 or 6 hours. In parallel, equal amounts of protein were separated on 12% SDS polyacrylamide gels and immunoblotting was performed using cyclin D1 and Cdk4 antibodies.

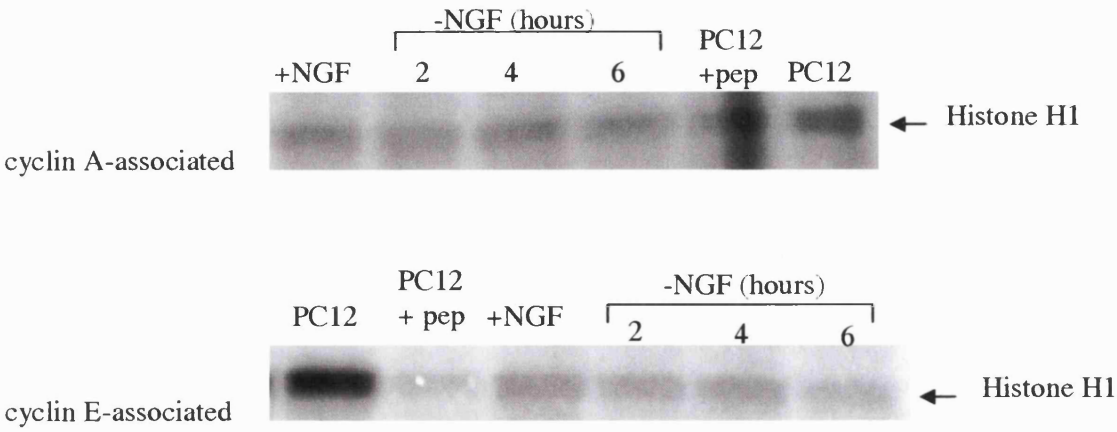
Figure 4.9 Cyclin A and cyclin E levels and cyclin A- and cyclin E-associated kinase activity do not change during the NGF withdrawal-induced apoptosis of sympathetic neurons.

Sympathetic neurons were cultured in medium containing NGF for 7 days. Cells were then refed with medium containing fresh NGF (+NGF), or medium lacking NGF (-NGF) for 2, 4, or 6 hours and cell extracts were prepared. The cell extracts were divided in two, half was used in kinase reactions and the other half was used in SDS-PAGE analysis to measure the amount of protein.

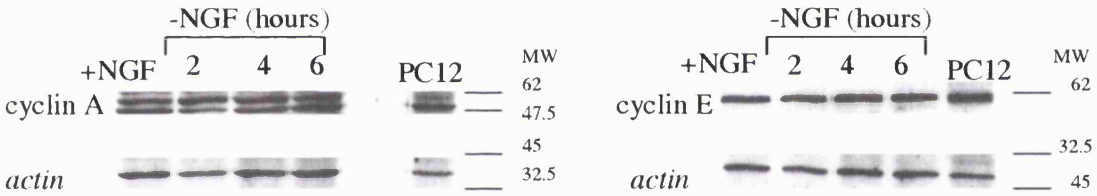
(A) Equal amounts of protein were used per immunoprecipitation. Extracts from proliferating PC12 cells were used as a positive control (PC12), and blocking peptide was added to positive control immunoprecipitations to show that the kinase activity was specific to that kinase (+pep). Kinase activity in immune complexes was measured using histone H1 as a substrate. Reaction products were separated on 12% SDS polyacrylamide gels. Histone H1 phosphorylation was detected by phosphorimaging.

(B) Equal amounts of protein were separated on 12% SDS polyacrylamide gels. Immunoblotting was performed using antibodies specific for cyclin A and cyclin E. Membranes were reprobed with an actin antibody to control for differences in loading. The positions and sizes in kilodaltons of molecular weight markers that were run in parallel are shown on the right. Immunoblots were scanned on a densitometer and the relative amount of cyclin A protein in extracts from cells cultured in NGF (+NGF), or in the absence of NGF for 4 hours (-NGF 4hrs) was determined. The data shown represents the mean of two independent experiments ± range.

(A) Kinase assays



(B) Immunoblots



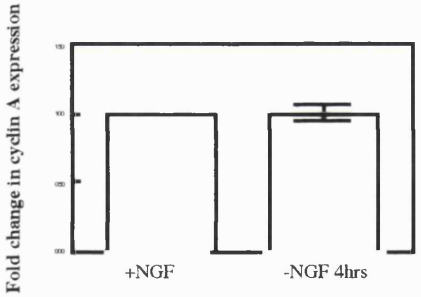


Figure 4.10A shows that there was little or no change in p35-associated kinase activity in sympathetic neurons following NGF withdrawal. At 4 hours after NGF withdrawal there was a 1.2 ± 0.2 fold increase in p35-associated kinase activity which was not a significant increase compared to basal levels (+NGF) (p = 0.5, Student's t-Test).

Figure 4.10B shows that cyclin D1-associated kinase activity remained constant in sympathetic neurons after NGF withdrawal and Figure 4.10C shows that there was no change in cyclin D1 protein levels. Figure 4. 10C also shows that Cdk4 protein levels do not change in sympathetic neurons following NGF withdrawal.

In summary, these results indicate that the increase in Cdk5 kinase activity in sympathetic neurons following NGF withdrawal is not due to increased association with p35. Cyclin D1-associated kinase activity is unaltered during the timecourse of NGF withdrawal that I looked at.

4.2.9 Expression of the Cdk inhibitors, p21 and p27, do not change following NGF withdrawal.

Cdk inhibitors negatively regulate Cdk activity so it was important to look at what happens to the expression of these proteins after NGF withdrawal. Sympathetic neurons were deprived of NGF for 0, 2, 4, and 6 hours and equal amounts of protein were resolved on 12% SDS polyacrylamide gels and immunoblotting performed using antibodies specific for p21 and p27.

Figure 4.11 shows that p21 and p27 protein levels remain constant in sympathetic neurons after NGF withdrawal.

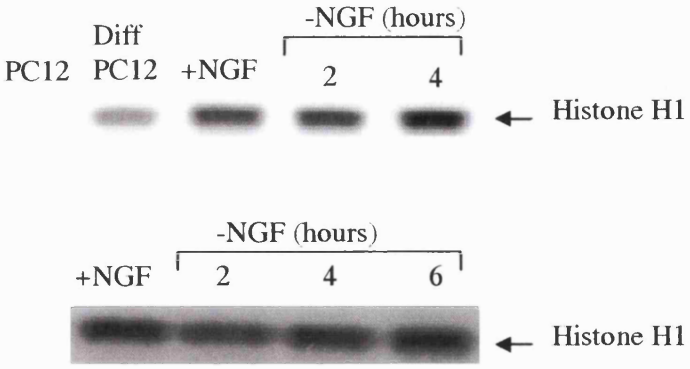
Figure 4.10 p35- and cyclin D1-associated kinase activity do not change in sympathetic neurons after NGF withdrawal.

Sympathetic neurons were deprived of NGF for 0, 2, 4, or 6 hours. Cell extracts were made and equal amounts of protein were used per immunoprecipitation. The kinase activity of immune complexes was measured using (A) histone H1 or (B) Rb-GST as the substrate.

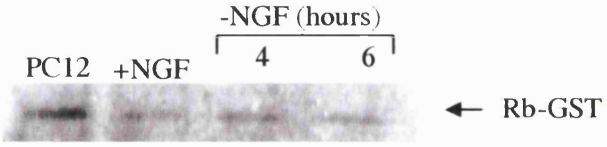
(C) Equal amounts of protein were separated on 12% SDS polyacrylamide gels. Immunoblotting was performed using antibodies specific for Cdk4 and cyclin D1. The membrane was reprobed with an actin antibody to control for differences in loading. The positions and sizes in kilodaltons of molecular weight markers are shown on the right.

(D) Immunoblots were scanned on a densitometer and the relative amount of protein in extracts from cells cultured in the presence of NGF (+NGF), and in the absence of NGF for 4 hours (-NGF 4hrs), was determined. The data shown represents the average of 2 independent experiments ± range.

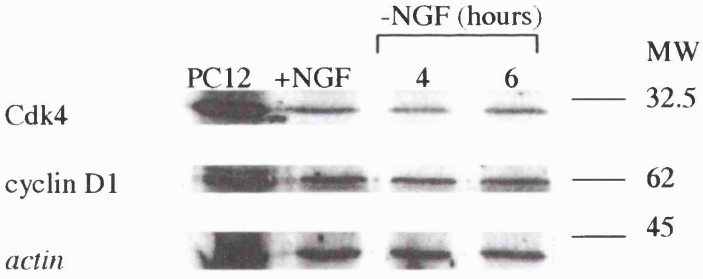
(A) p35-associated kinase activity



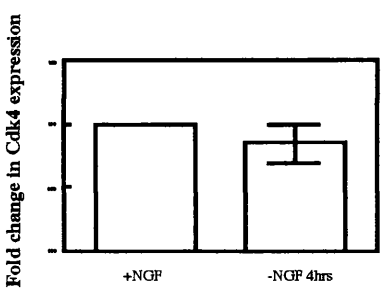
(B) cyclin D1-associated kinase activity

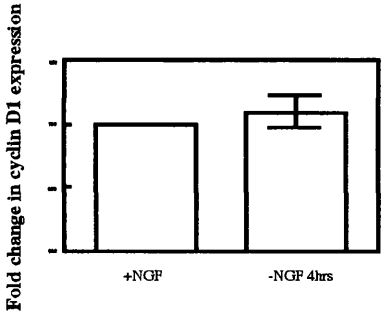


(C) Immunoblots



(D) Graphical representation of immunoblots





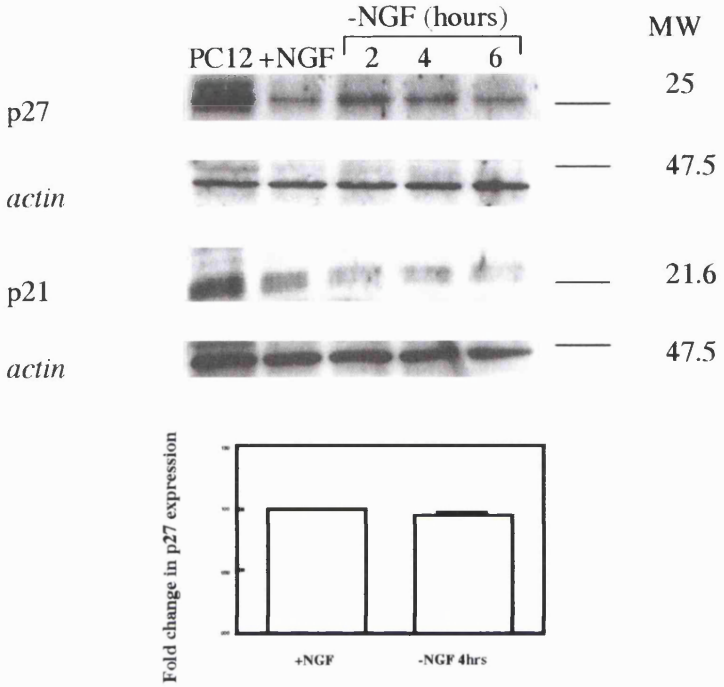


Figure 4.11 The Cdk inhibitor proteins, p21 and p27, do not change in level in sympathetic neurons after NGF withdrawal.

Sympathetic neurons were cultured in the presence of NGF for 7 days. Cells were then deprived of NGF for 0, 2, 4, or 6 hours. Cell extracts were prepared and equal amounts of protein were separated on 12% SDS polyacrylamide gels. Immunoblotting was performed using antibodies specific for p21 and p27. The membranes were reprobed with an actin antibody to control for any differences in protein loading. The positions and sizes of molecular weight markers are shown on the right. p27 immunoblots were scanned on a densitometer and the relative amount of protein in extracts from cells cultured in NGF (+NGF), or in the absence of NGF for 4 hours was determined. The data shown represents the average of two independent experiments ± range.

4.3 Discussion

The results presented in this chapter further demonstrate that cell cycle regulators play a role in neuronal apoptosis. Roscovitine, HD, and NG-75 were able to protect sympathetic neurons from NGF withdrawal-induced death. Roscovitine, a purine analogue, selectively inhibits Cdc2, Cdk2, and Cdk5 (Meijer *et al.*, 1997). NG-75 (Chang *et al.*, 1999) and HD (Meijer *et al.*, 2000), are two newly developed Cdk inhibitors that specifically inhibit Cdk2, Cdc2 and Cdk5 and are more potent than roscovitine. It has been previously reported that flavopiridol and olomoucine, which are pharmacological Cdk inhibitors, promote survival of sympathetic neurons deprived of NGF (Park *et al.*, 1996) and rescue sympathetic and cortical neurons from camptothecin-induced death (Park *et al.*, 1997b). Flavopiridol inhibits Cdc2, Cdk2, and Cdk4, but can also efficiently inhibit cGMP-dependent protein kinase and tyrosine kinase activity. Olomoucine, although less potent compared to flavopiridol, is a more selective inhibitor of Cdk2, Cdc2 and Cdk5 (Meijer *et al.*, 1997). Both roscovitine and olomoucine were shown to prevent apoptosis of central and peripheral neurons (Maas *et al.*, 1998). This data would suggest that Cdc2, Cdk2, and/or Cdk5 activities are important in neuronal apoptosis because agents that specifically block these kinases can prevent apoptosis.

Cell cycle regulatory proteins have previously been implicated in neuronal cell death. In sympathetic neurons, trophic factor withdrawal-induced death is associated with an increase in the level of cyclin D1 mRNA (Freeman *et al.*, 1994). Expression of dominant-negative (DN) mutants of Cdk4/6 has been reported to inhibit the death of sympathetic neurons deprived of NGF (Park *et al.*, 1997a) and can protect cortical neurons against death induced by the DNA damaging agent camptothecin (Park *et al.*, 1998a). This would suggest that Cdk4 activity is required for the NGF withdrawal-induced death of sympathetic neurons. Cdk inhibitors fall into three classes: those which are not selective for any Cdk, those which inhibit Cdk1, Cdk2, and Cdk5, and those which are selective for Cdk4 and Cdk6 (Meijer *et al.*, 1997). Roscovitine, olomoucine, NG-75, and HD are from the class which inhibit Cdk1, Cdk2, and Cdk5 most effectively, which implies that other Cdks may be involved in neuronal apoptosis. Cyclin B protein levels increase during apoptosis induced by oxidative stress in chick post-mitotic sympathetic neurons (Shirvan *et al.*, 1998). Induction of cyclin B expression and cyclin B-associated kinase activity has

been reported to occur in neuronally differentiated PC12 cells undergoing apoptosis following withdrawal of NGF (Gao and Zelenka, 1995).

Because I observed an increase in Cdk2 kinase activity in PC12 cells undergoing apoptosis, and because the pharmacological Cdk inhibitors that block sympathetic neuron apoptosis inhibit Cdk2 activity, I thought that it would be interesting to specifically inhibit the expression of this protein during sympathetic neuron apoptosis. An antisense Cdk2 oligonucleotide was able to partially, but significantly, protect sympathetic neurons from NGF withdrawal-induced death (Figure 4.5). This suggests that Cdk2 protein has a functional role in neuronal apoptosis. Although Cdk2 protein levels do not change in sympathetic neurons after NGF withdrawal there is an early and significant increase in Cdk2 kinase activity (Figure 4.6). This increase in Cdk2 activity is observed at 2, 4, and 6 hours after NGF withdrawal and returns to basal levels by 9 and 16 hours (data not shown). When NGF is added back to sympathetic neurons within 24 +/- 4 hours of NGF deprivation, death is prevented in half the neurons. Hence, 50% of the cells are committed to cell death at 24 +/- 4 hours after NGF withdrawal (Martin *et al.,* 1992; Edwards and Tolkovsky, 1994). This would suggest that when NGF is added back after the observed activation of Cdk2 activity, i.e. 2, 4, and 6 hours after NGF withdrawal, cell death would be prevented. An explanation for this would be that Cdk2 activation is an early reversible event in the cell death pathway, and that other pathways may need to be activated in order for cell death to occur (e.g. JNK pathway). Activation of Cdk2 requires binding of cyclin A, cyclin E, or cyclin D. Cyclin A, cyclin E, or cyclin D1-associated kinase activity did not change during the timecourse of Cdk2 activation after NGF withdrawal (Figure 4.9 and 4.10). This implies that a different, or unknown, regulator activates Cdk2 after NGF withdrawal in sympathetic neurons.

Roscovitine, HD, and NG-75 can also inhibit Cdc2 activity. Cdc2 protein levels remain constant after NGF withdrawal but there is a rapid transient increase in Cdc2 activity (Figure 4.7). Cyclin A or cyclin B association activates Cdc2. The increase in Cdc2 activity is not associated with cyclin A (Figure 4.9); therefore cyclin B or some unknown regulator could regulate Cdc2 after NGF withdrawal.

The levels of the Cdk inhibitors, p21 and p27, remain constant following NGF withdrawal from sympathetic neurons at the timepoints I looked at. It would be interesting to determine if there is any change in the association of these Cdk

inhibitors with Cdk-cyclin complexes following NGF withdrawal. This would determine if the increase in Cdk2 and Cdc2 kinase activity observed could be attributed to a reduction in inhibition by p21 and/or p27.

Although it was previously reported that there is an induction in cyclin D1 mRNA levels in sympathetic neurons following NGF withdrawal (Freeman *et al.*, 1994), I did not observe any change in cyclin D1 protein levels. The reason for this discrepancy could be because I looked at earlier timepoints after NGF withdrawal. In addition Freeman *et al* measured changes in *cyclin D1* mRNA levels, which need not necessarily lead to an increase in cyclin D1 protein levels.

Cdk5 is not a cell cycle regulator, however, it was important to look at Cdk5 regulation after NGF withdrawal because roscovitine can also inhibit Cdk5 activity. Although Cdk5 is widely expressed, its kinase activity is detected only in the post-mitotic neurons of the central nervous system (Nikolic *et al.*, 1996). More importantly, it has been reported that the expression of Cdk5 and its activator p35 is associated with apoptotic cell death in both adult and embryonic tissues (Ahuja *et al.*, 1997). My results show that although Cdk5 protein levels do not change in sympathetic neurons following NGF withdrawal, there is a significant and rapid increase in Cdk5 kinase activity (Figure 4.8). Association of Cdk5 with p35, its regulatory subunit, is critical for kinase activation (Lew *et al.*, 1994; Tsai *et al.*, 1994). p35-associated kinase activity does not change during the timecourse of Cdk5 activation after NGF withdrawal (Figure 4.10). This suggests that Cdk5 activity is regulated by a different activator in sympathetic neurons.

The fragmentation of chromosomal DNA into oligonucleosome-sized fragments is one of the characteristics of apoptosis. Roscovitine blocks DNA fragmentation in sympathetic neurons deprived of NGF as determined by TUNEL analysis (Figure 4.3). An important event in the NGF withdrawal-induced death pathway is the release of cytochrome c from the mitochondria, which precedes DNA fragmentation and chromatin condensation (Deshmukh and Johnson, 1998; Neame *et al.*, 1998). Roscovitine prevented the release of cytochrome c from the mitochondria of sympathetic neurons after NGF deprivation (Figure 4.4). However, although roscovitine prevented cytochrome c release, the nuclei of neurons cultured in medium containing roscovitine appeared to show some of the morphological changes of apoptosis. This data suggests that although roscovitine inhibits cytochrome c release some caspase activation may still occur, perhaps by a cytochrome c

independent pathway. A possible pathway may be via Smac/Diablo release from the mitochondria, which competes with caspase-9 for IAP binding, thereby alleviating the inhibition of caspase-9. Alternatively, the nuclear changes observed in the presence of roscovitine might depend on a biochemical pathway independent of cytochrome c and caspases e.g. the serine protease Omi/HtrA2. It would have been interesting to determine if these nuclear changes occurred when sympathetic neurons were cultured in NGF and roscovitine containing medium. This would ascertain whether the change in nuclear morphology is due to roscovitine or an apoptotic effect. The effect of HD, and NG-75, which are more potent Cdk inhibitors, on cyctochrome c release, and DNA fragmentation, could also have been determined. These inhibitors could have been used at lower doses in order to prevent cross inhibition of other kinases. Reports have shown that roscovitine is more potent in rescuing neurons than in inhibiting Jun kinase (Maas *et al.*, 1998), and that the IC_{50} value for JNK inhibition is 50 μM (Markus *et al.*, 1997). These studies suggest that the mechanism by which roscovitine is protecting sympathetic neurons from NGF withdrawal-induced apoptosis is not due to the inhibition of JNK activation.

Important future experiments include 1) determining whether these pharmacological Cdk inhibitors specifically block the observed increases in Cdk2, Cdc2, and Cdk5 activity. 2) Determining what occurs if you remove the drugs and add back NGF, to find out if these neurons are still viable. 3) Determining at what time after NGF withdrawal these inhibitors need to be added in order to rescue cells from cell death, i.e. when are their "commitment points".

The results described in this chapter suggest that Cdk2, Cdc2, and Cdk5 kinase activity has a functional role in the NGF withdrawal-induced apoptosis of sympathetic neurons because when these kinase activities are blocked by antisense oligonucleotides or pharmacological Cdk inhibitors apoptosis is blocked. These kinases function upstream of cytochrome c release and do not seem to be regulated by their canonical cyclins during the NGF withdrawal-induced death of sympathetic neurons.

Chapter 5: Discussion

The results described in this thesis indicate that NGF regulates the expression and activity of key regulators of cell cycle progression during the differentiation and apoptosis of neuronal cells. NGF causes a G_1 block in the cell cycle in PC12 cells by reducing Cdk4 and Cdk6 protein levels, and by increasing the level of the Cdk inhibitors, p21 and p27. Although there is an increase in cyclin D1 levels, there is a decrease in Cdk4 kinase activity that could be due to the inhibitory action of p21 and p27. An accumulation of cyclin D1/Cdk4/p21 complexes has previously been observed in PC12 cells following NGF treatment and this coincides with a reduction in cyclin D1-associated kinase activity (Yan and Ziff, 1995; van Grunsven *et al.*, 1996a). The reduction in cyclin-D1 activity is associated with accumulation of unphosphorylated Rb and an increase in cyclin D1/Rb complexes, which is evidence to support a G1 phase block (Yan and Ziff, 1995). PC12 cells accumulate in the G1 phase of the cell cycle following treatment with NGF (Rudkin *et al.*, 1989; Yan and Ziff, 1995; van Grunsven *et al.*, 1996b), and overexpression of cyclin D1 in PC12 cells can cause arrest in G1 phase, prevent entry into S phase, and inhibit expression of PCNA (Yan and Ziff, 1995).

NGF treatment of PC12 cells also leads to a reduction in the expression and activity of Cdk2 and cyclin E-associated kinases, which are required for the G_1/S transition. A decrease in cyclin A expression and cyclin A-associated kinase activity, along with a decrease in PCNA levels, suggests that NGF treatment reduces the number of cells in S phase. NGF treatment of PC12 cells also causes a reduction in the level of Cdc2 protein and Cdc2 activity, which is required during mitosis. A reduction in the level and activity of Cdk2 and Cdc2 in NGF-treated PC12 cells has been reported before and this coincides with a decrease in the DNA binding activity of the E2F/DP transcription factor (Buchkovich and Ziff, 1994). The decreases in Cdk2 and Cdc2 kinase activity can be attributed to the reduction in their protein levels, as well as the decrease in expression of their cyclin subunits. NGF induction of the Cdk inhibitors, p21 and p27, provides an additional mechanism for NGF-specific repression of Cdk kinase activity. Overexpression of either Cdc2 or Cdk2 can completely inhibit differentiation of PC12 cells whereas, simultaneous suppression of both Cdc2 and Cdk2 can induce neuronal differentiation (Dobashi *et al.*, 1995; Dobashi *et al.*, 2000). In the developing CNS, Cdc2 mRNA is down-

regulated, concurrent with the cessation of cell proliferation in the CNS (Hayes *et al.*, 1991; Okano *et al.*, 1993). This suggests that a down-regulation of Cdk2 and Cdc2 protein expression is required for cell cycle arrest and neuronal differentiation. Inducible expression of p21 in PC12 cells has been shown to induce permanent growth arrest and enhance differentiation by NGF (Erhardt and Pittman, 1998a). Expression of p21 led to an accumulation of cyclin D1 and a decrease in Cdk4, Cdc2, cyclin A, and cyclin B expression along with a decrease in cell proliferation (Erhardt and Pittman, 1998a). Taken together these findings suggest that the down-regulation of cell cycle activators, and selective induction of the Cdk inhibitors is responsible for the post-mitotic arrest of NGF-treated PC12 cells and may be required for their survival.

NGF mediates its effects by binding to the TrkA receptor, inducing receptor dimerisation and autophosphorylation. Activation of Trk receptors leads to stimulation of a number of intracellular signalling cascades including, among others, the ras/extracellular regulated kinase (ERK) and the phosphatidylinositol-3-kinase (PI3K) cascades (Klesse and Parada, 1999). Ras regulates neuronal differentiation and promotes neuronal survival either through the PI3K or the mitogen activated protein kinase (MAPK)/ERK pathways (see Figure 5.1). Among the targets of ERK are the ribosomal S6 kinases (RSKs). Both RSK and MAPK-activating protein kinase 2 phosphorylate CRE-binding protein (CREB) and other transcription factors (Xing *et al.*, 1998). CREB has been shown to regulate genes that are essential for survival of sympathetic and cerebellar granule neurons (Bonni *et al.*, 1999; Riccio *et al.*, 1999). Trk receptors can activate PI3K through at least two distinct pathways, ras-dependent activation and through adaptor proteins (Patapoutian and Reichardt, 2001). PI3K activates the Akt kinases which phosphorylate several proteins important in controlling cell survival (see Figure 5.1). These include BAD, IκB, the forkhead transcription factor FKHRL1, and GSK3-β (Patapoutian and Reichardt, 2001). Phosphorylation of BAD results in its association with 14-3-3 proteins, preventing binding to Bcl-x_L (Datta *et al.*, 1997). Phosphorylation of IκB activates the transcription factor NF-κB which has been shown to promote neuronal survival (Middleton *et al.*, 2000). The transcription factor FKHRL1 controls the expression of apoptosis-promoting genes, such as FasL (Brunet *et al.*, 1999). Elevated GSK3-β has been shown to promote apoptosis in cultured neurons (Hetman *et al.*, 2000). In my

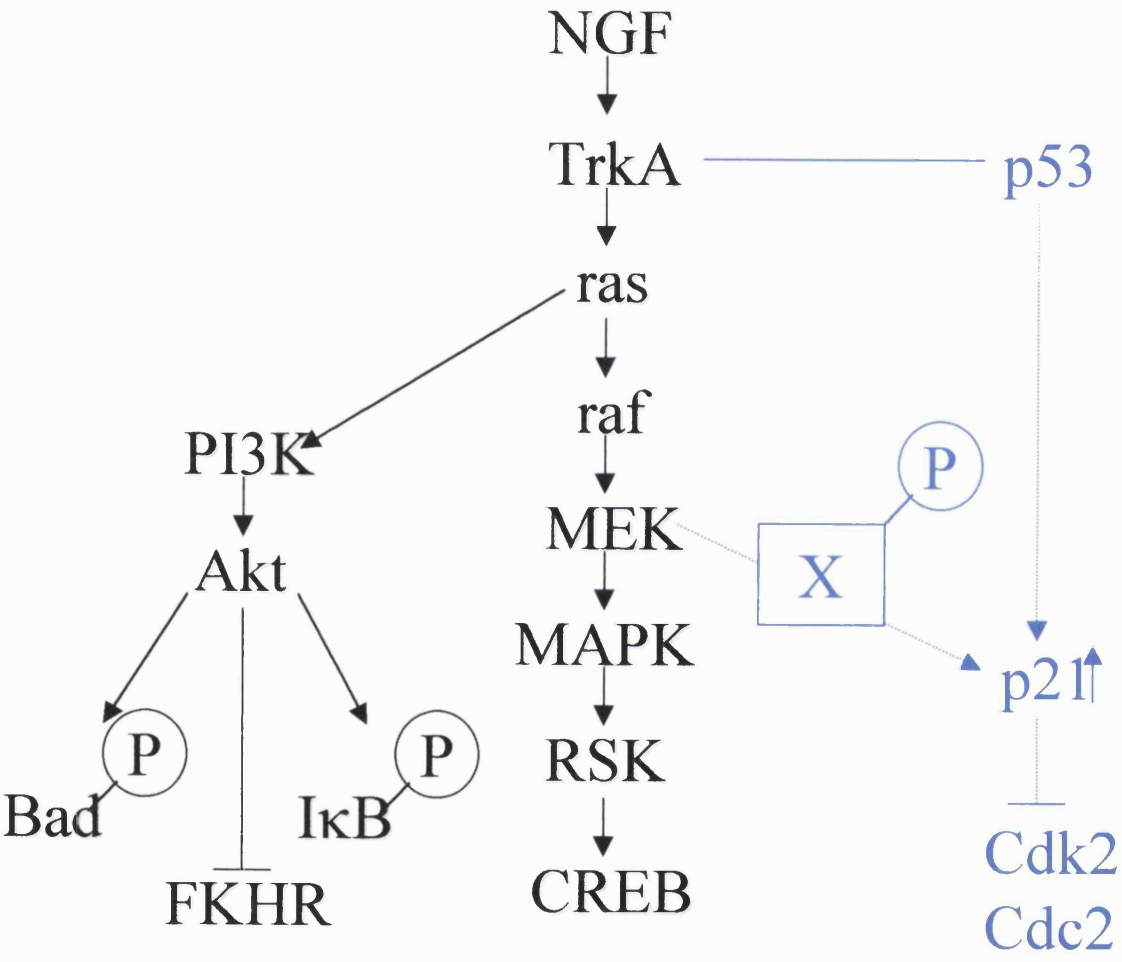


Figure 5.1 Diagram of NGF signal transduction pathways mediated by TrkA.
The different pathways are described in the text.

studies NGF suppresses the activity of Cdks. This inhibition appears to be critical in the ability of NGF to induce differentiation, and act as a survival factor in both PC12 cells and sympathetic neurons. Figure 5.1 indicates certain points in the NGF survival pathway whereby cell cycle regulators may be acting to induce differentiation and cell cycle arrest. The MEK/MAPK pathway has been shown to negatively regulate Cdk activity and mediate cell cycle arrest. The MEK inhibitor, PD98059, was able to prevent NGF-induced cell cycle arrest as well as the NGF-induced reduction in Cdk2 and Cdk4 activity. PD98059 was also able to reduce the levels of p21 protein induced by NGF to 50% (Pumiglia and Decker, 1997). MEK/MAPK may be phosphorylating a protein or proteins that are important in the regulation of Cdk2, Cdc2, PCNA, and p21 e.g transcription factor, transcriptional corepressor. NGF inhibits Cdk2 and Cdc2 activity in PC12 cells by signalling through the TrkA receptor to the tumour suppressor protein p53, which activates the transcription of p21 and induces cell cycle arrest (Gollapudi and Neet, 1997; Hughes *et al.*, 2000). TrkA binds to the amino-terminus of the oligomerisation domain of p53 in vitro and in vivo (Browes *et al.*, 2001).

NGF also induces Cdk5 expression and activity during PC12 differentiation. Cdk5 expression is widely distributed in differentiated tissues, however Cdk5-associated kinase activity has only been found in the brain (Lew *et al.*, 1995). On the other hand, expression of p35 and p25 (the regulatory subunits of Cdk5) is detected exclusively in the brain (Lew *et al.*, 1994; Tsai *et al.*, 1994; Lew *et al.*, 1995), suggesting that p35/p25 expression is the limiting factor for Cdk5 activity. In the forebrain of developing mice, Cdk5 expression and activity correlates with terminal differentiation of neurons of the embryonic brain (Tsai *et al.*, 1993b). Like Cdk5, p35 localises to the soma and along the entire length of neurites in developing neurons (Nikolic *et al.*, 1996). The developmental expression patterns and activity of Cdk5 and p35 suggest that one of the physiological roles of the Cdk5/p35 subunit is in neuronal differentiation. In support of this possibility, it has been shown that neurons co-transfected with Cdk5 and p35 produce longer neurites, whereas those transfected with dominant negative forms of Cdk5 or with antisense p35 produce fewer and shorter neurites (Nikolic *et al.*, 1996). Moreover, targeted disruption of the *Cdk5* gene in mice produces a lethal phenotype with structural abnormalities in the CNS (Ohshima *et al.*, 1996). To find out if the increase in Cdk5 expression and activity has a functional role in PC12 cell differentiation, dominant negative Cdk5 could be

inducibly expressed to determine whether inhibition of Cdk5 activity would alter NGF-induced differentiation. It would also be interesting to measure p35/p25-associated kinase activity to ascertain if these regulators are responsible for activating Cdk5 during PC12 differentiation.

When post-mitotic neurons are deprived of NGF they undergo apoptosis and there is increasing evidence to support the potential involvement of cell cycle regulators in this process. Agents known to prevent cell cycle progression promote neuronal survival. For example, the Cdk inhibitors, flavopiridol and olomoucine, suppressed the death of post-mitotic PC12 cells and sympathetic neurons caused by NGF deprivation (Park *et al.*, 1996). Roscovitine, another Cdk inhibitor which inhibits Cdk2, Cdc2, and Cdk5, can protect primary neurons from neurotrophin withdrawal-induced death (Maas *et al.*, 1998). Although roscovitine was able to block c-Jun N-terminal kinase in this study, it was more potent in rescuing neurons than in inhibiting Jun kinase. These findings support the potential role of Cdks in the process of neuronal apoptosis.

The results presented in this thesis show that Cdk2 kinase activity increases during the NGF-withdrawal-induced apoptosis of differentiated PC12 cells and sympathetic neurons. Induction of Cdk2 activity occurred prior to the commitment point of sympathetic neurons to NGF withdrawal-induced death i.e. approximately 7 hours after NGF withdrawal. The commitment point to cell death is defined as the time after which readdition of trophic support will no longer prevent cell death (Martin *et al.*, 1992; Edwards and Tolkovsky, 1994). Differentiated PC12 cells become gradually committed to cell death over a period extending from 10-20 hours after NGF withdrawal (Mesner *et al.*, 1995). Thus, the increase in Cdk2 activity in differentiated PC12 cells after NGF withdrawal occurs after the cell death commitment point. There was no increase in Cdk2 kinase activity at 12 hours after NGF withdrawal; however, it would have been interesting to look at Cdk2 activity before the cell death commitment point to determine if there is an early transient increase in Cdk2 activity as observed in sympathetic neurons. The temporal differences in Cdk2 activation after NGF withdrawal from sympathetic neurons and differentiated PC12 cells could be due to the fact that PC12 cells are a transformed cell line, and may have aberrant cell cycle properties. Another possible explanation could be due to differential expression of high and low affinity Trk/NGF receptors between the two model systems, resulting in different signalling properties. Eilers *et*

al (1998) report a 12 hour difference in Jun kinase activity between sympathetic neurons and differentiated PC12 cells. In PC12 cells the increase in Cdk2 kinase activity can be partly attributed to the increase in Cdk2 protein expression, however, Cdk2 requires cyclin E or cyclin A binding to be activated. Cyclin A and cyclin E protein levels remain constant in sympathetic neurons and PC12 cells following NGF withdrawal. Cyclin E- and cyclin A-associated kinase activity do not change following NGF withdrawal in sympathetic neurons. Although there is an increase in cyclin E-associated kinase activity in PC12 cells 72 hours after NGF withdrawal this cannot account for the increase in Cdk2 kinase activity observed at 24 hours after NGF withdrawal. These results suggest that a different or unknown regulator during neuronal apoptosis may regulate Cdk2 activity. A role for Cdk2 kinase in apoptosis has been suggested by previous studies. Cdk2 activity increased in thymocytes in response to several different treatments that induce apoptosis: dexamethasone, anti-Fas crosslinking, heat shock, and γ-irradiation (Gil-Gomez *et al.*, 1998; Hakem *et al.*, 1999). One study showed that the elevated Cdk2 kinase activity is not associated with either cyclin E or cyclin A and requires *de novo* protein synthesis (Gil-Gomez *et al.*, 1998). However, the other study shows that both cyclin A and cyclin E-associated kinase activities increase in response to γ-irradiation- and dexamethasone-induced apoptosis (Hakem *et al.*, 1999). Similar findings in cardiomyocytes, which are terminally differentiated cells, show that Cdk2-cyclin A activity mediates hypoxia-induced apoptosis (Adachi *et al.*, 2001).

The decrease in the level of the Cdk inhibitor, p21 may also contribute to the increase in Cdk2 kinase activity in PC12 cells after NGF withdrawal. Cleavage of p21, following induction of apoptosis in human hepatoma cells treated with ginsenoside Rh2 results in the activation of Cdk2-cyclin A kinase activity, but not Cdk2-cyclin E kinase activity (Jin *et al.*, 2000). Similarly, cleavage of p21 and p27 mediates growth factor withdrawal-induced apoptosis of endothelial cells through activation of Cdk2-cyclin A activity (Levkau *et al.*, 1998). However, the results described in this thesis show that in sympathetic neurons Cdk2 activity is not regulated by changes in p21 or p27 protein levels after NGF withdrawal. This suggests that in sympathetic neurons novel mechanisms may exist for regulating Cdk2 activity. However, it would be interesting to look at the association of these inhibitors with Cdk-cyclin complexes following NGF withdrawal, to determine if there is a decrease in association. A difference in the signalling pathways that are

activated by NGF withdrawal in PC12 cells and sympathetic neurons has been reported before. c-Jun N-terminal kinase and p38 kinase activity both increased in differentiated PC12 cells after NGF withdrawal, but in the case of sympathetic neurons activation of p38 could not be detected (Eilers *et al.*, 1998). Expression of a dominant negative mutant of SEK1 (MKK4) protected differentiated PC12 cells against NGF withdrawal-induced death (Xia *et al.*, 1995), but it did not prevent NGF deprivation-induced death of sympathetic neurons (Eilers *et al.*, 1998). With regard to Cdk inhibitors, it has been shown, however, that expression of p21 or p27 can protect sympathetic neurons from apoptotic death evoked by withdrawal of NGF (Park *et al.*, 1997a). Loss of p27 precedes cell death in neocortical neurons subjected to oxygen-glucose deprivation *in vitro*, which is followed by upregulation of cyclin D1, activation of Cdk2, and subsequent cytoskeletal disintegration. Olomoucine can significantly reduce this cell death (Katchanov *et al.*, 2001).

Cdc2 kinase activity transiently increases in sympathetic neurons after NGF withdrawal, whereas Cdc2 protein levels do not change. In contrast, Cdc2 protein levels increase in PC12 cells after NGF withdrawal, but Cdc2 kinase activity remains constant. The increase in Cdc2 kinase activity in sympathetic neurons is not associated with cyclin A, however, it may be associated with cyclin B. Induction of cyclin B protein and mRNA levels occurs prior to the commitment of chick sympathetic neurons to both dopamine- and peroxide-induced apoptosis (Shirvan *et al.*, 1998). Freeman *et al* (1994) show that there is a decrease in cyclin B RNA levels in sympathetic neurons following NGF withdrawal. However, this decrease occurs 15 hours after NGF deprivation and therefore cyclin B cannot be ruled out as the activator of Cdc2 in neuronal apoptosis. If more time had of been available cyclin B-associated kinase activity following NGF withdrawal from sympathetic neurons would have been measured. More recently it has been reported that neuronal activity deprivation stimulates Cdc2-cyclin B activity in granule neurons, and once activated, Cdc2 triggers neuronal apoptosis. In this system Cdc2 induces the phosphorylation of Bad at serine 128, which inhibits the ability of 14-3-3 proteins to sequester growth factor-induced serine 136-phosphorylated Bad and thus promotes the apoptotic effect of Bad (Konishi *et al.*, 2002). It would be interesting to determine whether this phosphorylation of Bad occurs in sympathetic neurons after NGF withdrawal and whether it is dependent on Cdc2 activation.

Cdc2 kinase activity does not increase in dying PC12 cells even though there is an increase in Cdc2 protein levels. This could be due to the inhibitory action of the Cdk inhibitor p27, or to reduced levels of cyclin B after NGF withdrawal. However, other studies have previously shown that cyclin B levels and cyclin B-associated activity increase in PC12 cells after NGF withdrawal, and this activity is associated with Cdc2 (Davis *et al.*, 1997; Gao and Zelenka, 1995). One reason for the discrepancy between their results and my results could be due to the timecourse studied. One study observed an increase in cyclin B-associated kinase activity at 96 hours after NGF withdrawal, whereas in the experiments described in this thesis the time course of kinase activity was measured up until 72 hours (Gao and Zelenka, 1995). Therefore the increase in Cdc2 activity during NGF withdrawal-induced apoptosis of PC12 cells occurs after the activation of Cdk2. In the other study, Cdc2 was precipitated from cell extracts by using $p13^{suc1}$ agarose beads. $p13^{suc1}$ binds to both Cdc2 and Cdk2 and this study did not determine whether the increase in kinase activity measured was specific to Cdc2 (Davis *et al.*, 1997).

A novel regulator of Cdk2 and Cdc2, named RINGO, which plays an important role in the meiotic cell cycle of *Xenopus oocytes* has been identified (Ferby *et al.*, 1999). RINGO (**r**apid **i**nducer of **G_2**/M progression in **o**ocytes) can bind and activate Cdk2 and Cdc2, but has no sequence homology to cyclins. The phosphorylation of Thr 160/161 is not required for full activation of Cdk-RINGO complexes. Moreover, RINGO-bound Cdk2 and Cdc2 are both less susceptible to inhibition by p21 (Karaiskou *et al.*, 2001). It has been suggested that the Cdk-RINGO complexes may be active under conditions in which cyclin-bound Cdks are inhibited and can therefore play different regulatory roles. It would be interesting to determine whether the increase in Cdk2 or Cdc2 activity during NGF withdrawal-induced apoptosis of sympathetic neurons is associated with RINGO.

Cdk5 kinase activity increases in sympathetic neurons prior to the commitment point to NGF withdrawal-induced apoptosis. The increase in Cdk5 activity is not associated with an increase in Cdk5 protein level or p35-associated kinase activity. This suggests that Cdk5 activity during sympathetic neuronal apoptosis may be regulated by a different or unknown activator. p39 (Tang *et al.*, 1995), and p67 (Shetty *et al.*, 1995) are also known activators of Cdk5. However, it has been reported that p35, but not p39 or p67, expression is associated with the increase in Cdk5 kinase activity during apoptosis in neurons of the substantia nigra *in*

vivo (Neystat *et al.*, 2001). Expression of Cdk5 and p35 is associated with apoptotic cell death in both adult and embryonic tissues (Ahuja *et al.*, 1997; Henchcliffe and Burke, 1997; Zhang *et al.*, 1997). p25, a truncated form of p35, has been shown to accumulate in neurons in the brains of patients with Alzheimer's disease. This accumulation correlates with an increase in Cdk5 kinase activity (Patrick *et al.*, 1999). The increase in Cdk5 kinase activity observed in sympathetic neurons is not associated with p25, as the antibody used to immunoprecipitate p35 also recognises p25 (Patrick *et al.*, 1999). Cdk5 RNA levels have been shown to decrease in sympathetic neurons 15 hours after NGF withdrawal, which is long after the observed increase in Cdk5 activity (Freeman *et al.*, 1994). Cdk5 kinase activity does not change in PC12 cells after NGF withdrawal which is further evidence of a difference in the NGF withdrawal signalling pathways in these two systems.

I found that there was no change in Cdk4 or cyclin D1 expression during NGF withdrawal-induced death of sympathetic neurons or PC12 cells. Moreover, there was no change in Cdk4 kinase activity in PC12 cells, or cyclin D1-associated kinase activity in sympathetic neurons after NGF withdrawal. A previous study reported that cyclin D1 mRNA levels increase in sympathetic neurons after NGF withdrawal (Freeman *et al.*, 1994). This increase occurred 19 hours after NGF deprivation and no measurement of cyclin D1 protein levels or cyclin D1-associated activity was presented in the same paper. Cyclin D1 activates Cdk4 and Cdk6, and it has been reported that expression of dominant-negative (DN) Cdk4 and DN Cdk6 proteins can protect sympathetic neurons from death caused by NGF withdrawal (Park *et al.*, 1997a). However, Park *et al.*, did not show that DN Cdk4 or DN Cdk6 specifically inhibited Cdk4 and Cdk6 activity respectively. The observations of Park *et al.*, suggest that cyclin D1-Cdk4/6 activity has a functional role in neuronal apoptosis, but these authors did not show any cyclin D1-Cdk4/6 activation after NGF withdrawal. DN Cdk4 and DN Cdk6 protect sympathetic neurons against UV-irradiation- and AraC-induced death (Park *et al.*, 1998a). Cyclin D1-associated activity is elevated in cortical neurons upon camptothecin treatment, and expression of DN Cdk4 and DN Cdk6 protects against apoptosis in this system (Park *et al.*, 1998a).

It therefore appears that Cdk2, Cdc2, and Cdk5 kinase activity is induced during NGF deprivation-induced apoptosis. Consistent with the observations that Cdk activation plays a required role in neuronal death, the Cdk inhibitors roscovitine,

NG-75, and HD protect sympathetic neurons from NGF withdrawal-induced apoptosis, and roscovitine inhibits neuronal PC12 death. This correlates with the findings that the Cdk inhibitors, flavopiridol and olomoucine, suppress the death of PC12 cells and sympathetic neurons after NGF-withdrawal (Park *et al.*, 1996). These agents can also protect against camptothecin-induced apoptosis of PC12 cells, sympathetic neurons, and cerebral cortical neurons (Park *et al.*, 1997b; Park *et al.*, 1998b). Roscovitine and NG-75, like olomoucine, are purine analogues which specifically inhibit Cdk2, Cdc2, and Cdk5, however, they are more potent than olomoucine (see Table 1.1). HD, contains both bromopyrrole and guanidine groups, and is a very potent inhibitor of Cdc2, Cdk2, and Cdk5, GSK-3β and CK1. Although I have emphasised the highly selective nature of these Cdk inhibitors, it remains conceivable that they may inhibit kinases yet to be examined or other kinases that play a required role in cell death. Roscovitine blocks the increase in Cdk2 kinase activity that normally occurs after NGF-withdrawal in PC12 cells. However it cannot be ruled out that roscovitine may block the activation or activity of the apoptosis-required c-Jun N-terminal kinase (JNK). Roscovitine has been shown to inhibit JNK activity in primary neurons however, it was concluded that roscovitine was more potent at preventing death than inhibiting JNK activity (Maas *et al.*, 1998). The IC_{50} value for roscovitine to inhibit JNK activity is 50μM, which is higher than the concentration I used in the cell death inhibitory experiments (Markus *et al.*, 1997). It is still important to ensure that roscovitine is not promoting its survival effect by inhibiting JNK activity following NGF withdrawal. HD, as well as inhibiting Cdks, potently inhibits GSK-3β and CK1. GSK-3β has been shown to be involved in the apoptosis of cultured cortical neurons induced by trophic factor withdrawal (Hetman *et al.*, 2000), however, it has not been determined whether CK1 has a role in neuronal apoptosis. Pharmacological Cdk inhibitors have limitations because no inhibitor selective for a single Cdk has been discovered, and this is probably due to the conservation of the amino acids lining the Cdk ATP-binding pocket (personal communication with L. Meijer). To overcome this problem, I used an anti-sense Cdk2 oligonucleotide, which specifically blocks Cdk2 expression and activity, to investigate the role of Cdk2 in sympathetic neurons. The fact that this increased survival further supports the functional role of Cdk2 activation in neuronal apoptosis.

It would be interesting to find out if antisense Cdc2 and Cdk5 oligonucleotides can also protect sympathetic neurons from NGF withdrawal-induced death.

Release of cytochrome c from the mitochondria has been shown to be an important event in sympathetic neuron apoptosis (Neame *et al.*, 1998). Thus it was important to determine whether Cdk activation occurred upstream or downstream of this process. Roscovitine prevented the loss of cytochrome c from the mitochondria after NGF withdrawal, which implies that Cdk activation occurs upstream of cytochrome c release. However, the nuclei of these neurons did exhibit early morphological changes characteristic of apoptosis. This would suggest that although roscovitine prevents cytochrome c release there may be residual caspase activation occurring.

In some systems it has been reported that Cdk activity is regulated in a caspase-dependent manner. For example, Cdk2 activation during staurosporine-induced apoptosis of HeLa cells is blocked by the peptide caspase inhibitor z-VAD-fmk (Harvey *et al.*, 2000). Similarly, Cdk2 activation during ginsenoside Rh-2-induced apoptosis of human hepatoma cells is dependent on the cleavage of p21 by caspase 3 (Jin *et al.*, 2000) and, caspase cleavage of p21 and p27 is required for Cdk2 activation during growth factor deprivation-induced apoptosis of endothelial cells (Levkau *et al.*, 1998). It is important to note, however, that these cells are cycling, so Cdks will be involved in apoptosis as well as regulating the cell cycle in these systems. On the other hand, p21 or p27 do not appear to be cleaved during NGF withdrawal-induced death of neuronal cells (this study). More importantly, caspase activation has been shown to be downstream of mitochondrial cytochrome c release in NGF withdrawal-induced apoptosis of sympathetic neurons (Deshmukh and Johnson, 1998). Another explanation for my result is that roscovitine is not completely efficient in inhibiting cytochrome c release and small amounts of cytochrome c are sufficient to activate caspases. Alternatively, a mitochondria-independent apoptotic pathway may be activated, that is not dependent on Cdk activation.

A hypothetical model of the role of Cdks in neuronal apoptosis is presented in Figure 5.2. The results described in this thesis support a model in which Cdk2, Cdc2, and Cdk5 activation plays a causal role in NGF withdrawal-induced death of sympathetic neurons and this Cdk-dependent step is upstream of cytochrome c release. The activation of these kinases is not dependent on their usual activators –

cyclin A, cyclin E, or p35/p25 - and activation by these novel regulators may be responsible for targeting Cdk activity to apoptotic substrates.

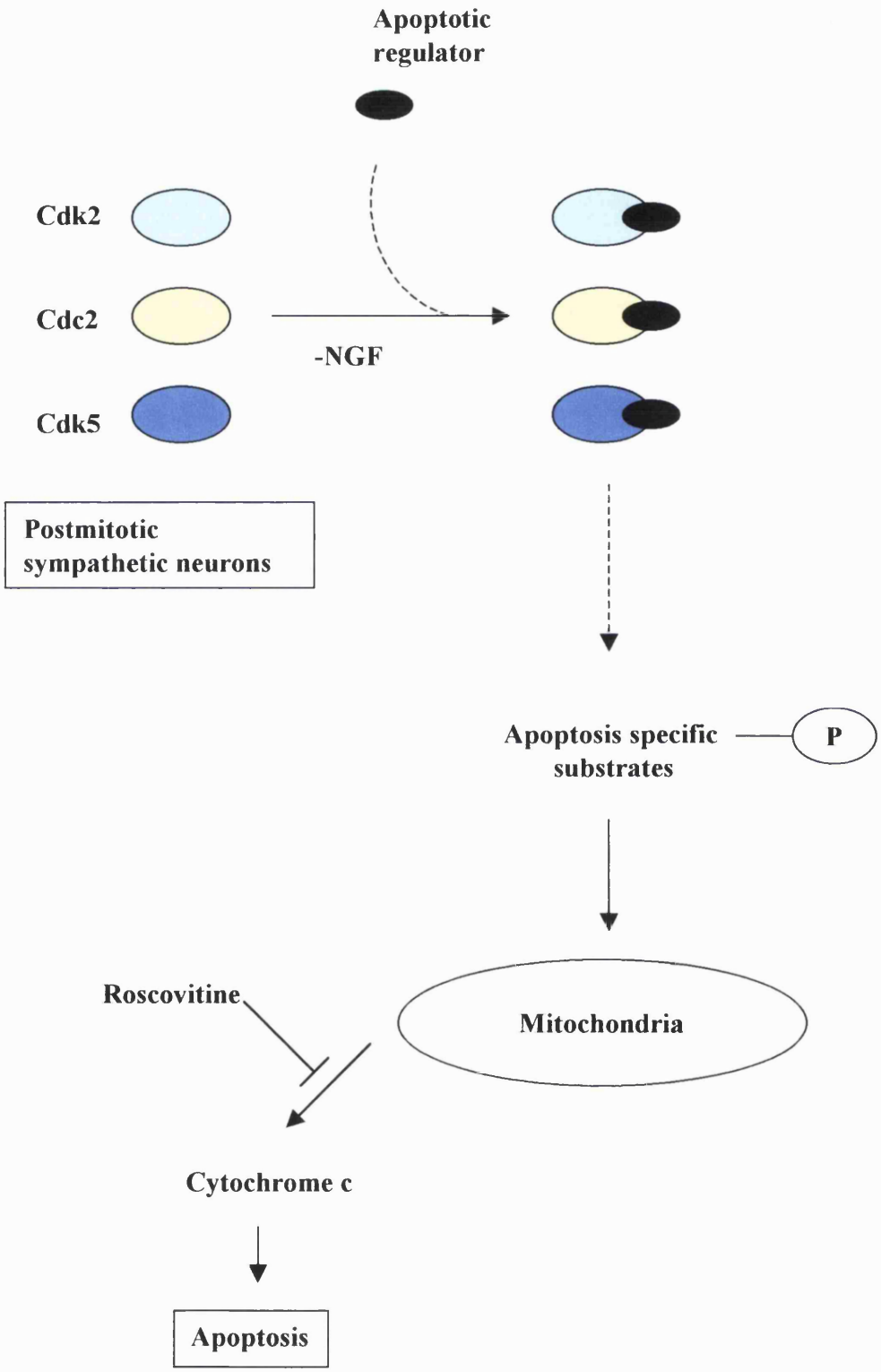
It would be interesting to determine whether activation of the Cdk pathway is in anyway connected to the NGF withdrawal-induced JNK signalling pathway that is activated in sympathetic neurons. This could be determined by measuring c-Jun phosphorylation in sympathetic neurons treated with NGF, or anti-NGF antibody, or anti-NGF antibody and roscovitine. c-Jun is phosphorylated after NGF withdrawal. Therefore, if this phosphorylation was blocked by roscovitine it would indicate that Cdk activation is upstream of c-Jun phosphorylation. On the other hand, if c-Jun was still phosphorylated when cells were treated with roscovitine this could mean that: 1) Cdk activation is downstream of c-Jun phosphorylation, or 2) Cdk activation does not affect the JNK signalling pathway.

Figure 5.2 Proposed model for the participation of Cdk2, Cdc2 and Cdk5 in sympathetic neuron apoptosis

Cdk2, Cdc2 and Cdk5 kinase activity increases in sympathetic neurons after NGF withdrawal. The increase in activity of these kinases is not associated with their usual activators, and it is hypothesised that they may be regulated by novel regulators during apoptosis. Cdk2, Cdc2 and Cdk5 may also phosphorylate apoptosis specific substrates. Cdk activation is upstream of mitochondrial events because the pharmacological Cdk inhibitor, roscovitine, blocks cytochrome c release and hence apoptosis.

——— lines represent known pathways,

- - - - - - lines represent hypothetical pathways.


Apoptotic
regulator
Cdk2
Cdc2
Cdk5
-NGF
Postmitotic
sympathetic neurons
Apoptosis specific
substrates
P
Roscovitine
Mitochondria
Cytochrome c
Apoptosis

Cdk5 has been shown to directly phosphorylate JNK3 and this inhibits its kinase activity, leading to reduced c-Jun phosphorylation. Expression of Cdk5 and p35 in HEK293T cells inhibits c-Jun phosphorylation induced by UV irradiation. Moreover, Cdk5-deficient cultured cortical neurons exhibit increased sensitivity to apoptotic stimuli, as well as elevated JNK3 activity and c-Jun phosphorylation (Li *et al.*, 2002). These findings indicate that Cdk5 may exert its role as a key regulator of neuronal survival by negatively regulating the JNK/SAPK signalling pathway during neuronal apoptosis. This does not agree with the data in this study which suggests that Cdk5 activation is required for neuronal apoptosis. A possible explanation for these differences could be that a novel regulator is responsible for activating Cdk5 during neuronal apoptosis, and this allows Cdk5 to phosphorylate apoptosis specific substrates. This theory is supported by the finding that p25, a truncated form of p35, accumulates in neurons in the brains of patients with Alzheimer's disease. p25 is not readily degraded, and binding of p25 to Cdk5 constitutively activates Cdk5, changes its cellular location and alters its substrate specificity (Patrick *et al.*, 1999). Another factor which has been shown to determine the proliferative or apoptotic action of Cdk2 is its subcellular localisation. In proliferating mesangial cells Cdk2-cyclin A is nuclear, but in UV-treated cells there is an increase in protein and kinase activity for Cdk2-cyclin A in the cytoplasm (Hiromura *et al.*, 2002).

Cdks allow progression through the different phases of the cell cycle by phosphorylating critical serines and threonines on their target substrates. It is clear that Cdks have an important role in regulating certain apoptotic pathways, but to understand how Cdks influence apoptosis it will be important to identify the substrates that they phosphorylate. These substrates could be proteins that contribute to the morphological similarities between apoptotic and mitotic cells, for example, proteins involved in cell rounding, nuclear membrane breakdown, or chromatin condensation. Alternatively Cdks may be phosphorylating apoptosis-specific substrates. During NGF withdrawal-induced apoptosis of PC12 cells, p21 degradation coincides with the observed increase in Cdk2 kinase activity. Ubiquitination of p27 during the cell cycle requires direct phosphorylation by Cdk2-cyclin E (Pagano *et al.*, 1995; Vlach *et al.*, 1997). However, it is not clear whether p21 degradation is dependent on (Yu *et al.*, 1998b), or independent (Sheaff *et al.*, 2000) of ubiquitination. One possibility is that during NGF withdrawal-induced apoptosis of PC12 cells, Cdk2 kinase phosphorylates p21, which targets it for

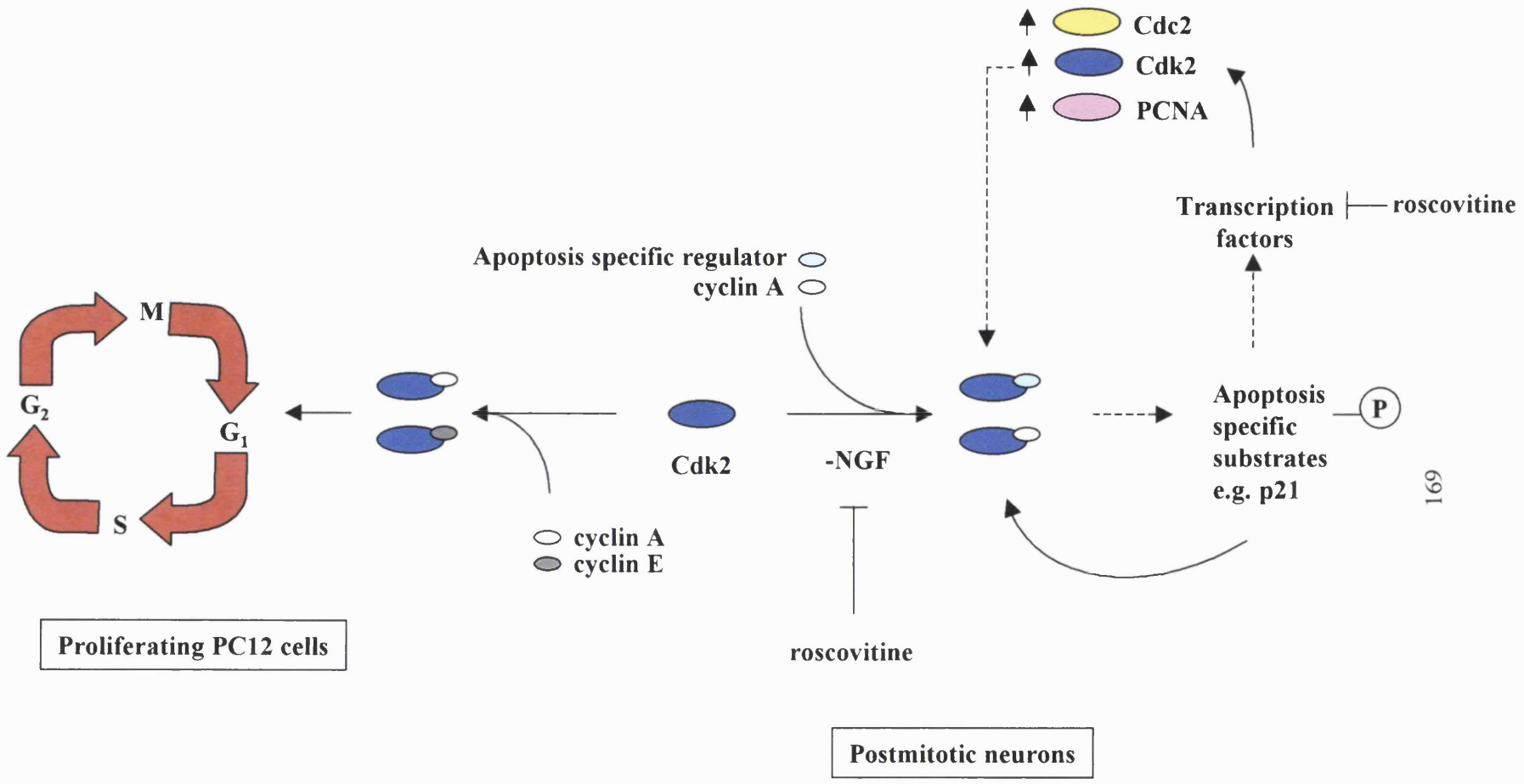
degradation and this leads to a further increase in Cdk2 activity. The increase in Cdk2 protein levels in PC12 cells after NGF withdrawal may also contribute to the increase in Cdk2 activity. The decrease in p21 levels also correlates with an increase in PCNA expression. The carboxy-terminal domain of p21 associates with PCNA, a subunit of DNA polymerase δ, and inhibits its role in DNA replication (Li *et al.*, 1994). It has recently been reported that PCNA binding can be modulated by reversible phosphorylation of p21 at its C-terminus (Scott *et al.*, 2000). Therefore, the phosphorylation and degradation of p21 removes its inhibitory action on PCNA, which is then capable of participating in DNA replication. The increase in PCNA and Cdc2 levels would suggest that cells may be re-entering the cell cycle, however, there is no significant increase in DNA synthesis after NGF withdrawal in PC12 cells. Roscovitine inhibits the increase in Cdk2 kinase activity in PC12 cells after NGF deprivation, and in doing so prevents the increase in Cdk2, Cdc2, and PCNA expression. This would suggest that Cdk2 is phosphorylating proteins that are involved in modulating Cdk2, Cdc2, and PCNA expression e.g. the transcription factors C/B-myb or Sp1. A model consistent with these observations is presented in Figure 5.3.

Figure 5.3 Model of Cdk2 activation in cycling and apoptotic PC12 cells

In proliferating PC12 cells Cdk2 activity is regulated by cyclin A and cyclin E. During NGF withdrawal-induced apoptosis of differentiated PC12 cells, Cdk2 activity increases and this is associated initially with an unknown regulator, and subsequently with cyclin A. The increase in Cdk2 kinase activity is associated with an increase in Cdk2, Cdc2 and PCNA protein levels, and a decrease in p21 levels. The pharmacological Cdk inhibitor, roscovitine, blocks the increase in Cdk2 activity, as well as the increase in Cdk2, Cdc2 and PCNA levels. A possible explanation for these observations is that Cdk2 may be phosphorylating apoptosis specific substrates, which leads to the transcription of Cdk2, Cdc2 and PCNA. The increase in Cdk2 protein levels could act in a positive feedback manner to further induce Cdk2 activity. The decrease in p21 levels, through possible phosphorylation, also contributes to the increase in Cdk2 activity.

_____ represent known pathways,

------ represent hypothetical pathways.


Cdc2
Cdk2
PCNA
Transcription factors
roscovitine
Apoptosis specific regulator
cyclin A
M
G2
G1
S
Cdk2
-NGF
Apoptosis specific substrates e.g. p21
P
cyclin A
cyclin E
roscovitine
Proliferating PC12 cells
Postmitotic neurons

A recent report shows that the elevation of cyclin D1-associated kinase activity in camptothecin-treated cortical neurons is associated with an increase in pRb and p107 phosphorylation, and flavopiridol can suppress this phosphorylation. Expression of pRb mutated at sites of phosphorylation, and DN versions of DP-1, inhibited camptothecin-induced apoptosis (Park *et al.*, 2000a). These results suggest that the Cdk-pRb/E2F/DP pathway is an essential component in the neuronal death evoked by DNA damage. Furthermore, the mRNA and protein levels of E2F-1 increase during K^+ deprivation-evoked death of cerebellar granule neurons, and flavopiridol can block this increase in E2F-1 expression (O'Hare *et al.*, 2000). Overexpression of E2F-1 was sufficient to evoke death, whereas E2F-1 deficient neurons were moderately resistant to death induced by low K^+ levels. Further identification of substrates phosphorylated by Cdks during apoptosis could be determined by a proteomics approach. For example, the differences in protein phosphorylation between PC12 cells treated with NGF, anti-NGF antibody, or anti-NGF antibody and roscovitine could be determined by 2-dimensional gel electrophoresis and mass spectrometry.

The identification of Cdk target substrates that are important for cell death will help resolve the apparent paradox that, as well as playing a role in neuronal cell death, Cdks are involved in cell proliferation. Furthermore, if Cdks prove to be important for cell death *in vivo*, an understanding of Cdk regulation and the mechanism by which Cdks induce cell death may lead to the development of novel strategies for treating diseases of the nervous system in which neuronal apoptosis occurs. The data described in this study and other reports indicate the requirement of Cdk signals for death of cultured post-mitotic neurons exposed to select death insults. However, the required role of cell cycle signalling molecules has largely been examined in cultured neuronal systems, and their involvement in the death of neurons *in vivo* and their potential as therapeutic targets is unknown. Several groups have reported that abnormal expression of cell cycle regulators (Cdc2, cyclin B, D, E, Cdc25, Cdk4), and mitosis-specific antigens (TG-3 and MPM-2), in post-mitotic neurons, correlates with the onset of neurodegeneration in Alzheimer's disease (Busser *et al.*, 1998; Nagy *et al.*, 1997; Raina *et al.*, 2000). The characteristic neuroplaques in the Alzheimer's disease brain are composed of β-amyloid, a 39-43 amino acid residue hydrophobic peptide that assembles into insoluble aggregates (Jarrett and Lansbury, 1993). Flavopiridol and DN Cdk4 and DN Cdk6, protect

cortical neurons from death induced by β-amyloid treatment, and inhibit phosphorylation of pRb that occurs during β-amyloid-induced death (Giovanni *et al.*, 1999). p25, the Cdk5 activator, accumulates in the brains of patients with Alzheimer's disease and this correlates with an increase in Cdk5 activity. *In vivo*, the p25/Cdk5 complex hyperphosphorylates tau, which reduces tau's ability to associate with microtubules and may lead to cytoskeletal abnormalities (Patrick *et al.*, 1999). These findings suggest that the combined inhibition of Cdc2/Cdk2/Cdk5/Cdk4 might have a therapeutic advantage in the treatment of Alzheimer's disease. The use of Cdk inhibitors in the treatment of Alzheimer's disease would have to be targeted to the affected area (e.g. hippocampus), in order prevent any side-effects on healthy proliferating cells. It has recently been reported that the Cdk4/6/pRb pathway is activated in a rat model of focal stroke and that these signals are required for death of neurons induced by ischaemia (Park *et al.*, 2000b). It has been demonstrated that loss of endogenous p16 is an early event in neuronal death in striatal neurons after mild cerebral ischaemia *in vivo,* and this results in an increase in nuclear cyclin D1 expression (Katchanov *et al.*, 2001). Cdk5 activity has been shown to increase in rat brain following ischaemia (Green *et al.*, 1997). This data would suggest that pharmacological inhibitors that inhibit Cdk5 and Cdk4, may be useful therapeutic agents in the treatment of stroke. This indicates that clinically active Cdk inhibitors need not be highly selective, but instead possess a combination of enzymatic effects which may sometimes yield better therapeutic agents.

In conclusion, I have demonstrated that in sympathetic neurons NGF withdrawal leads to Cdk2, Cdc2, and Cdk5 activation, and that the activated kinases are not associated with their canonical cyclins. This data suggests Cdk activation has a functional role in neuronal apoptosis because blocking Cdk activity with pharmacological Cdk inhibitors or antisense oligonucleotides blocks cell death.

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