An investigation of the regulation and role of c-Jun during neuronal apoptosis

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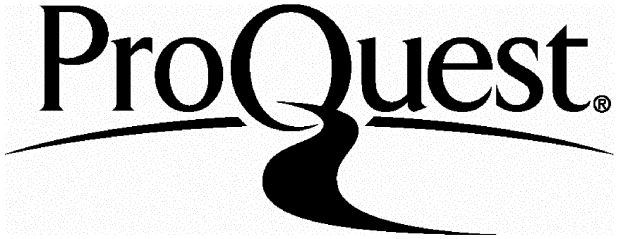

Abstract

Apoptotic cell death plays a key role during the development of the nervous system. Sympathetic neurons die by apoptosis when deprived of nerve growth factor (NGF). It is known that expression of a c-Jun dominant negative mutant promotes sympathetic neuron survival following NGF withdrawal, whereas overexpression of c-Jun induces apoptosis in the presence of NGF. The aims of this thesis were to investigate how c-Jun expression is regulated in sympathetic neurons and to determine the mechanism by which c-Jun activates the cell death programme.

In microinjection experiments, a *c-jun* promoter/CAT reporter gene was activated following NGF withdrawal and this required c-Jun/ATF-2 binding sites within the *c-jun* promoter. Expression of dominant negative c-Jun blocked the NGF withdrawal-induced increase in endogenous c-Jun protein, suggesting that c-Jun positively regulates its own synthesis. Expression of activated MEK kinase 1 (MEKK1), an upstream component of the c-Jun N-terminal kinase (JNK) pathway, increased the level of N-terminally phosphorylated (activated) c-Jun and induced apoptosis in the presence of NGF. Therefore, stimulation of the c-Jun pathway increases the level of phosphorylated c-Jun and triggers apoptosis.

To investigate how c-Jun promotes neuronal apoptosis, an adenoviral gene delivery system was developed. Infection of sympathetic neurons with a virus that expressed dominant negative c-Jun prevented cytochrome c release from mitochondria, a key step in neuronal apoptosis. Expression of MEKK1 caused cytochrome c to be released in the presence of NGF. The pattern of expression of several potential regulators of cytochrome c efflux was studied. A large increase in the level of *bim* mRNA and protein was observed and this was reduced by expression of dominant negative c-Jun. Overexpression of Bim in microinjection experiments induced both cytochrome c release and apoptosis in the presence of NGF. Thus, the effect of c-Jun on cytochrome c release is mediated, at least in part, by Bim.

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This thesis is dedicated to the memory of my Grandad.

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Abbreviations

AIF	apoptosis-inducing factor
AP-1	activator protein-1
Apaf-1	apoptotic protease activating factor-1
ATF	activating transcription factor
BAF	BOC-aspartyl(OMe)-fluoromethylketone
CAT	chloramphenicol acetyl transferase
CPE	cytopathic effect
CREB	cAMP response element binding protein
ERK	extracellular signal-regulated kinase
gpIgG	guinea pig IgG
HEK 293	human embryonic kidney 293 cells
JNK	c-Jun N-terminal kinase, also known as SAPK
MAPK	mitogen-activated protein kinase
MEKK1	MAPK/ERK kinase kinase 1
MOI	multiplicity of infection
NF-κB	nuclear factor-κB
NGF	nerve growth factor
PFU	plaque forming unit
PI-3-K	phosphatidylinositol-3-kinase
PS	phosphatidylserine
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SAPK	stress-activated protein kinase
SCG	superior cervical ganglion
SEK1	SAPK/ERK kinase 1
$TCID_{50}$	50% tissue culture infectious dose
TNFα	tumour necrosis factor α
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRE	TPA-responsive element
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling
ZVADfmk	N-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone

Chapter 1: Introduction

1.1 Apoptosis

1.1.1 History of cell death

Apoptosis was first defined in 1972 as a "basic biological phenomenon with wide-ranging implications in tissue kinetics" (Kerr *et al.*, 1972). Since that time, and particularly in the last 10 years, the number of researchers in this field has grown enormously. Over 8000 papers were published on cell death in 1999 (Samali *et al.*, 1999b). Cell death was first observed by Vogt in 1842 during amphibian metamorphosis (reviewed in Clarke and Clarke, 1996). A paper published early in the 20th century entitled "A new point of view regarding the elimination of cells" was the first step to understanding the genetic basis of cell death (Gräper, 1914). However, the significance of this work was lost during the war years, and also perhaps because embryologists at that time found it difficult to accept the idea of cells dying during embryogenesis, particularly in actively growing regions (Rich *et al.*, 1999). In addition, degeneration can result in the loss of a cell from tissue within a matter of hours, and so to find cells undergoing cell death can be difficult. It was not until 1951 that the importance of cell death during development was stated (Glücksmann, 1951). Glücksmann summarised all of the published work on cell death in various tissues and attempted to classify degenerations according to their function (e.g. changes in the form of organs and tissues, or the regression of vestigial structures). He stated that "there can be no doubt that cell deaths occur regularly at certain developmental stages of all vertebrate embryos" and pointed out that some previous work had misinterpreted cell deaths as "mitotic metabolites" or blood cells. Then Kerr noted that cells in mildly hypoxic livers showed different characteristics to the structural changes that had previously been 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 emphasise the potential importance of this form of cell death as a complementary process to mitosis in regulating cell populations, the term "apoptosis" was coined, derived from the Greek meaning the naturally-occurring falling of leaves from trees, or petals from flowers (Kerr *et al.*, 1972).

It took almost another 20 years, however, before the idea that animal cells have a built-in "suicide" programme became generally accepted. It has become clear that death by apoptosis, along with the processes of proliferation and differentiation, is of crucial importance during the life of all vertebrates and invertebrates. The term "programmed cell death" was originally used to describe cell deaths that occurred at predictable times and places during development, to emphasise that this is often an inherently programmed event in the developmental plan of the organism (Lockshin and Beaulaton, 1974). However, it became clear that these cell deaths could be modified by environmental cues and were not always inevitable. Now the term implies simply that a pre-existing genetic programme is present within cells. This programme appears to be present in all nucleated cells, a fact supported by research showing that staurosporine (a protein kinase inhibitor) can rapidly induce apoptosis in the presence of cycloheximide (which prevents new protein synthesis) in all of the various mammalian cell types tested so far (Ishizaki *et al.*, 1995; Weil *et al.*, 1996). This result extends to the fly, *Drosophila melanogaster,* and the nematode worm, *Caenorhabditis elegans,* suggesting that it is probably true for all animal cells (Steller, 1995; Shaham Horvitz, 1996). The only exception discovered so far is the human red blood cell which does not have a nucleus or other organelles (Weil *et al.*, 1996). Interestingly, anucleated cytoplasts undergo apoptosis when treated with staurosporine, showing that a nucleus is not a necessary part of the suicide machinery (Jacobson *et al.*, 1994). Thus, components of the cell death programme seem to be present in the cytoplasm of all animal cells. However, many forms of cell death, both *in vivo* and *in vitro*, from insect metamorphosis (Lockshin, 1969) to developmental death in the chick embryo (Oppenheim *et al.*, 1990), are inhibited by protein synthesis inhibitors, suggesting that macromolecular synthesis is required for the activation of pre-existing cell death machinery.

1.1.2 Apoptosis and necrosis

A defined set of characteristics are common to different cell types undergoing apoptosis. These include morphological and biochemical changes such as shrinkage of the cytoplasm and nucleus, membrane blebbing, a fall in the rates of RNA and protein synthesis, the involvement of caspases, degradation of the DNA into uniform 180 base pair oligonucleosomal fragments (DNA "laddering"), separation of the cell into membrane-bound fragments, and rapid phagocytosis by surrounding cells (Wyllie *et*

al., 1980). Indeed, in tissues, apoptotic cells are almost always found inside other cells and are degraded so rapidly (often within an hour or less) that surprisingly few dead cells are seen even when large numbers of cells are undergoing apoptosis. In contrast to this naturally-occurring cell death found during development and tissue homeostasis, where there can be extensive deletion of cells with little tissue disruption, the term "necrosis" is used to refer to the cell death more associated with the response to noxious or pathological stimuli, or injury. Necrotic cell death is characterised by swelling of the cell body and organelles, rupture of the plasma and organellar membranes, leakage of cellular contents, and an inflammatory response in the surrounding tissue.

Variations in the characteristics of apoptosis have been described and in recent years, the boundaries between apoptotic and necrotic forms of cell death have blurred, and cells may manifest aspects of both types of death (Clarke, 1998). It is now generally assumed that both forms of cell death make up two extremes of a continuum (Kroemer *et al.*, 1997; Portera-Cailliau *et al.*, 1997). For example, the same toxin can induce apoptosis or necrosis depending on the applied dose (Kroemer, 1995). Moreover, classic anti-apoptotic proteins such as Bcl-2 can inhibit death in some models of necrosis (Kroemer *et al.*, 1997). Apoptosis and necrosis are used generally to differentiate between processes that involve activation of dedicated cellular suicide machinery (programmed cell death, apoptosis), and the destruction of cells by external insults that does not necessarily involve the activation of an intracellular programme (necrosis).

1.2 The roles of apoptosis

1.2.1 Physiological apoptosis

Apoptosis plays a critical role during normal embryonic development in many different types of cells, tissues, and organs. While mutant nematodes whose cells do not die can have a normal lifespan (albeit with 15% more cells) they function less well than wild-type worms (Ellis *et al.*, 1991b). Mutant flies, on the other hand, die early during development with a large excess of cells (White *et al.*, 1994). Mutant vertebrates, for example those without caspase-3 (an important apoptotic enzyme), die perinatally with a vast excess of cells in their central nervous system and consequently a distended brain (Kuida *et al.*, 1996).

Programmed cell death serves many functions in animal development. These have been classified in different ways (see, for example, Glücksmann, 1951; Oppenheim, 1991). Jacobson *et al.* (1997) defined 5 functions of programmed cell death during animal development. (1) Certain structures must be sculpted: for example, eliminating the cells between developing digits, and creating the lumina of tubular structures. (2) Unneeded structures are deleted. This occurs in animals that undergo changes during development, such as the metamorphosis of a tadpole into a frog. Cell death in this animal removes structures- the tadpole's tail and lateral line organs- that are no longer needed in the adult form. Similarly, degeneration may be used to remove vestigial structures that are evolutionary "hang-overs", such as the pronephric tubules that will form functioning kidneys in the fish, but which are not used in mammals. (3) Cell death plays a role in controlling cell numbers. This is particularly apparent in the nervous system, where both neurons and oligodendrocytes are produced in excess and approximately half are eliminated by apoptosis. (4) Abnormal, misplaced, or harmful cells must be removed. In the immune system, self-reactive cells are deleted, while cancerous or virally-infected cells must be removed to protect the organism. (5) Cell death may be involved in the production of differentiated cells without organelles. Cell types such as skin keratinocytes and mammalian red blood cells lose their nucleus and other organelles in the process of differentiation. There are suggestions that these highly specialised differentiation processes may be modified forms of apoptosis (Nataraj *et al.*, 1994; Weil *et al.*, 1999).

The removal of harmful cells continues in the adult organism. This includes cells that are infected, or in which the DNA has become damaged, and also newly-generated autoreactive T cells. Apoptosis also plays a role in homeostasis, maintaining tissues such as bones and gastric epithelium, and in the immune system, removing excess cells after an infection.

1.2.2 Pathological apoptosis

Recent evidence suggests that apoptosis, as well as necrosis, underlies the aetiology of many diseases. Disorders characterised by the accumulation of cells include cancer, autoimmune diseases, and viral illnesses. Traditionally, attention has been directed towards studying the mechanisms of increased proliferation, but mounting evidence suggests that inhibition of programmed cell death is also important (reviewed in Thompson, 1995). For example, cells from a wide variety of human

tumours have a decreased ability to undergo apoptosis (Hoffman and Liebermann, 1994). In addition, genes such as *c-myc, v-rel,* and *bcl-2,* which are oncogenic, are involved in controlling the decision to undergo apoptosis (Guo and Hay, 1999). Physiological cell death is essential for removing autoreactive lymphocytes during development or those that arise as a result of somatic mutation during an immune response. Failure to remove autoreactive cells, however, leads to autoimmune disease (Thompson, 1995). Cells may respond to viral infection by initiating cellular suicide or by being induced to do so by T lymphocytes that recognise infected cells (Levine *et al.*, 1993). A number of viruses have therefore developed mechanisms to overcome the host cell defences by disrupting the normal regulation of apoptosis. For example, adenoviral infection depends on the E1B 19k protein (Rao *et al.*, 1992) and this is a structural homologue of the anti-apoptotic protein Bcl-2.

In contrast to diseases caused by inhibition of apoptosis, excessive, abnormal cell death can also lead to a variety of disorders. Virus-induced lymphocyte depletion occurs during AIDS, which is caused by the human immunodeficiency virus (Meyaard *et al.*, 1992). Acute loss of blood flow (ischaemia) is associated with myocardial infarction and stroke. Whereas cells within the central ischaemic area undergo necrosis, there is evidence that the more delayed cell deaths that occur outside this area are due, at least in part, to apoptosis (Thompson, 1995). The gradual loss of specific populations of neurons is the hallmark of neurodegenerative diseases, and there is evidence that apoptosis plays a role during neurodegeneration (see section 1.5.2).

1.3 Genes involved in the apoptotic pathway

1.3.1 Programmed cell death in the nematode worm

Around 30 years ago, Robert Horvitz began studying developmental cell death in the nematode worm, *Caenorhabditis elegans.* These deaths show many morphological similarities to mammalian apoptosis. Of the 1090 cells created in the hermaphrodite worm, precisely 131 undergo programmed cell death during development. In every animal the same cells die, each at its characteristic time (reviewed in Ellis *et al.*, 1991b). Nematode strains showing aberrant developmental death were found to result from mutations in a hierarchy of cell death genes. Initially, *ced-3* and *ced-4* (*ce*ll *d*eath abnormal genes) were identified as being necessary for cell death, because mutations in either *ced-3* or *ced-4* caused the survival of almost all of the

cells that normally die (Ellis and Horvitz, 1986). These genes are negatively regulated by *ced-9* (Hengartner *et al.*, 1992). The *egl-1* gene (for *egg laying* abnormal) was discovered because gain-of-function mutations resulted in the death of the two hermaphrodite-specific neurons that are required for egg laying (Conradt and Horvitz, 1998). Loss of this gene, however, permitted the survival of all of the 131 cells that normally die. These genes operate in all cells in a genetic pathway, such that *egl-1* inhibits *ced-9*, which itself inhibits *ced-4*; *ced-4* activates *ced-3*. Several other *ced* genes play a role in the engulfment of a dead cell by a neighbouring cell (Hedgecock *et al.*, 1983; Ellis *et al.*, 1991a).

The *ces-1* and *ces-2* genes (*ce*ll death *s*pecification abnormal) regulate programmed cell death in a cell-specific manner (Ellis and Horvitz, 1991). *ces-1* can act to prevent the death of the sister cells of two neurosecretory motoneurons that normally die; *ces-2,* however, encodes a basic/leucine zipper transcription factor that is a negative regulator of *ces-1*. Thus, in these two cells, cell death is transcriptionally regulated.

Structural and functional homologues of many of these genes have been found in mammals and *Drosophila*, and they also play a pivotal role in apoptosis. For example, CED-3 is the nematode homologue of the mammalian caspase family of cysteine proteases. CED-4 has a region of similarity with Apaf-1 (although Apaf-1 is a more complicated molecule; Zou *et al.*, 1997). CED-9 homologues in mammals are the anti-apoptotic Bcl-2 family, and EGL-1 is a pro-apoptotic member of the Bcl-2 family. These will be discussed in the following sections.

1.3.2 The mitochondrial apoptotic cascade

Cytochrome c is a crucial component of the mitochondrial electron transport chain, where it shuttles electrons from complex III to IV. Michael Hengartner wrote in 1998 that "to general stupefaction" a new role for cytochrome c has become apparent (Hengartner, 1998). This reflects the fact that in response to a variety of death stimuli, cytochrome c is released from its normal location within the intermembrane space of mitochondria, into the cytosol (X. Liu *et al.*, 1996; P. Li *et al.*, 1997). Once in the cytosol, cytochrome c binds to Apaf-1 (mammalian CED-4) with strong affinity, and this triggers the oligomerisation of Apaf-1, cytochrome c, pro-caspase-9, and dATP in an "apoptosome" complex (Hu *et al.*, 1999; Rodriguez and Lazebnik, 1999; Zou *et al.*, 1999). Autocatalytic processing of the pro-caspase-9 zymogen then occurs (Zou *et al.*, 1999). Activated caspase-9 cleaves and activates caspase-3 (mammalian CED-3),

caspase-6, and caspase-7, which function as downstream effectors in the death program (reviewed by Thornberry and Lazebnik, 1998). Regulation of cytochrome c release is controlled by the Bcl-2 family (mammalian CED-9 and EGL-1 homologues). As well as being found in nematodes and man, components of this pathway have been conserved in *Drosophila.* The CED-4 homologue is variously called Dapaf-1 (Kanuka *et al.*, 1999), HAC-1 (Zhou *et al.*, 1999), or Dark (Rodriguez *et al.*, 1999). CED-3 homologues also exist: DRONC (Dorstyn *et al.*, 1999), drICE (Fraser and Evan, 1997), Dredd (Chen *et al.*, 1998), and DCP-1 (Song *et al.*, 1997). CED-9 homologues have very recently been reported: Drob-1 (Igaki *et al.*, 2000), also called dBorg1 (Brachmann *et al.*, 2000), and Debcl (Colussi *et al.*, 2000), thus demonstrating complete evolutionary conservation of the core molecules in the cell death process.

Cells derived from Apaf-1 or caspase-9-deficient mice demonstrate defects in response to a wide range of apoptotic stimuli and die before or around birth with a prominent overgrowth of neural structures, due to a lack of apoptosis (Cecconi *et al.*, 1998; Hakem *et al.*, 1998; Kuida *et al.*, 1998; Yoshida *et al.*, 1998). However, T lymphocytes derived from these mice undergo apoptosis normally in response to TNFα or Fas death receptor activation (Hakem *et al.*, 1998; Yoshida *et al.*, 1998). These results support the hypothesis that there are discrete apoptotic signalling pathways: a "cellular stress" or "mitochondrial" pathway dependent on cytochrome c, Apaf-1, and caspase-9; and a "death receptor" pathway mediated by the action of death ligands on their cell surface receptors, and caspase-8. (Green and Reed, 1998; Vaux and Korsmeyer, 1999). Both pathways converge on the effector caspases (such as caspase-3) that are responsible for the execution phase of programmed cell death. There is also evidence that while these pathways function in parallel, in some cell types the death receptor pathway must be amplified by the mitochondrial pathway through caspase cleavage of the Bcl-2 family member Bid, which then leads to cytochrome c release and increased activation of caspases (Li *et al.*, 1998; X. Luo *et al.*, 1998). Amplification loops are therefore possible, whereby caspase-mediated cleavage of proteins such as Bid feeds back to the mitochondria to cause further release of apoptogenic factors. It is also possible to directly activate the caspases, for example by granzyme B secreted from T lymphocytes.

1.3.3 Caspases

The effector molecules of cytotoxic T lymphocytes, which are responsible for inducing apoptosis, are proteases (called granzymes) with a specific and unusual preference for cleaving proteins at sites adjacent to aspartates (Shi *et al.*, 1992). Junying Yuan and Robert Horvitz noticed that the CED-3 protease has the same specificity (Yuan *et al.*, 1993). Caspase-1, then called interleukin-1-β-converting enzyme (ICE), was shown to have sequence similarity with CED-3 (Yuan *et al.*, 1993; Xue *et al.*, 1996). Caspases (meaning cysteine proteases with specificity for cleaving after aspartate residues; Alnemri *et al.*, 1996) are an expanding family of proteases that currently includes 14 mammalian members, with several homologues in *Drosophila.* Caspases are expressed as inactive pro-enzyme zymogens and are activated by cleavage, either by themselves or by upstream caspases (for a review, see Thornberry and Lazebnik, 1998). Caspases are considered to be the primary executioners or "foot-soldiers" of apoptosis, and cleavage by caspases is irreversible. Caspases share similarities in amino acid sequence, structure, and substrate specificity (Nicholson and Thornberry, 1997). A variable tetrapeptide recognition sequence amino-terminal to the cleavage site confers selectivity among different caspases. Moreover, not all proteins that contain an optimal tetrapeptide sequence are cleaved, suggesting that tertiary structure may influence substrate recognition.

Cells dying by apoptosis undergo a number of morphological and biochemical changes that are stereotypical for this type of death. These effects are brought about by the caspases. The final stage of the death act is mediated by the executioner caspases: caspase-3 (originally called CPP32, Yama, and Apopain) being the major caspase, along with caspases-6 and -7. These caspases are responsible for cleaving a number of substrates that neatly dismantle the cell and produce the apoptotic phenotype (for a review, see Cryns and Yuan, 1998; Earnshaw *et al.*, 1999). Caspase-3, for example, cleaves iCAD (inhibitor of caspase-activated DNase), releasing CAD (Enari *et al.*, 1998). This DNase cuts the chromosomes between nucleosomes to produce DNA fragments that are 180 base pairs long or multiples thereof. This type of DNA fragmentation is found in many cell types undergoing apoptosis, and appears as a DNA ladder on a gel. Caspase cleavage of acinus activates this protein which then causes chromatin condensation (Sahara *et al.*, 1999). Caspase-3 also cleaves and activates gelsolin, a protein involved in the regulation of actin dynamics, which can produce the

phenotypic characteristic of blebs (Kothakota *et al.*, 1997). As well as dissolution of cell integrity and digestion of structural proteins, caspases can also cleave anti-apoptotic proteins such as Bcl-2 and Bap31, converting them into pro-apoptotic molecules and thus amplifying the death signal (Ng *et al.*, 1997; Kirsch *et al.*, 1999). Cleavage of other proteins blocks macromolecular synthesis and cellular repair mechanisms. Experiments with cells lacking caspases or treated with caspase inhibitors show that other effects also depend on these proteases, such as loss of mitochondrial membrane potential, loss of cell adhesion, and loss of plasma membrane integrity. Another caspase-mediated event is the loss of plasma membrane lipid asymmetry. Some lipids such as phosphatidylserine (PS) that are normally localised on one side of the plasma membrane (in this case, inside) lose their asymmetry (Green, 2000a). In the case of PS, externalisation is a significant step in the death pathway because macrophages and other phagocytic cells possess a PS receptor, which results in a PS-expressing cell being rapidly cleared (Fadok *et al.*, 2000). In summary, caspases co-ordinate the reorganisation of the cytoskeleton, the shutting down of DNA replication and repair, the destruction of DNA, the induction of cells to display signals that mark them for phagocytosis, and the disintegration of the cell into apoptotic bodies.

It should be noted, however, that not all caspases are involved in apoptosis. The first caspase discovered (ICE, caspase-1) is involved in inflammation (Thornberry *et al.*, 1992). Whereas many of the caspase knockout mice die perinatally due to profound defects in developmental cell death, caspase-1-deficient mice develop normally but have defective interleukin-1β-production (reviewed in Green, 1998). Several lines of evidence suggest that caspase-independent mechanisms may exist. For example, caspase inhibitors do not inhibit the normally-apoptotic cell death in cerebellar neurons induced by low K+ concentrations, although they do block caspase activity and DNA fragmentation (Miller *et al.*, 1997). However, other authors point out that one should question whether the caspase inhibitors used are potent and selective enough to conclude that all caspases are inhibited and that no other enzymes are affected (Vaux, 1999). These authors suggest, therefore, that apoptosis should be used to refer to caspase-mediated cell death, to distinguish it from other cell deaths in which the underlying biochemical changes are not yet clear (Samali *et al.*, 1999b; Vaux, 1999).

1.3.4 Mitochondria and the release of apoptogenic factors

With the knowledge that members of the Bcl-2 family are frequently located at the mitochondrial membrane, and the discovery that a critical factor needed to form the apoptosome was cytochrome c, it became clear that the mitochondria were key regulators of apoptosis. Apoptosis research has shifted from a paradigm in which the nucleus is the key regulator, to one in which the mitochondria are at the centre of the death decision.

Only recently have functional studies of cytochrome c-deficient cells been possible. The involvement of this protein in mitochondrial respiration means that cytochrome c knockout mice die at mid-gestation (Li *et al.*, 2000). Cell lines were therefore derived from early embryos and cultured under conditions that compensated for their defect in mitochondrial respiration. These cells were resistant to death induced by UV irradiation, staurosporine, and, to a lesser extent, serum withdrawal (Li *et al.*, 2000). However, the response to TNFα treatment was not reduced (and was in fact augmented) supporting the hypothesis that the death receptor pathway can be independent of the mitochondria. These authors even suggested that a defect in one pathway may lead to upregulation of the other. Cytochrome c release has recently been detected *in vivo* in some diseases states, such as during cardiomyopathy (Narula *et al.*, 1999), traumatic brain injury (Morita-Fujimura *et al.*, 1999), and ischaemic brain injury (Fujimura *et al.*, 1998), suggesting that what has been proved *in vitro* may well be important during physiological and pathological cell deaths.

The mechanism by which cytochrome c translocates to the cytosol is the subject of much debate. Interest has focussed on the Bcl-2 family of proteins (discussed in the next section). In addition to triggering the activation of caspases, release of cytochrome c may well have other consequences, such as the loss of oxidative phosphorylation and the generation of reactive oxygen species. These could contribute to cell death even if caspase function is inhibited and thus may explain why caspase inhibitors do not block death in some apoptotic settings (Green and Reed, 1998). Typically, cytochrome c release has been studied by western blotting. However a recent paper from Douglas Green's group described this release in individual cells expressing green fluorescent protein-tagged cytochrome c (Goldstein *et al.*, 2000). In response to a variety of apoptotic agents under various conditions, cytochrome c release was completed within 5 minutes. The strength of the apoptotic signal affected the onset of release but did not

affect the duration. Similarly, there were variations in the time between cytochrome c relocalisation and downstream events such as phosphatidylserine exposure and the loss of membrane integrity. This suggests that while differences of death stimulus and cell type can alter the timecourse of cell death, release of cytochrome c is an "all-or-nothing" event, consistent with the proposal that cytochrome c decides the fate of the cell (Green and Reed, 1998).

Other apoptotic factors, such as apoptosis-inducing factor (AIF), are also released by the mitochondria. AIF is not a caspase but is responsible for some of the changes observed during apoptosis, namely chromatin condensation and cleavage of the DNA into 50 kilobase pair fragments (Susin *et al.*, 1999b). There is also evidence to suggest that pro-caspases-2 and -9 are released from mitochondria (Susin *et al.*, 1999a), and pro-caspase-3 appears to have both a cytosolic and mitochondrial distribution (Mancini *et al.*, 1998). In addition, the Hsp60 protein appears to be localised to the mitochondria and released along with other apoptogenic factors such as cytochrome c (Samali *et al.*, 1999a). This protein has been shown to accelerate processing of the caspase-3 zymogen (Xanthoudakis *et al.*, 1999). Very recently, a protein named Smac (Du *et al.*, 2000) or DIABLO (Verhagen *et al.*, 2000) has been discovered. This is also released from mitochondria during apoptosis and appears to function by inhibiting the IAP family of caspase inhibitors (see section 1.3.6). Taken together, these results show that other mitochondrial factors are important in the decision for a cell to die, although the results from the cytochrome c knockout mice suggest that these other factors cannot fully compensate for the absence of cytochrome c to sustain the mitochondrial death pathway (Li *et al.*, 2000).

Other organelles in addition to the mitochondria have been implicated in triggering death, but currently their role is not clear. For example, Bcl-2 specifically targeted to the endoplasmic reticulum (ER) can prevent mitochondrial cytochrome c release and can protect cells from drug treatments that affect the secretory system (Hacki *et al.*, 2000). In addition, tethering proteins such as Bap31 in the ER can bind both Bcl-2 family members and the caspases and may therefore co-ordinate their activities at this site (Ng *et al.*, 1997).

The "double-life" of cytochrome c in both the sustenance and the suicide of the cell appears to have arisen in metazoa, since it is not a feature of death in yeast, which do not appear to have Apaf-1 or caspase-9 (Mewes *et al.*, 1997). It has been postulated

as a means of eliminating cells with damaged mitochondria (Li *et al.*, 2000). Other authors have proposed that it is a vestige of evolutionary conflicts between the protomitochondrial endosymbiont and the host cell (Blackstone and Green, 1999).

1.3.5 The Bcl-2 family

In 1988, David Vaux, Suzanne Cory and Jerry Adams noted that the principal function of Bcl-2 is to prevent cell death, and they coined the term "survival factor" (Vaux *et al.*, 1988). Bcl-2 is the mammalian homologue of CED-9, and approximately 18 members of this family are currently recognised, with homologues in worms (CED-9), flies (e.g. Debcl, Drob-1), and viruses (e.g. E1B 19k, BHRF1). The human Bcl-2 protein is even able to inhibit cell death in the worm (Vaux *et al.*, 1992). The Bcl-2 family consists of both pro- and anti-apoptotic members that can homodimerise or form heterodimers with other members of the Bcl-2 family. Changing the relative levels of different Bcl-2 proteins alters whether the cell lives or dies, which led Stanley Korsmeyer to propose the "rheostat" model (Oltvai *et al.*, 1993). This states that the primary decision to live or die is based on the level of Bax homodimers; anti-apoptotic members such as Bcl-2 and Bcl-x_L inactivate Bax by heterodimerisation.

Bcl-2 family members possess up to four conserved Bcl-2 homology (BH) domains but otherwise show little sequence similarity (for a review, see Adams and Cory, 1998; Antonsson and Martinou, 2000). Many of the anti-apoptotic members show conservation of all four domains (e.g. Bcl-2, Bcl-x_L, Bcl-w, Boo/Diva,). On the other hand, pro-apoptotic members lack the BH4 domain. Some contain the other three domains, BH1-3 (e.g. Bax, Bak, Bok). The BH3 domain appears to be critical for their death-promoting function, and several BH3-only proteins exist (e.g. Bid, Bad, Bim/BOD, DP5/Hrk, Noxa), which are all pro-apoptotic.

Several mechanisms have been put forward to account for the regulation of the pro-apoptotic Bcl-2 family members. Activation of Bax appears to involve translocation of cytosolic, monomeric Bax to the mitochondrial membrane and homodimerisation within the membrane (Wolter *et al.*, 1997; Gross *et al.*, 1998). The presence of Bcl-2 or Bcl-x_L can inhibit the activation of Bax (Gross *et al.*, 1998). Another mechanism of regulation is cleavage. Bid, for example, is cleaved by caspase-8 following TNF receptor or Fas activation, and this generates a fragment that translocates to the mitochondria (Li *et al.*, 1998; X. Luo *et al.*, 1998). There it induces the release of cytochrome c, due to its interaction with Bax (Goping *et al.*, 1998; Desagher *et al.*,

1999). Cleavage of anti-apoptotic members, such as Bcl-2, can generate pro-apoptotic fragments (Kirsch *et al.*, 1999). Bad, on the other hand, is regulated by phosphorylation. In the presence of survival factor, Akt (protein kinase B) or cAMP-dependent protein kinase (protein kinase A) can phosphorylate Bad on two serines (Datta *et al.*, 1997; Harada *et al.*, 1999), which results in its sequestration in the cytosol by 14-3-3 proteins (Zha *et al.*, 1996). Very recently, Bad has been shown to be phosphorylated on an additional site within the BH3 domain, raising the intriguing possibility that other pro-apoptotic members may be similarly phosphorylated (Tan *et al.*, 2000; Zhou *et al.*, 2000). Bcl-2 can be phosphorylated within the loop region, and this has been shown to abrogate its anti-apoptotic activity by inducing a conformational change (Haldar *et al.*, 1995; Chang *et al.*, 1997). In addition to the post-translational changes described, another method of regulation is by transcription. For example, DP5/Hrk gene products are upregulated during apoptosis in sympathetic neurons (Imaizumi *et al.*, 1997). Anti-apoptotic genes such as *bcl-*x_L can also be regulated transcriptionally (Boise *et al.*, 1995).

In the nematode, CED-9 binds to CED-4. Studies from the laboratories of Vishva Dixit and Gabriel Nuñez suggested that Bcl-x_L and Boo may similarly bind to and inhibit Apaf-1 (Hu *et al.*, 1998; Pan *et al.*, 1998; Song *et al.*, 1999). However, this issue is controversial, as more recent work from other laboratories suggests that these proteins do not associate (Moriishi *et al.*, 1999; Hausmann *et al.*, 2000; Newmeyer *et al.*, 2000).

The mechanism by which this family functions at the mitochondria is also an area of intense research (Antonsson and Martinou, 2000; Loeffler and Kroemer, 2000). Pro-apoptotic Bcl-2 family proteins appear to regulate the release of cytochrome c from the mitochondria and alter the mitochondrial membrane potential, either by forming pores in the outer mitochondrial membrane, or by regulating the activity of existing channels. The origin of this model lies in the fact that the Bcl-x_L molecule structurally resembles pore-forming bacterial toxins (Muchmore *et al.*, 1996), and recombinant Bcl-x_L, Bcl-2, Bax, and Bak can form ion channels in artificial membranes. This model, proposed by Stanley Korsmeyer, suggests that proteins such as Bax form channels in the outer mitochondrial membrane that allow the release of cytochrome c (for a review, see Gross *et al.*, 1999). Other workers, such as Yoshihide Tsujimoto, John Reed, and Guido Kroemer, suggest that these proteins function by binding to proteins already

located in the mitochondrial membrane. Direct binding of Bax to proteins in the mitochondrial permeability transition pore (PTP) such as the adenine nucleotide translocator (Marzo *et al.*, 1998) and the voltage-dependent anion channel (Narita *et al.*, 1998), support work which shows that Bax may stimulate PTP opening (Zamzami *et al.*, 1998; Shimizu *et al.*, 1999). Opening of this pore occurs during apoptosis and necrosis and results in the loss of mitochondrial membrane potential, uncoupling of oxidative phosphorylation, and swelling of the mitochondria (Green and Reed, 1998).

1.3.6 Caspase inhibitors

The only cellular inhibitors of caspase activity identified to date are the inhibitor of apoptosis (IAP) family of proteins (reviewed by Deveraux and Reed, 1999). These evolutionarily-conserved proteins were first identified in baculovirus (Crook *et al.*, 1993; Clem and Miller, 1994), and are characterised by a 70 amino acid baculoviral IAP repeat (BIR) domain, at least one copy of which is essential for the caspase-inhibiting function (Deveraux *et al.*, 1997; Roy *et al.*, 1997). Overexpression of various members of this family suppresses apoptosis induced by a number of different stimuli.

The *Drosophila* Hid, Grim and Reaper proteins function, at least in part, by inhibiting the function of the DIAPs (Wang *et al.*, 1999; Goyal *et al.*, 2000). This triggers activation of the caspases. Recently, a mammalian functional homologue of these proteins has been discovered, adding a missing link to the evolutionary conservation of death mechanisms. This protein, called Smac/DIABLO, is released into the cytosol when apoptosis is triggered, binds to IAPs, and prevents them from inhibiting caspase activity (see section 1.3.4; for a review, see Green, 2000b).

Other caspase inhibitors found only in viruses include p35 and CrmA. p35 is a baculovirus protein that has no known cellular homologues. p35 is a "suicide" inhibitor of most caspases (Komiyama *et al.*, 1994; Bump *et al.*, 1995), and can suppress apoptosis when overexpressed in insect, nematode and mammalian cells. It can functionally overlap with other viral IAP proteins, although it shares no sequence homology with them (Clem and Miller, 1994; Hawkins *et al.*, 1996). CrmA is a serpin family protease inhibitor from the cowpox virus, which specifically inhibits caspases-1 and -8, and shares no sequence homology with either p35 or the IAPs (Zhou *et al.*, 1997).

1.4 Regulation of survival and death by signalling pathways

1.4.1 Survival pathways

Survival signals from the environment and internal sensors of cellular integrity prevent activation of the apoptotic machinery. Martin Raff proposed that the basic components of the cell death machinery are present in all cells, and that growth factors promote the survival of cells primarily by keeping the cell death pathway inhibited (Raff, 1992).

A major mechanism by which a variety of signalling molecules promote survival is by activation of the Ras/PI-3-K/Akt pathway (reviewed by Marte and Downward, 1997). Binding of growth factors to receptor tyrosine kinases leads to activation of the GTPase Ras. PI-3-K is a Ras effector kinase. The downstream effector of PI-3-K signalling is the serine/threonine kinase Akt (protein kinase B). Phosphorylation of Akt results in its activation and the subsequent regulation of several cellular processes (reviewed in Datta *et al.*, 1999; Khwaja, 1999). Akt can protect a variety of cells against a diverse array of apoptotic stimuli, and even functions in *Drosophila,* suggesting an evolutionarily conserved mechanism (Bergmann *et al.*, 1998; Staveley *et al.*, 1998). One function of Akt is to phosphorylate the pro-apoptotic Bcl-2 family member Bad (Datta *et al.*, 1997; del Peso *et al.*, 1997). This leads to the sequestering of Bad by 14-3-3 proteins (see section 1.3.5). Akt can also prevent cytochrome c release by an unknown mechanism that is independent of Bad phosphorylation (Kennedy *et al.*, 1999). In addition to these post-translational mechanisms, Akt can regulate the transcription of death genes. For example, Akt phosphorylates FKHRL1, a member of the Forkhead transcription factor family (Brunet *et al.*, 1999). FKHRL1 is then sequestered in the cytosol by 14-3-3 proteins, and is therefore prevented from translocating to the nucleus and activating target genes such as *fas ligand* (Brunet *et al.*, 1999). In addition, Akt can regulate signalling via the NF-κB transcription factor pathway (reviewed by Khwaja, 1999). For example, Akt can phosphorylate a subunit of IKK, which leads to its degradation and the subsequent release of NF-κB (Ozes *et al.*, 1999). NF-κB then translocates to the nucleus and turns on the expression of survival genes such as the IAPs (section 1.3.6; Wang *et al.*, 1998).

Another pathway that may play a role in the transduction of survival signals is the Ras/Raf/MEK/ERK pathway. The most compelling evidence comes from genetic studies. Ras and ERK block apoptosis in the *Drosophila* eye (Sawamoto *et al.*, 1998), while experiments with mice lacking B-Raf suggest that this Raf family member plays a role in endothelial cell survival (Wojnowski *et al.*, 1997). Furthermore, overexpression of components of the ERK pathway prevents apoptosis after NGF deprivation from PC12 cells (Xia *et al.*, 1995; Parrizas *et al.*, 1997) or cardiotrophin deprivation from cardiac myocytes (Sheng *et al.*, 1997). However, ERK activity is not necessary for the survival of hippocampal neurons (Marsh and Palfrey, 1996) and some reports suggest that ERK does not play a role in sympathetic neurons (Creedon *et al.*, 1996; Virdee and Tolkovsky, 1996). Therefore, a requirement for ERK signalling appears to be more cell-type specific than for the PI-3-K/Akt pathway.

1.4.2 Death pathways

A mechanism has evolved in mammals to allow individual cells to be directed to self-destruct. This kind of apoptosis is especially important in the immune system. Death ligands bind to death receptors on the cell surface, and can rapidly activate the caspases. Death receptors belong to the TNF receptor superfamily, which is defined by cysteine-rich extracellular domains and a "death domain" sequence found in the cytoplasmic side of the receptor (Smith *et al.*, 1994). The "death domain" enables the receptor to engage the apoptotic machinery (Nagata, 1997).

The best characterised death receptors are TNFR1 and Fas (also called CD95 and Apo1), with their activating ligands TNF and Fas ligand respectively. The ligands, which are structurally related to each other, bind as homotrimers to induce trimer receptor complex formation (for reviews, see Smith *et al.*, 1994; Nagata, 1997). The death domains tend to associate with each other, and this recruits other death-domain proteins such as FADD (Fas-associated death domain) or TRADD (TNFR1-associated death domain) to the receptor complex. FADD also contains a death effector domain (DED), which is responsible for recruiting pro-caspase-8. The DED is an example of an interaction domain called the CARD (caspase recruitment domain) that is found in several caspases with large prodomains. Recruitment and subsequent oligomerisation of caspase-8 leads to its autocatalytic processing, and the activation of downstream caspases. The same basic mechanisms probably also apply to the more recently discovered receptors.

In contrast to the Fas receptor which mediates physiological apoptosis in the immune system, TNFR1 activation by TNF only triggers apoptosis in the presence of protein synthesis inhibitors. Typically, TNF is produced in response to infection, and activation of TNFR1 induces the transcription of proinflammatory and immunomodulatory genes (Tartaglia and Goeddel, 1992). Like Fas receptor signalling, TNFR1 can recruit TRADD and FADD, which leads to the activation of caspase-8. However, TRADD can also bind RIP and TRAF2, which stimulate pathways leading to NF-κB expression and JNK/AP-1 activation (these proteins will be discussed later). Transcriptional targets of NF-κB include the genes for the inhibitor of apoptosis proteins, c-IAP1 and c-IAP2, suggesting a mechanism by which apoptosis in response to TNF is normally suppressed (Wang *et al.*, 1998).

Another member of the TNF receptor superfamily, the p75 neurotrophin receptor, may play a role in triggering apoptosis in the nervous system (for reviews see Friedman and Greene, 1999; Kaplan and Miller, 2000). p75 can bind all members of the neurotrophin family but with different affinities. This receptor has been suggested to mediate a variety of functions, such as influencing ligand binding and ligand discrimination of the other neurotrophin receptors, inhibiting neuronal growth, and enhancing survival in the presence of Trk receptors, possibly via increased NF-κB expression (Kaplan and Miller, 2000). In addition, the p75 receptor contains an intracellular death domain, and in neuronal cell lines it can mediate the induction of apoptosis in response to NGF, in the absence of a TrkA receptor (Rabizadeh *et al.*, 1993). Since then it has been shown to cause apoptosis in many other types of neurons both in culture and *in vivo*. p75 only activates apoptosis in the absence of, or with suboptimally activated, Trk. Thus, NGF can be pro-apoptotic for cells that do not express the NGF receptor TrkA (Yoon *et al.*, 1998) and BDNF can be pro-apoptotic for those cells that do not express the BDNF-receptor TrkB (Bamji *et al.*, 1998). The outcome of p75 signalling, therefore, depends on the expression of particular Trk receptors, suggesting that Trk activation suppresses p75 apoptotic signalling. In some types of neuron, signalling from p75 appears to be essential for death to occur, so that apoptosis is delayed after growth factor withdrawal in sensory neurons deficient in p75 (Barrett and Bartlett, 1994). *In vivo*, the naturally-occurring cell death of sympathetic neurons in the superior cervical ganglia is delayed in the absence of p75 expression (Bamji *et al.*, 1998). However, death is not permanently blocked, and other authors

have proposed that p75 is not acting pro-apoptotically in this case, but rather is influencing the responsiveness to NT-3 (Brennan *et al.*, 1999). They suggest that the death of sympathetic neurons could be delayed in *p75* (–/–) mice due to compensation by NT-3 in the target tissues of those neurons receiving suboptimal NGF.

One of the mechanisms by which p75 may mediate apoptosis is via activation of the JNK/c-Jun pathway, which has been implicated in some models of neuronal cell death (see section 1.9).

1.5 Neuronal apoptosis

1.5.1 Death and development

In the nervous system, cell death plays a particularly significant role, since, depending on the neuronal population, approximately 20-80% of the neurons produced during neurogenesis subsequently die by apoptosis (Oppenheim, 1991). Early work on neuronal apoptosis was hindered by the fact that it seemed inconceivable that such widespread (but non-pathological) cell death might occur during development. It was not until the quantitative studies in the chick embryo by Viktor Hamburger and Rita Levi-Montalcini during the 1940s and 1950s, that the appearance of massive cell death in the nervous system began to be recognised (for a review, see Hamburger, 1992).

A common role for neuronal death in both vertebrates and invertebrates is the development of sexually dimorphic structures, such as the neural song circuit in birds, where the male can learn to reproduce complex vocalisations, but the female cannot (Bottjer and Arnold, 1997). This is also an example of synaptic plasticity, whereby there is seasonal proliferation and cell death of projecting neurons involved in the learning of birdsong. Other explanations for the function of neuronal deaths include the elimination of incorrect projections, removal of cells that serve a transient developmental function (e.g. rohon-beard cells in frogs), pattern formation and morphogenesis (such as the death of neural crest cells during closure of the neural tube), and numerical limitations because successive cell doublings lead to an excess of neurons (Oppenheim, 1991; Burek and Oppenheim, 1996). The most widely applicable hypothesis for the function of neuronal cell death in vertebrates, however, appears to be that excess neurons are produced to ensure that their targets are sufficiently and precisely innervated (reviewed in Pettmann and Henderson, 1998). According to the neurotrophin hypothesis, neurons produced in excess compete for and depend on

limiting amounts of a neurotrophin derived from their target; neurons that do not obtain adequate amounts die (Barde, 1989; Oppenheim, 1989).

1.5.2 Death and disease

In recent years, similarities have emerged between the mechanisms of cell death during development and those which occur during neurodegenerative diseases, stroke, and trauma. The presence of apoptosis has been detected in post-mortem brains of patients with Alzheimer's disease (J. Su *et al.*, 1994), Huntington's disease (Portera-Cailliau *et al.*, 1995), Parkinson's disease (Anglade *et al.*, 1997), and cerebral ischaemia (MacManus *et al.*, 1993). The contribution of apoptotic cell death to disease progression remains to be established. The majority of cases of neurodegenerative diseases are sporadic, although occasionally familial forms appear that are similar, but not identical, to the sporadic forms. Identification of the affected genes has been the focus of intense effort in the last few years. For example, gain-of-function mutations in copper/zinc superoxide dismutase (Cu/ZnSOD) can cause the loss of cortical and spinal motoneurons that is characteristic of familial amyotrophic lateral sclerosis (Kunst *et al.*, 1997). It was also demonstrated that mutants of Cu/ZnSOD can have a pro-apoptotic effect when expressed in cultured neuronal cells (Rabizadeh *et al.*, 1995). Since then, mutant proteins associated with other neurodegenerative diseases have proved to have a similar effect. For example, mutants of presenilins-1 and -2, and also β-amyloid, which are associated with Alzheimer's disease, are all pro-apoptotic (Yamatsuji *et al.*, 1996; Guo *et al.*, 1998; Mattson *et al.*, 1998), while polyglutamine expansion diseases such as Huntington's disease, have gene products that can induce apoptosis in cultured cells (Martindale *et al.*, 1998).

Several proteins associated with neurodegenerative diseases are potential caspase substrates and cleavage has, in some cases, been shown to increase their apoptotic potential. For example, the protein huntingtin has been linked to Huntington's disease. Both wild-type and mutant huntingtin, for example, are cleaved by caspases-1 and -3 (Goldberg *et al.*, 1996; Wellington *et al.*, 1998). A caspase-resistant form of huntingtin is less neurotoxic than the wild-type protein (Wellington *et al.*, 2000). Interestingly, expression of dominant negative caspase-1 in a transgenic mouse model of Huntington's disease significantly reduced symptoms of the disease, and prevented the generation of huntingtin cleavage products normally detected in these mice (Ona *et al.*, 1999). On the other hand, degenerating neurons in transgenic models of

Huntington's disease as well as in brains from human patients do not exhibit the classic morphological features of apoptosis (Turmaine *et al.*, 2000). Moreover, in a *Drosophila* model of Huntington's disease, similar non-apoptotic degenerations were observed and death could not be prevented by the caspase inhibitor p35 (Jackson *et al.*, 1998). Therefore, in Huntington's disease, as for some other neurodegenerative diseases, the role of apoptosis is currently unclear.

Clearer results have been obtained with other disease models. For example, expression of p35 blocks retinal degeneration in *Drosophila* caused by a mutation in the gene for rhodopsin, which is mutated in human retinitis pigmentosa (Davidson and Steller, 1998). In addition, transgenic mice expressing p35 showed attenuated neurodegeneration after kainic acid injection, suggesting that excitatory amino acid-induced death involves caspases and is apoptotic (Viswanath *et al.*, 2000). Moreover, transgenic mice overexpressing Bcl-2 show reduced death in several models of disease, such as axotomy-induced motoneuron loss (Sagot *et al.*, 1995). On the other hand, axonal degeneration was not prevented and lifespan was not prolonged in this model (Sagot *et al.*, 1995). Similarly, Bcl-2 expression delayed the onset of motoneuron disease and prolonged survival in a mouse model of amyotrophic lateral sclerosis, but only modestly, suggesting that apoptosis plays only a minor role in such degenerations (Kostic *et al.*, 1997). Taken together, the results suggest that apoptosis plays a role in neurodegeneration, although its importance may vary considerably between different diseases.

1.6 Sympathetic neurons as a model of apoptotic cell death

1.6.1 History of the model

The molecular mechanisms of programmed cell death have been studied extensively in sympathetic neurons. These neurons depend on the prototypic neurotrophic factor NGF for survival from approximately embryonic day 16 to 1 week postnatally (Coughlin and Collins, 1985). Deprivation of NGF during this period causes apoptosis both *in vivo* and *in vitro* (Levi-Montalcini, 1987). Evidence for this comes from experiments in which addition of exogenous NGF increased the survival of sympathetic neurons in neonatal rats (Hendry and Campbell, 1976), whereas reduction of the amount of NGF with blocking antibodies (Levi-Montalcini and Booker, 1960) or induction of autoimmunity against NGF (Gorin and Johnson, 1979) decreased the

number of sympathetic neurons. Deletion of the gene for NGF (Crowley *et al.*, 1994) or its receptor, TrkA (Smeyne *et al.*, 1994) also resulted in extensive sympathetic neuron loss *in vivo*.

Since the discovery and characterisation of NGF, several other neurotrophic factors and their receptors have been identified (reviewed in Friedman and Greene, 1999). The corresponding knockout mice have significant losses of neurons in many central and peripheral nervous system populations, demonstrating the widespread dependence of neurons on trophic factors during development (Snider, 1994). This suggests that sympathetic neurons are a valid paradigm of developmental cell death.

In the best characterised model of cell death, NGF-dependent sympathetic neurons are isolated from the superior cervical ganglia 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 induces apoptotic cell death in most of the neurons by 48-72 hours (Martin *et al.*, 1988). Advantages of this model are that a relatively homogenous population of neurons is obtained, and the neurons die in a synchronous and reproducible manner. In addition, cell death in culture is representative of the physiological cell death in which neurons are deprived of growth factor *in vivo*. Cell counts for developing ganglia show that there is a relatively constant number of neurons between embryonic day 19 and postnatal day 3 (39,000 ±3300), while 33% are then lost between postnatal day 3 and 7 (the number of sympathetic neurons decreased to 26,500 ±3900; Wright *et al.*, 1983). This period, postnatal days 3 to 7, represents the major time of developmental cell death for these neurons. A disadvantage of this model is that it is difficult to obtain large numbers of sympathetic neurons and there has been no efficient transfection method for the expression of foreign genes. Typically, sympathetic neurons have been microinjected with expression plasmids or antibodies (for example, see Garcia *et al.*, 1992; Estus *et al.*, 1994; Ham *et al.*, 1995), although recently viral vectors have also been used (Slack and Miller, 1996). Other investigators have used PC12 cells, which are derived from rat pheochromocytoma cells, since these attain a sympathetic neuron-like phenotype if differentiated in the presence of NGF, and then die by apoptosis after NGF deprivation (Rukenstein *et al.*, 1991; Mesner *et al.*, 1992). These cells display many of the biochemical changes characteristic of sympathetic neurons after NGF deprivation (Mesner *et al.*, 1992).

1.6.2 Cellular changes during apoptosis in sympathetic neurons

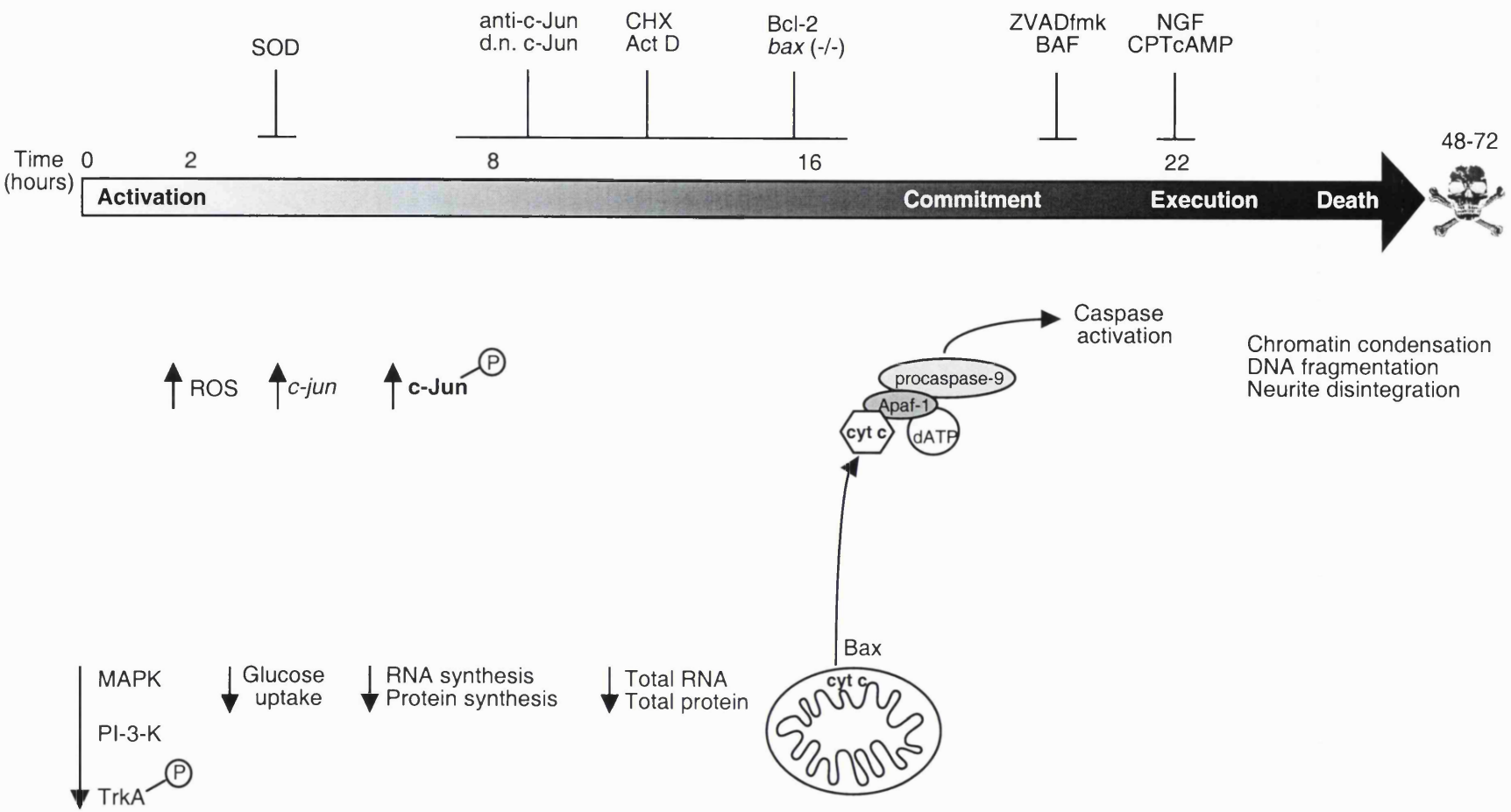
Sympathetic neurons undergoing apoptosis *in vitro* do not show any morphological changes until 12 hours after NGF deprivation, when the neurites begin to degenerate, the plasma membrane loses its smooth appearance, and the cell body begins to atrophy (Martin *et al.*, 1988; Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994). Before this time however, several biochemical changes occur (see Figure 1.1), such as a fall in glucose uptake to 35% of control levels within 6 hours of NGF removal (Deckwerth and Johnson, 1993). Rates of RNA and protein synthesis fall rapidly, with both reduced to 30% by 12 hours. NGF deprivation also decreases purine efflux two- to three-fold within 10 hours (Tolkovsky and Buckmaster, 1989). 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, and most neurons are dead by 48 hours after NGF removal (Martin *et al.*, 1988; Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994). The organelles, however, remain intact and only degenerate during the later stages of death (Martin *et al.*, 1988). Some of these changes have also been noted in sympathetic neurons undergoing apoptosis *in vivo* during development (Wright *et al.*, 1983) and after injection of anti-NGF into newborn mice (Levi-Montalcini *et al.*, 1969), demonstrating that cell death occurring in culture appears to resemble physiological and naturally-occurring cell death. These metabolic changes are likely to be part of the death program because these events occur well before the cell becomes committed to die. However, which of these events are essential for cell death is not clear.

The death pathway initiated by NGF withdrawal can be aborted if NGF is added back to the neurons prior to an irreversible "commitment point". For NGF, the "commitment point" has been defined as the time at which half of the neurons can no longer be rescued, and corresponds to approximately 22 hours after NGF deprivation (Edwards *et al.*, 1991; Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994). NGF can abort the death pathway even in the presence of cycloheximide (Edwards *et al.*, 1991; Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994) and therefore must be having a post-translational effect.

Significantly, many of the changes described for sympathetic neurons also occur in cerebellar granule neurons undergoing apoptosis after survival signal withdrawal (D'Mello *et al.*, 1993; Watson *et al.*, 1998) and in other neuronal cell types.

Figure 1.1 Temporal sequence of events during apoptosis in sympathetic neurons

The known biochemical and genetic changes that occur after the removal of NGF are shown. Critical events that must occur for the cell to undergo apoptosis include an increase in ROS, the expression of c-Jun, a Bax-dependent step, cytochrome c release from the mitochondria, and caspase activation (particularly caspase-2). Re-addition of NGF can abort apoptosis by a post-translational mechanism up to 22 hours after its removal. This figure is compiled from work performed in the laboratories of Eugene Johnson, Aviva Tolkovsky, Jean-Claude Martinou and Jonathan Ham (see text for references). Key: d.n. c-Jun - dominant negative c-Jun; CHX - cycloheximide; Act D - actinomycin D


SOD
anti-c-Jun
d.n. c-Jun
CHX
Act D
Bcl-2
bax (-/-)
ZVADfmk
BAF
NGF
CPTcAMP
Time (hours)
0
2
8
16
22
48-72
Activation
Commitment
Execution
Death
ROS
c-jun
c-Jun
P
Caspase activation
procaspase-9
Apaf-1
cyt c
dATP
Chromatin condensation
DNA fragmentation
Neurite disintegration
Bax
cyt c
MAPK
PI-3-K
TrkA
P
Glucose uptake
RNA synthesis
Protein synthesis
Total RNA
Total protein

1.6.3 Upstream signalling events during apoptosis in sympathetic neurons

NGF induces both neurite outgrowth and survival by binding to and stimulating autophosphorylation of the TrkA receptor (Friedman and Greene, 1999). Signalling through this receptor activates several pathways, including the Ras, ERK, and PI-3-K pathways (reviewed in Greene and Kaplan, 1995; see section 1.4.1), Neuronal survival is critically dependent on the PI-3-K signalling pathway because overexpression of PI-3-K or its downstream effector Akt kinase in sympathetic neurons blocks cell death after NGF withdrawal (Philpott *et al.*, 1997). Similarly, in neuronally-differentiated PC12 cells, inhibitors of PI-3-K, such as wortmannin or LY294002 reduce the ability of NGF to promote survival (Yao and Cooper, 1995). However, neither LY294002 nor a dominant negative PI-3-K cause sympathetic neurons to die if they are maintained in NGF (Philpott *et al.*, 1997). Thus, although NGF may regulate multiple pathways involved in neuronal survival, stimulation of the PI-3-K pathway is sufficient to promote survival in the absence of trophic factor.

One of the early events observed in sympathetic neurons upon NGF removal is an increase in reactive oxygen species (ROS). Levels of ROS peak at three hours and return to basal levels after 8 hours (Greenlund *et al.*, 1995a). Expression of copper/zinc superoxide dismutase (Cu/ZnSOD) in sympathetic neurons delays death (Greenlund *et al.*, 1995a; Jordan *et al.*, 1995). Furthermore, inhibition of NADPH oxidase, an enzyme that generates ROS, also promotes survival in NGF-deprived sympathetic neurons (Tammariello *et al.*, 2000). However, no protection occurs if Cu/ZnSOD is expressed after 8 hours, while NGF and other survival agents can prevent death long after the increase in ROS, suggesting that ROS act as early signalling events in the death pathway and do not simply cause irreversible cellular damage (Greenlund *et al.*, 1995a). The upstream activators and downstream targets of ROS during neuronal death have not been identified, although ROS can activate JNK in other systems (Lo *et al.*, 1996). In addition, Rac1, which is required for maximal NADPH oxidase activation (Bokoch, 1994), is implicated in the death of sympathetic neurons (Bazenet *et al.*, 1998).

The removal of NGF leads to a slow and sustained increase in JNK activity both in sympathetic neurons (Virdee *et al.*, 1997; Eilers *et al.*, 1998) and PC12 cells (Xia *et al.*, 1995; Park *et al.*, 1996b). In the case of differentiated PC12 cells, a study

from Michael Greenberg's group demonstrated that the JNK pathway plays a role in apoptosis because dominant negative mutants of SEK1 and c-Jun promoted survival after NGF withdrawal (Xia *et al.*, 1995). In addition, there appears to be a role for the p38 pathway in these cells, since p38 kinase is also activated after NGF withdrawal, and an inactive form of MKK3 (a p38 kinase) prevented apoptosis (Xia *et al.*, 1995). However, in sympathetic neurons there was no detectable increase in p38 kinase activity or its level of phosphorylation, suggesting that this kinase does not play a role in the death of these primary neurons (Eilers *et al.*, 1998). The role of the JNK pathway in sympathetic neurons will be examined in this thesis.

1.6.4 Downstream biochemical and molecular events during apoptosis in sympathetic neurons

One of the most terminal points in many models of apoptosis is the redistribution of phosphatidylserine from the inner to the outer surface of the plasma membrane, where it can be recognised by receptors on phagocytic cells (Fadok *et al.*, 1992). This event occurs in sympathetic neurons deprived of NGF and has been used as a marker for apoptosis in these cells by staining with fluorescently-labelled annexin V, which binds to PS (Martin *et al.*, 1995; Rimon *et al.*, 1997). PS "flipping" is a caspase-dependent event (Martin *et al.*, 1996). Several observations demonstrate the importance of caspases for mediating apoptosis in sympathetic neurons as in many other models of death. Viral proteins such as p35 and crmA, which are naturally-occurring inhibitors of caspases, promote survival after NGF deprivation (Gagliardini *et al.*, 1994; Martinou *et al.*, 1995). In addition, peptide inhibitors that contain caspase-substrate cleavage sites prevent apoptosis. For example, the cell-permeable, pan-caspase inhibitor BOC-aspartyl(OMe)-fluoromethylketone (BAF) completely blocks the death of sympathetic neurons *in vitro* (Deshmukh *et al.*, 1996), while an inhibitor more specific for caspase-3, acetyl-Asp-Glu-Ala-Asp-aldehyde (Ac-DEVD-CHO) inhibited death when microinjected into NGF-deprived neurons (McCarthy *et al.*, 1997). However, caspase-3 does not appear to be activated during sympathetic neuron death (Deshmukh *et al.*, 1996). On the other hand, caspase-2 activation has been demonstrated (Deshmukh *et al.*, 1996), and antisense RNA targetted to NEDD2 (now known as caspase-2) transcripts protected sympathetic neurons from cell death (Troy *et al.*, 1997). In contrast, neurons cultured from caspase-2-deficient mice are still sensitive to NGF deprivation (Bergeron *et al.*, 1998) although this could be due to

developmental compensation by another caspase. Most significant, perhaps, is the fact that neurons cultured from caspase-9 knockout mice show reduced cell death after NGF withdrawal, suggesting that the apoptosome complex is involved in the death of these neurons (Deshmukh *et al.*, 2000). Thus, caspases-2 and -9 are currently implicated in the death of sympathetic neurons, but it is unclear how many other caspases may contribute to apoptosis in this system.

Caspase-9 is thought to be activated by autocatalytic cleavage of the pro-caspase-9 zymogen, following cytochrome c release from the mitochondria (see section 1.3.2). Indeed, recent work has shown that cytochrome c is a critical mediator of cell death in sympathetic neurons. Microinjection of neurons with an antibody against cytochrome c promoted survival in the absence of NGF (Neame *et al.*, 1998). Interestingly, cytoplasmic injection of cytochrome c in the presence of NGF did not kill the neurons, in contrast to other cell types studied to date (Deshmukh and Johnson, 1998; Neame *et al.*, 1998). However, when neurons were withdrawn from NGF in the presence of cycloheximide, microinjection of cytochrome c was then able to induce caspase-dependent apoptosis (Deshmukh and Johnson, 1998). These authors suggested that sympathetic neurons need to acquire "competence-to-die", a protein synthesis-independent step that must occur in conjunction with cytochrome c release.

Bcl-2 is another protein that plays a role in sympathetic neuron apoptosis, and this family of proteins is thought to accomplish this by controlling cytochrome c release. Neurons microinjected with Bcl-2 expression vectors show increased survival in response to NGF withdrawal (Garcia *et al.*, 1992; Ham *et al.*, 1995). In addition, overexpression of Bcl-2 using transgenic mice protects neurons from naturally-occurring developmental apoptosis (Martinou *et al.*, 1994). Perhaps surprisingly therefore, *bcl-2* (–/–) mice show no decrease in the number of sympathetic neurons at birth (Michaelidis *et al.*, 1996). However, these Bcl-2 deficient mice had 58% fewer sympathetic neurons at postnatal day 44 than wild-type littermates, and neurons cultured from these mice underwent apoptosis more rapidly than cells from wild-type littermates (Greenlund *et al.*, 1995b). Also, the most significant period of naturally-occurring cell death in sympathetic neurons is between postnatal days 3 and 7. The anti-apoptotic Bcl-2 family member Bax appears to have an opposing role to Bcl-2. Sympathetic neurons derived from Bax-deficient mice do not undergo apoptosis (although the soma atrophy and the neurites do not extend), while the mice have a

three-fold increase in the number of sympathetic neurons per ganglion (Deckwerth *et al.*, 1996).

Inhibitors of RNA and protein synthesis prevent NGF withdrawal-induced cell death in sympathetic neurons (Martin *et al.*, 1988) as well as death induced by trophic factor withdrawal in many other neuronal cell types, both *in vitro* and *in vivo* (Oppenheim *et al.*, 1990; Scott and Davies, 1990; D'Mello *et al.*, 1993). Based on results such as these, it was proposed that developmental apoptosis in these neurons is an active programme requiring the synthesis of new RNA and protein (Martin *et al.*, 1988; Johnson and Deckwerth, 1993). This has led to a search for genes upregulated by NGF withdrawal, since it seems likely that some of these might be important for mediating cell death.

Table 1 shows a list of the changes in the various gene products that have been studied in sympathetic neurons undergoing programmed cell death. Most of the mRNAs that have been studied substantially decrease in level over 24 hours, in line with the general fall in RNA and protein synthesis that occurs after NGF withdrawal. General neuronal markers such as *neurofilament-M* and *tyrosine hydroxylase*, or cellular markers such as *cyclophilin*, have been used as controls in much of the published work and these transcripts declined in level as would be expected. Much of this work came from the laboratory of Eugene Johnson, and in one paper over 70 mRNAs were reported to decline in level (Estus *et al.*, 1994). Other studies show that the transcripts of various genes, including *p53, Rb, c-myc, cyclins, Cu/Zn or MnSOD* also declined (Freeman *et al.*, 1994; Greenlund *et al.*, 1995a).

The gene for the protein called SM-20 was recently shown to be upregulated in sympathetic neurons within 5 hours of NGF withdrawal (Lipscomb *et al.*, 1999). Overexpression of SM-20 by microinjection of sympathetic neurons induced apoptosis, although the mechanism of action of this protein is not yet clear (Lipscomb *et al.*, 1999). Other genes such as *fosB* and *junB* showed a delayed response, increasing at 10-15 hours. However, c-Fos protein was not detected in neurons by immunoblotting, and intriguingly JunB protein actually declined in level (Ham *et al.*, 1995). The increase in *cyclinD1* initially provoked interest since it had been suggested that neurons undergo apoptosis due to an abortive attempt to re-enter the cell cycle (Heintz, 1993; Rubin *et al.*, 1993). Support for this model in sympathetic neurons comes from work carried out in Lloyd Greene's laboratory, which shows that cell cycle blockers, inhibitors of

Table 1.1 Changes in gene expression in sympathetic neurons after NGF withdrawal

The change in the level of expression of various mRNA species in NGF-deprived sympathetic neurons is shown, with the time of induction and maximal expression time indicated where appropriate. Induced genes have been divided into those upregulated at early or late timepoints; the "late" genes *transin* and *collagenase* are putative AP-1 target genes. Most other gene products studied (more than 70 different mRNA species) declined in level over 24 hours, including neuronal-specific or general cellular markers. *S-100β* is specifically expressed in contaminating Schwann cells present in sympathetic neuron cultures. Schwann cells do not die after NGF withdrawal.

Gene		Induced / Maximal	Reference
c-jun	Early	<5h / 15h	1
mkp-1		<5h / 5-25h	1
SM-20		<5h / 10-12h	5
c-myb	Delayed	5-10h / 10-15h	1
cyclin D1		5-10h / 10-20h	2
c-fos		10-15h / 15h	1
fosB		10-15h / 15h	1
junB		10-15h / 15h	1
ngf1-a		10-15h / 15h	1
collagenase	AP-1 targets	20-25h / 20-25h	1
transin		20-25h / 20-25h	1
bax	Bcl-2 family	Decreases	4
bcl-x_L		Decreases	4
bcl-2		Decreases	4
Cu/Zn or Mn-SOD	Others	Decreases	3
cdk 4/5		Decreases	2
cyclin D2/D3/E		Decreases	2
NF-κB		Decreases	1
fra1/fra2		Decreases	1
c-myc		Decreases	2
p53		Decreases	2
Rb		Decreases	2
neurofilament-M	Neuronal markers	Decreases	1 2
neuron specific enolase		Decreases	1
tyrosine hydroxylase		Decreases	1 3 4
cyclophilin	Cellular marker	Decreases	1 2 5
S-100β	Schwann cell marker	Constant	1 2

1: Estus *et al.* (1994) J. Cell Biol., 127 (6), 1717-27
2: Freeman *et al.* (1994) Neuron, 12, 343-55
3: Greenlund *et al.* (1995) Neuron, 14, 303-315
4: Greenlund *et al.* (1995) Neuron, 15, 649-661
5: Lipscomb *et al.* (1999) J. Neurochem, 73, 429-32

cyclin-dependent kinases such as olomoucine, and dominant negative cyclin-dependent kinase 4 and 6 all promote survival in sympathetic neurons (Park *et al.*, 1996a; Park *et al.*, 1997). However, the genetic and molecular bases of apoptosis share no obvious mechanisms with cell division in most cases. More recently, it has been suggested that proliferative and apoptotic pathways are coupled, such that proliferating cells (which are potentially dangerous to the multicellular organism) are more sensitive to apoptotic stimuli (Evan and Littlewood, 1998).

One gene that was rapidly induced in sympathetic neurons was *mkp-1* (Estus *et al.*, 1994). The phosphatase MKP-1 can inhibit the MAPK/ERK pathway, and it is possible that this pathway may mediate some of the protective functions of NGF (Szeberenyi and Erhardt, 1994). There are conflicting reports about the role of ERK in sympathetic neurons. ERK activity does not seem to be necessary for the survival-promoting effects of NGF on sympathetic neurons (Creedon *et al.*, 1996; Virdee *et al.*, 1997). However, other authors report that CREB phosphorylation is necessary for sympathetic neuron survival, and this may be mediated by ERK/Rsk (Riccio *et al.*, 1999; Bonni *et al.*, 1999). It could be that downregulation of MAPK activity after NGF withdrawal plays a role in apoptosis, as is the case in PC12 cells (Xia *et al.*, 1995). Increased expression of MKP-1 could be responsible for inhibiting MAPK activity. Another gene induced rapidly and in a sustained manner was *c-jun* and this has been the most extensively studied. Induction begins within 5 hours of NGF deprivation and is maximal at approximately 15 hours (Estus *et al.*, 1994). A more detailed discussion of c-Jun will begin in section 1.7.

The p53 tumour suppressor gene, which can induce apoptosis in a number of cell types (for a review, see White, 1996), has been suggested to have a role in the death of sympathetic neurons. In cultured neurons, p53 protein levels are elevated in response to NGF withdrawal or activation of the p75 neurotrophin receptor (Aloyz *et al.*, 1998), and adenoviral-mediated expression of p53 induces apoptosis in the presence of NGF (Slack *et al.*, 1996). Interestingly, in *p53* (–/–) mice, there are increased numbers of sympathetic neurons at postnatal day 15, and this appears to be an effect on reduced apoptosis rather than increased proliferation because reduced numbers of TUNEL-positive neurons are detected in *p53* (+/–) mice compared with wild-type littermates (Aloyz *et al.*, 1998). These authors suggested that p53 could be a

transcriptional target of c-Jun. In other cell types, p53 can be stabilised and activated by JNK (Milne *et al.*, 1995; Hu *et al.*, 1997; Fuchs *et al.*, 1998b).

1.6.5 Anti-apoptotic proteins in sympathetic neurons

A recently discovered mechanism by which NGF exerts its survival effect in chick sympathetic neurons, is through the upregulation of a protein called ITA (Wiese *et al.*, 1999). ITA is a chick homologue of the IAP caspase inhibitors. Antisense ITA constructs introduced into the neurons reduced their survival in the presence of NGF, whereas overexpression of ITA itself supported survival in the absence of NGF (Wiese *et al.*, 1999). The increase in ITA expression in response to NGF was due to PI-3-K activation since it was blocked by wortmannin. As discussed in Section 1.6.3, PI-3-K inhibitors induce caspase-dependent death of sympathetic neurons, consistent with the hypothesis that this is due to downregulation of ITA. In other systems Akt acts downstream of PI-3-K, and a constitutively active mutant of Akt can prevent the death of sympathetic neurons after NGF deprivation (Philpott *et al.*, 1997). Interestingly, Akt has been suggested to activate the NF-κB pathway in fibroblasts (Ozes *et al.*, 1999; Romashkova and Makarov, 1999). NF-κB is activated by the binding of NGF to TrkA in PC12 cells (Wood, 1995). Work from Robert Freeman's group has implicated NF-κB as a survival protein in sympathetic neurons. A specific NF-κB inhibitory peptide (SN50) induced cell death in the presence of NGF, while expression of NF-κB promoted survival after NGF deprivation (Maggirwar *et al.*, 1998). According to these authors, NF-κB may function by inhibiting the transactivating potential of c-Jun, a protein strongly implicated in the death of these neurons (Maggirwar *et al.*, 2000; see section 1.7). However, this recent finding suggests that a survival pathway involving NGF⇒PI-3-K⇒Akt⇒NF-κB⇒ITA may exist as well (Wiese *et al.*, 1999; Degterev and Yuan, 1999). One should note that NGF can exert its survival effect in the presence of protein synthesis inhibitors (Edwards *et al.*, 1991; Deshmukh and Johnson, 1997; Deshmukh and Johnson, 1998) so it is currently unclear how this can be reconciled with the facts that NGF can induce a large increase in ITA protein and that antisense ITA can protect sympathetic neurons.

The cAMP response element binding protein (CREB) can be phosphorylated on its transcriptional regulatory site in distal axons of sympathetic neurons exposed to NGF (Riccio *et al.*, 1997), suggesting that this protein may play a role in these neurons. Indeed, CREB-mediated gene expression was necessary for NGF-dependent

survival since expression of CREB mutants that either inhibit CREB DNA-binding activity or are transcriptionally inactive reduced the survival of sympathetic neurons in the presence of NGF (Riccio *et al.*, 1999). Furthermore, a transcriptionally active form of CREB promoted survival in the absence of NGF (Riccio *et al.*, 1999).

Recently, work from Freda Miller's group showed the surprising result that the p53 family member, p73, serves an anti-apoptotic role in sympathetic neurons (Pozniak *et al.*, 2000). A truncated form of p73 that is normally present in sympathetic neurons was dramatically downregulated after NGF withdrawal, while overexpression of this isoform could promote survival in the absence of NGF. In *p73* (–/–) mice, apoptosis of developing neurons was greatly enhanced. It is possible that this truncated form of p73 counteracts the pro-apoptotic function of p53 which they have previously suggested is a critical component of the death pathway in sympathetic neurons (Slack *et al.*, 1996; Aloyz *et al.*, 1998).

1.7 c-Jun and apoptosis

Some of the earliest and most compelling evidence for a pro-apoptotic role for c-Jun came from sympathetic neurons. In this model, a substantial increase in the level of *c-jun* mRNA was observed within 5 hours of NGF withdrawal and remained elevated for 24 hours, in contrast to the general reduction in the prevalence of other RNA species (Estus *et al.*, 1994). Induction of *c-jun* mRNA was not dependent on new protein synthesis and hence was mediated by post-translational mechanisms. In contrast, the later induction of other mRNA species such as *c-fos* and *transin* was inhibited by the presence of cycloheximide, suggesting that upregulation of these genes during death was part of a genetic program and required ongoing protein synthesis. Microinjection of neutralising antibodies to c-Jun protected these cells from apoptosis, whereas antibodies to JunB or JunD did not promote survival (Estus *et al.*, 1994). In immunoblotting experiments, increased c-Jun expression and a mobility shift due to phosphorylation of the c-Jun transactivation domain was observed after NGF withdrawal and this occurred before the cell death commitment point (Ham *et al.*, 1995). Other AP-1 factors generally remained constant in level (Ham *et al.*, 1995). c-Fos and FosB, which were upregulated at the mRNA level (Estus *et al.*, 1994), showed no increase in protein level (Ham *et al.*, 1995). In addition, c-Fos was detected by immunocytochemistry in neurons that already displayed pyknotic nuclei, whereas

c-Jun expression was seen in neurons before the point at which they showed any morphological changes characteristic of apoptosis (Ham *et al.*, 1995). c-Jun expression was detected in most of the neurons after NGF withdrawal. Microinjection of an expression vector for dominant negative c-Jun, which is able to dimerise and bind to DNA, but which lacks the transactivation domain, protected these neurons from apoptosis, while overexpression of wild-type c-Jun was able to induce apoptosis in the presence of NGF. These data, in conjunction with the fact that antibodies specific for c-Jun also promote survival, are the most compelling results that show the importance of c-Jun in the death of sympathetic neurons. However, an absolute requirement for c-Jun in sympathetic neurons has been impossible to show due to the embryonic lethality of *c-jun* (–/–) mice, which die at mid-gestation (Hilberg *et al.*, 1993; Johnson *et al.*, 1993), before the time of naturally-occurring cell death in sympathetic neurons.

c-Jun immunoreactivity and phosphorylation have also been detected in many other models of neuronal apoptosis, such as differentiated PC12 cells (Mesner *et al.*, 1995), granule neurons (Miller and Johnson, 1996; Watson *et al.*, 1998), and hippocampal neurons exposed to β-amyloid (Anderson *et al.*, 1995). c-Jun is induced during naturally-occurring and radiation-induced apoptosis in the developing rat brain (Ferrer *et al.*, 1996a) and is associated with apoptotic areas in several human tumours of the central nervous system (Ferrer *et al.*, 1996b). Furthermore, c-Jun immunoreactivity was detected in neurons *in vivo* after ischaemia (Dragunow *et al.*, 1993). Antisense oligonucleotides were used by Schlingensiepen *et al.* (1994) to show that inhibition of c-Jun expression promoted survival in cultured hippocampal neurons. In cerebellar granule neurons, transfection of an expression vector for a c-Jun dominant negative mutant lacking the transactivation domain protected the cells from survival factor withdrawal (Watson *et al.*, 1998). In addition, a c-Jun mutant with the activating serines changed to alanines protected the neurons, whereas an activated mutant in which the serines were mutated to aspartate promoted apoptosis (Watson *et al.*, 1998). Transfection of an expression vector for dominant negative c-Jun also protected differentiated PC12 cells from NGF withdrawal-induced death (Xia *et al.*, 1995). Furthermore, a pro-apoptotic role for c-Jun has been demonstrated in striatal neurons treated with dopamine (Y. Luo *et al.*, 1998).

Other studies in different cell systems have supported a role for c-Jun in some forms of apoptosis. Colotta *et al.* (1992) showed that lymphoblastoid cells express

c-jun and *c-fos* mRNA when undergoing apoptosis after growth factor deprivation. Antisense oligonucleotides to *c-jun* promoted survival in this model. Qian *et al.* (1997) showed that c-Jun is a critical factor in vitamin E succinate induced apoptosis of lymphoblastoid cells. Increased c-Jun expression was associated with cell death in this model, and a dominant negative form of c-Jun reduced AP-1 activity and promoted survival. Furthermore, overexpression of c-Jun in 3T3 fibroblasts induced apoptosis (Bossy-Wetzel *et al.*, 1997).

1.8 The transcription factor c-Jun

The proto-oncogene *c-jun* is the mammalian homologue of *v-jun*, the transforming oncogene of avian sarcoma virus 17 (Maki *et al.*, 1987). In the few years following its identification, a number of studies were published on c-Jun and its contribution to AP-1 activity by Peter Angel (working with Michael Karin), Dirk Bohmann (in Robert Tjian's group), and by the laboratory of Peter Vogt. It was soon discovered that c-Jun participates in a number of diverse cellular responses including transformation (Schutte *et al.*, 1989a; Bos *et al.*, 1990), proliferation (Castellazzi *et al.*, 1991; Lloyd *et al.*, 1991), differentiation (Bengal *et al.*, 1992; Treier *et al.*, 1995; Hou *et al.*, 1997; Riesgo-Escovar and Hafen, 1997), and the response to toxic agents (van Dam *et al.*, 1995; see section 1.7). In support of these *in vitro* studies, mice lacking c-Jun exhibited impaired hepatogenesis and altered red blood cell production (Hilberg *et al.*, 1993), and fibroblasts cultured from early embryos showed proliferative defects (Johnson *et al.*, 1993). The mice died mid-gestation at approximately E12.5 (Johnson *et al.*, 1993).

In addition to its role in proliferation, differentiation, and promoting apoptosis (discussed in the section before), c-Jun can also have an anti-apoptotic role. For example, phosphorylated (activated) c-Jun is critical for protection against UV-induced apoptosis (Wisdom *et al.*, 1999), and mice deficient in c-Jun had increased cell death in the liver (Hilberg *et al.*, 1993). The diversity of cellular processes in which Jun has been demonstrated to function may reflect the ability of c-Jun to associate with itself or with other members of the AP-1 family, and also with the ATF/CREB family of proteins (Diamond *et al.*, 1990; Angel and Karin, 1991; Hai and Curran, 1991; Deng and Karin, 1993). Further complexity is derived from a variety of transcriptional regulatory events (kinases, phosphatases, tissue specific cofactors) that are influenced

by cell type and environmental stimuli (Deng and Karin, 1993; Pfarr *et al.*, 1994). Thus it seems likely that the level of c-Jun and the ratio of the various AP-1 and ATF factors provide a cell with the capability to integrate signals from multiple pathways and determine whether a cell proliferates, exits from the cell cycle, differentiates, or undergoes apoptosis by modulating the expression of specific target genes. Identification of the target genes of c-Jun is therefore of utmost importance.

There are three cellular members of the Jun family: c-Jun, JunB, and JunD, and four members of the Fos family: c-Fos, FosB, Fra1, and Fra2. All of these factors contain a highly basic domain, responsible for DNA binding, and a leucine zipper motif that mediates dimer formation. These basic/leucine zipper (bZIP) transcription factors constitute the activity known as AP-1 (Angel and Karin, 1991; Curran and Vogt, 1992), which binds to the consensus sequence 5'-TGA$^{C}/_{G}$TCA-3'. JunB and JunD show less transactivating potential and can function as negative regulators of c-Jun activity (Chiu *et al.*, 1989; Schutte *et al.*, 1989b; Deng and Karin, 1993). Changes in the relative level of different AP-1 factors can have important biological consequences, which shows the importance of studying all of the members of the family in a particular model system. For example, inhibition of JunB protein synthesis, using antisense oligonucleotides, increased the rate of proliferation and reduced the rate of differentiation of PC12 cells, while inhibition of c-Jun expression had the opposite effect (Schlingensiepen *et al.*, 1993; Pfarr *et al.*, 1994).

Other bZIP transcription factors exist, such as the ATF/CREB family. c-Jun can bind to certain members of this family. Dimerisation is highly selective between AP-1 factors and ATF members: c-Jun, for example, can bind to ATF-2, -3, and -4, but not ATF-1 (Hai and Curran, 1991). The recognition sequence of c-Jun/ATF-2 heterodimers (5'-TTACCTCA-3') differs slightly from AP-1 or ATF/CREB sites.

1.9 Regulation of c-Jun

1.9.1 JNK, ERK, and p38 kinase

The MAPK superfamily of serine/threonine kinases are themselves activated by phosphorylation on specific threonine and tyrosine residues. Kinase cascades (see Figure 1.2) are formed whereby each MAPK (i.e. ERK, JNK, or p38) is activated by a corresponding dual-specificity MAPK kinase (e.g. MEK1 or 2 for ERK, SEK1 or MKK7 for JNK). These, in turn, are activated by MAP kinase kinases (e.g. c-Raf for

ERK, MEKK1 and ASK1 for JNK). This cascade of three or four kinases can provide a considerable degree of amplification from low-level surface signals. In the case of MAPK, ligand binding to the growth factor receptors results in autophosphorylation of the tyrosine kinase domain, recruitment of signalling mediators such as Grb2 and the guanine nucleotide exchange factor Sos, and activation of Ras. This leads to binding of Raf to Ras, and then phosphorylation and activation of the MAPK upstream kinases.

There is the possibility of cross-talk between these upstream kinases. Specificity can be achieved through the use of scaffold proteins (see Figure 3). These may either facilitate the activation of one kinase by another, or may hold the kinase in a latent state close to the receptor that will induce its activation (Pawson and Scott, 1997; (Whitmarsh and Davis, 1998). MAPKs also have specific interaction sites on their substrates (Holland and Cooper, 1999).

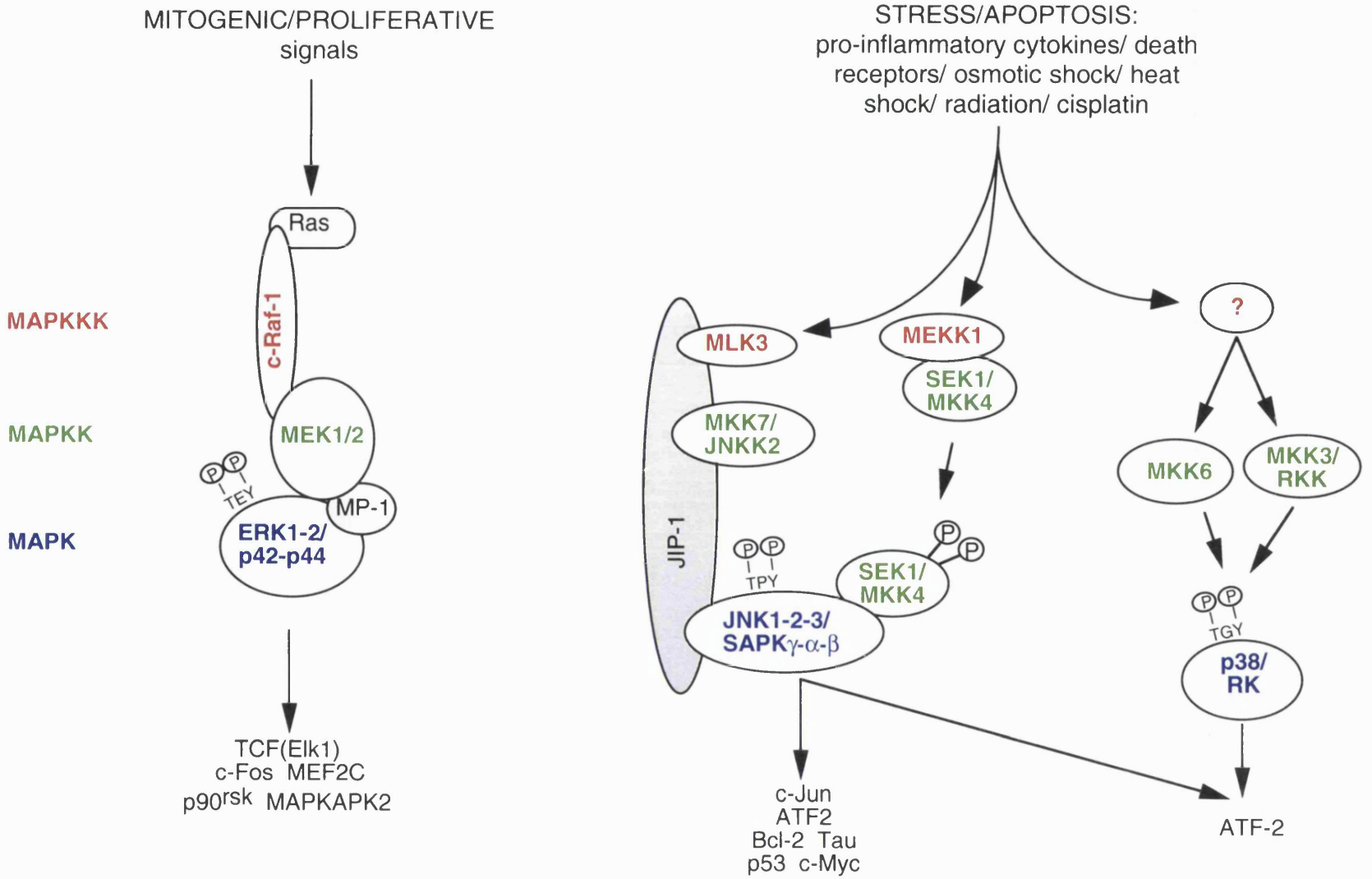
MAPK can contribute to increased AP-1 activity because ERK phosphorylates TCF/Elk1 which leads to increased *c-fos* expression (Gille *et al.*, 1995). p38 kinase phosphorylates and activates ATF-2 (as does JNK) providing another way in which kinases other than JNK can alter the outcome of c-Jun activation, in this case increasing the expression of c-Jun/ATF-2 targets rather than AP-1-responsive genes. However, the only kinase currently recognised that efficiently phosphorylates and activates c-Jun directly is JNK.

1.9.2 JNK and c-Jun

c-Jun amino-terminal kinase (JNK) was first identified in human cell lines by the laboratories of Roger Davis and Michael Karin (Derijard *et al.*, 1994; Sluss *et al.*, 1994), and also in rat cells by John Kyriakis and James Woodgett (who named them stress-activated protein kinases, SAPK; Kyriakis *et al.*, 1994). There are three different mammalian *Jnk* genes, which code for 10 known isoforms of approximately 46 and 54 kDa (Gupta *et al.*, 1996). Isoforms vary in their substrate specificity and activity in response to different stimuli (Gupta *et al.*, 1996). JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is restricted to the brain, heart and testis (Mohit *et al.*, 1995); Ip and Davis, 1998; Kumagae *et al.*, 1999). JNK is 40% identical to ERK, but is activated by stressful stimuli such as UV irradiation, protein synthesis inhibitors, and TNFα (see Figure 1.3), which have only a small effect on ERK.

Figure 1.2 Mammalian MAPK superfamily kinase cascades

ERK, JNK, and p38 kinase are activated by upstream MAPK kinases, which are themselves activated by MAPK kinase kinases. MAPKs and their activators may be brought into close proximity by interactions with scaffold proteins such as MP-1 or JIP-1, or by direct binding of a kinase to its substrate kinase. SEK1, for example, binds to its activator MEKK1 and, once phosphorylated, SEK1 dissociates and binds to JNK. Examples of the various stimuli that activate each pathway are shown. Some of the phosphorylation targets of these pathways are indicated.


MITOGENIC/PROLIFERATIVE
signals
MAPKKK
MAPKK
MAPK
Ras
c-Raf-1
MEK1/2
TEY
MP-1
ERK1-2/
p42-p44
TCF(Elk1)
c-Fos MEF2C
p90rsk MAPKAPK2
STRESS/APOPTOSIS:
pro-inflammatory cytokines/ death
receptors/ osmotic shock/ heat
shock/ radiation/ cisplatin
MLK3
MEKK1
SEK1/
MKK4
?
MKK7/
JNKK2
MKK6
MKK3/
RKK
JIP-1
TPY
SEK1/
MKK4
JNK1-2-3/
SAPKγ-α-β
TGY
p38/
RK
c-Jun
ATF2
Bcl-2 Tau
p53 c-Myc
ATF-2

JNK is responsible for the phosphorylation of c-Jun on serines 63 and 73 within the transactivation domain (Pulverer *et al.*, 1991; Smeal *et al.*, 1991). Activation of c-Jun then leads to the activation of c-Jun target genes, including *c-jun* itself, thus forming a positive autoregulatory loop (Angel *et al.*, 1988b). c-Jun can also be phosphorylated on threonines 91 and 93 by JNK, and this may contribute to its increased transcriptional potential (Papavassiliou *et al.*, 1995). Although both serines are followed by prolines and are therefore potential MAPK targets, these sites are not effectively phosphorylated by ERK or p38 kinase (Alvarez *et al.*, 1991; Minden *et al.*, 1994; Rouse *et al.*, 1994). ERK instead appears to phosphorylate a site located in the carboxy-terminal domain of c-Jun, which is associated with the inhibition of DNA binding (Alvarez *et al.*, 1991; Minden *et al.*, 1994). Phosphorylation of this carboxy-terminal site can also be catalysed by casein kinase II (Lin *et al.*, 1992) or GSK3 (Boyle *et al.*, 1991).

Deletion of the JNK binding site (the δ domain), located between amino acids 30-60 of c-Jun, diminishes the ability of c-Jun to be phosphorylated *in vivo* in response to a variety of JNK activators (Adler *et al.*, 1992; Hibi *et al.*, 1993; B. Su *et al.*, 1994). Interaction of JNK and c-Jun *in vivo* also appears to require a carboxy-terminal region (May *et al.*, 1998). JNK phosphorylation also increases the stability of c-Jun by reducing its ubiquitination (Fuchs *et al.*, 1996; Musti *et al.*, 1997).

There are conflicting reports about the localisation of JNK. In some cell types JNK is present both in the cytoplasm and the nucleus under normal conditions but becomes concentrated in the nucleus after certain stresses (Cavigelli *et al.*, 1995; Kawasaki *et al.*, 1996; Mizukami *et al.*, 1997). In other cells, JNK becomes activated in the nucleus after stimulation (Chen *et al.*, 1996a).

1.9.3 The JNK/c-Jun signalling pathway

Many potential upstream kinases in the JNK/c-Jun signalling pathway have been identified to date. The first kinase that was shown to phosphorylate JNK was SEK1 (for SAPK/ERK kinase 1), also known as MKK4 and JNKK1 (Derijard *et al.*, 1994; Sanchez *et al.*, 1994; Lin *et al.*, 1995). More recently, MKK7 (also known as JNKK2) has been identified as a JNK activator (Tournier *et al.*, 1997; Yao *et al.*, 1997). MEKK1 was the first kinase shown to activate SEK1 (Yan *et al.*, 1994). Since then, however, this pathway has become much more complex. There are currently over

10 JNK kinase kinases that have been shown to activate SEK1 or MKK 7 (for a review, see Chen and Tan, 2000).

1.9.4 Other JNK substrates

In addition to c-Jun, JNK appears to be able to phosphorylate various other substrates. ATF-2 is an efficient substrate for JNK, and phosphorylation stimulates its transcriptional activity (Gupta *et al.*, 1995; van Dam *et al.*, 1995). Bcl-2 and Bcl-x_L can also be phosphorylated by JNK; in both cases phosphorylation inactivates the anti-apoptotic effect of these proteins (Maundrell *et al.*, 1997; Kharbanda *et al.*, 2000). This suggests a further mechanism by which JNK can be pro-apoptotic. Phosphorylation of the tau protein by JNK3 (SAPKβ) has also been demonstrated (Reynolds *et al.*, 1997). Tau is hyperphosphorylated when found in the neurofibrillary tangles of Alzheimer's patients (Ksiezak-Reding *et al.*, 1992; Kopke *et al.*, 1993). It is interesting that tau can be phosphorylated by a stress-activated kinase, and also one that is mostly found in the brain. In non-stressed cells, p53 is targetted by JNK for ubiquitination and degradation (Fuchs *et al.*, 1998a). Conversely, the p53 protein can be phosphorylated by JNK (Milne *et al.*, 1995). Moreover, activation of the JNK signalling pathway (by an upstream activating kinase, MEKK1) phosphorylated and stabilised p53, increasing its transcriptional activity and potentiating p53-dependent apoptosis (Fuchs *et al.*, 1998b).

1.10 Aims of this thesis

The transcription factor c-Jun has been strongly implicated in the programmed cell death of sympathetic neurons withdrawn from NGF, as well as in several other models of apoptosis. In this study, the regulation of c-Jun expression in sympathetic neurons will be examined by looking at the activity of the *c-jun* promoter during NGF withdrawal-induced death to determine what role this plays in the increased expression of c-Jun. In addition, the putative upstream kinases that lead to activation of c-Jun in other systems will be investigated to determine whether they have a role in sympathetic neuron cell death. To this end, the effect of triggering or blocking the c-Jun pathway on neuronal survival will be examined.

A key issue with many transcription factors, including c-Jun, is the identification of the targets of transactivation. The direct targets of c-Jun are not clear. In the death pathway of sympathetic neurons c-Jun appears to function upstream of Bcl-2, Bax, and the caspases. Overexpression of Bcl-2 promotes survival but does not

block the increase in endogenous c-Jun (Ham *et al.*, 1995), while Bax-deficient sympathetic neurons are prevented from dying but show increased *c-jun* mRNA (Easton *et al.*, 1997). Several reports suggest that c-Jun functions upstream of caspase activity. The caspase inhibitors BAF and ZVADfmk both promote survival in sympathetic neurons and PC12 cells but do not block JNK activation (Park *et al.*, 1996b; Stefanis *et al.*, 1996). Furthermore, the Bax-dependent step and the caspase-inhibitable events are transcription-dependent, suggesting that the transcriptional target of c-Jun is a more distal event. Here, I aim to connect c-Jun to the general cell death machinery to determine its role during cell death and then determine what might be the downstream targets of c-Jun transactivation.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals and equipment

Laboratory reagents were of AnalaR quality.

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

ABI

ABI Prism 310 Genetic Analyser

Agar Scientific

Number 5 Dumont forceps

Amersham Pharmacia Biotech

[^{35}S]dATP, [α^{32}P]dCTP, Hyperfilm MP, ^{35}S-labelled methionine, ECL chemiluminescence reagent, USB Sequenase Version 2.0 DNA sequencing kit, Hybond ECL nitrocellulose membrane, Rainbow protein markers

Becton-Dickinson and Co.

Falcon tissue culture flasks and dishes

Bio-Rad Laboratories

Gel dryer, Mini-Protean II Electrophoresis Cell, Mini Trans-Blot Electrophoretic Transfer Cell, Tris, Tween-20, acrylamide:bisacrylamide (30:0.8), acrylamide:bisacrylamide (19:1), NAP Sephadex G25 columns, ammonium persulphate (APS), glycine, sodium dodecyl sulphate (SDS), Bradford protein assay kit, ethylenediamine tetra-acetic acid (EDTA), N'N'N'N' tetramethylethylene diamine (TEMED), Coomassie Brilliant Blue G-250

Boehringer Mannheim

TUNEL assay kit, calf intestine alkaline phosphatase (1 unit/μl)

Boots

Non-fat milk powder

Carl Zeiss

Axiovert 100 and 135M inverted fluorescence microscopes

Citifluor

Citifluor

Costar

0.22 μM Spin-X centrifuge tube filters, 12 well tissue culture dishes

Difco Laboratories

Bacto-agar, bacto-yeast extract, bacto-tryptone

Dow Chemical Company

Saran wrap

Eppendorf

Microinjection equipment: micromanipulator (5171), transjector (5246), heater (TRZ 3700), CO_2 controller (CTI 3700), environmental chamber

Eurogentec

Smart DNA ladder

Fisons Scientific Equipment

Boric acid

FMC Bioproducts

SeaPlaque agarose

Gibco BRL Life Technologies

Horizon agarose gel electrophoresis tanks, trypsin/EDTA, L-glutamine, penicillin/streptomycin, 1 kb DNA ladder, buffer-saturated phenol, CsCl optical grade, electrophoresis grade agarose

ICN Biomedicals

BSA Path-o-cyte

Intracel

1.2 mm od x 0.8 mm id x 10 cm glass capillaries

John Weiss

Dissection scissors and forceps

Kodak

TMY 400 Film, 1D gel electrophoresis documentation and analysis system

Kopf Instruments

Vertical pipette puller model 720

Merck Ltd. BDH

Acetone, ethanol, glacial acetic acid, glycerol, HCl, KCl, $MgCl_2$, methanol, NaCl, NaOH, coverslips, microscope slides, chloroform, isoamyl alcohol, Ponceau-S

MJ Research

Peltier Thermal Cycler

Molecular Probes

Live/dead assay kit, Texas Red-dextran 70,000 MW neutral

New England Biolabs

Restriction endonucleases, modifying enzymes, Lambda DNA *Hin*d III digest

Nikon Co.

Microphot FXA fluorescence microscope

PAA Laboratories

Foetal calf serum

Promega

2.5S NGF, TNT Coupled Reticulocyte Lysate System

Qiagen

RNeasy kit, PCR gel extraction kit

Quantum Biotechnologies

HEK 293A cells, Adeno-Quest kit, Adeno-LacZ

Sigma-Aldrich Chemical Company

Bovine serum albumin, bromophenol blue, dimethyl sulphoxide (DMSO), ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N',-tetraacetic acid (EGTA), glutaraldehyde, leupeptin, sterile phosphate buffered saline ($\pm Ca^{2+}/Mg^{2+}$), xylene cyanol FF, ethidium bromide, ribonuclease A, goat serum, laminin, poly-L-lysine, aprotinin, pepstatin A, phenyl methyl sulphonyl fluoride (PMSF), Hoechst 33342, sodium selenite, transferrin, insulin, progesterone, putrescine, L-thyroxine, selenium, tri-iodo-thyronine, diethyl pyrocarbonate (DEPC), Triton X-100, tetracycline, ampicillin, chloramphenicol, β-mercaptoethanol, Dulbecco's Modified Eagle's Medium (DMEM), uridine, 5-fluoro-2-deoxyuridine, sodium acetate, guinea pig IgG, trypsin

Stratagene

XL1-Blue MRF' Epicurian Coli supercompetent cells

Upstate Biotechnology

Collagen type 1

Whatman Scientific

3MM paper

Worthington Biochemicals

Collagenase type II

2.1.2 Antibodies

Affinity-purified rabbit polyclonal anti-Bax antibody was kindly provided by Dr. K. Vekrellis, Eisai London Research Laboratories (Vekrellis *et al.*, 1997).

Affinity-purified rabbit polyclonal anti-c-Jun and anti-JunB antibodies (Lallemand *et al.*, 1997), and mouse monoclonal anti-phospho-c-Jun (serine 63) antibody (Lallemand *et al.*, 1998) were kindly provided by Dr. D. Lallemand, Institut Pasteur.

5'-3'

Rabbit polyclonal anti-β-galactosidase antibody

Rabbit polyclonal anti-CAT antibody

Amersham Pharmacia Biotech

Goat anti-rabbit horseradish peroxidase conjugated Ig

Goat anti-mouse horseradish peroxidase conjugated Ig

Sheep anti-mouse biotinylated Ig

Sheep anti-rabbit biotinylated Ig

Streptavidin horseradish peroxidase conjugated Ig

Boehringer-Mannheim

Mouse monoclonal anti-Myc epitope tag (epitope EQKLISEEDL) antibody, clone 9E10

Mouse monoclonal anti-HA tag (epitope YPYDVPDYA) antibody

Mouse monoclonal anti-NGF antibody

Chemicon

Rabbit polyclonal anti-Bim/BOD antibody

Dako

Mouse monoclonal anti-human Bcl-2 antibody

Jackson Immunoresearch Laboratories

Donkey anti-guinea pig IgG, TRITC-conjugated

Goat anti-mouse IgG, FITC-conjugated

Goat anti-mouse IgG, TRITC-conjugated

Goat anti-rabbit IgG, FITC-conjugated

Goat anti-rabbit IgG, TRITC-conjugated

PharMingen

Mouse monoclonal anti-cytochrome c antibody, clone 6H2.B4

Promega

Mouse monoclonal anti-β-galactosidase antibody

Santa Cruz

Rabbit polyclonal anti-Bad antibody

Goat polyclonal anti-Bid antibody

Goat polyclonal anti-Hrk antibody

anti-goat horseradish peroxidase

Sigma

Mouse monoclonal M2 anti-FLAG octapeptide (epitope DYKDDDDK) antibody

Transduction Laboratories

Mouse monoclonal anti-Bcl-x_L antibody

Mouse monoclonal anti-c-Jun antibody

Upstate Biotechnology

Rabbit polyclonal anti-Bak antibody

Rabbit polyclonal anti-Myc epitope tag (epitope MEQKLISEEDLN) antibody

Zymed

Rabbit polyclonal anti-FLAG octapeptide (sequence DYKDDDDK) antibody

2.1.3 Bacterial strains

DH5α F'*psi80d, lacZM15, endA1, recA1, hsdR17,*($r_k^- m_k^-$)*supE44, thi-1k-, gyrA96, relA, (lacZYA-argF)U69*

XL1-Blue MRF' *Δ((mrcA)183 Δ(mrCB-hsdSMR-mrr)173 recA1, endA1, gyrA96, thi-1, supE44, relA1, lac, [*F'*, proAB, lacI*^{q}*ZΔM15, Tn10(tet*^{r}*)]*

2.1.4 Plasmids

CMVlacZ was constructed by Alberts *et al.*(1994).

pcD-FLAGΔ169 and pcD-Bcl-2 were described by Ham *et al.* (1995).

pMT-SM-MEKK1 (Myc epitope-tagged MEKK1 c-terminus) was constructed by Olson *et al.* (1995).

pMT-2-SEK*AL* (HA-tagged SEK*AL*) was constructed by Yan *et al.* (1994).

c-jun CAT, j1j2 CAT, and 6xjun2 SVe CAT were described by Eilers *et al.* (1998) and van Dam *et al.* (1995).

pSG5 was described by Green *et al.* (1988).

pAd-CMVpolyA adenoviral shuttle vector was kindly provided by Dr. Freda Miller.

pcDNA1 was purchased from Invitrogen.

pcD-Bax and pcD-Bcl-x_L were previously described (Vekrellis *et al.*, 1997).

2.1.5 Stock solutions

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

5 x DNA loading buffer	0.2% w/v bromophenol blue 0.2% w/v xylene cyanol 25% glycerol 50 mM EDTA pH 8.0
Plasmid preparation buffer	50 mM glucose 25 mM Tris-Cl pH 8.0 10 mM EDTA
10x TBE	890 mM Tris 890 mM boric acid 20 mM EDTA pH 8.0
TE	10 mM Tris-Cl pH 8.0 1 mM EDTA pH 8.0
RF I	100 mM RbCl 50 mM $MnCl_2$ 30 mM KOAc 10 mM $CaCl_2$ 12% v/v glycerol pH adjusted to 5.8 with CH_3COOH
RF II	10 mM MOPS 10 mM RbCl 75 mM $CaCl_2$ 12% v/v glycerol pH adjusted to 6.8 with NaOH
SOB	2% w/v bacto-tryptone 0.5% w/v bacto-yeast extract 9 mM NaCl

	2.5 mM KCl 10 mM $MgCl_2$
LB	1% w/v bacto-tryptone 0.5% w/v yeast extract 17 mM NaCl
LB-agar	LB containing 1.5% w/v bacto-agar
Ampicillin	100 mg/ml in water
Chloramphenicol	34 mg/ml in EtOH
Phosphate buffered saline (PBS)	137 mM NaCl 2.7 mM KCl 1.47 mM KH_2PO_4 8.1 mM Na_2HPO_4
Western blot transfer buffer	39 mM glycine 48 mM Tris base 0.037% w/v SDS 20% v/v methanol
SDS running buffer	192 mM glycine 25 mM Tris base 0.1% w/v SDS pH 8.3
Western blot stripping solution	100 mM β-mercaptoethanol 2% w/v SDS 62.5 mM Tris-Cl pH 6.8

4 x Laemmli buffer	125 mM Tris-Cl pH 6.8 8% w/v SDS 40% v/v glycerol 4% v/v β-mercaptoethanol 0.04% w/v bromophenol blue
TBS-T	10 mM Tris-Cl, pH 8.0 150 mM NaCl 0.1% v/v Tween-20
SDS lysis buffer	10 mM Tris-Cl pH 7.6 150 mM NaCl 0.5 mM EDTA 1 mM EGTA 1% w/v SDS 1 mM PMSF 1 µg/ml aprotinin 2 µg/ml leupeptin 1 µg/ml pepstatin
4x Tris-Cl/SDS pH 6.8	0.5 M Tris base 0.4% w/v SDS
4x Tris-Cl/SDS pH 8.8	1.5 M Tris base 0.4% w/v SDS
Coomassie Brilliant Blue solution	50% MeOH 0.05% Coomassie 10% HAc
Ponceau-S solution	0.2% Ponceau-S 3% trichloroacetic acid 3% sulfosalicylic acid

3% paraformaldehyde	3% paraformaldehyde in PBS 0.1 mM $CaCl_2$ 0.1 mM $MgCl_2$ pH 7.4
DMEM/FCS X%	X% foetal calf serum 100 units/ml penicillin 100 µg/ml streptomycin 2 mM glutamine in Dulbecco's Modified Eagle's Medium (with 4.5 g/l glucose, 0.11 g/l pyruvate)
SATO mix	45% v/v BSA Path-o-cyte 2.82 µg/ml progesterone 0.73 mg/ml putrescine 18.2 µg/ml L-thyroxine 1.76 µg/ml selenium 15.3 mg/ml tri-iodo-thyronine
SATO medium	2.75% SATO 0.0125 % w/v Transferrin 1.25 mM Glutamine 100 units/ml Penicillin 100 µg/ml Streptomycin in DMEM

2.2 Methods

2.2.1 DNA manipulations

2.2.1.1 Phenol-chloroform extraction

To remove protein from solutions of DNA, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the sample vortexed for 10 seconds. After centrifugation for one minute at 10,000 x g, the upper aqueous phase was retained and any traces of phenol then removed by the addition of choroform:isoamyl alcohol (24:1), followed by vortexing and centrifuging as above. DNA was precipitated from the solution by the addition of sodium acetate pH 5.5 to 0.3 M and 2.5 volumes of ethanol followed by incubation at -20°C for 15 minutes or longer. Precipitated DNA was recovered by centrifugation at 10,000 x g for 10 minutes, and the pellet was then washed with cold 70% v/v ethanol. After centrifugation again, the pellet was air dried and dissolved in TE or water. DNA was quantitated as described in section 2.2.1.4.

2.2.1.2 Bacterial transformation

To produce transformation-competent DH5α bacteria a single colony was transferred from a freshly streaked SOB agar plate into 10 ml LB and shaken overnight at 37°C. 0.5 ml of this culture was diluted into 50 ml of pre-warmed SOB and shaken at 37°C until the culture had reached an A_{600} of 0.5 (approximately 4-7 x 10^7 cells/ml). The culture was transferred into pre-chilled 50 ml polypropylene centrifuge tubes, cooled on ice for 10 minutes and centrifuged at 1,400 x g for 10 minutes at 4°C. The bacterial pellet was resuspended in 16 ml RF I by gentle pipetting and incubated on ice for 10 minutes. The cells were re-pelleted and resuspended in 4 ml RF II by gentle mixing. Following a 15 minute incubation on ice, the competent cells were snap frozen in 200 µl aliquots in liquid nitrogen and stored at -80°C. In addition, XL1-Blue MRF' Epicurian Coli Supercompetent cells were purchased from Stratagene.

To transform bacteria, 100 µl of competent cells were thawed on ice and 100 ng of DNA (in 10 µl of TE) was added. The bacteria were incubated on ice for 20 minutes, heat shocked at 42°C for 90 seconds and chilled for a further two minutes on ice. Cells were mixed with 400 µl of LB and incubated at 37°C to allow the transformed cells time to acquire antibiotic resistance. After one hour, 30-300 µl was spread on LB agar plates

containing a final concentration of 100 μg/ml ampicillin. Bacterial plates were allowed to dry and were incubated overnight at 37°C.

2.2.1.3 Small scale plasmid preparations (minipreps)

Single colonies were inoculated into 10 ml of LB containing 100 μg/ml ampicillin and shaken vigorously overnight at 37°C. A portion of each culture (9 ml) was transferred to a falcon tube and centrifuged at 4,000 x g in a bench-top centrifuge for 10 minutes. After removing the supernatant, the cell pellet was resuspended in 100 μl of ice cold plasmid preparation buffer. After 5 minutes at room temperature 200 μl of a 1% SDS, 0.2 M NaOH solution was added and the solutions were gently mixed (at this stage the solution should be clear, indicating complete cell lysis). After a further 5 minutes on ice, 150 μl of ice cold potassium acetate (3 M potassium, 5 M acetate) was added. The solutions were gently mixed and left on ice for 5 minutes. Cellular debris was removed by centrifugation for 10 minutes at 10,000 x g and the supernatant transferred to a fresh microcentrifuge tube. Residual protein contamination was removed by phenol:chloroform:isoamyl alcohol extraction and plasmid DNA was then precipitated by the addition of 0.7 volumes of isopropanol. The solutions were mixed and left at room temperature for 20 minutes. DNA was recovered by centrifugation at 10,000 x g for 10 minutes. The supernatant was removed and the pellet washed in 70% v/v ethanol and air dried. The DNA was resuspended in 30 μl of TE and approximately 5-10 μl was digested with the appropriate restriction enzyme (with ribonuclease A added to the digest) before being subjected to electrophoresis on a TBE-agarose gel.

2.2.1.4 Large scale plasmid preparations (maxipreps)

This protocol uses alkaline lysis to isolate plasmid DNA for subsequent purification through a caesium chloride (CsCl)-ethidium bromide gradient. CsCl gradient purification makes use of the differing buoyancy of supercoiled and linear plasmid DNA after intercalation of ethidium bromide to yield highly purified supercoiled plasmid DNA, which is essential for single-cell microinjection experiments.

A single bacterial colony from a freshly streaked LB-agar plate was transferred into 10 ml of LB containing ampicillin at 100 μg/ml and incubated overnight at 37°C with vigorous shaking. An aliquot (4 ml) of culture was used to inoculate 400 ml of LB in a 2 litre conical flask. Cultures were grown at 37°C with shaking until they had reached an A_{600} of 1 (1 ml aliquots were tested at intervals). Chloramphenicol was

added to 170 μg/ml for amplification of the plasmid, and the cultures were grown for a further 16 hours.

Bacterial cultures were harvested by centrifugation at 4,000 x g for 15 minutes at 4°C. The supernatants were discarded and the pellets resuspended in 10 ml of ice cold plasmid preparation buffer. One ml of freshly prepared lysozyme solution (10 mg/ml in water) was added and the suspension was mixed gently and incubated for 5 minutes at room temperature. A 1% SDS, 0.2 M NaOH solution (20 ml) was added, mixed and incubated at room temperature for 10 minutes until the solution became clear. To neutralise the bacterial lysate and precipitate genomic DNA, proteins, and RNA, 15 ml of ice cold potassium acetate (3 M with respect to potassium, 5 M with respect to acetate) was added and the lysate was incubated on ice for 10 minutes. The lysate was centrifuged at 4,000 x g for 15 minutes at 4°C and the resultant supernatant filtered through sterile gauze. Isopropanol (0.7 volumes) was added for 20 minutes at room temperature to precipitate plasmid DNA. The DNA was recovered by centrifugation at 7,500 x g for 15 minutes at room temperature. The pellet was washed in 70% v/v ethanol, air dried and resuspended in 4 ml of sterile TE buffer.

Plasmid DNA was further purified by centrifugation through a CsCl-ethidium bromide gradient. Solid CsCl was added (5 g) to the DNA solution, which was mixed until the salt had dissolved, and then 0.4 ml of ethidium bromide (10 mg/ml) was added. Triton X-100 was added (55 μl of 1%) and the solution was gently mixed. The solution was transferred to a 5 ml quick seal tube, balanced, heat sealed and centrifuged at 420,000 x g for 16 hours in a Beckman NVT-90 near vertical rotor (corresponds to 78,000 rpm) at 20°C. After centrifugation, the lower ethidium bromide-stained band containing supercoiled DNA was collected by drawing out the DNA solution with a 2 ml syringe through a 19 G needle. The DNA was then centrifuged overnight on a second CsCl-ethidium bromide gradient and again collected as above. To remove ethidium bromide after the second gradient, an equal volume of sodium chloride-saturated isopropanol:water was vigorously mixed with the aqueous DNA solution. After allowing the two phases to separate the upper phase was discarded. This extraction procedure was repeated until the DNA solution was colourless. The DNA solution was diluted in a Corex tube with three volumes of water, precipitated with two volumes of ethanol for 20 minutes at 4°C, and then centrifuged for 20 minutes at

10,000 x g. The pellet was washed in 70% v/v ethanol, air dried and resuspended in distilled water or TE.

DNA concentration was determined by measuring the absorbance of a diluted sample at 260 and 280 nm using a UV spectrophotometer. DNA concentration was calculated using the following formula: [nucleic acid] = (A_{260}) x (dilution) x (Σ), where A_{260} is absorbance at 260 nm and Σ = 50 μg/ml for double stranded DNA. DNA was considered to be without significant contaminants if the A_{260}/A_{280} ratio was between 1.5 and 2.0. In addition, the DNA was checked for purity on a TBE-agarose gel.

2.2.1.5 Restriction endonuclease digestion

To analyse plasmid DNA, 1 μg of DNA was mixed with an excess of restriction enzyme, the appropriate volume of enzyme buffer, and made up to a final volume of 30 μl with distilled H_2O. The reaction mix was incubated at the appropriate temperature (generally 37°C) for 1-2 hours. In the case of miniprep DNA, DNase-free RNase was added to the digestion mix at a final concentration of 10 μg/ml. Preparative reactions for isolation of DNA fragments were also digested as above but with the volumes scaled up.

2.2.1.6 Agarose gel electrophoresis of DNA

To analyse digested DNA by agarose gel electrophoresis, a 1/4 volume of 5 x DNA loading buffer was added to the restriction digest mix. DNA fragments were resolved on 1% agarose gels, prepared in 1 x TBE with 0.5 μg/ml ethidium bromide. DNA size markers were loaded in adjacent lanes. DNA was visualised on a UV transilluminator and the appropriate DNA fragments were cut out of the gel. DNA was extracted from the gel by snap freezing the gel slice in a 0.22 μM Spin-X column in liquid nitrogen for 5 minutes, thawing, and then centrifuging at 10,000 x g for 10 minutes at 4°C. DNA was extracted twice with phenol:chloroform:isoamyl alcohol, once with chloroform:isoamyl alcohol and then precipitated with ethanol. After spinning at 10,000 x g for 10 minutes the DNA was washed with 70% v/v ethanol, air dried, and then resuspended in water. DNA was quantitated by running a known volume through a 1% agarose gel alongside 500 ng of Lambda DNA digested with *Hin*d III. The DNA was visualised on a UV transilluminator and the intensity of the DNA to be quantitated was compared to each fragment of the Lambda digest.

2.2.1.7 Ligation of DNA

The recombinant shuttle vectors for the production of adenoviruses were constructed by digesting the plasmid pAd-CMV-polyA (Lamarche *et al.*, 1990; Acsadi *et al.*, 1994) with the required restriction enzyme for one hour at 37°C, followed by dephosphorylation of the restricted ends with 2 units/μg calf intestinal alkaline phosphatase for 30 minutes at 37°C, followed by a further 30 minutes at 37°C (for cohesive ends) or at 50°C (for blunt ends). The DNA was subsequently purified from agarose gels as described above. Where necessary, cohesive ends were filled in using 10 units of the large fragment of DNA polymerase I (Klenow).

The inserted cDNAs were: FLAGΔ169 (excised from pcD-FLAGΔ169), Myc-tagged MEKK1 (excised from pMT-SM-MEKK1), and Bcl-2 (excised from pcD-Bcl-2). Ligations were performed overnight at 16°C, using T4 DNA ligase, approximately 50 ng of vector, and a 2:1 molar ratio of insert:vector. Ligated DNA was then used to transform competent cells as detailed in section 2.2.1.2.

2.2.1.8 DNA sequencing

Sequencing of double stranded plasmid DNA was performed using the Sequenase Version 2.0 DNA sequencing kit with the T7 primer as detailed in the Sequenase kit. Briefly, 5 μg of DNA was denatured by adding 0.1 volumes of 2 M NaOH, 2 mM EDTA for 10 minutes at room temperature, neutralised with 0.1 volumes of 3M sodium acetate (pH 5.5), then precipitated with three volumes of ethanol. After washing with 70% v/v ethanol, the denatured DNA was resuspended in 7 μl of distilled water and 2 μl of sequenase reaction buffer was added together with 1 μl of T7 primer (500 pmoles/ml). Primer and DNA were annealed by heating the mixture to 65°C for three minutes and then allowing it to cool slowly to room temperature. The annealed DNA and primer were chilled on ice and stored at -20°C until required. To label DNA, primer and template were mixed with 1 μl of 0.1 M DTT, 2 μl of labelling mixture, 0.5 μl of [^{35}S]dATP and 2 μl of Sequenase T7 polymerase at room temperature for 5 minutes. Labelling mixture (3.5 μl) was then incubated with 3.5 μl of each of the four termination mixes (G, A, T, C) at 37°C for a further 5 minutes. Reactions were stopped by the addition of 4 μl of STOP solution and heated at 90°C for 5 minutes prior to loading onto a 6% polyacrylamide sequencing gel (6% acrylamide:bis-acrylamide [19:1], 7M urea, 1 x TBE, 0.05% APS, 0.05% TEMED). Gels were run for 2-4 hours

at 100V, fixed in 20% methanol/7% glacial acetic acid and dried for 90 minutes at 80°C on a BioRad gel dryer before exposure to X-ray film overnight.

In addition, an automated ABI Prism 310 Genetic Analyser was used for sequencing when available.

2.2.2 Tissue culture

2.2.2.1 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 the addition of 5 ml of 0.2% collagenase in PBS (containing Mg^{2+} and Ca^{2+}) and a further 30 minute incubation at 37°C. The enzymatic reaction was stopped by the addition of 5 ml of SCG medium (DMEM/FCS 10% plus 200 ng/ml NGF) and then spun for 10 minutes at 200 x g. NGF (2.5S, purified from adult male mouse submaxillary glands) was kindly provided by Dr. S. Brooks. The ganglia were dissociated by trituration through the tip of a Gilson P1000, and then further dissociated through a 21 gauge needle if necessary. The SCG neuron population was enriched by pre-plating for three hours on tissue culture dishes, to allow non-neuronal cells to attach (Deckwerth and Johnson, 1993). After pre-plating, neurons were gently rinsed off, centrifuged at 200 x g for 10 minutes and resuspended in 1 ml of culture medium. The number of cells was determined by mixing 10 µl of cells with an equal volume of 0.1% trypan blue dye and counting the cell suspension using a modified Fuchs Rosenthal haemocytometer. Cells were plated onto poly-L-lysine/laminin-coated 13 mm glass coverslips at 5,000 cells per coverslip in a volume of 50 µl. To limit the growth of non-neuronal cells, fluorodeoxyuridine and uridine were added to the SCG medium to final concentrations of 20 µM. Cells were cultured in 10% v/v CO_2 for 5-7 days prior to microinjection, or for 3-5 days before adenoviral infection. Fresh medium was added after 1, 2, 4, and 6 days in culture. For NGF withdrawal experiments, the cells were washed once with SCG medium lacking NGF, and then refed with SCG medium lacking NGF, supplemented with 100 ng/ml of anti-NGF antibody.

2.2.2.2 PC12 cell culture

Undifferentiated PC12 cells were grown in SATO medium containing insulin at 10 μg/ml (stock 2 g/l in 10 mM HCl) and 2% foetal calf serum, in collagen-coated 75 cm^2 flasks in 7.5% v/v CO_2. Collagen was batch tested and diluted 1:25 in PBS. Flasks were coated for one hour and then washed with PBS. PC12 cells were subcultured every 7 days by rinsing the cells with PBS and incubating for 5 minutes with 1 x trypsin/EDTA. After harvesting the cells by centrifugation, the cell pellet was resuspended, and the cells dissociated by trituration through a 19G needle. Cells were plated out at a density of 2 x 10^6 per flask in 10 ml of basic SATO medium. To differentiate the cells and obtain a sympathetic neuron-like phenotype, PC12 cells were cultured in SATO medium plus 100 ng/ml NGF (Promega, 0.1 g/l in 0.1% BSA in DMEM). In this case the flasks were first coated with poly-L-lysine (10 μg/ml in water) for one hour, and after washing twice with water, were then coated with collagen as above.

2.2.2.3 HEK 293A cell culture

HEK 293A cells were maintained in DMEM/FCS 10%, and kept under 5% v/v CO_2. Cells were routinely passaged before reaching 70% confluency (typically every 3-4 days) by harvesting the cell monolayer after treatment with 1 x trypsin/EDTA, and pelleting the cells at 200 x g for 10 minutes. Cells were then resuspended in fresh medium and diluted 1:10 into new flasks.

For long term storage, 1 ml aliquots of 1 x 10^6 cells were kept in liquid nitrogen. Cells to be frozen were resuspended in DMEM/FCS 10% containing 10% dimethyl sulphoxide (DMSO) and slowly cooled overnight in an insulated box at -80°C before placing in liquid nitrogen. Cells were recovered from storage by rapid thawing at 37°C followed by the addition of 9 ml of fresh medium. After centrifugation at 200 x g for 5 minutes, these cells were plated out in fresh medium in a 75 cm^2 flask. For amplification of adenoviral stocks, 293 cells were grown in 175 cm^2 flasks.

2.2.3 Adenoviruses

All work with adenoviruses was carried out according to ACGM Containment Level 2 rules, with additional precautions taken to minimise the risk of contamination by aerosols.

2.2.3.1 Generation of recombinant adenoviruses

The generation of a recombinant adenovirus is a two-step process involving the insertion of the desired cDNA into a shuttle vector, followed by transfer into the adenoviral genome by homologous recombination (the genome is too large to be easily manipulated). This is followed by identification and purification of the recombinant adenovirus and then amplification to produce high titre viral stocks. Replication-defective recombinant adenoviruses lack the E1 region which is crucial for propagation of the virus within host cells. Adenoviruses are made and propagated in HEK 293 cells since this cell line has been stably transfected with the entire E1 region of the adenoviral genome, and hence supplies the E1 proteins in *trans*.

The following recombinant adenoviruses were made in collaboration with Dr. Freda Miller's group at the Montreal Neurological Institute: FLAG-tagged dominant negative c-Jun (FLAGΔ169), Bcl-2, and Myc-tagged MEKK1 (C-terminus). Initially, the cDNA's were inserted into the multiple cloning site of the shuttle vector pAd-CMVpolyA (Lamarche *et al.*, 1990; Acsadi *et al.*, 1994) using standard molecular biological techniques (section 2.2.1.7), which generates an expression cassette containing the CMV enhancer and promoter upstream of the cDNA, and then SV40 polyadenylation and splice signals downstream of the insert. The vector also contains sufficient adenovirus DNA for efficient homologous recombination with the adenoviral genome, as well as the viral inverted terminal repeat (ITR) necessary for initiation of viral replication. The shuttle vector is co-tranfected into 293 cells along with the long-arm viral DNA (which lacks the viral ITR and E1 region), and when homologous recombination occurs between the viral DNA and the shuttle vector, the expression cassette is inserted in place of the E1 region, and the ITR is restored to the viral genome, generating a replication-defective recombinant adenovirus.

Recombinant shuttle vectors were linearised using *Cla* I and then purified by agarose gel electrophoresis. Standard calcium phosphate transfection was carried out according to the manufacturer's instructions with materials supplied in the Adeno-Quest kit. Briefly, 5 μg of recombinant shuttle vector was mixed with long-arm viral DNA and calcium chloride, and this mixture was then added dropwise to Hepes-buffered saline while constantly vortexing. The precipitate formed was added dropwise to 293 cells which had been plated at a density of 7.5 x 10^5 cells per 60 mm dish 24 hours earlier. After incubation overnight, the cells were washed once with PBS plus 1 mM

EGTA and twice with PBS, and then split 1:4 followed by incubation overnight. The medium was then removed and 1.25% SeaPlaque agarose in DMEM/FCS 5% carefully added. Additional SeaPlaque agarose medium was added every four days. When homologous recombination occurs, recombinant adenoviruses with a restored ITR are able to propagate in the 293 cells, and plaques of infected cells appear after 5-10 days. Infected cells round up and form grape-like clusters, which detach but are prevented from spreading because of the agarose medium. To screen plaques for the presence of recombinant adenoviruses, cells were removed from the plaque using a Gilson P200 and incubated in 1 ml of DMEM/FCS 5% for 24 hours to elute the virus. 200 µl of this viral suspension was added to 1 x 10^5 293 cells per well of a 24 well plate in duplicate. After two days, the cells in one of the 24 well plates were fixed and stained with an antibody which recognised the recombinant protein. The other plate was incubated for 5-10 days until complete cytopathic effect was observed (all of the cells had rounded up and detached). This plate was then freeze-thawed three times (–20°C/+37°C) to release viral particles from the cells, and those wells in which recombinant protein had been detected on the other plate were then further screened to detect the presence of wild-type adenoviruses (generated very rarely by homologous recombination between long-arm viral DNA and E1 DNA from the genome of the 293 cells). This was performed by adding a serial dilution of the viral solution to 1 x 10^6 293 cells plated on 60 mm dishes. After incubation for 90 minutes, the cells were gently overlayed with 1.25% SeaPlaque agarose in DMEM/FCS 5% and incubated for a further 10 days. Cells from a pure round plaque were then collected into a 24 well plate as before, and the plate frozen/thawed three times. This viral solution was then further amplified as detailed in 2.2.3.2.

The Adeno-LacZ virus was obtained from Quantum Biotechnologies, and then re-purified from pure plaques and amplified at Eisai. This virus contains the same serotype 5 adenovirus backbone and the strong CMV promoter as those generated above.

2.2.3.2 Amplification and purification

This involves successive rounds of infection with increasing cell culture size. A suspension of recombinant adenovirus particles as from 2.2.3.1 was thawed and 0.5 ml (in 10 ml DMEM/FCS 5%) was used to infect 2.5 x 10^6 293 cells in a 75 cm^2 tissue culture flask. After incubation for 72 hours, the cells and medium were harvested by

centrifugation at 200 x g, frozen/thawed three times, and the cell debris pelleted at 200 x g. Three ml of the supernatant (in 75 ml of DMEM/FCS 5%) was used to infect 3 x 175 cm^2 flasks, each containing 1 x 10^7 293 cells. After 48 to 72 hours (when the cells had detached and formed grape-like clusters) the cells and medium were harvested and then treated as above to release the viral particles. An aliquot of this solution (50 ml put into 500 ml of DMEM/FCS 5%) was used to infect 20 x 175 cm^2 flasks, each containing 1 x 10^7 293 cells. The cells were harvested and pelleted after 48 hours, and then resuspended in 5 ml of TE. After three cycles of freeze/thaw the cell debris was removed by centrifugation, and the resulting viral solution was purified by centrifugation on a discontinuous CsCl gradient (Precious and Russell, 1985; Kanegae *et al.*, 1994). The gradient was made by pouring CsCl 1.2 (1.59 M CsCl in 10 mM Tris-Cl pH 7.9) slowly onto CsCl 1.4 (3.15 M CsCl in 10 mM Tris-Cl pH 7.9) in cellulose nitrate tubes with a ratio of 8:6 CsCl 1.4:1.2. Up to 3 ml of viral solution was added to the top of the gradient, and the tubes spun at 100,000 x g in an SW40 swinging bucket rotor for 90 minutes with minimal deceleration. A milky blue band containing infectious viral particles was harvested, and the solution was desalted through a Sephadex G25 column. Eight drops per well were collected in a 96 well plate and the absorbance at 595 nm was read. Only fractions with the highest absorbance were pooled, diluted in PBS, and stored at -80°C.

2.2.3.3 Assay of infectious viral particle number

Two methods were used to assay the number of infectious viral particles. Plaque assays, the standard method for viral titration (Graham and Prevec, 1991), were performed by making serial dilutions of viral stocks, and adding 1 ml of each dilution from 10^{-5} to 10^{-12} to 60 mm dishes which each contained 5 x 10^5 293 cells. After incubation for 90 minutes, the monolayer was carefully overlaid with 1.25% SeaPlaque agarose in DMEM/FCS 5% and incubated for 21 days. Fresh SeaPlaque/ DMEM/FCS 5% was added every 5 days. The number of plaques on each dish was then scored, and hence a measure of the number of infectious viral particles per ml was obtained.

A cytopathic effect (CPE) assay was also performed. This method is quicker and more reliable than the plaque assay (Nyberg-Hoffman *et al.*, 1997). A serial dilution of the viral stock from 10^{-5} to 10^{-12} was prepared in duplicate in DMEM/FCS 2%. 10^4 293 cells in 100 µl of DMEM/FCS 2% were plated in each well of a 96 well plate in duplicate. For each row, 100 µl of each viral dilution was added to wells 1 to

10. Wells 11 and 12 were uninfected controls. After 10 days, the number of wells in a row showing cytopathic effects was scored. It is important that no infection is observed at the highest dilution, while 100% of cells are infected at the lowest dilution. The number of wells showing CPE out of the total number of wells (where dilutions 10^{-1} to 10^{-4} were 10 out of 10 each, i.e. a total of 4) gives the ratio S, and viral titre = $10^{S+0.8}$. This represents the 50% Tissue Culture Infectious Dose ($TCID_{50}$).

The average results from both assays were similar, although the $TCID_{50}$ for the MEKK1 virus was several-fold higher than the titre as determined by plaque assay. The viral titre as determined by CPE assay was more consistent between assays and much simpler to score and hence was used as the final titre.

2.2.3.4 Infection of sympathetic neurons

Neurons were cultured for 4-5 days and then infected with the required number of viral particles per cell (termed the multiplicity of infection, MOI) by refeeding with SCG medium containing recombinant adenoviruses. The neurons were refed after 24 hours with virus-free SCG medium, and then incubated for a further 24 hours, at which time the neurons were refed with SCG medium or were withdrawn from NGF. Immunofluorescence was performed 24 hours later.

2.2.4 Protein Analysis

2.2.4.1 Protein extraction from cells

All steps were performed at 4°C. SCG neurons were removed from coverslips by rinsing with ice-cold PBS (so as to leave behind any contaminating fibroblasts) and the cells and medium spun for 10 minutes at 1,000 x g. For PC12 extracts the cells were treated with 1 x trypsin/EDTA for 5 minutes, and then harvested and pelleted by centrifugation at 200 x g for 10 minutes. The cell pellets were washed once in ice-cold PBS, resuspended in 1 x SDS lysis buffer (containing 0.2 mM phenyl methyl sulphonyl fluoride (PMSF), 0.1% aprotinin, 0.7 μg/ml pepstatin and 1 μg/ml leupeptin) and incubated at 90°C for 20 minutes with occasional pipetting. After centrifugation at 10,000 x g for 20 minutes, the supernatant was transferred to a new tube. After estimation of protein content the samples were boiled for 5 minutes prior to loading on an SDS-polyacrylamide gel.

2.2.4.2 Protein estimation

Concentration of proteins was estimated by the method of Bradford (1976), using a BioRad assay kit, with γ-globulin as the standard.

2.2.4.3 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 MiniProtean II system. Resolving gels for SDS-PAGE were made of 10% or 12% acrylamide:bis-acrylamide [30:0.8] in 0.375 M Tris-Cl, pH 8.8, 0.1% SDS, 0.07% N'N'N'N' tetramethylethylene diamine (TEMED) and 0.03% ammonium persulphate (APS). The resolving gel was poured to fill approximately 70% of the space between two vertical glass plates separated by 1 mm spacers and overlaid with water until polymerised. The stacking gel consisted of 4% acrylamide:bis-acrylamide [30:0.8], 0.125 M Tris-Cl, pH 6.8, 0.1% SDS, 0.1% TEMED and 0.1% APS and was poured on top of the resolving gel. SDS running buffer (1x) was added to the gel apparatus. Samples were mixed with Laemmli SDS loading buffer, heated to 90°C for 5 minutes, and then the gel was electrophoresed at 200 V until the bromophenol blue dye had reached the bottom of the gel. After electrophoresis, the gels were immunoblotted as outlined below.

2.2.4.4 Immunoblotting of proteins

Following electrophoresis, proteins were transferred to Hybond ECL nitrocellulose membrane by electroblotting using a BioRad Mini Trans-Blot Cell. Prior to transfer the gel was equilibrated with transfer buffer for 20 minutes and the membrane pre-soaked in transfer buffer for 10 minutes. The polyacrylamide gel was placed onto the membrane and sandwiched between three pieces of Whatmann 3MM paper on either side. The sandwich was carefully rolled with a pipette to remove air bubbles and placed into the submerged blotting apparatus containing 1 x transfer buffer. Protein gels were allowed to transfer for 1 to 1.5 hours at 4°C with a constant voltage of 100V. After the transfer, protein loading and transfer was checked by staining membranes for 5 minutes in 0.2% w/v ponceau-S solution, and then washing in distilled water.

To detect immobilised proteins, membranes were blocked overnight at 4°C in blocking solution (TBS-T containing 5% non-fat milk powder). Membranes were either

incubated for one hour at room temperature or overnight at 4°C with the primary antibody diluted in blocking solution (usually between 1:100-1:2,000). Membranes were washed three times for 10 minutes each in TBS-T before incubation with a 1:2,000 dilution of a secondary anti-mouse or anti-rabbit HRP-conjugated antibody in blocking solution for one hour at room temperature. Following three washes in TBS-T (each for 10 minutes, or for longer if required) the membranes were treated with ECL reagent for one minute and exposed to Hyperfilm. For re-probing, membranes were stripped for 30 minutes at 50°C in stripping solution. After extensive washing with water and then TBS-T, membranes were wrapped in Saran and kept at 4°C until needed.

2.2.5 Microinjection

Microinjection was carried out using a Zeiss Axiovert 135M microscope fitted with a heated stage and CO_2 chamber, using an Eppendorf transjector (model 5246) and micromanipulator (model 5171). Microinjection needles were pulled from glass capillaries using a Kopf Instruments gravity puller. DNA expression vectors were injected into the nucleus in 0.5 x PBS (-Ca^{2+}, Mg^{2+}) at a DNA concentration of between 0.05-0.4 mg/ml depending on the construct. Reporter genes were injected at lower concentrations (*c-jun* CAT and j1j2 CAT at 0.01 mg/ml, 6 x jun2 SVeCAT or SVeCAT at 0.005 mg/ml). When different vectors were co-injected, the concentration of all injection mixes was adjusted using an empty vector to equalise final DNA concentrations. To analyse the effects of expression constructs on survival, SCG neurons were injected 5-7 days after plating. To follow the survival of neurons over the course of several days neutral 70,000 MW Texas Red-dextran was added to the injection mixture at a final concentration of 5 mg/ml so as to mark the injected cells. For cells which were to be fixed, guinea pig IgG was injected at a final concentration of 5 mg/ml. After injection of neurons with Texas Red-dextran the number of cells surviving the injection procedure was scored after four hours. Typically more than 80% of cells survived injection. Phase morphology was used to assess neuronal viability on subsequent days. Viable cells typically retained Texas Red-dextran within the nucleus. Apoptotic neurons had shrunken cell bodies and nuclei which were distorted or no longer visible. In addition, the Texas Red-dextran had leaked out of the nucleus and

was seen throughout the cell. More than 150 neurons were injected per construct, and each experiment was repeated at least three times.

2.2.6 Immunofluorescence

Neurons were fixed with 3% paraformaldehyde at room temperature for 15 minutes and then permeabilised with 0.5% Triton X-100 in PBS for 5 minutes. For the detection of Myc-tagged MEKK1 expression, neurons were fixed with 50:50 methanol:acetone at -20°C for 20 minutes. Non-specific binding was blocked using 50% goat serum/ 0.05% BSA in PBS for one hour. Cells were incubated with primary antibody for one hour at room temperature, or overnight at 4°C. The cells were washed in PBS before incubation with the appropriate FITC- or TRITC-conjugated secondary antibodies. In the case of neurons co-injected with guinea pig IgG, TRITC-conjugated goat anti-guinea pig IgG antibody was added for one hour at room temperature. After washing in PBS, nuclei were detected by staining with 1 μg/ml Hoechst 33342 dye for 5 minutes. 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.

Chapter 3: Regulation of c-Jun expression

3.1 Introduction

Programmed cell death in neurons often proceeds in a macromolecular synthesis-dependent manner, both *in vitro* (Martin *et al.*, 1988) and *in vivo* (Oppenheim *et al.*, 1990). In the sympathetic neuron model of developmental apoptosis, RNA and protein synthesis inhibitors block NGF withdrawal-induced death (Martin *et al.*, 1988). This suggests that new gene expression is required for cell death and that transcription factors are likely to play a crucial role in initiating the death process. To identify genes whose expression increases during neuronal death, RT-PCR and immunoblotting has been performed to examine selective gene and protein induction after NGF withdrawal. Members of the AP-1 family of transcription factors have been studied, since these are immediate early genes that are rapidly and transiently induced in response to a variety of extracellular signals (Karin and Hunter, 1995) and which have already been shown to play an important role in other major cellular processes such as proliferation and differentiation (Angel and Karin, 1991). Also, previous reports had associated AP-1 mRNA induction with apoptosis induced by growth factor withdrawal (Colotta *et al.*, 1992).

c-jun mRNA was found to be induced in sympathetic neurons withdrawn from NGF before any of the morphological signs of apoptosis (Estus *et al.*, 1994). The maximum level of *c-jun* mRNA was detected after NGF withdrawal for 16 hours, which coincides with the apoptotic commitment point of these neurons for rescue by cycloheximide (Deckwerth and Johnson, 1993; Estus *et al.*, 1994). This induction did not depend on new protein synthesis. There was also an increase in the amount of c-Jun protein in neurons whereas other members of the Jun and Fos family did not change in level (Ham *et al.*, 1995). In addition, there was a mobility shift, detected by SDS-polyacrylamide gel electrophoresis, which corresponds to increased phosphorylation of c-Jun on serines and threonines within the transactivation domain (Ham *et al.*, 1995). Such phosphorylation increases the transcriptional activity of c-Jun (Pulverer *et al.*, 1991; Smeal *et al.*, 1991; Karin, 1994). Expression of a c-Jun dominant negative mutant, which inhibits AP-1 activity, protected neurons from NGF withdrawal-induced death (Ham *et al.*, 1995). Since c-Jun was the only AP-1 family

protein to increase in level, this strongly suggests that c-Jun is an important component of this AP-1 activity. Microinjection of antibodies specific for c-Jun also inhibited cell death whereas antibodies against JunB and JunD did not (Estus *et al.*, 1994). Overexpression of wild-type c-Jun was sufficient to kill sympathetic neurons even in the presence of NGF (Ham *et al.*, 1995). These studies suggest that c-Jun is important for the death of sympathetic neurons after NGF withdrawal. However, it was not clear whether increased transcription of the *c-jun* gene occurs after NGF withdrawal, and, if so, by what mechanism the promoter was activated.

Overexpressed c-Jun protein can activate transcription of the *c-jun* gene by binding to TRE (AP-1) sites within the *c-jun* promoter (Angel *et al.*, 1988a). The proximal TRE, termed the jun1 TRE, is a variant AP-1 binding site differing from the classic *collagenase* TRE by a single nucleotide insertion (5'-TGACATCA-3'; Angel *et al.*, 1988a). Although originally suggested to function as a high-affinity AP-1 binding site (Angel *et al.*, 1988a), later work showed that while the jun1 TRE does bind c-Jun/c-Fos complexes, it binds c-Jun/ATF-2 with much greater affinity (Herr *et al.*, 1994). A distal AP-1-like site (5'-TTACCTCA-3'), designated the jun2 TRE, is a classic c-Jun/ATF-2 binding site (Stein *et al.*, 1992). This suggests that both c-Jun and ATF-2 could be involved in the regulation of *c-jun* transcription. In the following studies I looked at how the *c-jun* promoter was regulated in sympathetic neurons deprived of NGF. The microinjection experiments involving the *c-jun* promoter constructs were performed in collaboration with my supervisor (Dr. Jonathan Ham, Institute of Child Health, UCL).

3.2 Results

3.2.1 Endogenous c-Jun protein increases in level and becomes more phosphorylated on serine 63 during NGF withdrawal-induced death of sympathetic neurons.

Sympathetic neurons cultured from the superior cervical ganglia have been used for many years as a model of apoptosis. After removal from new-born rats these neurons are cultured in the presence of NGF, and die by apoptosis in its absence. The cells display the classic morphological features of programmed cell death, such as shrunken cell bodies, membrane blebbing, shrunken and distorted nuclei, and condensed chromatin. Figure 3.1 shows sympathetic neurons that were cultured in the

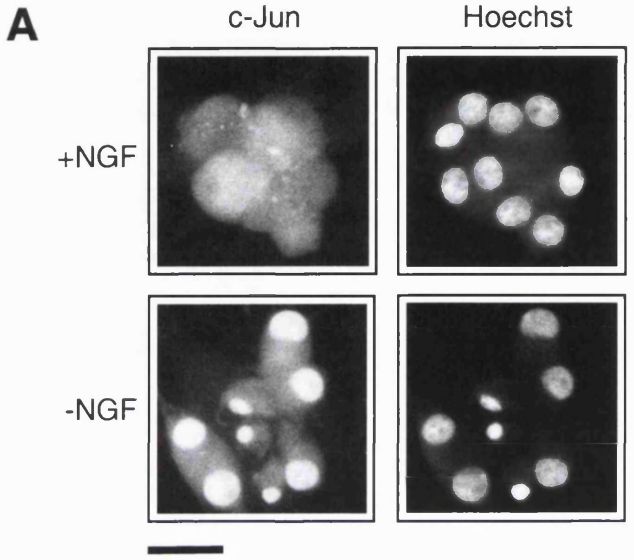
presence or absence of NGF for 24 hours, and then fixed and stained with affinity-purified anti-c-Jun and anti-phospho-c-Jun antibodies. The anti-c-Jun antibody specifically recognises c-Jun, and does not cross-react with JunB or JunD (Lallemand *et al.*, 1997). The phospho-c-Jun antibody specifically recognises c-Jun protein phosphorylated on serine 63 and does not recognise unphosphorylated c-Jun, or phosphorylated JunB or JunD (Lallemand *et al.*, 1997; Watson *et al.*, 1998). Hoechst dye was used to stain the nuclear DNA. In the presence of NGF, very little c-Jun protein or phosphorylated c-Jun was detected (typically less than 10% of cells showed nuclear staining with either antibody; data not shown). However, after NGF withdrawal, c-Jun protein increased in level so that most neurons had brightly stained nuclei, and c-Jun became more phosphorylated on serine 63, which increases its transcriptional activity. This confirms previously reported results which showed that c-Jun protein levels increased and that there was a mobility shift detected by immunoblotting which was due to N-terminal phosphorylation (Ham *et al.*, 1995). Furthermore, these results show that these increases are specifically seen in neurons, and not in any contaminating cells that may be present in the cultures.

3.2.2 Activation of the *c-jun* promoter occurs after NGF withdrawal and requires the jun1 and jun2 TRE elements

c-Jun protein can regulate transcription of the *c-jun* gene by binding with ATF-2 to the jun1 and jun2 TRE sites within its own promoter (Angel *et al.*, 1988a; van Dam *et al.*, 1995). To investigate the importance of these elements during the death of sympathetic neurons the following experiments were performed. Regulation of the *c-jun* promoter during apoptosis was examined by microinjecting a construct in which promoter sequences from -1600 to +170 had been cloned upstream of the bacterial chloramphenicol acetyl transferase (CAT) gene, together with guinea pig IgG (gpIgG) to mark injected cells (see Figure 3.2A for the structure of the constructs). A construct in which the jun1 and jun2 TRE sites had been mutated (j1j2 CAT) was also injected to investigate whether these elements were required for promoter activity. The neurons were withdrawn from NGF for 24 hours, and then fixed and stained with an anti-CAT antibody and also with an anti-gpIgG antibody to identify the injected cells. The percentage of cells expressing CAT was scored. Figure 3.2B shows some typical immunofluorescence pictures of neurons stained with an anti-CAT antibody after

Figure 3.1 Sympathetic neurons express increased levels of phosphorylated c-Jun after NGF withdrawal

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), or with fresh NGF-containing medium (+NGF). 24 hours later, the cells were fixed and stained with antibodies that recognise either c-Jun (A) or c-Jun phosphorylated on serine 63 (B), and also with Hoechst dye. In the presence of NGF, weak background staining of the cell body was observed with both the c-Jun and phospho-c-Jun antibodies. After NGF withdrawal, bright c-Jun and phospho-c-Jun staining was seen in the nuclei. Representative cells are shown. The scale bar represents 30 μm.


A
c-Jun
Hoechst
+NGF
-NGF

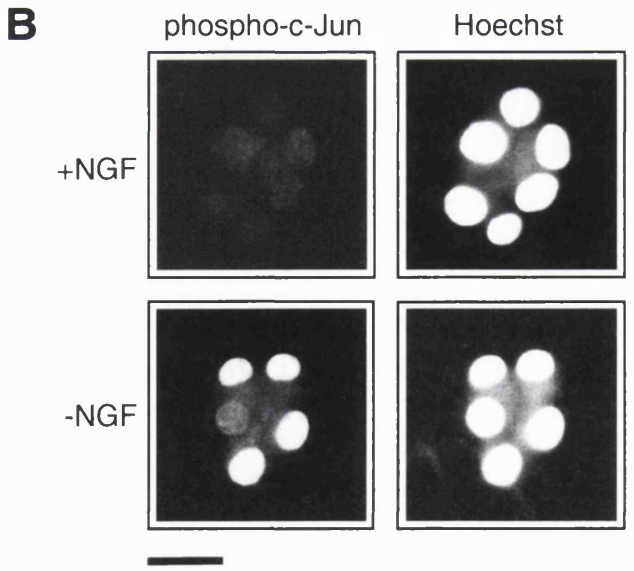
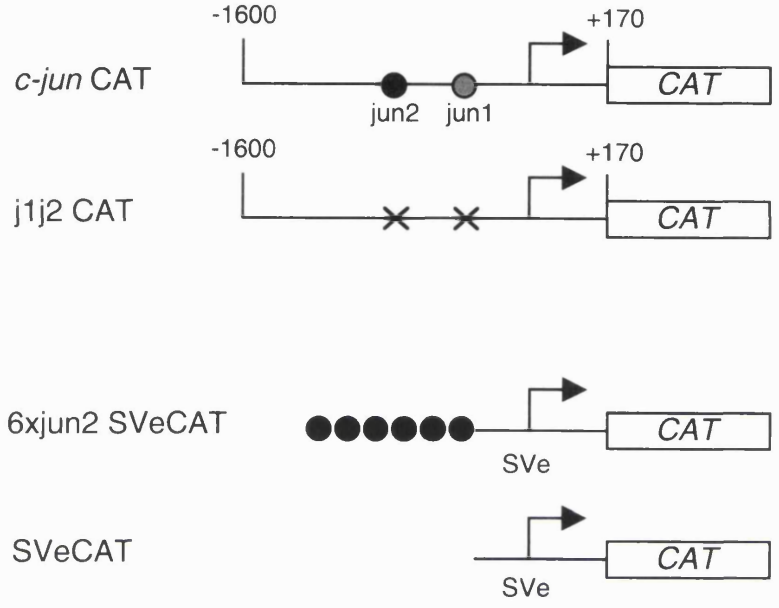

B
phospho-c-Jun
Hoechst
+NGF
-NGF

Figure 3.2 Structure of the *c-jun* reporter gene constructs

(A) *c-jun* CAT contains wild-type *c-jun* promoter sequences from -1600 to +170 cloned upstream of the bacterial CAT gene (van Dam *et al.*, 1995). The position of the jun1 and jun2 TRE elements is indicated. In j1j2 CAT, these sites have been mutated so that they are non-functional. 6xjun2 SVeCAT was constructed by cloning 6 copies of the jun2 TRE element (5'-TTACCTCA-3') upstream of the SV40 early promoter in SVeCAT (Eilers *et al*, 1998).

(B) *c-jun* CAT was microinjected into sympathetic neurons at a concentration of 0.01 mg/ml together with guinea pig IgG (2.5 mg/ml). The cells were refed with medium containing (+NGF) or lacking NGF (-NGF). 18 hours later, the cells were fixed and stained with an anti-CAT antibody and with TRITC-conjugated anti-gpIgG to identify injected neurons. Typical immunofluorescence pictures are shown.

A


-1600
+170
c-jun CAT
jun2
jun1
CAT
-1600
+170
j1j2 CAT
CAT
6xjun2 SVeCAT
CAT
SVe
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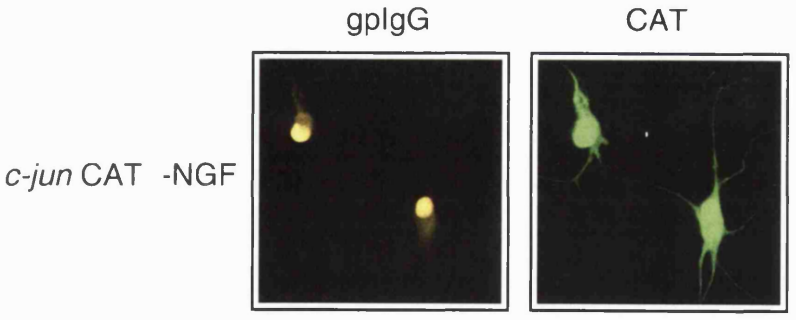
CAT
SVe

B


gpIgG
CAT
c-jun CAT -NGF

microinjection of the *c-jun* CAT reporter gene and withdrawal of NGF from the neurons. In the presence of NGF, the activity of the *c-jun* promoter was relatively low with only 10% of the injected cells expressing detectable CAT protein (Figure 3.3A). After NGF withdrawal, however, over 60% of the cells expressed CAT. NGF deprivation thus causes a significant increase in the activity of the *c-jun* promoter. This is consistent with previous data showing that there was a large increase in *c-jun* mRNA levels after NGF withdrawal (Estus *et al.*, 1994), and therefore demonstrates that this increase is due, at least in part, to increased promoter activity. In those neurons in which the j1j2 CAT construct was injected, there was no increase in CAT expression after NGF withdrawal (Figure 3.3A). This indicates that the jun1 and jun2 elements are crucial for the increase in activity of the *c-jun* promoter and hence the transcription of the gene which is detected after NGF withdrawal. In addition, the level of CAT expression in the presence of NGF was also significantly lower when the cells were injected with j1j2 CAT suggesting that these sites are important for the basal level of *c-jun* transcription in these cells ($p<0.05$, Student's t-test).

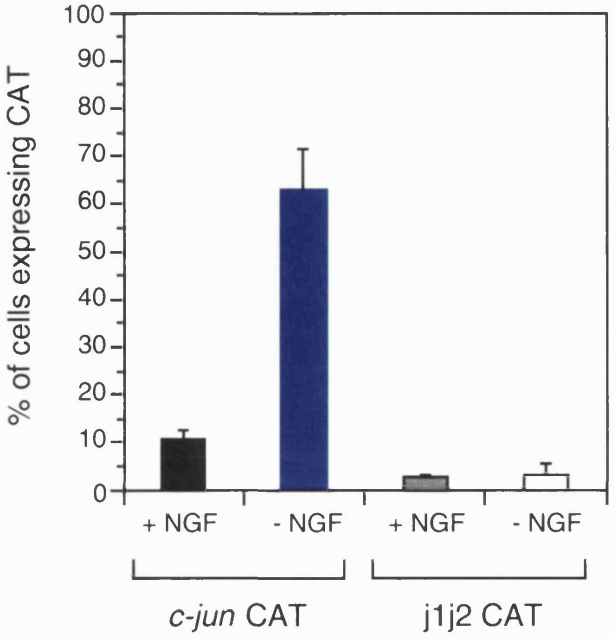
To further examine the function of the jun2 TRE, 6 copies of this site (5'-TTACCTCA-3') were cloned upstream of the SV40 early promoter linked to the bacterial CAT gene (see Figure 3.2A for structure of the constructs). As before, the neurons were injected, withdrawn from NGF for 18 hours, and then fixed and stained with anti-CAT and also anti-gpIgG antibodies to detect injected cells. In this case, there was an increase in CAT expression from 17% to 82% after NGF withdrawal when the cells were injected with 6 x jun2 SVeCAT (Figure 3.3B). A control construct which contained only the SV40 early promoter linked to CAT was not activated at all after NGF deprivation. Multiple copies of the jun2 TRE can therefore function as a transcriptional enhancer which is activated after NGF withdrawal. This lends further support to the hypothesis that the jun2 TRE is a crucial site involved in the increase in *c-jun* promoter activity seen after NGF withdrawal, and therefore that ATF-2 and c-Jun itself are potentially important regulators of *c-jun* gene transcription.

Figure 3.3 The jun1 and jun2 TRE sites are critical for activation of the *c-jun* promoter after NGF withdrawal

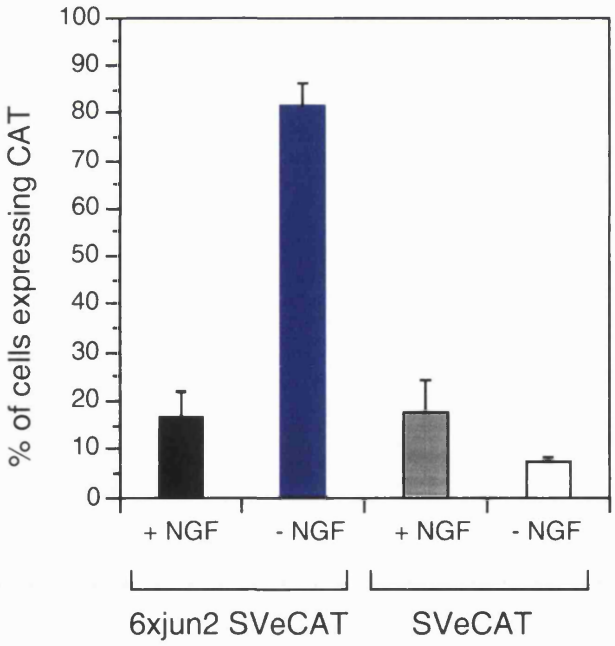
(A) *c-jun* CAT or j1j2 CAT were microinjected into sympathetic neurons at a concentration of 0.01 mg/ml together with guinea pig IgG (2.5 mg/ml). The cells were refed with medium containing (+NGF) or lacking NGF (-NGF). 18 hours later, the percentage of cells expressing CAT was determined in immunofluorescence experiments with an anti-CAT antibody. The data shown represent the average of three independent experiments with the standard error. The *c-jun* promoter was activated after NGF withdrawal, and the jun1 and jun2 TRE sites were essential for this.

(B) 6 x jun2SVeCAT was microinjected into sympathetic neurons to determine whether the jun2 TRE was activated after the withdrawal of NGF. The control plasmid contained only the SV40 minimal promoter and the CAT gene, and both constructs were injected at 0.005 mg/ml. After injection, the cells were withdrawn from NGF for 18 hours and then were fixed and stained with an antibody against CAT. The results show that there was a 7 fold induction of CAT expression upon withdrawal of NGF when the cells were injected with the jun2 TRE reporter gene. This induction was not seen with the control plasmid which contains no jun2 TRE sites.

A


% of cells expressing CAT
100
90
80
70
60
50
40
30
20
10
0
+ NGF
- NGF
+ NGF
- NGF
c-jun CAT
j1j2 CAT

B


% of cells expressing CAT
100
90
80
70
60
50
40
30
20
10
0
+ NGF
- NGF
+ NGF
- NGF
6xjun2 SVeCAT
SVeCAT

3.2.3 AP-1 activity is required for the increase in the level of endogenous c-Jun protein after NGF withdrawal

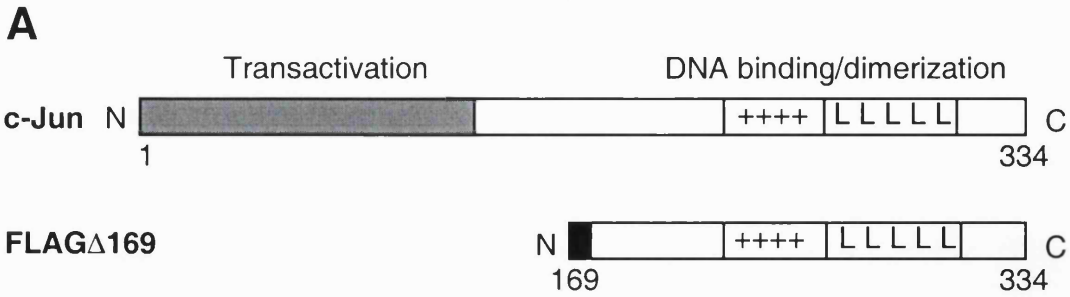
To further investigate the involvement of AP-1 activity in the regulation of *c-jun* transcription, the effect of a c-Jun dominant negative mutant on the induction of c-Jun protein was studied. Dominant negative c-Jun (FLAGΔ169) was constructed by deleting the N-terminal transactivation domain so that only the leucine zipper and DNA binding domains remain (Figure 3.4A; Ham *et al.*, 1995). The protein can therefore bind to other Jun or Fos proteins, or ATF-2, and also bind to DNA as a homo- or heterodimer, but is unable to activate transcription of target genes. An expression vector for FLAGΔ169 was microinjected into sympathetic neurons along with gpIgG; the cells were withdrawn from NGF for 24 hours, and then fixed and stained with the anti-c-Jun antibody and also anti-gpIgG to identify injected cells. The number of injected cells that expressed c-Jun was scored. Cells were considered c-Jun positive when the nuclear c-Jun staining was brighter than the cytoplasm. In the presence of NGF typically less than 10% of the neurons express c-Jun (Ham *et al.*, 1995). After NGF withdrawal 65% of the neurons expressed c-Jun (Figure 3.4B). Microinjection of the empty vector alone reduced this value to 37%. This decrease could be due to non-specific effects of the strong CMV promoter in the vector, because 60% of the neurons expressed c-Jun when an empty vector containing the weaker SV40 promoter was injected (see Figure 4.5A). However, the effect of microinjecting an expression vector for FLAGΔ169 was dramatic, reducing the expression of c-Jun to just 1.7% of the injected cells. This result demonstrates that AP-1 activity is crucial for the induction of c-Jun expression.

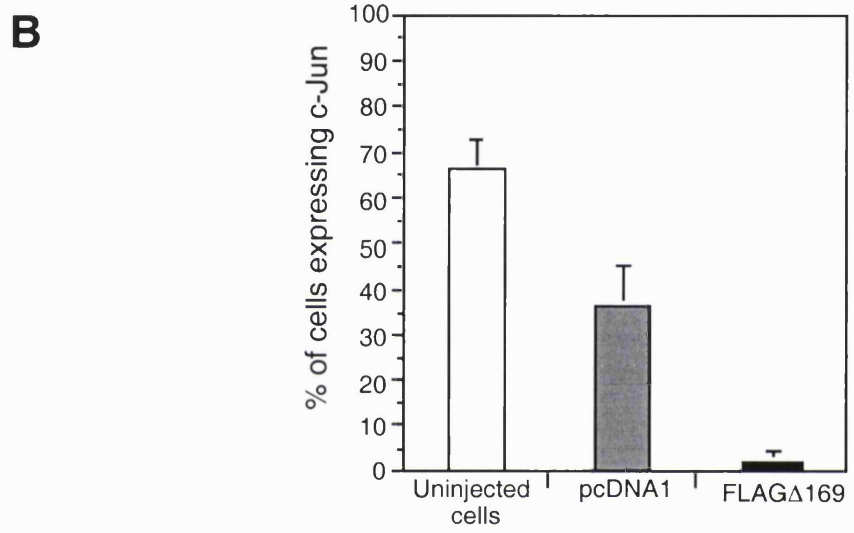
To show that this repression was not a general effect on gene transcription, an expression vector for FLAGΔ169 was injected as before, but instead, the expression of JunB was examined using an affinity-purified JunB polyclonal antibody. This AP-1 family member is expressed in sympathetic neurons but is regulated differently to c-Jun: there was no significant change in JunB levels after NGF withdrawal as detected by immunoblotting (Ham *et al.*, 1995). In the absence of NGF 99% of the cells expressed JunB (Figure 3.4C). When neurons were injected with the empty vector, 88% of the cells expressed JunB, compared to 86% when neurons were injected with FLAGΔ169. Thus, expression of dominant negative c-Jun does not reduce the level of JunB protein. AP-1 activity is therefore specifically required for the induction of c-Jun

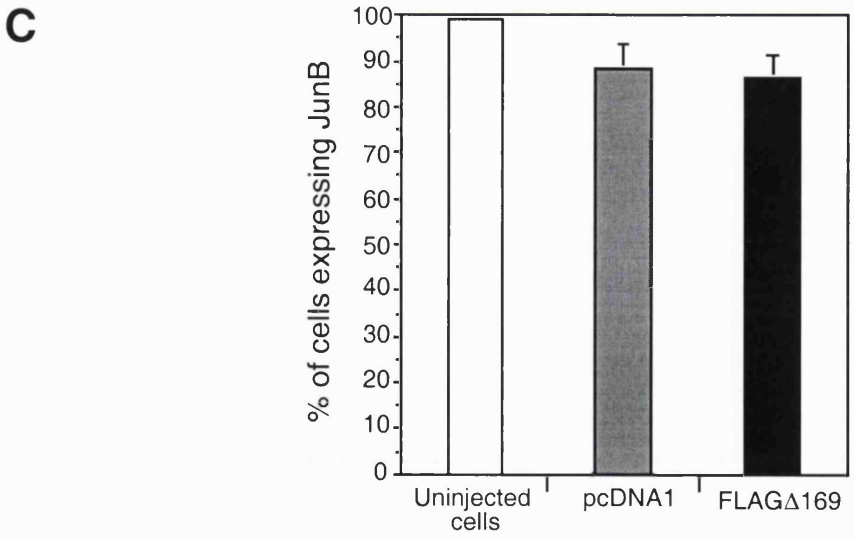
Figure 3.4 Dominant negative c-Jun (FLAGΔ169) prevents the induction of endogenous c-Jun after NGF withdrawal but does not affect JunB protein levels

(A) Structure of FLAGΔ169. The mouse c-Jun open reading frame is shown at the top. The amino-terminal transactivation domain is shaded. ++++ represents the basic region that contacts DNA. LLLLL represents the leucine zipper dimerization domain. FLAGΔ169 lacks the transactivation domain but contains the basic/leucine zipper region. The eight amino acid FLAG epitope added to the amino terminus is represented by a black box. The construction of pcD-FLAGΔ169 is detailed in (Ham *et al.*, 1995).

(B) and (C) Sympathetic neurons were maintained *in vitro* for 5 to 7 days and then microinjected with an expression vector for the c-Jun dominant negative mutant (pcD-FLAGΔ169) or with the empty CMV vector (pcDNA1) as a negative control. Plasmid DNA was injected at 0.2 mg/ml. Guinea pig IgG was also injected to mark the cells. After injection, the cells were withdrawn from NGF for 24 hours, and then fixed and stained with rabbit polyclonal antibodies specific for either c-Jun or JunB. The number of injected cells that expressed either c-Jun (B) or JunB (C) protein was scored. Uninjected cells were also scored for comparison. Cells were only considered to be expressing protein if nuclear staining was more intense than the background cytoplasmic staining. In each experiment 200 cells were injected per construct. Coverslips were scored in a blinded manner. Each graph shows the average result for three experiments with the standard error. (B) FLAGΔ169 inhibited the expression of endogenous c-Jun protein normally seen after NGF withdrawal. (C) FLAGΔ169 did not affect the level of JunB.


A
Transactivation
DNA binding/dimerization
c-Jun N
++++
L L L L L
C
1
334
FLAGΔ169
N
++++
L L L L L
C
169
334


B
% of cells expressing c-Jun
100
90
80
70
60
50
40
30
20
10
0
Uninjected cells
pcDNA1
FLAGΔ169


C
% of cells expressing JunB
100
90
80
70
60
50
40
30
20
10
0
Uninjected cells
pcDNA1
FLAGΔ169

protein after NGF withdrawal, and FLAGΔ169 does not seem to generally suppress promoter activity. In conjunction with the *c-jun* promoter work this supports the hypothesis that c-Jun can regulate its own synthesis in sympathetic neurons.

3.3 Discussion

The results described in this chapter demonstrate that AP-1 activity is necessary for the increase in the level of endogenous c-Jun protein that occurs after NGF withdrawal in sympathetic neurons. The increase in c-Jun expression is due, at least in part, to an increase in activity of the *c-jun* promoter. It is also possible that stabilization of the *c-jun* mRNA or c-Jun protein could account for some of the increase. The increase in *c-jun* promoter activity is mediated by the jun1 and jun2 TRE sites suggesting that c-Jun/ATF-2 heterodimers may be the critical regulators of promoter activation. Furthermore, several copies of the jun2 TRE alone can function as a transcriptional enhancer after NGF withdrawal.

In other cell types, it has been shown that transcription of *c-jun* is rapidly induced in response to stimuli such as cytokines, growth factors and tumour promoters (Angel and Karin, 1991). This enhanced transcription returns to basal levels after two to three hours. In sympathetic neurons induced to die by the removal of NGF, increased levels of *c-jun* mRNA are detected after a few hours and remain high until a peak at 16 hours (Estus *et al.*, 1994). This is a remarkably delayed and protracted response for an immediate early gene (reviewed in Angel and Karin, 1991). The delay suggests that the induction is in response to intracellular signalling events. The induction still has the immediate early gene characteristic in that it is independent of macromolecular synthesis since cycloheximide did not block the increase in *c-jun* mRNA level (Estus *et al.*, 1994). Hence, post-translational events must be responsible for the increase in *c-jun* promoter activity. This is also the case for the induction of *c-jun* during the G_0/G_1 transition in fibroblasts (Ryseck *et al.*, 1988) and during the response of HeLa cells to UV or phorbol esters (Stein *et al.*, 1992). Phosphorylation of c-Jun protein on serine 63, which occurs between 4 and 8 hours after NGF withdrawal (Eilers *et al.*, 1998) would increase the transcriptional activity of c-Jun, which could then increase transcription of the *c-jun* gene by binding to the jun1 and jun2 TRE sites. It would thus be expected that such autoregulation would form a positive feedback loop, possibly accounting for the extended expression of *c-jun* after NGF withdrawal.

It should be noted, however, that phosphorylation of pre-existing c-Jun protein, rather than the synthesis of new c-Jun protein, must be sufficient for the increase in *c-jun* mRNA, since this increase occurs even in the presence of cycloheximide (Estus *et al.*, 1994).

Analysis of deletion mutants of the *c-jun* promoter has previously identified two AP-1-like binding sites that are involved in the transcriptional response to UV (Devary *et al.*, 1991; Stein *et al.*, 1992), phorbol esters (Angel *et al.*, 1988a), and the adenovirus E1A product (van Dam *et al.*, 1993). Both sites differ from the consensus *collagenase* promoter AP-1 site (5'-T^{G}/$_{T}$A^{C}/$_{G}$TCA-3') by the addition of a single nucleotide (jun1 TRE: TGAC<u>A</u>TCA; jun2 TRE: TTAC<u>C</u>TCA), and consequently resemble the consensus ATF/CREB binding site (5'-T^{G}/$_{T}$ACGTCA-3'; Montminy *et al.*, 1986). The protein complexes that bind to the jun1 and jun2 TREs *in vitro* appear to be different from the typical AP-1 complexes (Jun/Jun homodimers, or Jun/Fos heterodimers). For example, in HeLa cells both sites are important for mediating *c-jun* induction in response to UV, but bind factors different to those bound by the *collagenase* TRE (Stein *et al.*, 1992). In addition, different proteins were shown to bind to the jun1 and 2 TREs than to the *collagenase* TRE in 3T3 cells (van Dam *et al.*, 1993). In this case the jun2 TRE was shown to specifically bind c-Jun/ATF-2 heterodimers after E1A-mediated transformation. The jun1 TRE can also be recognised by Jun/Fos heterodimers, although with much lower affinity (Herr *et al.*, 1994). However, the response to UV and TPA was mediated in this study specifically by c-Jun/ATF-2 heterodimers (Herr *et al.*, 1994). In sympathetic neurons, very little *c-fos* was detected until 10-15 hours after NGF withdrawal, and only 1% of neurons expressed c-Fos after 24 hours (Estus *et al.*, 1994; Ham *et al.*, 1995). Therefore it seems unlikely that the level of c-Jun/c-Fos heterodimers would exceed that of c-Jun/ATF-2 dimers sufficiently to compete for binding. Since the jun1 and 2 TRE sites are necessary for the basal level of *c-jun* transcription in sympathetic neurons and also for the increase seen after NGF withdrawal (Figure 3.3A) it seems likely that ATF-2 and c-Jun itself are the important transcriptional regulators of the *c-jun* promoter in these cells as well as those described above.

To investigate the mechanism of transcriptional activation, studies have been performed to look at the composition of complexes bound to the *c-jun* promoter. In unstimulated cells, the jun1 and jun2 sites appear to be fully occupied, and the DNA

binding activity of the factors already bound does not alter after stimulation with UV or TPA (Hagmeyer *et al.*, 1993; Rozek and Pfeifer, 1993; Herr *et al.*, 1994). Thus, perhaps the composition of pre-bound factors alters to more transcriptionally-active dimers upon stimulation, and then returns to factors with a lower transactivation potential (such as JunB and JunD) to shut off transcription. However, in the case of the response to UV or TPA no change in the nature of the complex is found (Herr *et al.*, 1994). In addition, JunB and JunD are not able to heterodimerise with ATF-2 (Benbrook and Jones, 1990; Hai and Curran, 1991). Another possibility is that post-translational modification of pre-bound factors occurs. In sympathetic neurons phosphorylation of c-Jun on serine 63 within the transactivation domain is observed after NGF withdrawal (Figure 3.1B). This increases the transactivating potential of c-Jun and therefore could account for the increase in activity of the jun2 (and presumably also jun1) TRE sites seen in the present study. It is currently unknown whether there is any modification of ATF-2 in sympathetic neurons. Indeed, ATF-2 could simply be involved in directing these heterodimers to the correct site. This appears to be the case for the highly related ATF/CREB family member ATFa which stably binds JNK2 but is not a substrate for this kinase, and hence serves as a JNK2-docking site for ATFa-associated partners like JunD, allowing them to be phosphorylated by the kinase (De Graeve *et al.*, 1999). Furthermore, ATF-2 is not always required for the expression of *c-jun* mRNA. *c-jun* mRNA induction in the lungs of *ATF-2* (–/–) mice was normal in response to lipopolysaccharide, which suggests that either ATF-2 was not involved in *c-jun* induction, or that other ATF-like proteins may compensate for its absence (Reimold *et al.*, 1996). It would be interesting to analyse *c-jun* expression in response to various stimuli and in different cell types in these mice.

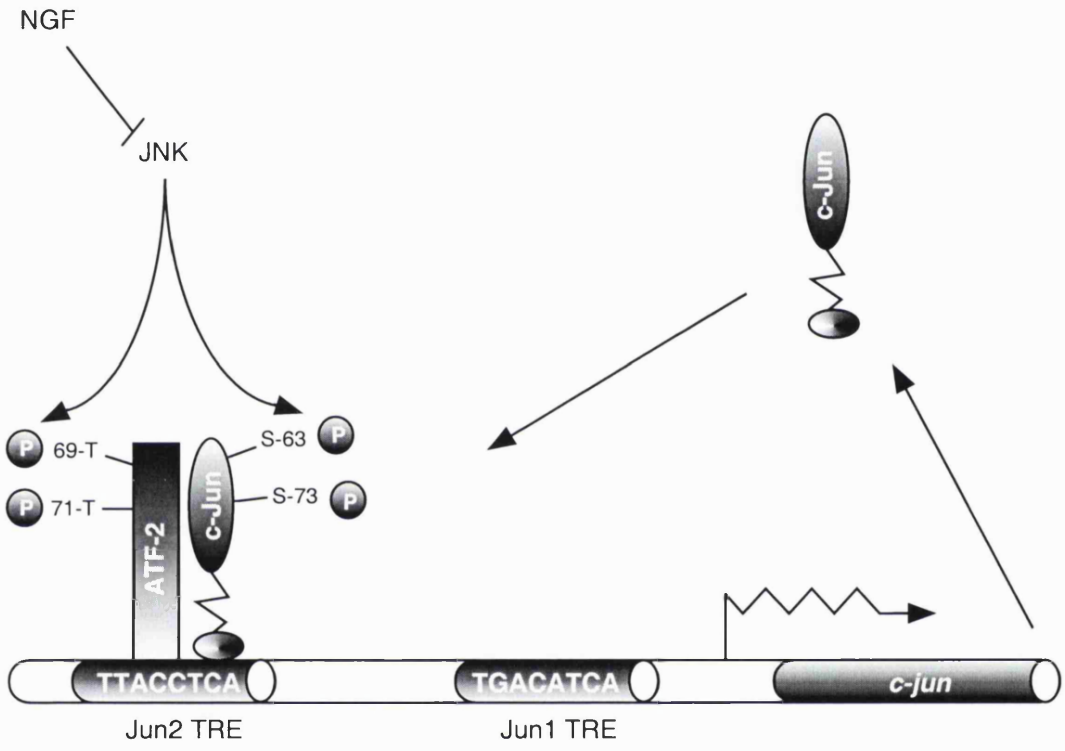
ATF-2 can be phosphorylated on threonines 69 and 71 by c-Jun N-terminal kinase (Gupta *et al.*, 1995; Livingstone *et al.*, 1995), which is the kinase responsible for activating c-Jun and which is itself activated during sympathetic neuron apoptosis (Eilers *et al.*, 1998; Virdee *et al.*, 1997). Furthermore, ATF-2 is activated in response to a variety of genotoxic agents and this depends on threonines 69 and 71 (van Dam *et al.*, 1995). ATF-2 pre-bound to the *c-jun* promoter became phosphorylated in response to UV (van Dam *et al.*, 1995) and it was phosphorylated on threonine 71 after okadaic acid treatment of PC12 cells or hypoxic-ischaemic insult *in vivo* (Walton *et al.*, 1998). Hence it is possible that ATF-2, if present in sympathetic neurons, would become

phosphorylated after NGF withdrawal. In addition, *c-jun* transcription can be initiated in cells that do not contain basal levels of c-Jun such as those from *c-jun* (–/–) mice (Hilberg *et al.*, 1993; Johnson *et al.*, 1993). These studies suggest that other factors, such as ATF-2, can function as a transcriptional activator of the *c-jun* promoter. Indeed, in F9 carcinoma cells which lack c-Jun, *c-jun* gene induction after UV treatment depends on the activation of pre-existing and pre-bound factors (Auer *et al.*, 1994; van Dam *et al.*, 1995). The predominant complex contains ATF-2 which may be bound as a homodimer, or as a heterodimer with uncharacterised non-c-Jun factors (van Dam *et al.*, 1995). It will be interesting to study the phosphorylation state of ATF-2 in sympathetic neurons deprived of NGF. Also, cells derived from mice lacking both ATF-2 and c-Jun might be useful for finding out what other factors are necessary for *c-jun* induction.

Since c-Jun becomes phosphorylated on serine 63, and it is possible that ATF-2 is similarly activated during sympathetic neuronal apoptosis, interest has focussed on the upstream kinases. c-Jun N-terminal kinases (JNK, also termed SAPK) are proline-directed serine/threonine kinases that phosphorylate c-Jun on several residues within the transactivation domain, principally on serine 63 and 73 (Hibi *et al.*, 1993; Derijard *et al.*, 1994; Kyriakis *et al.*, 1994). JNK can also phosphorylate ATF-2 on threonine 69 and 71 (Livingstone *et al.*, 1995). Figure 3.5 shows a hypothetical model, with NGF withdrawal leading to activation of JNK and increased phosphorylation of c-Jun (and possibly ATF-2) bound to the jun2 (and probably jun1) TRE sites. This increases transcription of the *c-jun* gene, and the c-Jun protein produced then binds to target genes, including *c-jun* itself. The role of the JNK pathway in the activation of c-Jun and the control of apoptosis in sympathetic neurons will be investigated in the next chapter.

Figure 3.5 Hypothetical model showing regulation of the *c-jun* promoter by phosphorylated c-Jun and ATF-2

Withdrawal of NGF from sympathetic neurons triggers activation of JNK, which in turn phosphorylates c-Jun (and possibly ATF-2) pre-bound to the jun1 and jun2 TRE sites within the *c-jun* promoter. This enhances transcription of the *c-jun* gene and increases the endogenous level of c-Jun protein, thus forming a positive regulatory loop.


NGF
JNK
c-Jun
P
69-T
P
71-T
ATF-2
c-Jun
S-63
P
S-73
P
TTACCTCA
TGACATCA
c-jun
Jun2 TRE
Jun1 TRE

Chapter 4: Regulation of c-Jun expression and activity by upstream kinases

4.1 Introduction

c-Jun N-terminal kinase (JNK) is a member of the MAPK superfamily of proline-directed serine/threonine kinases, which also includes the extracellular signal-regulated kinase (ERK) and p38 kinase subgroups. c-Jun is specifically activated by JNK (also known as SAPK) which binds to the ∂ domain and phosphorylates serines 63 and 73, and threonines 91 and 93 within the transactivation domain (Karin and Hunter, 1995; Papavassiliou *et al.*, 1995). Phosphorylation of c-Jun on serines 63 and 73 increases the ability of c-Jun to activate the transcription of target genes (Karin and Hunter, 1995). To date no other kinases that efficiently phosphorylate these sites have been identified (Hibi *et al.*, 1993; Minden *et al.*, 1994). There are three mammalian *Jnk* genes which are alternately spliced to give 10 isoforms. These have varying abilities to bind and phosphorylate c-Jun (Gupta *et al.*, 1996). The expression and regulation of individual JNKs has not been extensively studied. JNK is activated by phosphorylation on threonine 183 and tyrosine 185, and the first JNK kinase (JNKK) to be isolated was SEK1 (also known as MKK4/JNKK1) (Derijard *et al.*, 1994; Sanchez *et al.*, 1994; Lin *et al.*, 1995). Other kinases that directly activate either JNK, or p38, or both kinases have been cloned. These include MKK7 (also known as JNKK2) which specifically activates JNK (Tournier *et al.*, 1997; Yao *et al.*, 1997), and MKK3 which is selective for p38 (Derijard *et al.*, 1995; Raingeaud *et al.*, 1995). Further upstream in the JNK signalling pathway lies MEKK1 which activates SEK1 (Yan *et al.*, 1994). Many other JNKK kinases (JNKKKs) have been cloned, including MEKK2, 3, and 4 (Blank *et al.*, 1996; Gerwins *et al.*, 1997), ASK1 (Ichijo *et al.*, 1997), and MLK3 (Ing *et al.*, 1994; Rana *et al.*, 1996). A parallel MAPK signalling cascade leads to phosphorylation of ATF-2 by p38 kinase (Raingeaud *et al.*, 1995; Raingeaud *et al.*, 1996). p38 is activated by MKK3 (Derijard *et al.*, 1995; Raingeaud *et al.*, 1995) or MKK6 (Cuenda *et al.*, 1996; Han *et al.*, 1996; Raingeaud *et al.*, 1996). In addition, JNK can phosphorylate and activate ATF-2 (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995).

c-Jun can bind as a heterodimer with ATF-2 to the jun2 TRE site within the *c-jun* promoter. I showed in Chapter 3 that the jun2 TRE can function as a transcriptional enhancer in sympathetic neurons after NGF withdrawal, hence JNK, and also p38, could potentially contribute to increases in *c-jun* gene expression. However, p38 kinase activity did not increase in sympathetic neurons withdrawn from NGF, while JNK activity increased two-fold (Eilers *et al.*, 1998), suggesting that JNK, but not p38, is involved in activating c-Jun in these neurons. To investigate the regulation of c-Jun by upstream kinases during apoptosis, expression vectors for mutated SEK1 and MEKK1 were microinjected into sympathetic neurons.

4.2 Results

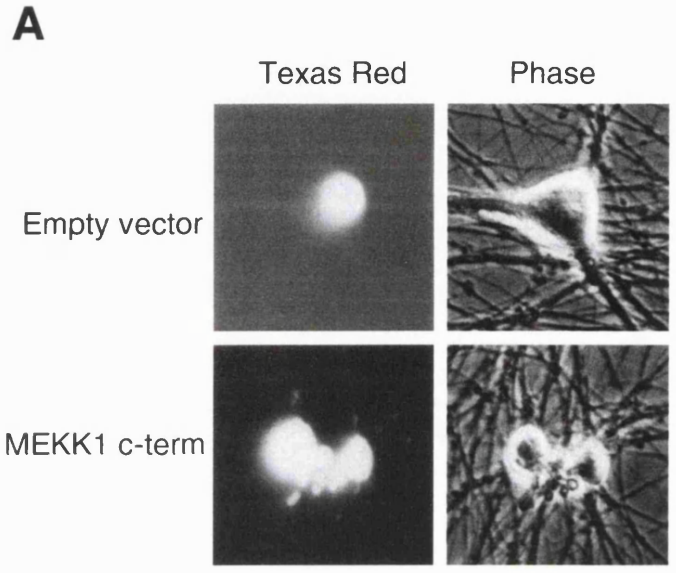
4.2.1 Overexpression of activated MEKK1 induces cell death by apoptosis

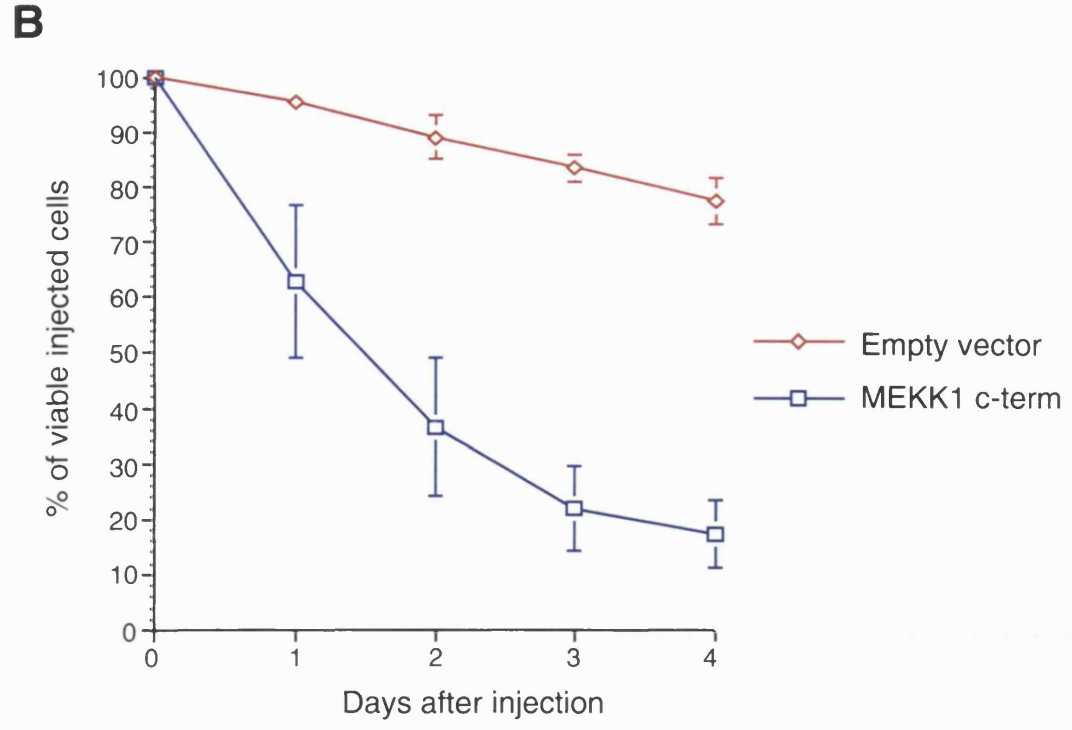
To determine whether activation of the JNK pathway was sufficient to induce apoptosis in sympathetic neurons in the presence of NGF, an expression vector for a constitutively active form of MEKK1 was microinjected into neurons. This expression vector encodes a Myc epitope-tagged, truncated form of MEKK1 which consists of the C-terminal kinase domain and which is constitutively active. This protein has been shown to strongly activate the JNK pathway (Olson *et al.*, 1995), and the catalytic domain of MEKK1 is known to preferentially activate the JNK pathway (Minden *et al.*, 1995). Expression of MEKK1 in microinjected neurons was confirmed by immunofluorescence with the anti-Myc 9E10 monoclonal antibody. When the expression vector was injected at 0.4 mg/ml, 40% of the neurons expressed the Myc-tagged protein throughout the cell. The reasons for this low level of expression (compared to other plasmids I have injected) are not clear, but could be due to the weaker promoter within this vector (an SV40 enhancer, with the adenovirus major late promoter). To determine whether expression of the kinase affected the survival of neurons, the MEKK1 expression plasmid was microinjected into the nucleus at 0.1 mg/ml along with a fluorescent injection marker (Texas Red-dextran). Control neurons were injected with an empty SV40 vector. The number of cells surviving the injection procedure was scored after four hours. The neurons were maintained in NGF and counted over the following four days on an inverted fluorescent microscope. Most of

Figure 4.1 Overexpression of constitutively active MEKK1 in sympathetic neurons induces apoptosis

(A) Morphology of neurons four days after injection with an expression plasmid for constitutively active MEKK1. Sympathetic neurons were microinjected with an empty vector or a MEKK1 expression plasmid together with Texas Red-dextran (5 mg/ml) to mark the injected neurons. The injected cells were left for four days in NGF-containing medium and then were examined on an inverted fluorescence microscope. The majority of cells injected with the empty vector had a normal morphology by phase microscopy and the injected Texas Red-dextran was still retained within the nucleus. A representative cell is shown. In contrast, the majority of cells injected with the MEKK1 plasmid had an apoptotic morphology by phase contrast (two cells injected with MEKK1 are shown) and the dextran marker was no longer retained within the nucleus.

(B) Kinetics of cell death after microinjection of the MEKK1 expression vector. Sympathetic neurons were microinjected with the MEKK1 vector or the control vector, each at 0.1 mg/ml together with Texas Red-dextran (5 mg/ml). The percentage of viable injected cells that remained at different times after injection was determined by scoring the number of cells that had a normal appearance by phase contrast and that still retained the Texas Red-dextran within the nucleus. The results shown are the average for four independent experiments with the standard error. In each experiment, 200 neurons were injected per construct. Expression of MEKK1 induced apoptosis in sympathetic neurons.


A
Texas Red
Phase
Empty vector
MEKK1 c-term


B
% of viable injected cells
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
Days after injection
Empty vector
MEKK1 c-term

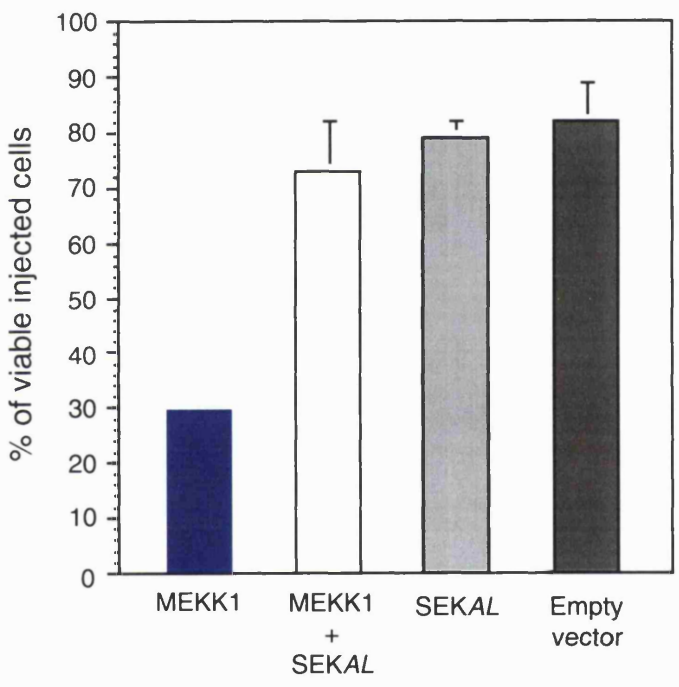
the neurons (80%) injected with the control plasmid were viable after four days (Figure 4.1B). These cells had a normal morphology and the Texas Red-dextran was retained within the nucleus (Figure 4.1A). The small decrease in the percentage of viable cells could be due to the presence of plasmid DNA within the cell, or side effects of the microinjection process. In contrast, only 20% of the neurons injected with the vector for activated MEKK1 were still viable after four days. Most of the cells had shrunken cell bodies and nuclei that were distorted or no longer visible. In addition, the Texas Red-dextran had leaked out of the nucleus and was seen throughout the cell. Chromatin condensation as visualised by Hoechst staining, and DNA strand breaks detected by TUNEL staining, were also significantly increased in neurons injected with MEKK1 (J. Ham, personal communication). Thus, expression of activated MEKK1 in sympathetic neurons leads to cell death and this has the features of apoptosis.

4.2.2 Dominant negative SEK1 blocks MEKK1-induced c-Jun expression and death

To determine whether the death induced by MEKK1 required SEK1 activity, a dominant negative form of SEK1 (SEK*AL*) was co-expressed with MEKK1. SEK1 is activated by phosphorylation on serine 220 and threonine 224 by MEKK1 (Yan *et al.*, 1994). In SEK*AL* these sites have been mutated to alanine and leucine respectively and hence this mutant kinase cannot be phosphorylated and is inactive. SEK*AL* acts as a dominant negative mutant and prevents JNK activation by MEKK1 (Yan *et al.*, 1994). SEK*AL* is tagged with the HA epitope tag and expression of the dominant negative kinase was detected in approximately 30% of the neurons injected with the SEK*AL* expression vector (at 0.4 mg/ml) when immunocytochemistry was performed with an anti-HA antibody. Expression was seen throughout the cytoplasm and along the neurites. Experiments were performed as before, with survival assessed after three days by examining the morphology of neurons marked with Texas Red-dextran. DNA concentrations were equalised by the addition of empty vector when necessary. When the SEK*AL* plasmid was injected at 0.4 mg/ml into neurons maintained in NGF, there was only a small effect on survival which was not significantly different to the effect of the empty vector injected as a control (Figure 4.2). Injection of the MEKK1 plasmid at 0.1 mg/ml induced apoptosis in more than 70% of the neurons. Co-injection of the SEK*AL* vector at 0.4 mg/ml along with the MEKK1 plasmid at 0.1 mg/ml

Figure 4.2 Co-expression of SEK*AL* prevents MEKK1-induced apoptosis

Sympathetic neurons were microinjected with expression vectors for MEKK1 (0.1 mg/ml), SEK*AL* (0.4 mg/ml) or the control vector pSG5 (0.1-0.5 mg/ml) in the combinations indicated. Where necessary, the total DNA concentration was adjusted to 0.5 mg/ml by the addition of pSG5. Texas Red-dextran was included in the injection mixes at 5 mg/ml. Several hours after injection, the number of Texas Red-containing cells was determined and the cells were left in NGF-containing medium. Three days later, the number of viable injected cells that remained was determined. The results shown are the average of three independent experiments with the standard error. Co-expression of SEK*AL* completely blocked MEKK1-induced death. Expression of SEK*AL* alone did not affect cell survival in the presence of NGF.


% of viable injected cells
100
90
80
70
60
50
40
30
20
10
0
MEKK1
MEKK1 + SEKAL
SEKAL
Empty vector

completely blocked the cell death which would have occurred after three days. This result suggests that MEKK1 induces apoptosis by activating SEK1 or a similar kinase. Less protection from MEKK1-induced death was seen at lower concentrations of the SEK*AL* vector (data not shown). It is possible that a high concentration of SEK*AL* is needed because the constitutively active form of MEKK1 is a very potent activator of the JNK pathway, or perhaps because the signal is amplified further down the kinase cascade.

To investigate whether MEKK1 could increase c-Jun protein levels in sympathetic neurons maintained in NGF, neurons were microinjected with the MEKK1 expression plasmid along with guinea pig IgG to mark injected cells. After 48 hours, the cells were fixed and stained with a polyclonal c-Jun antibody and with a TRITC-conjugated anti-gpIgG antibody. The number of injected cells that were c-Jun positive was scored. c-Jun positive neurons were considered to be those in which the nucleus was stained brighter than the cytoplasm. Microinjection of the empty vector alone did not significantly increase c-Jun expression above background levels (as seen in uninjected cells, data not shown). Overexpression of MEKK1 caused a 5-fold increase in the number of c-Jun positive neurons compared with the empty vector control (Figure 4.3A). This increase was completely blocked by co-expression of SEK*AL* (both plasmids were injected at the concentration used for the survival experiments).

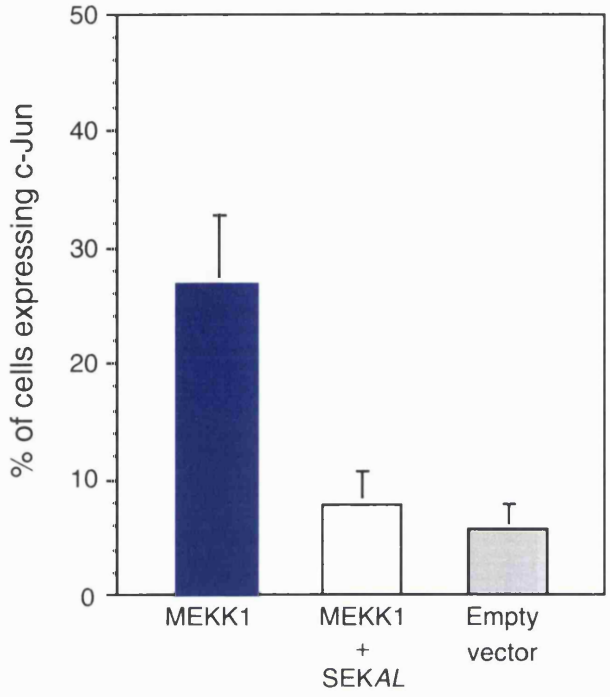
Similar results were obtained when the neurons were stained with a phospho-c-Jun antibody that only recognises c-Jun phosphorylated on serine 63 (Lallemand *et al.*, 1998). In this case, approximately 26% of the neurons injected with the MEKK1 vector showed phospho-c-Jun staining in the nucleus, whereas only 4% were stained when the empty vector was injected (Figure 4.3B). In addition, co-expression of SEK*AL* completely blocked the phosphorylation of c-Jun caused by MEKK1 expression (5% phospho-c-Jun positive). It is interesting to note that between 48 and 72 hours after injection approximately 35% of the neurons injected with the MEKK1 plasmid that were still viable at 48 hours proceed to die (from Figure 4.1B, decrease in viability of 37% to 23%). This is similar to the percentage of neurons expressing c-Jun and phospho-c-Jun at 48 hours, and thus there is a correlation between the number of neurons that are expressing c-Jun and phospho-c-Jun, and those which die in the following 24 hours. There is, however, no direct evidence to show that those cells which are expressing c-Jun then proceed to die.

Figure 4.3 Overexpression of MEKK1 induces c-Jun expression and phosphorylation of c-Jun on serine 63

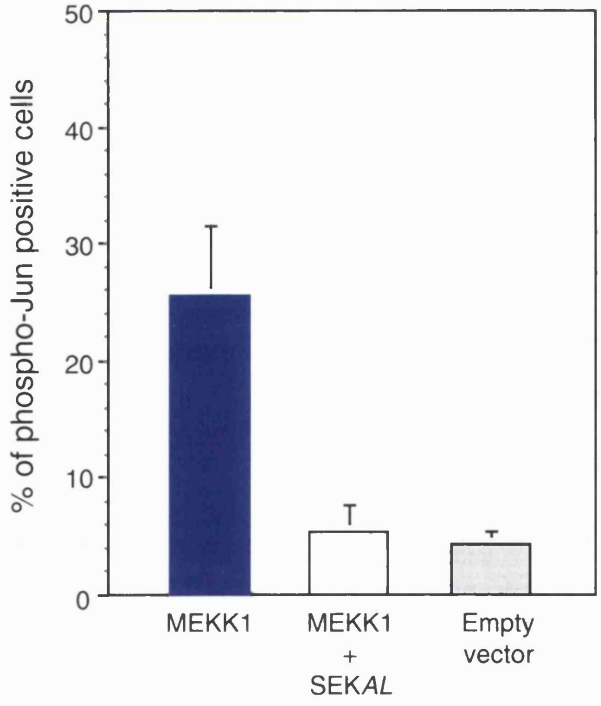
(A) Sympathetic neurons were microinjected with expression vectors encoding a constitutively active form of MEKK1, a dominant negative mutant of SEK1 (SEK*AL*), or an empty vector. The MEKK1 plasmid was injected at 0.1 mg/ml, and SEK*AL* at 0.4 mg/ml. Guinea pig IgG was added at 5 mg/ml to the injection mix to identify injected cells. The neurons were maintained in the presence of NGF for 48 hours, and then fixed and stained with an anti-c-Jun antibody and with an anti-gpIgG antibody. The number of neurons that expressed c-Jun protein above background levels was scored. The data shows the average of 7 independent experiments with the standard error indicated. Approximately 2500 cells were injected in total. Some of the experiments were scored in a blinded manner. MEKK1 expression induced an increase in the level of endogenous c-Jun.

(B) Neurons were injected as above but stained with an antibody specific for phospho-c-Jun. The number of neurons that were phospho-c-Jun positive was then scored. The data shows the average of four independent experiments and the standard error is shown. Approximately 1000 cells were injected in total. Some of the experiments were scored in a blinded manner. Expression of MEKK1 caused increased phosphorylation of c-Jun on serine 63.

A


% of cells expressing c-Jun
50
40
30
20
10
0
MEKK1
MEKK1 + SEK*AL*
Empty vector

B


% of phospho-Jun positive cells
50
40
30
20
10
0
MEKK1
MEKK1 + SEK*AL*
Empty vector

Thus, expression of activated MEKK1 in sympathetic neurons increases the level of c-Jun protein and also causes increased phosphorylation of c-Jun on serine 63. These effects are completely blocked by co-expression of dominant negative SEK1. This suggests that MEKK1 activation increases JNK activity in a SEK1-dependent manner.

4.2.3 Dominant negative SEK1 does not prevent induction of c-Jun or block apoptosis after NGF withdrawal

Since co-expression of SEK*AL* efficiently inhibits the activation of the JNK/c-Jun pathway and induction of apoptosis induced by the expression of MEKK1, it might also block this pathway in the absence of NGF and thus prevent cell death. To test this hypothesis, sympathetic neurons were injected with a SEK*AL* expression vector or an empty vector as a control. In addition, a Bcl-2 vector was injected as a positive control (along with the corresponding empty vector, pcDNA1). Texas Red-dextran was added to each injection mix and the number of cells surviving the injection process was scored immediately after the neurons were withdrawn from NGF. Cell viability was then determined after three days by counting the number of injected neurons that were morphologically normal by phase contrast. Bcl-2 expression significantly protected sympathetic neurons compared to the empty vector, as had been shown previously (Garcia *et al.*, 1992; Ham *et al.*, 1995). However, when injected at 0.4 mg/ml (the concentration at which co-injection of the SEK*AL* vector inhibited MEKK1-induced death) SEK*AL* did not protect neurons (Figure 4.4). In fact, there were fewer viable neurons when the SEK*AL* vector was injected than with the empty vector. A range of concentrations of the SEK*AL* plasmid were also tested (0.05 to 0.8 mg/ml), but no protection was observed (data not shown). These results suggest that the NGF withdrawal-induced death of sympathetic neurons occurs in a SEK1-independent manner.

Since expression of SEK*AL* did not protect neurons from death induced by NGF withdrawal it was important to determine whether it had any effect on the level of endogenous c-Jun protein. Neurons were injected with an expression vector for SEK*AL* or the empty vector, or with FLAGΔ169 which represses the induction of c-Jun after NGF withdrawal (see Figure 3.4B). In this case, the neurons were deprived of NGF for 24 hours and then fixed and stained with an anti-c-Jun antibody.

Figure 4.4 Dominant negative SEK1 (SEK*AL*) does not increase neuronal survival after NGF withdrawal

Sympathetic neurons were microinjected with an expression vector encoding a dominant negative form of SEK1 (SEK*AL*). As a control, either empty vectors or an expression vector encoding Bcl-2 were injected. SEK*AL* was injected at 0.4 mg/ml. Texas Red-dextran was also injected to mark the cells. After allowing the proteins to be expressed for several hours, the cells were withdrawn from NGF by re-feeding them with medium lacking NGF and supplemented with an anti-NGF antibody. The number of neurons that had survived injection was then scored. After three days, the number of neurons that were still viable was scored by examining morphology on phase contrast. The data shown represents the average of three experiments with standard error. Approximately 400 neurons were injected per construct. All of the experiments were scored in a blinded manner.

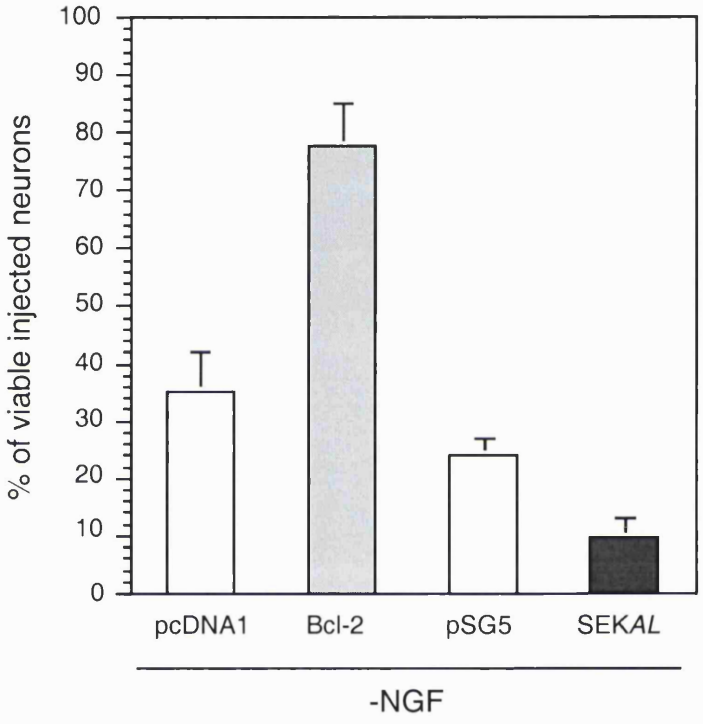
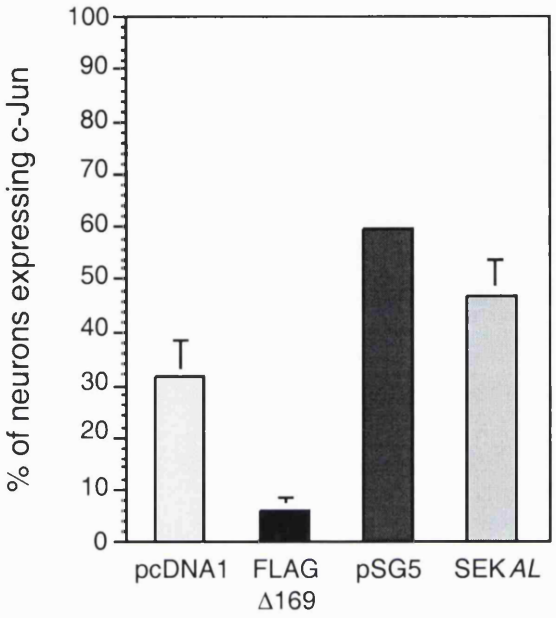

100
90
80
70
60
50
40
30
20
10
0
% of viable injected neurons
pcDNA1
Bcl-2
pSG5
SEKAL
-NGF

Figure 4.5 SEK*AL* does not inhibit the expression or phosphorylation of c-Jun protein after NGF withdrawal

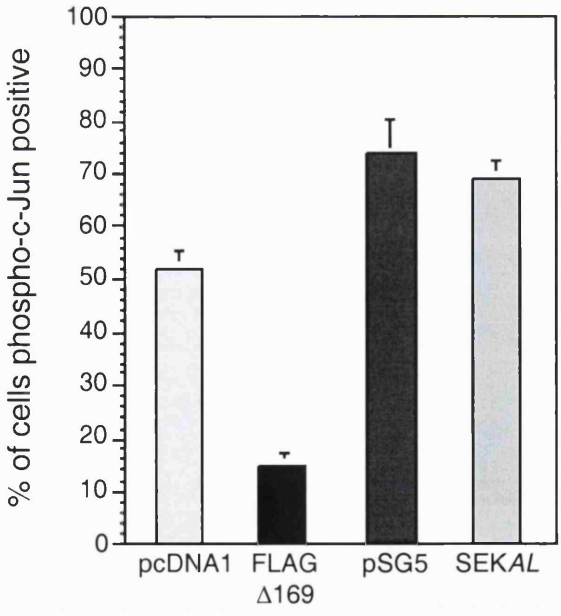
(A) Sympathetic neurons were injected with pcD-FLAGΔ169 or the empty vector pcDNA1 (0.2 mg/ml), or with the SEK*AL* expression plasmid or the empty vector pSG5 (0.4 mg/ml), together with guinea pig IgG at 5 mg/ml. After injection, the cells were withdrawn from NGF. 24 hours later, the neurons were fixed and stained with an anti-c-Jun antibody. The percentage of injected cells that expressed nuclear c-Jun protein was determined. c-Jun expression in uninjected cells on the same coverslips was scored for comparison. The data shown is the average for three independent experiments with the standard error. SEK*AL* did not prevent the induction of c-Jun after NGF withdrawal.

(B) Neurons were injected as above, but were stained with an antibody specific for c-Jun phosphorylated on serine 63. The data shows the average of three independent experiments and the standard error is indicated. Approximately 300 cells were injected per plasmid. All of the experiments were scored in a blinded manner. SEK*AL* did not prevent the phosphorylation of c-Jun on serine 63 after NGF withdrawal.

A


% of neurons expressing c-Jun
100
90
80
70
60
50
40
30
20
10
0
pcDNA1
FLAG Δ169
pSG5
SEK*AL*

B


% of cells phospho-c-Jun positive
100
90
80
70
60
50
40
30
20
10
0
pcDNA1
FLAG Δ169
pSG5
SEK*AL*

FLAGΔ169 dramatically reduced the number of neurons expressing c-Jun compared to the corresponding empty vector pcDNA1 (Figure 4.5A). In contrast, SEK*AL* had no significant effect on c-Jun levels compared to the empty vector.

When the cells were stained with the phospho-c-Jun antibody, expression of FLAGΔ169 greatly decreased the number of phospho-c-Jun positive neurons. Presumably FLAGΔ169 functions by preventing the induction of c-Jun protein itself, rather than actually blocking phosphorylation, since FLAGΔ169 is unable to bind to JNK, but could bind to and repress activation of the *c-jun* promoter. There was no significant effect of SEK*AL* compared to the empty vector. These results suggest that the increase in the level of c-Jun protein and its phosphorylation after NGF withdrawal occur via a SEK1-independent mechanism.

4.3 Discussion

In this chapter I have demonstrated that expression of a constitutively active form of MEKK1 in sympathetic neurons can induce apoptosis in the presence of NGF. MEKK1 expression causes an increase in endogenous c-Jun levels and an increase in the amount of c-Jun phosphorylated on serine 63. Interestingly, expression of MEKK1 also activates the *c-jun* promoter, as shown by co-injection experiments with the *c-jun* CAT reporter gene (J. Ham, personal communication). Thus, stimulation of the JNK pathway, which results in the increased expression and activation of c-Jun, can initiate apoptosis and overcome the protective effect of NGF. MEKK1-induced increases in c-Jun protein levels and cell death are completely blocked by dominant negative SEK1. However, expression of SEK*AL* prevents neither the death of neurons, nor the increases in c-Jun expression and phosphorylation that occur after NGF withdrawal. This suggests that NGF withdrawal-induced death occurs in a SEK1-independent manner. This contrasts with the effect of SEK1 dominant negative mutants in other systems. A dominant negative SEK1 mutant prevented the ceramide-induced death of endothelial cells (Verheij *et al.*, 1996), while SEK*AL* abrogated BRCA1-induced apoptosis (Harkin *et al.*, 1999) and promoted the survival of fibroblasts exposed to UV or *cis*-platinum (Zanke *et al.*, 1996). Most pertinent to this study, perhaps, is the fact that a SEK1 dominant negative mutant inhibited JNK activation by MEKK1 in HeLa cells, whereas the mutant did not prevent the activation of JNK by arsenite in the same cells (Cavigelli *et al.*, 1996). These results suggest that JNK can be activated in some cases by SEK1-independent pathways. Experiments with *Sek1* (–/–) mice support this

idea, because in *Sek1* (–/–) cells activation of JNK by some stimuli, such as anisomycin, is inhibited, whereas JNK activation by other stimuli, such as UV radiation, is unaltered (Nishina *et al.*, 1997).

After SEK1 was cloned, other kinases capable of activating JNK were discovered. In particular, MKK7 (also known as JNKK2) was found to be a specific activator of JNK but not p38 kinase (Tournier *et al.*, 1997; Wu *et al.*, 1997; Yao *et al.*, 1997). Gene knockout experiments in the mouse show that *Sek1* (*Mkk4*) is an essential gene and therefore MKK7 is unable to fully substitute for the function of SEK1, demonstrating that these kinases serve non-redundant functions as JNK activators *in vivo* (Nishina *et al.*, 1997; D. Yang *et al.*, 1997; Ganiatsas *et al.*, 1998). Similarly, D-MKK4 and D-MKK7 serve non-redundant functions in *Drosophila.* For example, loss of D-MKK7 cannot be substituted for by D-MKK4 during dorsal closure in the embryo (Glise *et al.*, 1995). It is also possible that other JNK kinases exist. For example, SAPKK4 and 5 were isolated from stressed cells and are potential activators of JNK but not p38 (Meier *et al.*, 1996). These kinases are immunologically different to MKK4, although one is possibly MKK7 (Meier *et al.*, 1996). Several distinct JNK-activating fractions have also been chromatographically isolated from osmotically-stressed fibroblasts (Moriguchi *et al.*, 1995). Therefore, it is possible that MKK7 (or another JNKK) could be the major JNK activator in sympathetic neurons. Dominant negative mutants of MKK7 have been described and it would be interesting to express such a mutant in sympathetic neurons.

Although MEKK1 is able to activate the JNK pathway and induce apoptosis, it is not clear whether this kinase is involved in the NGF withdrawal-induced death pathway. In other cell types, MEKK1 has been shown to have either a pro- or anti-apoptotic role in cell death. Activated MEKK1 induces cell death in fibroblasts (Johnson *et al.*, 1996). In T cells, activated MEKK1 induces apoptosis by upregulating the expression of Fas ligand via an AP-1 site in the *FasL* promoter (Faris *et al.*, 1998b). MEKK1 is also a substrate for caspase-3 (Cardone *et al.*, 1997; Widmann *et al.*, 1998). Cleavage at Asp^{874} generates a 91 kD carboxy-terminal kinase domain, which can amplify caspase activation and induce death (Widmann *et al.*, 1998). However, *Mekk1* (–/–) embryonic stem cells display reduced JNK activation and increased apoptosis in response to cold stress and microtubule disruption, demonstrating an anti-apoptotic function of MEKK1 and the JNK pathway in this context (Yujiri *et al.*, 1998). JNK activation in response to other signals, such as UV radiation, heat shock, and

* If SEK*AL* functions by sequestering JNK, the results described in this chapter would demonstrate that MEKK1-induced apoptosis in sympathetic neurons depends solely on activation of the JNK pathway. If, however, SEK*AL* sequesters MEKK1, it would not be possible to conclude that the JNK pathway is the key pathway activated by MEKK1, since overexpression of MEKK1 has been reported to activate other pathways, such as the p38 kinase and ERK pathways, in addition to activating JNK. It would therefore be interesting to examine which putative downstream pathways were activated in response to MEKK1 overexpression, perhaps by using phospho-specific antibodies to detect activated forms of JNK, p38, and ERK. In addition, inhibitors of each pathway could be used to determine the contribution of each to MEKK1-induced death. For example, co-expression of the JIP-1 JNK binding domain could be used to specifically inhibit JNK activity by sequestering JNK to the cytosol; PD098059 could be used to inhibit the ERK activator MEK, and dominant negative MKK3 or MKK6 could be used to block p38 activation.

anisomycin, was not altered in these cells, showing that other JNKKKs mediate the JNK response to these stimuli (Yujiri *et al.*, 1998). To elucidate the role of MEKK1 in the death of sympathetic neurons dominant negative mutants, neurons cultured from *Mekk1* (–/–) mice, or perhaps chemical inhibitors selective for MEKK1 are needed.

The parallel kinase cascade which leads to the activation of p38 could also affect *c-jun* transcription. p38 kinase phosphorylates ATF-2, a transcription factor that can activate *c-jun* gene expression (see chapter 3). Another substrate of p38 is the MEF2C transcription factor (Han *et al.*, 1997) which can increase activity of the *c-jun* promoter via a MEF2 site (Coso *et al.*, 1997). However, p38 kinase assays showed that in sympathetic neurons the basal level of p38 kinase activity does not alter after NGF withdrawal (Eilers *et al.*, 1998). In addition, phosphorylated (activated) p38 is present at low levels and does not increase after NGF withdrawal (Eilers *et al.*, 1998). Furthermore, microinjection of dominant negative MKK3 (a p38 kinase kinase) did not protect neurons after NGF withdrawal (data not shown). This contrasts with the situation in differentiated, postmitotic PC12 cells where p38 kinase appears to be involved in the apoptotic response (Xia *et al.*, 1995). In these sympathetic neuron-like cells, dominant negative MKK3, and also SEK*AL*, promoted survival after NGF deprivation (Xia *et al.*, 1995). The reasons for these differences are unclear, since in many respects differentiated PC12 cells resemble sympathetic neurons. It is worth noting that in our laboratory p38 kinase activity was found to increase in PC12 cells withdrawn from NGF, but not in NGF-deprived sympathetic neurons tested in the same assays (Eilers *et al.*, 1998).

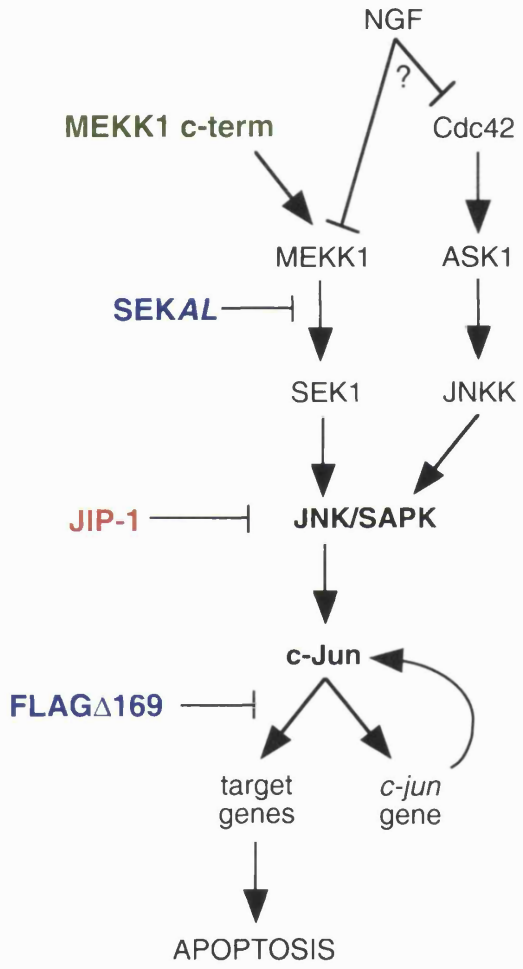
The mechanism of action of SEK*AL* is not known. It could function by sequestering JNKKKs that activate SEK1 such as MEKK1, which would imply that neither SEK1 nor any upstream kinase that SEK1 could bind to were physiologically relevant kinases in this case. If, on the other hand, SEK*AL* binds to JNK, this would suggest that JNK activity is not required for the induction of c-Jun and apoptosis after NGF withdrawal. Alternatively, SEK*AL* may be unable to bind to all of the different JNK isoforms. However, it has been suggested that activation of the JNK cascade occurs through sequential MEKK1:SEK1 then SEK1:JNK interactions since JNK and MEKK1 compete for binding with SEK1, and because phosphorylated SEK1 dissociates from MEKK1 (Xia *et al.*, 1998). One might therefore expect that a mutated form of SEK1 which cannot be phosphorylated would bind to and sequester MEKK1, rather than JNK.

*

To confirm the hypothesised role of JNK itself during neuronal cell death, direct inhibition of JNK activity is necessary, although this has proved difficult until recently. Dominant negative forms of JNK have worked in some cell types but have not been used in sympathetic neurons to date. A more direct strategy for examining the role of JNK uses JNK interacting protein-1. JIP-1 was cloned as a protein which binds to JNK and is believed to act as a scaffold protein that brings together JNK and its activating kinases MKK7 and MLK3/DLK (Dickens *et al.*, 1997; Whitmarsh *et al.*, 1998). Microinjection of the JIP-1 JNK binding domain into sympathetic neurons, which should directly inhibit JNK by sequestering it in the cytoplasm, protected neurons as effectively as Bcl-2 or FLAGΔ169 (Eilers *et al.*, manuscript submitted). This data strongly supports the previous studies which suggest that c-Jun and activation of the c-Jun pathway is important for the death of sympathetic neurons, and shows that JNK activity is critical for NGF withdrawal-induced death. Figure 4.6 shows a putative model for the apoptotic pathway in sympathetic neurons. The presence of NGF blocks activation of MEKK1 (or another JNKKK), but once NGF is removed a signalling cascade is triggered which ultimately leads to the activation of JNK and an increase in c-Jun phosphorylation. Activated c-Jun, bound to the jun1 and jun2 TRE sites within its own promoter, stimulates transcription of the *c-jun* gene (autoregulation). c-Jun can also bind to and activate the promoters of other target genes, some of which promote cell death. FLAGΔ169 binds to and prevents the activation of the promoters of c-Jun target genes (including *c-jun* itself), and therefore prevents cell death after NGF withdrawal. While activation of the JNK pathway by MEKK1 can induce cell death, the role of this kinase and SEK1 in NGF withdrawal-induced death is not clear. Another JNKKK which is strongly implicated in the death of these neurons is ASK1 (X. Wang *et al.*, 1996; Ichijo *et al.*, 1997). ASK1 is activated after NGF withdrawal and expression of active ASK1 induces apoptosis (Kanamoto *et al.*, 2000). Crucially, a dominant negative form of ASK1 blocks c-Jun induction and prevents death after NGF withdrawal (Kanamoto *et al.*, 2000). Upstream of ASK1 may lie Cdc42 or Rac1. Active forms of these Rho-like GTPases induce c-Jun expression and the death of sympathetic neurons in the presence of NGF, and both of these effects can be prevented by co-expression of FLAGΔ169 (Bazenet *et al.*, 1998). Dominant negative mutants of Cdc42 and Rac1 promote survival after NGF withdrawal (Bazenet *et al.*, 1998).

Figure 4.6 Putative kinase cascade leading to the activation of c-Jun in sympathetic neurons deprived of NGF

After NGF withdrawal, JNK activity, c-Jun levels and c-Jun phosphorylation increase. Expression of c-Jun protein and apoptosis are blocked by the c-Jun dominant negative mutant FLAGΔ169, which inhibits AP-1 activity (Ham *et al.*, 1995). NGF-withdrawal-induced death is also blocked by injection of antibodies against c-Jun but not Jun B or Jun D (Estus *et al.*, 1994). These results suggest that c-Jun activates target genes that promote apoptosis, as well as the *c-jun* gene itself. JNK/SAPK can be activated by SEK1, which in turn is activated by MEKK1. Overexpression of MEKK1 in sympathetic neurons increases the level of c-Jun protein and induces apoptotic cell death, suggesting that activation of the JNK pathway is sufficient to trigger apoptosis. The induction of c-Jun expression and apoptosis by MEKK1 is blocked by co-expression of the SEK1 dominant negative mutant (SEK*AL*). However, expression of SEK*AL* does not block the expression of endogenous c-Jun or the death of neurons that is induced by NGF withdrawal. Therefore, NGF withdrawal may activate a JNKKK and/or JNKK that cannot be inhibited by SEK*AL*. Cdc42 and ASK1 have recently been implicated in the regulation of this kinase pathway. Expression of activated forms of Cdc42 and ASK1 induce apoptosis in sympathetic neurons, and this can be blocked by the expression of FLAGΔ169 but not SEK*AL* (Bazenet *et al.*, 1998; Kanamoto *et al.*, 2000).


NGF
?
MEKK1 c-term
Cdc42
MEKK1
ASK1
SEKAL
SEK1
JNKK
JIP-1
JNK/SAPK
c-Jun
FLAGΔ169
target genes
c-jun gene
APOPTOSIS

Furthermore, dominant negative ASK1 prevents the death induced by activated Cdc42 (Kanamoto *et al.*, 2000). Dominant negative c-Jun blocks the death induced by both Cdc42 and ASK1 (Bazenet *et al.*, 1998; Kanamoto *et al.*, 2000).

Evidence for a pro-apoptotic role of the JNK pathway in other cell types includes: (1) induction of cell death by activated MEKK1 mutants in Jurkat cells (Chen *et al.*, 1996b; Faris *et al.*, 1998a), fibroblasts (Johnson *et al.*, 1996), and PC12 cells (Xia *et al.*, 1995); and (2) inhibition of apoptosis after induction by ceramide (Verheij *et al.*, 1996) or *cis*-platinum (Zanke *et al.*, 1996) by dominant negative SEK1 or MEKK1 mutants. In addition, direct inhibition of JNK activity using dominant negative mutants blocks Fas-induced apoptosis in neuroblastoma cells (Goillot *et al.*, 1997), and radiation-induced apoptosis in T cells (Chen *et al.*, 1996b). Most significant perhaps are the studies from knockout mice. *Jnk1* (–/–) T cells have decreased activation-induced cell death (Dong *et al.*, 1998), while apoptosis in immature thymocytes induced by anti-CD3 antibodies is reduced in the absence of *Jnk2* (–/–) (Sabapathy *et al.*, 1999). *Jnk3* (–/–) mice show markedly reduced apoptosis in the hippocampus after kainic acid injection (D.D. Yang *et al.*, 1997), while mice deficient in both *Jnk1* and *Jnk2* show reduced apoptosis in the hindbrain neural tube during development (Kuan *et al.*, 1999). There is clearly some strong evidence for the involvement of the JNK pathway in cell death. This is particularly true in neurons, where, in addition to the studies of knockout mice, apoptosis *in vitro* is inhibited by dominant negative mutants of SEK1 or c-Jun in differentiated PC12 cells after NGF withdrawal (Xia *et al.*, 1995) and striatal neurons after treatment with dopamine (Y. Luo *et al.*, 1998), while phosphorylation mutants of c-Jun promote survival in granule neurons (Watson *et al.*, 1998). Furthermore, a chemical inhibitor of components within the JNK pathway, CEP1347, protects several types of neuron from apoptosis (Maroney *et al.*, 1998; Maroney *et al.*, 1999).

However, c-Jun, JNK and the upstream kinases in the JNK pathway are by no means always involved in apoptosis. For example, the activated TNF receptor still induces apoptosis when its JNK-interacting component (TRAF2) is mutated so that it no longer binds JNK (Z. Liu *et al.*, 1996), and dominant negative SEK1 attenuates JNK activation but does not prevent detachment-induced apoptosis in epithelial cells (Khwaja and Downward, 1997). In some cases JNK activation follows activation of the caspases (Cahill *et al.*, 1996; Z. Liu *et al.*, 1996; Lenczowski *et al.*, 1997) suggesting that activation of the JNK pathway is redundant or a secondary response to the cellular damage associated with apoptosis. On the other hand, it has been suggested

that cleavage and activation of MEKK1 by caspases serves as a positive feedback mechanism which amplifies the apoptotic signal (Cardone *et al.*, 1997; Widmann *et al.*, 1998). JNK activation can also be part of the anti-apoptotic response of a cell. For example, *Sek1* (–/–) thymocytes show increased apoptosis in response to anti-CD3 antibodies (Nishina *et al.*, 1997), and studies with *Mekk1* (–/–) cells show that JNK activation is reduced while apoptosis is increased in response to various stressful stimuli (Yujiri *et al.*, 1998; Minamino *et al.*, 1999). Two recent studies show that the protective effect of TGF-β against either c-Myc induced apoptosis (Mazars *et al.*, 2000) or survival signal withdrawal-induced death (Huang *et al.*, 2000) depends on SEK1 activity and c-Jun phosphorylation.

In addition to regulating cell death and survival, the JNK pathway may also have a role in other cellular processes, including proliferation, differentiation, development, and transformation. During embryonic development in *Drosophila,* D-JNK is required for dorsal closure (Riesgo-Escovar *et al.*, 1996). JNK is required for the proliferative response of BAF3 cells stimulated with IL-3 (Smith *et al.*, 1997). The JNK pathway may also have a role in differentiation since PC12 differentiation is associated with increased JNK activity (Eilers *et al.*, 1998) and overexpressed c-Jun promotes neurite outgrowth (Heasley *et al.*, 1996) as does active ASK1 (Takeda *et al.*, 2000). JNK has also been associated with tumorigenesis: transformation by Bcr/Abl activates JNK and requires c-Jun (Raitano *et al.*, 1995), while transformation by the Met oncogene requires JNK activation (Rodrigues *et al.*, 1997). In addition, c-Jun itself was initially associated with cell proliferation, differentiation, and transformation (for reviews, see section 1.8; Angel and Karin, 1991).

How can activation of the JNK pathway lead to such diverse outcomes? Perhaps different JNK isoforms are responsive to different signals from the various upstream kinases. To date, SEK1 (MKK4) and MKK7 are capable of activating JNK, and there are a plethora of kinases upstream of these JNKKs. Scaffold proteins could be utilised to bring together particular upstream kinases and different JNK isoforms. The first JNK scaffold protein to be identified was JIP-1 (Dickens *et al.*, 1997). This protein functions by specifically binding MLK3, MKK7, and JNK (Whitmarsh *et al.*, 1998) and thereby facilitating signal transduction by the bound proteins. It is possible that other scaffold proteins contribute to the activation of JNK mediated by other upstream kinases. Indeed, the recently identified JSAP1 preferentially binds MEKK1, SEK1, and JNK3 (Ito *et al.*, 1999). The biological response to activation of the JNK

pathway may be cell-type specific, such that the outcome of JNK activation might depend on which other signalling pathways are active within the cell, since these would determine what c-Jun binding partners are present. For example, activation of JNK and subsequent c-Jun phosphorylation would lead to the expression of a different set of c-Jun target genes depending on whether ATF-2 or c-Fos is present in the nucleus, since heterodimers of c-Jun/ATF-2 have different recognition sites to those of c-Jun/c-Fos. Other factors that could determine the outcome are the duration and magnitude of JNK activation. Studies with the homologous protein MAPK have shown that transient versus sustained activation of MAPK leads to the expression of different subsets of AP-1 proteins (Cook *et al.*, 1999).

In conclusion, I have shown that stimulation of the JNK pathway and subsequent phosphorylation of c-Jun promotes apoptosis in sympathetic neurons and overcomes the survival effect of NGF. These effects can be completely blocked by co-expression of dominant negative SEK1. However, mutated SEK1 did not prevent apoptosis after NGF withdrawal. Since increases in c-Jun expression and phosphorylation still occurred when SEK*AL* was expressed, it seems likely that there are other kinases which can activate JNK during the death pathway in sympathetic neurons. Recently other such potential kinases have been identified, and currently a hypothetical pathway for cell death in response to NGF withdrawal from sympathetic neurons is Cdc42 $\Rightarrow$ ASK1 $\Rightarrow$? $\Rightarrow$ JNK $\Rightarrow$ c-Jun. Having examined the regulation of c-Jun by its upstream kinase activators, it would be interesting to investigate what lies downstream of c-Jun. Several possible target genes of c-Jun have been identified, and AP-1 sites are fairly common promoter elements. However, there is, as yet, no compelling evidence for targets of c-Jun transactivation during cell death. To enable easier identification of possible targets by biochemical or PCR-based techniques, it was essential to develop a new gene delivery system for sympathetic neurons due to the difficulties and limitations of single-cell microinjection experiments. In the next chapter I will describe the characterisation of a recombinant adenoviral transduction system, and following that the use of the viruses to identify targets of c-Jun transactivation.

Chapter 5: Construction and characterisation of recombinant adenoviruses

5.1 Introduction

The expression of foreign genes in neurons has proved to be a difficult task because these cells are not readily transfectable and also because conventional retroviral vectors, which are commonly used for gene transfer, cannot integrate into the genome of postmitotic neurons. Microinjection of expression vectors into the nucleus has been a successful method for introducing DNA into neurons, but this is a difficult and time-consuming process, and studies must be done at the single cell level, preventing the analysis of populations of neurons. Studies such as those in the previous two chapters demonstrate how microinjection can be used to introduce reporter genes or expression plasmids into single neurons, but to perform experiments on neuronal populations a method of easily transducing many more cells is required. Since the first use of replication-defective recombinant adenoviruses to express foreign genes in neurons, published by Le Gal La Salle *et al.* in 1993, and with subsequent corroboration by several other groups (Akli *et al.*, 1993; Bajocchi *et al.*, 1993; Davidson *et al.*, 1993) these vectors have proved to be very useful tools for neurobiological studies.

Research on the identification of the common cold virus led to the isolation of a cytopathic agent from human adenoids (Rowe *et al.*, 1953). Over 100 different serotypes have been isolated from a number of different species (both mammalian and fowl), all of which infect the ocular, respiratory, or gastro-intestinal tract (Graham and Prevec, 1991). However, adenoviruses are also able to infect a broad range of potential host cells, including both replicating and terminally differentiated cells, and hence they have gained popularity as gene transfer agents (for reviews, see Berkner, 1988; Trapnell and Gorziglia, 1994). In comparison with other viral vectors, adenoviruses have many advantages: foreign genes inserted into the viral genome are usually maintained, the virus replicates efficiently in permissive cells, and since it is not a lytic virus it remains concentrated within the cell making collection and purification easier. In addition, there is low pathogenicity in humans, and no insertional mutagenesis (the adenovirus DNA remains epichromosomal). Adenoviruses have never been associated with any type of tumour in humans. The first report of adenovirus infection of neurons

suggested that the majority of cultured sympathetic neurons could be infected with no apparent toxic effects or morphological changes (Le Gal La Salle *et al.*, 1993). Thus, to further the studies on sympathetic neurons and allow the examination of whole populations of neurons, an adenoviral gene delivery system was developed.

The adenoviral genome consists of linear, double-stranded DNA and is 36 kb long. The virion is an icosahedron of 70 nm diameter, and consists exclusively of DNA and protein. The genome contains four distinct early regions (E1 to E4) and a major late region which encode most of the viral structural components such as the capsid proteins (for review of the adenovirus genome structure, see Berkner, 1988). The E1 region codes for immediate early genes that are necessary for viral replication and that switch on the transcription of the other viral genes. In wild-type adenoviruses, the E1 genes are actively transcribed immediately upon entry of the viral genome into the nucleus due to the activity of ubiquitous cellular transcription factors. E1 products then activate the rest of the viral genetic program, infectious viral progeny are produced, and eventually the cell dies and there is release of new virus. Thus, to make recombinant adenoviruses the E1 region is replaced to prevent activation of major late gene expression and the production of more infectious viruses. This renders the adenovirus replication-defective, and viruses must therefore be grown in a permissive cell line which can *trans*-complement the E1 deficit. Human embryonic kidney (HEK) 293 cells are used, since this cell line is transformed with the left hand 11% of the adenovirus genome and expresses the E1 proteins (Graham *et al.*, 1977). The E2 region encodes proteins that are directly involved in viral DNA replication. E3 is the region responsible for evasion of the host immune response (by inhibition of the expression of class I major histocompatibility antigens on the surface of infected cells; Ginsberg *et al.*, 1989). The E3 region can be deleted when adenoviruses are used to infect cultured cells, since the host's immune response does not have to be considered. E4 proteins have multiple functions, including the control of viral transcription and replication, and the shutting off of host cell protein expression. With deletions in the E1 and E3 regions, and the fact that adenovirus can package 105% of its normal genome size, approximately 8 kb of foreign DNA can be inserted into the viral genome (Bett *et al.*, 1994). This is sufficient for the expression cassettes needed for the following studies.

The results shown in the previous two chapters have suggested the mechanism by which the *c-jun* promoter is regulated upon NGF withdrawal in sympathetic neurons, and have demonstrated how c-Jun and apoptosis are regulated by upstream

kinases. To further understand the events that occur during the death of sympathetic neurons, it would be interesting to investigate how c-Jun is connected to other putative apoptotic genes. To screen for genes that might be targets of c-Jun transactivation, an adenoviral vector could be used to express dominant negative c-Jun in a population of neurons for subsequent analysis of transcript expression. RT-PCR and immunoblotting could then be performed to identify which mRNA or protein species are upregulated by NGF withdrawal, and then to show which of these are repressed by the c-Jun mutant. It would also be useful to construct a MEKK1-expressing adenovirus so that it is possible to strongly activate the c-Jun signal transduction pathway.

The construction and purification of the recombinant adenoviruses was carried out in collaboration with Dr. Freda Miller's group at the Montreal Neurological Institute, Canada. Adenovirus serotype 5 has been the most extensively studied, and it is the type 5 viral DNA backbone that is used to produce most vectors. To begin the construction of a replication-defective recombinant adenovirus, the cDNA of interest is cloned into an expression cassette containing a promoter and polyadenylation signals, within a shuttle vector. This vector also contains sufficient adenoviral DNA for efficient homologous recombination to occur with the long-arm adenoviral DNA when both are transfected into 293 cells (Figure 5.1). Recombination generates viral DNA containing the expression cassette, and this is then replicated and packaged, and the infectious viral progeny are released. After several rounds of infection and release a plaque of infected cells is formed, and virus can be harvested and purified from the dead cells within this plaque. This chapter describes the preparation and characterisation of replication-defective recombinant adenoviruses encoding dominant negative c-Jun, activated MEKK1, Bcl-2, and β-galactosidase.

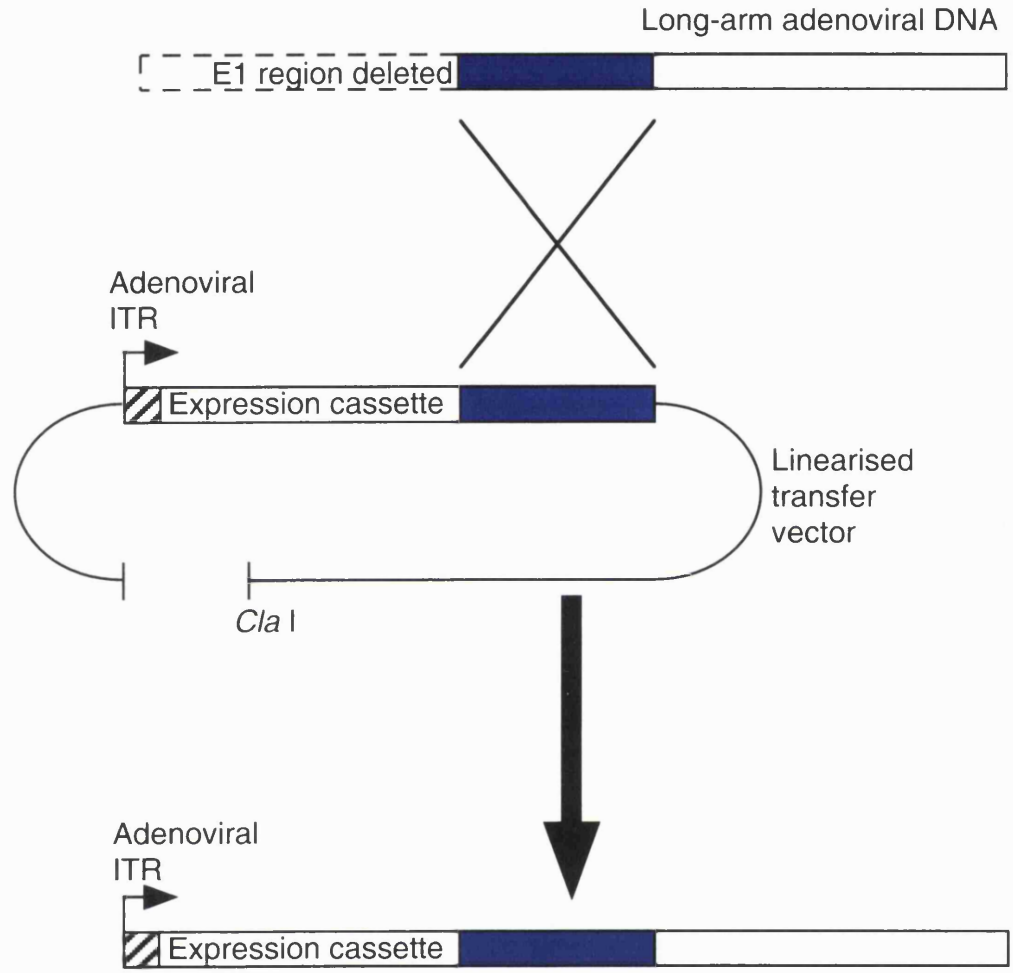
5.2 Results

5.2.1 Construction, amplification, and purification of recombinant adenoviruses

The first step in the construction of a new recombinant adenovirus is to clone the cDNA of interest into a shuttle vector (in this case, pAd-CMVpolyA, see Figure 5.2B). This vector contains the gene for ampicillin resistance and an origin of replication for amplification in bacteria, plus the strong cytomegalovirus (CMV)

Figure 5.1 Homologous recombination to generate a replication-defective recombinant adenovirus

HEK 293 cells were co-transfected with linearised adenoviral DNA which had been digested to remove the E1 and E3 regions, and with linearised pAd-CMVpolyA transfer vector. The transfer vector contains an expression cassette with the CMV enhancer and promoter, the coding sequence for the gene of interest, and SV40 polyadenylation and splice sequences downstream. The expression cassette is flanked by adenoviral DNA such that homologous recombination with the digested long-arm adenoviral DNA will generate a recombinant adenovirus containing the expression cassette in place of the E1 region, and with the inverted terminal repeat (viral origin of replication) and packaging signals restored.



Adenoviral DNA common to long-arm DNA and transfer vector

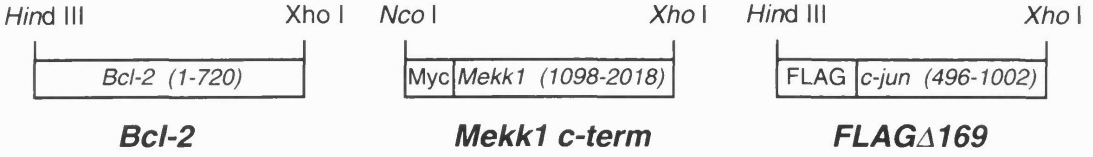
Adenoviral origin of replication and packaging signals

Figure 5.2 Structure of the cDNA inserts and the shuttle vector

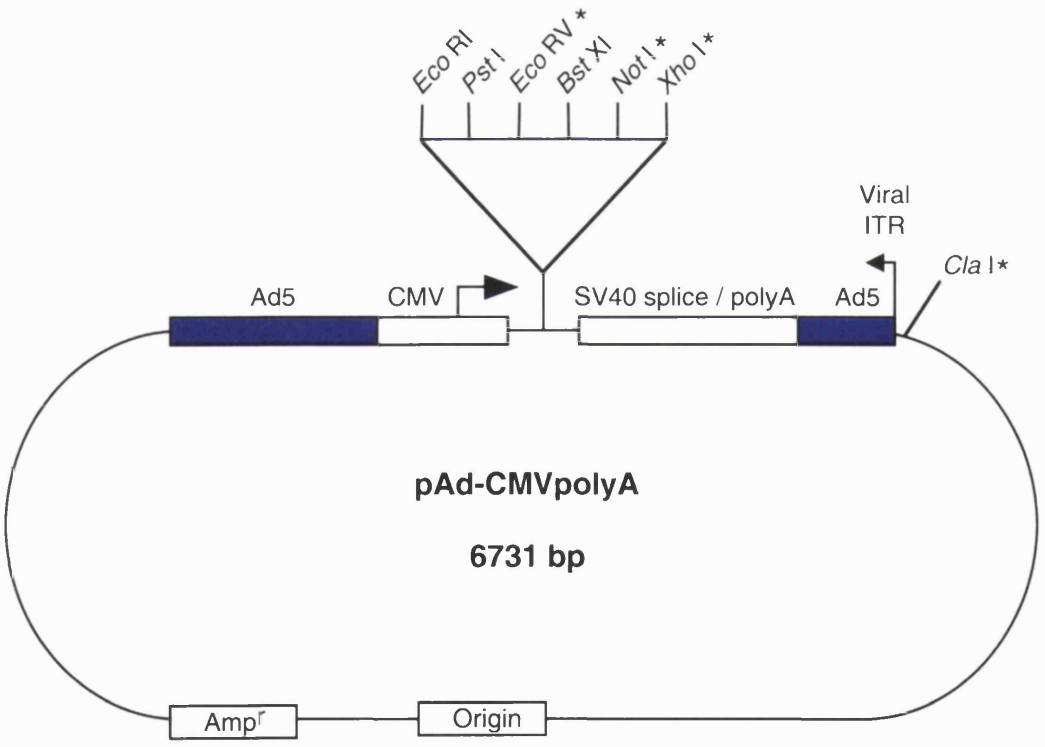
(A) The restriction enzymes used to clone each cDNA into the shuttle vector are shown. The 5' site was blunt-ended using Klenow polymerase and the cDNAs then inserted into the *Eco* RV/*Xho* I site in the transfer vector. The full *Bcl-2* coding sequence was inserted, while the truncated form of *Mekk1* encodes the C-terminal kinase domain and is tagged with the Myc epitope. FLAGΔ169 is FLAG epitope-tagged, and contains murine *c-jun* sequences from nucleotide 496 to 1002.

(B) Structure of the shuttle vector, pAd-CMVpolyA. This vector contains a bacterial origin of replication and the ampicillin resistance gene for amplification of the plasmid in *E. coli.* The expression cassette consists of the CMV promoter upstream of a multiple cloning site, and then polyadenylation and splice signals. In addition, the vector contains the adenoviral origin of replication and packaging signals, and serotype 5 DNA from map units 9.4 to 15.5 to enable homologous recombination with the linearised viral genome. *Cla* I is used to linearise the vector prior to co-transfection into 293 cells.

A


Hind III
Xho I
Bcl-2 (1-720)
Bcl-2
Nco I
Xho I
Myc
Mekk1 (1098-2018)
Mekk1 c-term
Hind III
Xho I
FLAG
c-jun (496-1002)
FLAGΔ169

B


Eco RI
Pst I
Eco RV *
Bst XI
Not I *
Xho I*
Viral
ITR
Cla I*
Ad5
CMV
SV40 splice / polyA
Ad5
pAd-CMVpolyA
6731 bp
Amp^r
Origin

enhancer and promoter, a multiple cloning site, and SV40 polyadenylation and splice signals. The vector also contains the inverted terminal repeat (viral origin of replication) and sufficient adenoviral DNA for efficient homologous recombination to occur with the adenovirus genome. The sequences for FLAGΔ169, MEKK1, and Bcl-2 were cloned into this shuttle vector by standard techniques using the restriction enzymes shown in Figure 5.2A. The vector was then linearised and co-transfected into HEK 293 cells along with adenoviral DNA from which the E1 region had been deleted (Figure 5.1). The E1 region is necessary for the initiation of transcription of the rest of the adenovirus genes and also contains the viral inverted terminal repeat and packaging signals which are critical for viral viability. Homologous recombination between the transfected DNAs leads to insertion of the expression cassette into the viral DNA in place of the E1 region, and restores the inverted terminal repeat to the viral genome. This recombinant DNA can then be packaged within the 293 cells, which supply the E1 protein in *trans*, to generate a recombinant adenovirus. Released viruses infect nearby cells and a plaque of infected cells slowly appears. After a first stage of screening to confirm the presence of recombinant adenovirus in these infected cells, a second round of screening was performed to isolate a pure recombinant virus.

Amplification steps were then performed to obtain stocks with a high titre of viral particles. This involved a sequential increase in cell culture size and several cycles of infection, culminating in the infection of 3 x 10^8 293 cells in 30 x 175 cm^2 flasks. After harvesting the cells, three cycles of freezing followed by complete thawing were performed to release the viral particles, and the virus was finally purified on a discontinuous CsCl gradient and desalting column.

A virus that expresses β-galactosidase (QBI-AdenoLacZ) was purchased from Quantum Biotechnologies. Expression of *E. coli* β-gal is driven from the strong CMV promoter, and is in the context of adenovirus serotype 5 DNA as for the other viruses used. This virus was used to infect 293 cells, and a plaque isolated and then amplified as above so that all viruses were purified by the same method.

To determine the concentration of infectious viral particles, two types of assay were carried out. The plaque assay is more traditional and is similar to the method for screening for the presence of recombinant viruses (Graham and Prevec, 1991). A serial dilution of the virus was made and added to monolayers of 293 cells. The number of plaques formed for a given dilution was scored after 21 days, and this gives a measure of the number of infectious viral particles per ml (termed plaque forming units). In

addition, an end-point cytopathic effect (CPE) assay was performed, and the Tissue Culture Infectious $Dose_{50}$ ($TCID_{50}$) calculated (Precious and Russell, 1985; Kanegae *et al.*, 1994). This assay is reported to be more sensitive, less labour intensive, and subject to less variability than plaque assays (Mittereder *et al.*, 1996; Nyberg-Hoffman *et al.*, 1997). In this case a serial dilution of the virus was made as before, but infection was carried out in duplicate in a 96-well plate. Ten wells were used for each dilution, and the number of wells that showed cytopathic effects (rounding up of the cells, and detachment from the dish) was scored after 10 days. Figure 5.3 shows the results from these assays. The average titres from both assays (and with other viral preparations also, data not shown) were reasonably similar, so that one $TCID_{50}$/ml corresponds to one plaque forming unit/ml, as previously reported (Kanegae *et al.*, 1994). Note that the titre of the MEKK1 virus was 5-fold higher in the CPE assay, and this result was confirmed by repeating this assay. However, the CPE assay was easier to score reliably, quicker to perform, and there was less variability between different CPE assays for most of the viruses that were tested (data not shown). Hence, the value obtained from the CPE assay was used in all cases.

5.2.2 Recombinant adenoviruses infect sympathetic neurons

Experiments were performed to verify that the adenovirally-encoded proteins were expressed, and correctly localised within the cells. It was important to determine the optimum viral titre to add to the cells to achieve a high level of infection without causing any morphological changes. Sympathetic neurons were cultured for 3 to 5 days and then infected with recombinant adenoviruses by adding an appropriate volume of viral solution to the cells. The cells were plated on coverslips at a similar density to that used for microinjection (5000 cells per coverslip). The multiplicity of infection (MOI) is a measure of the number of infectious viral particles (or plaque forming units) added per cell. Viruses were added at MOIs of 20, 100, and 500, and the neurons were then incubated for 24 hours to allow infection, at which time they were refed with regular medium. At this time there was very little expression of the recombinant proteins (data not shown). After a further 24 hours to allow time for protein expression, the cells were fixed and stained with antibodies that recognised the recombinant proteins, i.e. anti-β-gal, anti-Bcl-2, M2 anti-FLAG, 9E10 anti-Myc. The number of cells that expressed the protein encoded by the adenovirus was scored. Figure 5.4 shows typical immunofluorescence pictures of neurons infected at an MOI of 500 along with the

Figure 5.3 Determination of the concentration of purified viral stocks

(A) Plaque assays were performed to estimate the number of infectious viral particles in the viral suspensions. Serial dilutions of the virus were added to 60 mm dishes containing monolayers of 293 cells. After adsorption for 90 minutes, the monolayer was overlaid with 1.25% Seaplaque agarose medium and incubated for 21 days. The number of plaques for each viral dilution was scored, and the table shows the results from four independent assays. The titres are expressed in plaque forming units per ml (pfu/ml).

(B) Cytopathic effect assays were also performed to estimate viral titre. Serial dilutions from 10^{-5} to 10^{-12} of viral suspension were added to 10 wells each of a 96 well plate. Two wells per row were uninfected controls. After 10 days, those wells showing cytopathic effects were scored, and the 50% Tissue Culture Infectious Dose was calculated. A $TCID_{50}$ of 1 approximately equals 1 pfu/ml. Note that different preparations of the β-galactosidase virus were used in the assays shown in (A) and (B).

A

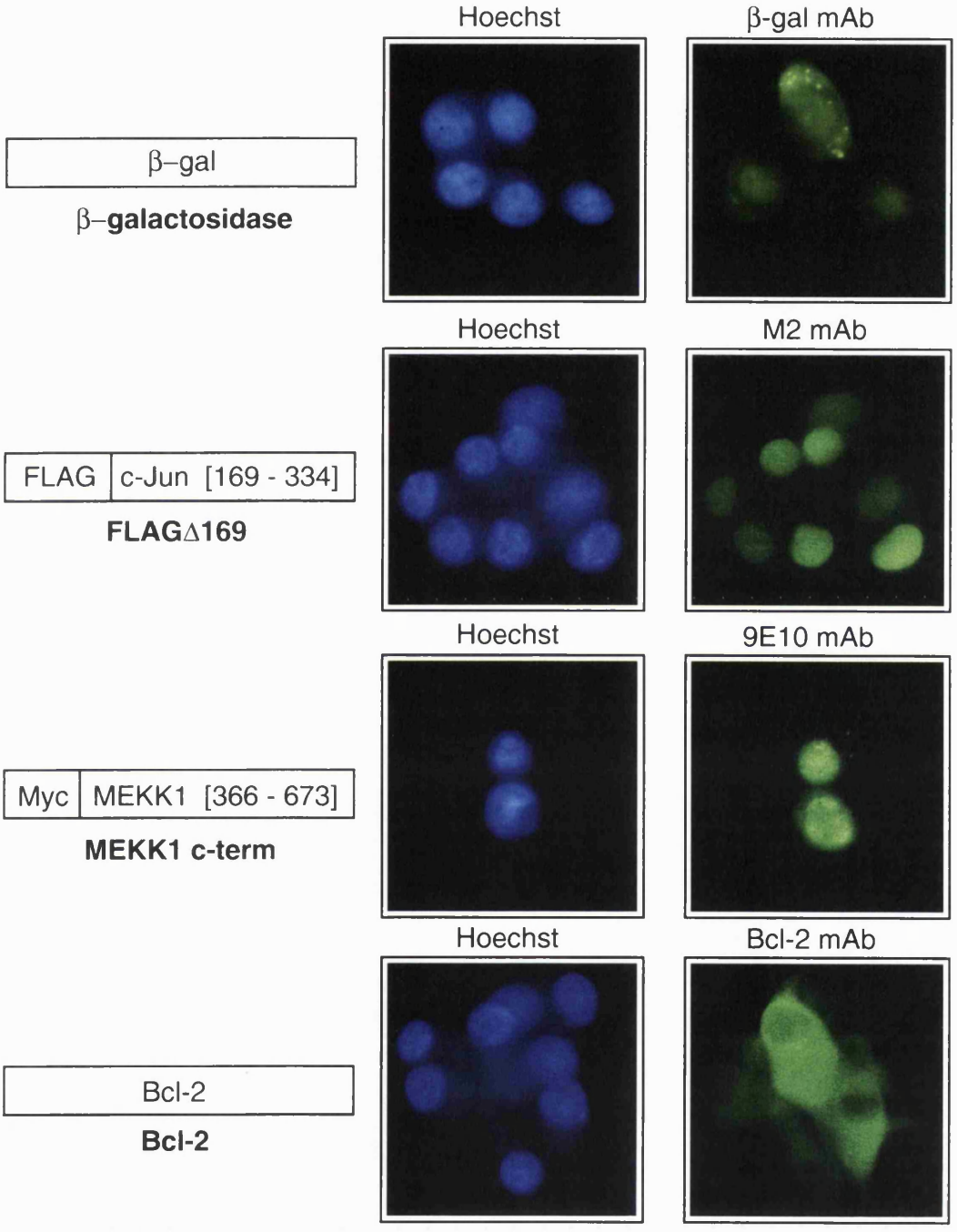
Virus	Plaque assay ($x10^8$)				Average
FLAGΔ169	0.8	2.5	2.9	1.9	**2.0×10^8**
Bcl-2	1.6	5.6	19	6.0	**8.0×10^8**
β-gal	ND	7.4	11	3.0	**7.1×10^8**
MEKK1	3.3	4.8	10	11	**7.3×10^8**

B

Virus	$TCID_{50}$ ($x10^8$)		Average
FLAGΔ169	3.2	4.4	**3.8×10^8**
Bcl-2	6.3	9.0	**7.7×10^8**
β-gal	160	150	**1.6×10^{10}**
MEKK1	35	32	**3.4×10^9**

Figure 5.4 Recombinant adenoviruses efficiently infect sympathetic neurons

Sympathetic neurons were maintained *in vitro* for 3 to 5 days, and then were infected with recombinant adenoviruses containing the coding sequences for FLAGΔ169, Bcl-2, a constitutively active form of MEKK1, or β-galactosidase. The amount of virus added was titrated from 20 to 500 viral particles per cell (a measure called the multiplicity of infection, MOI). Neurons were plated at a density of 5000 cells per coverslip. 24 hours after adding the viruses, the cells were refed with regular medium, and incubated for a further 24 hours, at which time they were fixed and stained with the appropriate monoclonal antibody (M2 anti-FLAG for FLAGΔ169, 9E10 anti-Myc for Myc-epitope tagged MEKK1, anti-Bcl-2, or anti-β-gal), and with Hoechst dye to stain the nuclear DNA. FLAGΔ169 was detected exclusively in the nucleus, whereas Bcl-2 was cytoplasmic. MEKK1 was detected throughout the cell, and the two cells shown had shrunken cell bodies and pyknotic nuclei. β-galactosidase was seen throughout the cell, sometimes in a punctate pattern. The structures of the recombinant proteins are shown, along with typical immunofluorescence pictures of infected neurons.


Hoechst
β-gal mAb
β–gal
β–galactosidase
Hoechst
M2 mAb
FLAG
c-Jun [169 - 334]
FLAGΔ169
Hoechst
9E10 mAb
Myc
MEKK1 [366 - 673]
MEKK1 c-term
Hoechst
Bcl-2 mAb
Bcl-2
Bcl-2

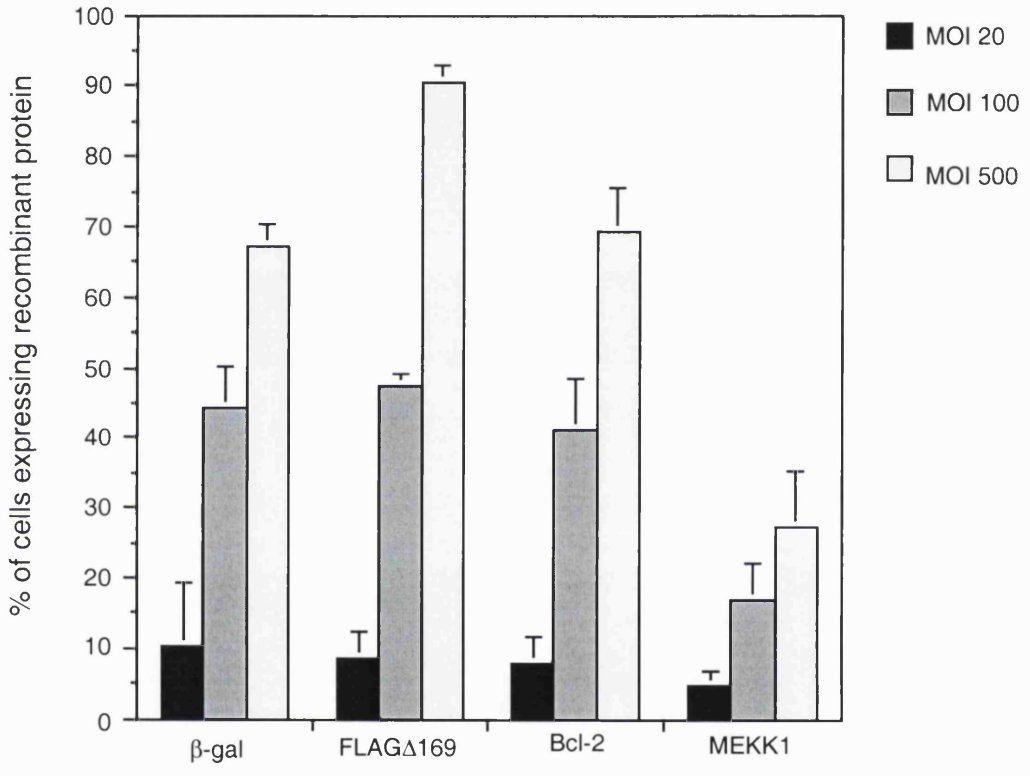
structures of the recombinant proteins. c-Jun is a nuclear protein, and FLAGΔ169 was detected exclusively in the nucleus, showing that the recombinant protein is being made after infection by the adenovirus and that it has the nuclear localisation signal and FLAG epitope tag. Bcl-2, on the other hand, is a cytoplasmic protein, and this was clearly expressed throughout the cell but excluded from the nucleus. MEKK1 and β-galactosidase were seen throughout the cell. The data shown in Figure 5.5 shows the percentage of infected cells that expressed the recombinant protein for each adenovirus. At an MOI of 20, less than 10% of the neurons expressed recombinant protein in all cases. At an MOI of 100, approximately 40-50% of the cells expressed FLAGΔ169, Bcl-2, or β-galactosidase. Expression of MEKK1 was restricted to fewer cells. However, I previously found in microinjection experiments that only about 50% of the injected cells expressed MEKK1 even when the plasmid was injected at a very high concentration (0.8 and 1.6 mg/ml, data not shown). At an MOI of 500, most of the neurons expressed recombinant protein (except for MEKK1-infected cells) and the staining was very bright. When neurons were infected at an MOI of 1000, most cells were very brightly stained, but it was clear that the nuclei were smaller (data not shown). This effect on morphology was not seen at lower viral titres.

5.2.3 Recombinant adenoviruses expressing FLAGΔ169 and Bcl-2 protect sympathetic neurons from apoptosis, whereas a MEKK1-expressing virus kills even in the presence of NGF

To further characterise the effect of the viruses, neurons were infected and then withdrawn from NGF to determine whether the Bcl-2 and dominant negative c-Jun recombinant adenoviruses could prevent apoptosis, as had previously been shown by microinjection experiments (Ham *et al.*, 1995). Sympathetic neurons were infected overnight with a viral titre of 100 viral particles per cell and then were refed with normal medium. After 24 hours, the cells were withdrawn from NGF for another 24 hours and then fixed and stained. Monoclonal antibodies specific for the recombinant proteins were used to identify infected cells and the nuclei were visualised by staining the DNA with Hoechst dye. The number of neurons with pyknotic nuclei was scored. Figure 5.6A shows the average result from 5 experiments. Approximately 50-60% of the nuclei were pyknotic after withdrawal of NGF for 24 hours when the cells were infected with β-galactosidase- or MEKK1-expressing adenoviruses. This is similar to the number of pyknotic nuclei seen in uninfected neurons. Fewer of the neurons

Figure 5.5 Relationship between the percentage of sympathetic neurons expressing recombinant protein and the multiplicity of infection

Neurons were infected, maintained in NGF-containing medium, and then fixed and stained as detailed in Figure 5.4. The amount of virus added was titrated from 20 to 500 viral particles per cell (a measure called the multiplicity of infection, MOI). The number of neurons that expressed recombinant protein was scored. The average of three experiments is shown plus the standard error. Approximately 200 neurons were counted per experiment. Increasing the MOI resulted in the infection of more neurons. A higher level of recombinant protein expression in each infected neuron was also evident (data not shown). No obvious effects on nuclear morphology were detected at these MOIs.


% of cells expressing recombinant protein
100
90
80
70
60
50
40
30
20
10
0
β-gal
FLAGΔ169
Bcl-2
MEKK1
MOI 20
MOI 100
MOI 500

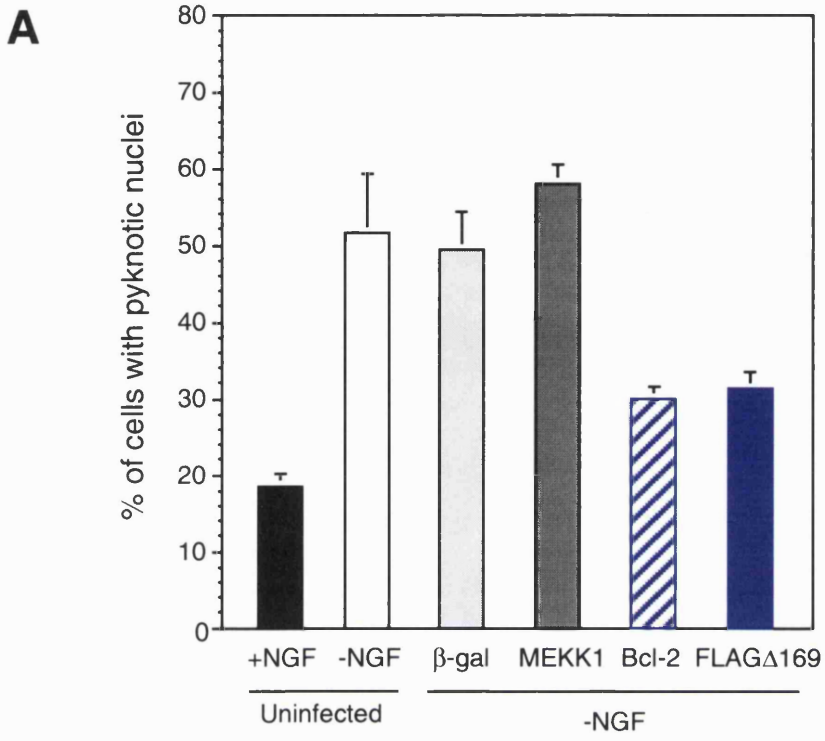
infected with either Bcl-2 or dominant negative c-Jun viruses had pyknotic nuclei (approximately 30%). In addition to counting all of the neurons, experiments were performed in which only the infected cells were scored, since approximately 50-60% of neurons are uninfected, at least as determined by immunofluorescence- it is, of course, possible that recombinant protein is present in these cells, but below the level of detection. In this case, protection was more significant (Figure 5.6B). Expression of FLAGΔ169 reduced the number of pyknotic nuclei from 53% for β-galactosidase-expressing cells to just 19%. Therefore more neurons were viable in the absence of survival factor when the cells were infected with recombinant adenoviruses expressing either Bcl-2 or dominant negative c-Jun. This confirms the result that had previously been demonstrated by microinjection of sympathetic neurons (Ham *et al.*, 1995). In an attempt to increase the survival effect of the adenoviruses by increasing the number of neurons infected and the level of expression of recombinant protein, cells were infected at MOIs of 250 and 500. However, no increase in survival was detected (data not shown). The reasons for this are unclear.

To further characterise the effect of the viruses and investigate any potential toxicity of adenoviral infection, neurons were infected in the presence of NGF with each of the recombinant adenoviruses. I was also interested to determine whether MEKK1 expression could induce to apoptosis as had previously been shown in microinjection experiments (Figure 4.1). Sympathetic neurons were infected overnight with an MOI of 100 viral particles per cell and then were refed with normal medium. The cells were maintained in NGF-containing medium for 48 hours to allow sufficient time for protein expression, and then were fixed and stained with monoclonal antibodies specific for the recombinant proteins. The nuclei were visualised by staining with Hoechst dye. The number of infected cells with pyknotic nuclei was scored. Figure 5.7 shows the average result from 5 experiments with the standard error. Between 10 and 20% of the nuclei were pyknotic when the cells were infected with FLAGΔ169, Bcl-2, or β-galactosidase. This is approximately equal to the background percentage of dying cells normally seen in the presence of NGF (19% of uninfected neurons had pyknotic nuclei, data not shown). When neurons were infected with MEKK1, 60% of the infected cells had pyknotic nuclei. Hence expression of MEKK1 by infection with adenoviruses can induce apoptosis and overcome the protective effect of NGF. Bcl-2, FLAGΔ169 and β-galactosidase do not appear to have toxic effects at this MOI. Thus, the adenovirus gene delivery system works as predicted.

Figure 5.6 Recombinant adenoviruses expressing FLAGΔ169 and Bcl-2 protect sympathetic neurons against NGF withdrawal-induced death

(A) Sympathetic neurons were maintained *in vitro* for 3 to 5 days, and then infected with recombinant adenoviruses containing expression cassettes coding for FLAGΔ169, Bcl-2, a constitutively active form of MEKK1, or β-galactosidase. Neurons were plated at a density of 5000 cells per coverslip. 24 hours after adding the viruses, the cells were refed with regular medium, and incubated for a further 24 hours. Neurons were then withdrawn from NGF for 24 hours, at which time they were fixed and stained with the appropriate monoclonal antibody and with Hoechst dye to visualise nuclear morphology. The number of neurons that had pyknotic nuclei out of the whole cell population was determined. The data represents the average of 5 experiments with the standard error.

(B) As above, but showing the percentage of infected neurons (i.e. those expressing detectable recombinant protein) that had pyknotic nuclei. The data represents the average of 5 experiments with the standard error.


A
% of cells with pyknotic nuclei
80
70
60
50
40
30
20
10
0
+NGF
-NGF
β-gal
MEKK1
Bcl-2
FLAGΔ169
Uninfected
-NGF

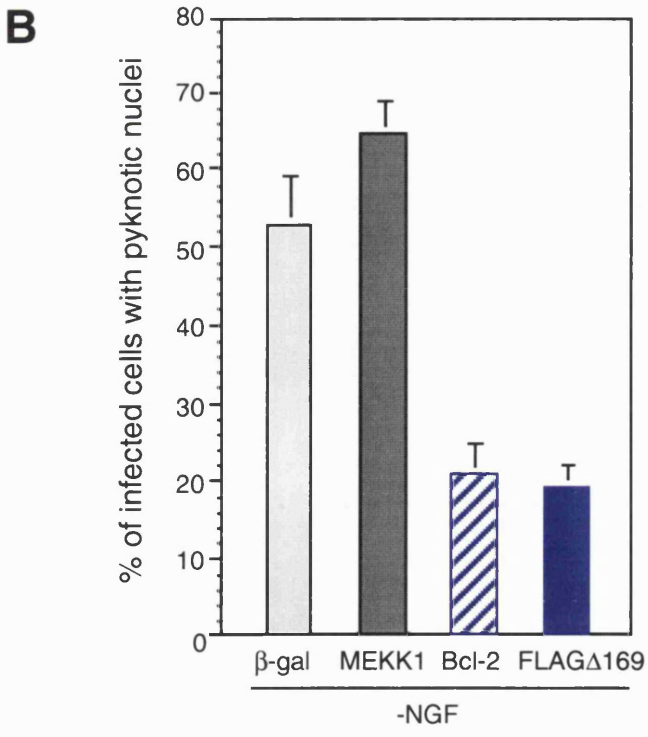
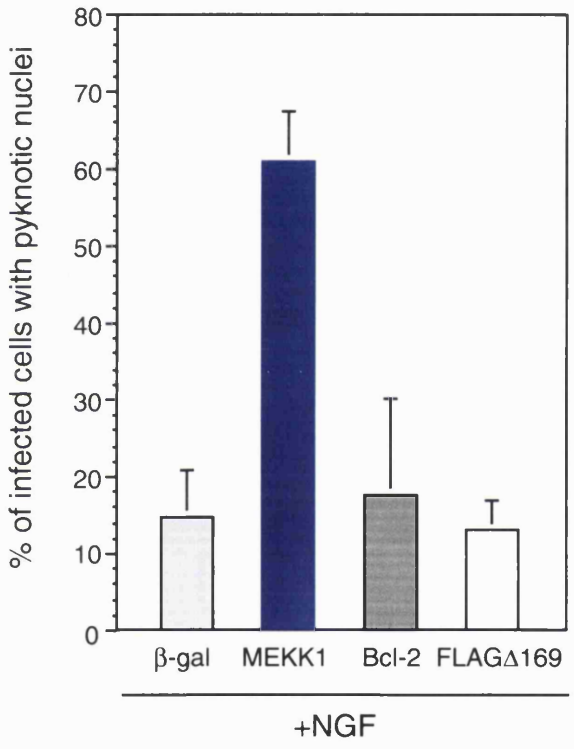

B
% of infected cells with pyknotic nuclei
80
70
60
50
40
30
20
10
0
β-gal
MEKK1
Bcl-2
FLAGΔ169
-NGF

Figure 5.7 A recombinant adenovirus expressing MEKK1 induces apoptosis in the presence of NGF

Sympathetic neurons were maintained *in vitro* for 3 to 5 days, and then infected with recombinant adenoviruses containing expression cassettes coding for FLAGΔ169, Bcl-2, a constitutively active form of MEKK1, or β-galactosidase. Neurons were plated at a density of 5000 cells per coverslip. 24 hours after adding the viruses, the cells were refed with regular medium, and were incubated for a further 48 hours in the presence of NGF, at which time they were fixed and stained with the appropriate monoclonal antibody and with Hoechst dye to visualise nuclear morphology. The number of infected neurons with pyknotic nuclei was determined. The data represents the average of four experiments with the standard error.


% of infected cells with pyknotic nuclei
80
70
60
50
40
30
20
10
0
β-gal
MEKK1
Bcl-2
FLAGΔ169
+NGF

5.3 Discussion

I have described the construction, purification, and characterisation of replication-defective recombinant adenoviruses that express dominant negative c-Jun, activated MEKK1, Bcl-2, and β-galactosidase. While adenoviral-mediated expression of FLAGΔ169 and Bcl-2 promotes the survival of neurons in the absence of NGF, MEKK1 does not and can even overcome the protective effect of NGF and induce apoptosis. These results confirm previous studies in which expression plasmids were microinjected into sympathetic neurons, and demonstrate that the adenoviral gene delivery system functions predictably.

One issue with the use of adenoviruses is how to quantitate the number of viral particles within a suspension. It is possible to measure the optical density of a viral suspension and therefore calculate the concentration of viral particles. This is a very accurate measure, but does not distinguish between infectious and defective viral particles. It is interesting to determine the relative number of defective viral particles in a suspension, particularly for the comparison of results between different laboratories. However, for infections, it is crucial to know the number of infectious viral particles. The plaque forming unit (PFU) has been the standard way of expressing the titre of viral stocks, relying on the formation of plaques in a monolayer of 293 cells. However, to obtain a plaque requires many cycles of infection, production of new adenoviruses and re-infection of neighbouring cells, and this takes three weeks before the results can be scored. Generally, there is poor reproduction between laboratories, and even within the same laboratory (Nyberg-Hoffman *et al.*, 1997). Indeed, I found some variation between different experiments (Figure 5.3A; data not shown). The cytopathic effect assay has been used to determine the titre of many other types of viruses but has only recently been applied to adenoviruses. In this case, dilutions of the virus are made in 96-well plates and the presence of cytopathic effects are scored after just 10 days. This method is quicker to perform, and is reported to be more consistent between assays (Nyberg-Hoffman *et al.*, 1997). I have observed that in the assays shown here and with the results from other viruses tested, 1 PFU approximately equals 1 $TCID_{50}$, as previously reported (Kanegae *et al.*, 1994). Assays, and hence the actual experiments, involving viral infection give variable results between laboratories and this is most commonly due to the way the virus is presented to the cells. The volume of virus used, the type of infection vessel, the incubation time, the number of cells, and the type of

medium can all influence the results. Studies of these parameters have suggested simple improvements to infection conditions: the minimum volume of medium for infection should be used, the cells should be continuously rocked during infection, the cells can be centrifuged for 90 minutes at 1000 x g after addition of the virus, and sufficiently long infection times should be employed (Mittereder *et al.*, 1996; Nyberg-Hoffman *et al.*, 1997). These conditions serve to increase the chance of a virus particle contacting a cell, since this relies entirely on Brownian motion within the medium. While some of these modifications were employed in the present study, for future studies it would be useful to investigate the effect of mechanical rocking on infection efficiency in sympathetic neurons. The use of 10% glycerol in the viral storage buffer increases stability of the virion, as does using 10 mM Hepes/1mM EDTA instead of PBS (Kanegae *et al.*, 1994) and so this modification should also be used for future viral preparations. In addition, alterations to the method of purification of neurons so that fewer contaminating cells are present in the cultures might increase infection efficiency, since I have observed in immunocytochemistry experiments that non-neuronal cells express recombinant protein and are therefore infected (data not shown).

Further to these methodological advances in the use of adenoviruses, new systems for the generation of recombinant adenoviruses have been developed. Previously, the construction of one virus was a lengthy process (approximately two to four months) and this limited the number of different viruses that could be made. Modifications such as performing the homologous recombination in *E. coli* rather than in 293 cells, and incorporating the gene for green fluorescent protein into the viral backbone, can decrease the time required to make a virus and allow viral production and infection to be easily monitored (He *et al.*, 1998).

It is important to note that at the MOI used in the above experiments, only 40-50% of the neurons expressed detectable recombinant protein (as determined by immunocytochemistry). Therefore, it might be expected that if more neurons could be infected an increased effect on cell survival would be observed. I therefore infected neurons with more viral particles per cell which increases the proportion of neurons transduced in the population and also the level of expression of recombinant protein per neuron (Figure 5.5). Adenoviruses can be used at MOIs in excess of 1000 in some cell lines without any toxic effects (Davidson and Hassell, 1987). However, no greater effects on the survival of neurons was seen at MOIs of 250 or 500 (data not shown). It may be that the addition of too many viral particles has non-specific effects. It has

previously been noted that sympathetic neurons do not tolerate MOIs greater than 100-200 without deleterious effects, such as a large decrease in total protein synthesis (Paquet *et al.*, 1996). Infection with recombinant adenoviruses at an MOI of greater than 1000 proved to be cytotoxic in other studies, including the infection of neurons from the dorsal root ganglion (Durham *et al.*, 1996). This could be due to direct toxicity of the capsid proteins that occurs during the uncoating of the virus (Caillaud *et al.*, 1993), or "leaky" expression of viral late genes such as those encoding capsid proteins (Durham *et al.*, 1996). Moreover, at high MOIs the E1 region becomes dispensable for replication and hence viral progeny could be produced (Jones and Shenk, 1979). The potential host immune response is, of course, a major concern *in vivo*, but does not apply to adenovirally-infected cells in culture. Cytotoxicity has also been associated with the expression of the transgene. Even when β-galactosidase was expressed in dorsal root ganglion neurons, toxicity was observed after one week in culture at MOIs of 100 and greater (Durham *et al.*, 1996). However, it would not be expected that this cytotoxicity would affect the experiments described in this chapter, since a relatively short time scale was used. Longer term studies *in vitro* and particularly *in vivo* would certainly need to address these issues though. To that end, second generation adenovirus vectors are being developed that contain either lethal mutations or deletions in regions additional to the E1 and E3 regions which are deleted in the first generation viruses. A promising example of this is the E1/E4 deleted virus. The E4 region is required for many events occurring at the onset of the late phase of the viral life cycle (Halbert *et al.*, 1985) and hence an E4-deleted adenovirus requires a cell line capable of complementing this lethal deletion. An E1/E4 deleted adenovirus should offer significant advantages towards eliminating cytopathic effects. Either of the E4-ORF3 or E4-ORF6 proteins were necessary and sufficient for the normal viral lytic cycle in the absence of the E4 region (Ketner *et al.*, 1989). After construction of a 293-ORF6 cell line, (Wang *et al.*, 1997) constructed an E1/E4-deficient adenovirus and demonstrated high-level persistent transgene expression *in vivo* for at least 6 months in immunocompetent mice, in the absence of cytopathic effects and with a significant reduction of the host immune response. The ultimate aim would be to produce "pseudo-adenovirus" or "gutless" vectors containing only the essential adenovirus packaging signals and the transgene, using a permissive cell line that could provide all of the complementing functions.

Having prepared and characterised the adenoviral gene transduction system, the next chapter will describe the infection of sympathetic neurons with dominant negative c-Jun to investigate where c-Jun functions in the death pathway, and what might be the targets of c-Jun transactivation.

Chapter 6: The function of c-Jun in neuronal apoptosis

6.1 Introduction

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 pro-caspase 9 (P. Li *et al.*, 1997). Caspase-9 then activates caspase-3 and other effector caspases. Cytochrome c added to cytosol can trigger apoptosis in cell-free apoptosis systems (X. Liu *et al.*, 1996; Kluck *et al.*, 1997), and microinjection of cytochrome c into the cytosol of living cells induces apoptosis (F. Li *et al.*, 1997). In sympathetic neurons, it has been shown that microinjection of an anti-cytochrome c antibody protects the cells from apoptosis induced by NGF withdrawal (Neame *et al.*, 1998).

Bcl-2 family proteins are important regulators of cytochrome c release (Reed *et al.*, 1998; Antonsson and Martinou, 2000). Pro-apoptotic members of this family, such as Bax and Bak, which share BH1, BH2, and BH3 domains with the anti-apoptotic members Bcl-2 and Bcl-x_L, cause the release of cytochrome c from isolated mitochondria (Jurgensmeier *et al.*, 1998; Narita *et al.*, 1998). Other members of the family, such as Bad, Bim and Bid, which share only the BH3 domain, may act by dimerising with and blocking the action of Bcl-2 or Bcl-x_L (Yang *et al.*, 1995; O'Connor *et al.*, 1998), or by acting directly on proteins such as Bax in the mitochondrial membrane (K. Wang *et al.*, 1996). Bcl-2 and Bcl-x_L can block the release of cytochrome c from mitochondria (X. Liu *et al.*, 1996; Kluck *et al.*, 1997; J. Yang *et al.*, 1997) and both proteins can inhibit the mitochondrial changes induced by Bax and Bak *in vitro* (Jurgensmeier *et al.*, 1998; Narita *et al.*, 1998).

How are the pro-apoptotic Bcl-2 family members regulated? Several different mechanisms have been described so far. Bid is activated by caspase-8-mediated cleavage (Li *et al.*, 1998; X. Luo *et al.*, 1998). Another pro-apoptotic Bcl-2 family member, DP5/Hrk, appears to be transcriptionally upregulated during apoptosis (Imaizumi *et al.*, 1997). Bax may also be regulated transcriptionally, since *bax* mRNA is induced in some cell types in response to p53 (Miyashita and Reed, 1995), or by a translocation event, since cytoplasmic Bax translocates to the mitochondria during

apoptosis (Wolter *et al.*, 1997; Gross *et al.*, 1998). Bax is known to be essential for the death of sympathetic neurons (Deckwerth *et al.*, 1996). Bad is phosphorylated and inactivated by Akt (Datta *et al.*, 1997), and overexpression of Akt blocks cell death after NGF deprivation in sympathetic neurons suggesting that Bad may be an important regulator of survival in these cells (Dudek *et al.*, 1997; Philpott *et al.*, 1997).

Studies investigating the relationship between the different events that occur during sympathetic neuron apoptosis have shown that Bcl-2 family proteins and caspases function downstream of c-Jun. Overexpression of Bcl-2 does not block the increase in c-Jun protein levels that occurs after NGF withdrawal (Ham *et al.*, 1995), while in sympathetic neurons cultured from *bax* (–/–) mice, increases in *c-jun* mRNA and phosphorylation of c-Jun protein still occur even though the neurons do not die after NGF withdrawal (Deckwerth *et al.*, 1998). Both the increase in JNK activity/*c-jun* mRNA levels and the functions of the Bcl-2 family appear to occur upstream of the caspases. BAF, a caspase inhibitor, prevents the death induced by NGF withdrawal in sympathetic neurons but does not prevent the increase in *c-jun* mRNA (Deshmukh *et al.*, 1996). It has not yet been demonstrated how c-Jun acts in the death pathway in sympathetic neurons or what death genes might be targets of c-Jun transactivation. Recent evidence suggests that the *fas ligand* gene, which contains an AP-1 site within its promoter (Faris *et al.*, 1998b; Kasibhatla *et al.*, 1998), may be a target of c-Jun transactivation in differentiated PC12 cells undergoing apoptosis induced by NGF withdrawal (Le-Niculescu *et al.*, 1999).

In this chapter I investigated whether adenoviral-mediated expression of dominant negative c-Jun could prevent the release of mitochondrial cytochrome c, and what might be the regulators of this release.

6.2 Results

6.2.1 Expression of dominant negative c-Jun prevents the release of cytochrome c from mitochondria after NGF withdrawal

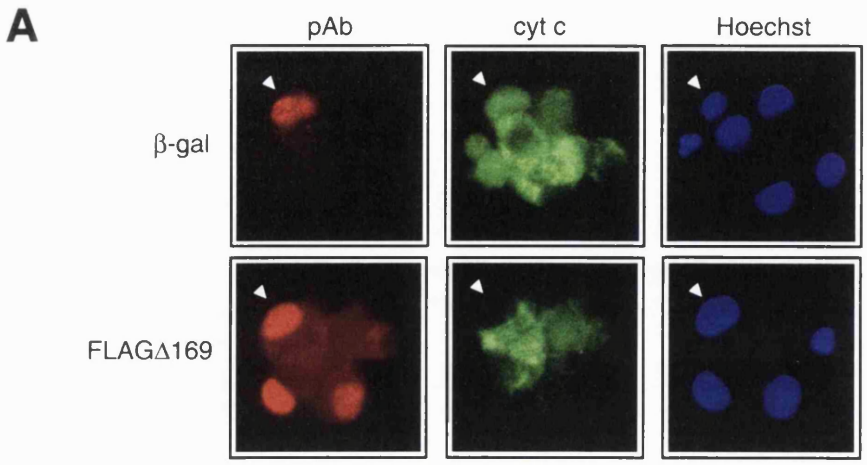
To understand how c-Jun promotes cell death, it is essential to identify the step in the neuronal death pathway at which c-Jun functions. This might suggest what type of genes would be relevant targets of c-Jun transactivation. I therefore investigated whether the c-Jun dominant negative mutant could block the release of cytochrome c from mitochondria, which has been shown to be critical for the death of sympathetic

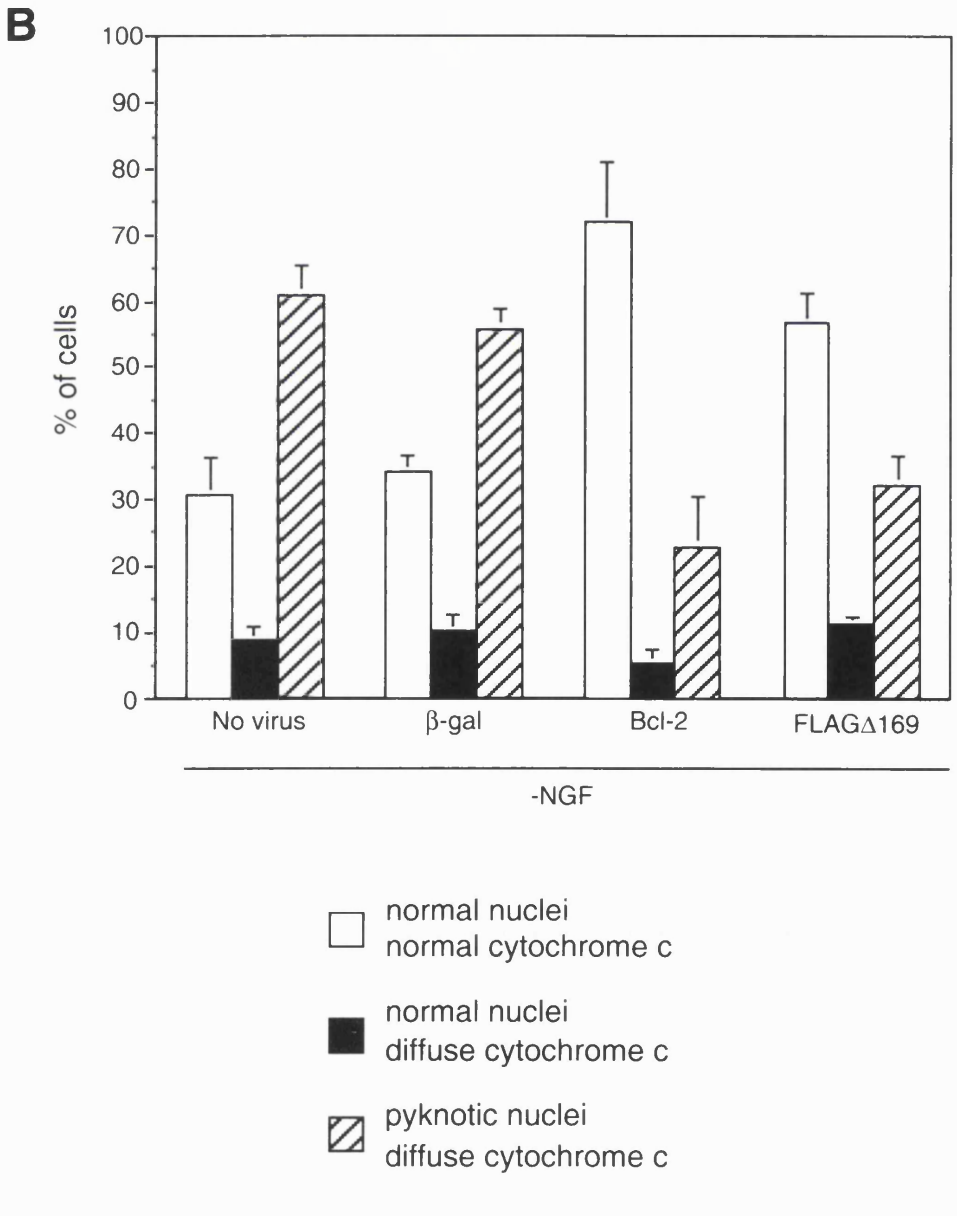
Figure 6.1 Expression of FLAGΔ169 prevents the release of cytochrome c from mitochondria

Sympathetic neurons were infected *in vitro* with recombinant adenoviruses expressing either Bcl-2, dominant negative c-Jun (FLAGΔ169), or β-galactosidase, at a multiplicity of infection of 100. The cells were withdrawn from NGF for 24 hours and then fixed and stained with a cytochrome c antibody and also with Hoechst to visualise the nuclei.

(A) Typical immunofluorescence pictures are shown. In the case of cells infected with the β-galactosidase adenovirus, the white arrowhead indicates a neuron overexpressing β-galactosidase that has a diffuse cytochrome c staining pattern and a pyknotic nucleus. For the dominant negative c-Jun adenovirus (FLAGΔ169), the white arrowhead indicates an infected neuron overexpressing FLAGΔ169 that has a punctate cytochrome c staining pattern and a normal nucleus.

(B) The number of cells with normal or pyknotic nuclei, and with normal or diffuse (redistributed) cytochrome c was scored. The average of the results from three experiments is shown with the standard error. Expression of Bcl-2 and FLAGΔ169 significantly increases the number of neurons with a normal cytochrome c staining pattern and normal nuclei ($p<0.05$, Student's t-test).


A
pAb
cyt c
Hoechst
β-gal
FLAGΔ169

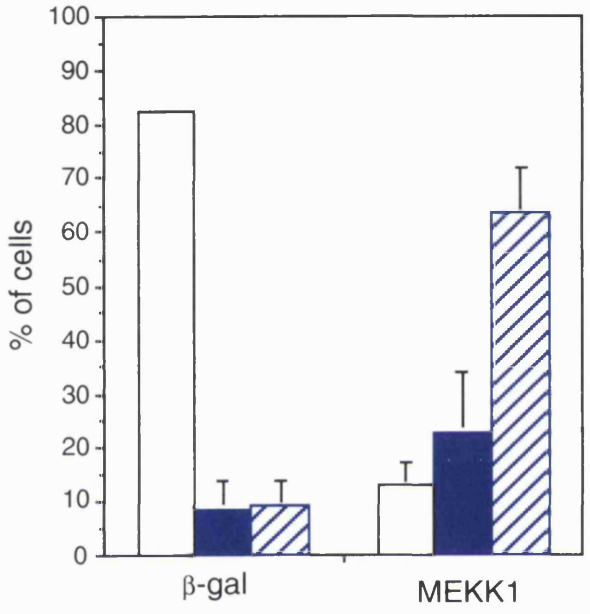

B
% of cells
100
90
80
70
60
50
40
30
20
10
0
No virus
β-gal
Bcl-2
FLAGΔ169
-NGF
normal nuclei
normal cytochrome c
normal nuclei
diffuse cytochrome c
pyknotic nuclei
diffuse cytochrome c

neurons deprived of NGF (Neame *et al.*, 1998). To this end, sympathetic neurons were infected *in vitro* with recombinant adenoviruses expressing either dominant negative c-Jun, β-galactosidase, or Bcl-2, at an MOI of 100. The cells were withdrawn from NGF for 24 hours and then fixed and co-stained with both a monoclonal antibody against cytochrome c and polyclonal antibodies specific for the recombinant proteins, and with Hoechst dye to visualise nuclear morphology. The number of cells with normal or pyknotic nuclei, and with normal (punctate) or redistributed (diffuse) cytochrome c was determined. The punctate cytochrome c staining pattern is consistent with localisation of cytochrome c to the mitochondria, whereas the diffuse distribution is observed when cytochrome c has been released into the cytosol. Figure 6.1A shows some representative immunofluorescence pictures of the cytochrome c staining pattern and nuclear morphology of neurons infected with either FLAGΔ169 or β-galactosidase expressing adenoviruses. Neurons expressing FLAGΔ169 frequently had normal (punctate) cytochrome c staining and normal nuclei, whereas those expressing β-galactosidase had diffuse cytochrome c and pyknotic nuclei. Figure 6.1B shows the average result from four experiments. In each infected population of cells approximately 5-10% of the neurons had diffuse cytochrome c staining but normal nuclei. No cells with pyknotic nuclei and normal cytochrome c were detected. This supports the idea that redistribution is an event which occurs prior to the nuclear morphological changes that occur during death. Crucially, expression of FLAGΔ169 or Bcl-2 increased the number of neurons with normal nuclei, i.e. prevented cell death, and also increased the number of neurons with normal cytochrome c staining, i.e. blocked cytochrome c release as well. There was no increase in the number of cells with normal nuclei and redistributed cytochrome c (Figure 6.1B, black bars), which would have occurred if nuclear pyknosis, but not cytochrome c release, was prevented. Such an effect is seen with the caspase inhibitor ZVADfmk, which blocks sympathetic neuron death after cytochrome c release (Neame *et al.*, 1998).

To determine whether activation of the JNK/c-Jun pathway was sufficient in itself to cause a redistribution of cytochrome c as well as causing cell death, sympathetic neurons were infected with recombinant adenoviruses expressing a constitutively active form of MEKK1, or as a control, β-galactosidase. After infection, the cells were maintained in NGF for three days and then fixed and stained with a cytochrome c antibody and also with antibodies to detect the recombinant proteins. Hoechst dye was

Figure 6.2 MEKK1 induces the release of cytochrome c from mitochondria and leads to cell death

Sympathetic neurons were infected *in vitro* for 24 hours with recombinant adenoviruses expressing either Myc epitope-tagged MEKK1 or β-galactosidase at an MOI of 100. The cells were maintained in NGF for a further three days, at which time they were fixed and stained with a cytochrome c antibody, and also with either anti-Myc or anti-β-galactosidase antibodies to identify infected cells. Hoechst was used to visualise the nuclear morphology. The number of infected cells with normal or pyknotic nuclei, or with normal or redistributed (diffuse) cytochrome c, was scored. The data shows the average result from two experiments with the standard error.



normal nuclei
normal cytochrome c

normal nuclei
diffuse cytochrome c

pyknotic nuclei
diffuse cytochrome c

used to visualise nuclear morphology. The number of infected cells with normal or pyknotic nuclei, and with normal or diffuse cytochrome c was scored. Figure 6.2 shows the average result for three experiments. Approximately 86% of the neurons expressing MEKK1 had diffuse cytochrome c staining, and 64% had pyknotic nuclei. In contrast, most of the cells expressing β-galactosidase had normal cytochrome c staining (under 20% of the cells had redistributed cytochrome c) and only 9% had pyknotic nuclei. Clearly, activation of the JNK/c-Jun signalling pathway by MEKK1 induces the release of cytochrome c from mitochondria, and also triggers cell death. Therefore, coupled with the fact that dominant negative c-Jun protects sympathetic neurons and prevents the relocalisation of cytochrome c, this suggests that c-Jun acts in the neuronal cell death pathway prior to the release of cytochrome c.

Figure 6.3 shows a hypothetical model of how the JNK/c-Jun pathway is connected to the mitochondrial death program. Activation of JNK/c-Jun by MEKK1 or NGF withdrawal induces the mitochondrial release of cytochrome c. Once in the cytosol, cytochrome c interacts with Apaf-1 and pro-caspase-9. In the presence of dATP pro-caspase-9 is then autocatalytically activated and initiates a caspase cascade which causes apoptosis. Possible regulators of cytochrome c release are the Bcl-2 family of pro- and anti-apoptotic proteins, and hence their expression in sympathetic neurons and their regulation by dominant negative c-Jun was investigated.

6.2.2 The pro-apoptotic Bcl-2 family member Bim is upregulated in sympathetic neurons after NGF withdrawal, whereas Bax, Bak, Bad and Bid levels remain constant

c-Jun could promote apoptosis by regulating the expression of the Bcl-2 family of proteins, since these proteins have been implicated in the control of cytochrome c release. The pattern of expression of pro-apoptotic members of the Bcl-2 family was examined. To carry out these experiments sympathetic neurons were cultured for 5 days and then infected with an adenovirus expressing β-galactosidase to control for any effect of adenoviral infection on cellular gene expression. After infection for 48 hours, the cells were withdrawn from NGF. Protein extracts were made either 16 or 24 hours later and equal amounts of protein were resolved using 12% SDS-polyacrylamide gels. Immunoblotting using antibodies specific for each pro-apoptotic Bcl-2 family member was performed. To control for protein loading, each blot was stripped and incubated with an anti-Bcl-x_L antibody since the level of Bcl-x_L has previously been shown to

Figure 6.3 Hypothetical model showing how the JNK/c-Jun pathway and cytochrome c are connected in the cell death pathway in neurons

Sympathetic neurons withdrawn from NGF have increased JNK activity, c-Jun protein levels, and c-Jun N-terminal phosphorylation. In addition, cytochrome c is released from the mitochondria and forms the apoptosome complex with pro-caspase-9, Apaf-1, and dATP. Autocatalytic processing of pro-caspase-9 then occurs. Active caspase-9 triggers a caspase cascade and apoptosis. Dominant negative c-Jun prevents cytochrome c release, and blocks cell death, whereas stimulation of the JNK pathway by MEKK1 phosphorylates c-Jun, causes cytochrome c efflux, and leads to cell death. Thus, c-Jun acts upstream of cytochrome c release. The targets of c-Jun transactivation are not clear.

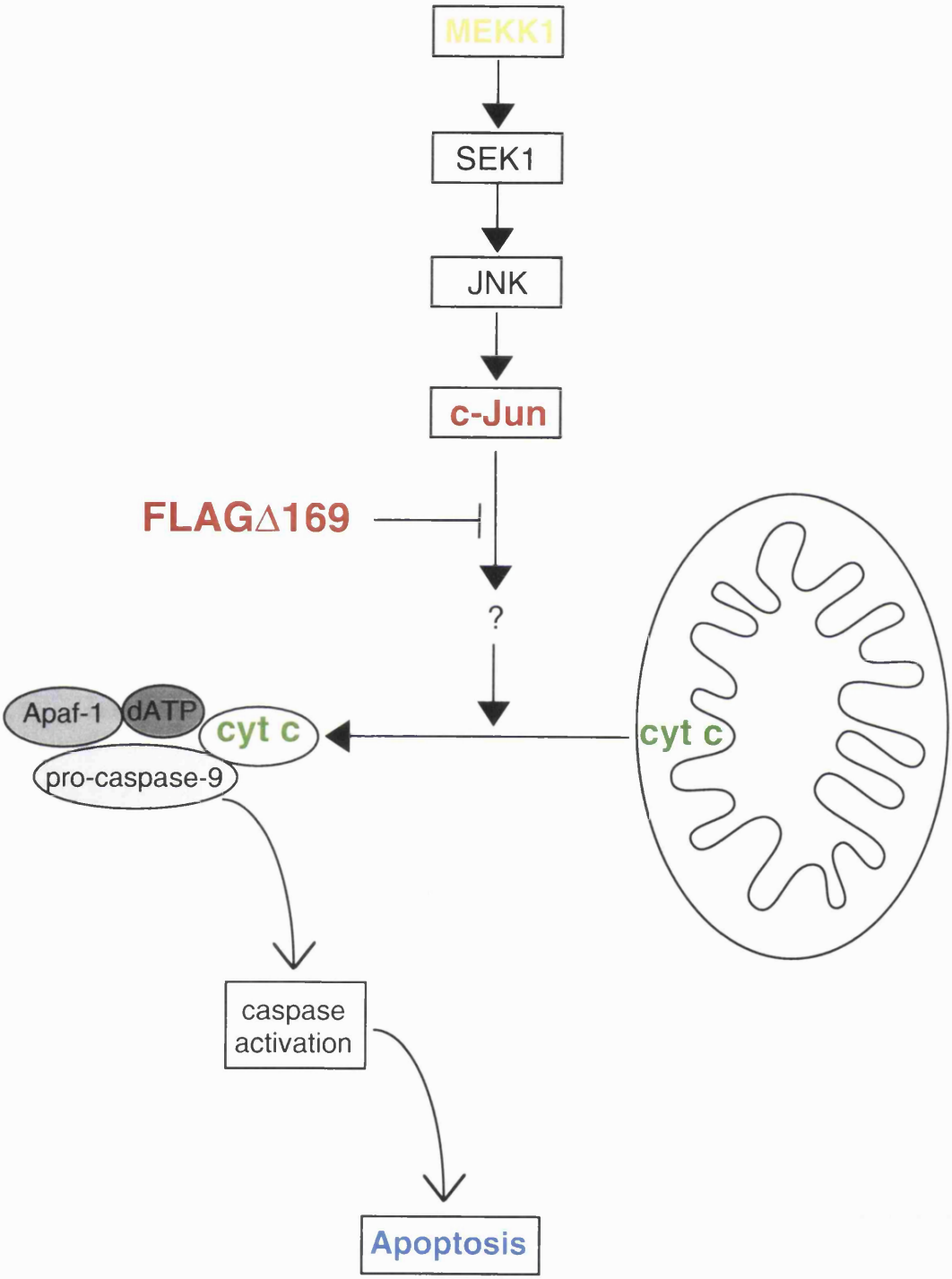
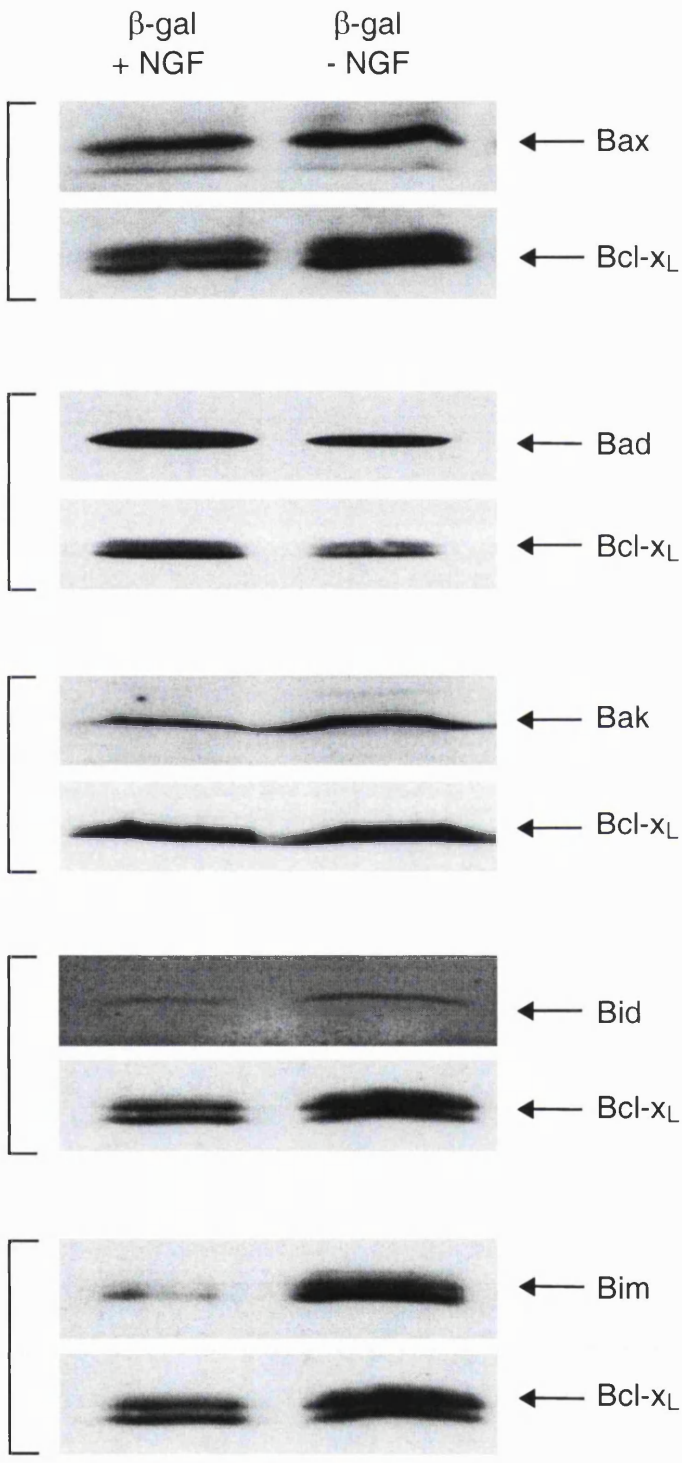

MEKK1
SEK1
JNK
c-Jun
FLAGΔ169
?
Apaf-1
dATP
cyt c
pro-caspase-9
cyt c
caspase activation
Apoptosis

Figure 6.4 Bax, Bak, Bad and Bid expression is not induced in sympathetic neurons after NGF withdrawal, whereas Bim levels increase significantly

Sympathetic neurons were infected with an adenovirus expressing β-galactosidase, and maintained in NGF-containing medium or deprived of NGF. After either 16 or 24 hours, protein extracts were made, and approximately 15 μg of + and – NGF extract was run on a 12% SDS-polyacrylamide gel and transferred to Hybond ECL nitrocellulose. Immunoblotting was performed using antibodies specific for Bax, Bak, Bad, Bid or Bim. Blots were stripped and reprobed with anti-Bcl-x_L as a control for protein loading. Each immunoblot was repeated three times, and typical results are shown. The level of Bcl-x_L remained more or less constant for 24 hours after NGF withdrawal. This was also the case for all of the other pro-apoptotic Bcl-2-family members examined, except Bim, which increased in level substantially.


β-gal
+ NGF
β-gal
- NGF
Bax
Bcl-xL
Bad
Bcl-xL
Bak
Bcl-xL
Bid
Bcl-xL
Bim
Bcl-xL

remain constant for 24 hours after NGF withdrawal (Aloyz *et al.*, 1998). In addition, immunoblots carried out with an anti-neuron specific enolase (NSE) antibody showed that when equal amounts of protein were loaded, both Bcl-x_L and NSE remained constant (data not shown). Immunoblots for each protein were performed with three sets of cell extracts prepared from independent experiments and Figure 6.4 shows some typical immunoblots. No change in the level of Bax, Bak, Bad and Bid was noted. In addition, there was no decrease in the level of Bid after NGF withdrawal, or the appearance of a Bid cleavage product. Cleavage of Bid by caspase-8 occurs in certain types of cells following activation of the Fas death receptor (Li *et al.*, 1998; X. Luo *et al.*, 1998). In contrast, there was a large increase in the level of Bim protein after NGF withdrawal, which shows that this protein could potentially be transcriptionally upregulated after NGF withdrawal. The anti-Bim antibody detected a protein that migrated between the 21 and 30 kD molecular weight standards. Three Bim isoforms generated by alternative splicing have been previously described (Hsu *et al.*, 1998; O'Connor *et al.*, 1998). The form detected in sympathetic neuron extracts corresponded in size to the largest isoform, Bim_{EL} (also known as BOD-L).

To further examine the increase in Bim protein level, a timecourse experiment was performed to determine the level of Bim protein at various times after NGF withdrawal. Figure 6.5A shows a typical luminogram, and quantitation is presented in Figure 6.5B, with Bim levels normalised to the levels of Bcl-x_L, to control for protein loading. The Bim protein increased in level significantly, peaking at approximately 16 hours after NGF withdrawal. It is interesting to note that this coincides with the timepoint at which only 50% of the neurons can be rescued by addition of actinomycin D/cycloheximide (16 hours) and precedes the cell death commitment point (22 hours) for rat sympathetic neurons (Martin *et al.*, 1988; Deckwerth and Johnson, 1993). To determine whether the increase in Bim protein was due to an increase in the level of *bim* mRNA, total RNA was isolated at several timepoints after NGF withdrawal, and reverse transcription PCR (RT-PCR) performed using *bim*-specific primers. This experiment was performed three times and Figure 6.6A shows a representative gel. The average results are presented in Figure 6.6B. There was a four-fold increase in the level of *bim* mRNA, which started between 4 and 8 hours, and which was maximal by 16 hours after NGF withdrawal. This contrasts with the level of a general neuronal marker, such as *neurofilament-M (NF-M)* which declined over the course of 24 hours.

Figure 6.5 Bim protein levels increase in sympathetic neurons after NGF deprivation

Sympathetic neurons were withdrawn from NGF and then protein extracts were prepared at several timepoints over the course of 24 hours. Approximately 15 μg of protein was run on a 12% SDS-polyacrylamide gel, blotted, and probed with an anti-Bim antibody. The membrane was then stripped and reprobed with anti-Bcl-x_L.

(A) A typical immunoblot is shown. Bim protein increases in level after NGF withdrawal, reaching a maximum level at around 16 hours.

(B) The blot shown in Figure 6.5A was scanned on a densitometer and Bim protein levels were normalised to the levels of Bcl-x_L at each timepoint.

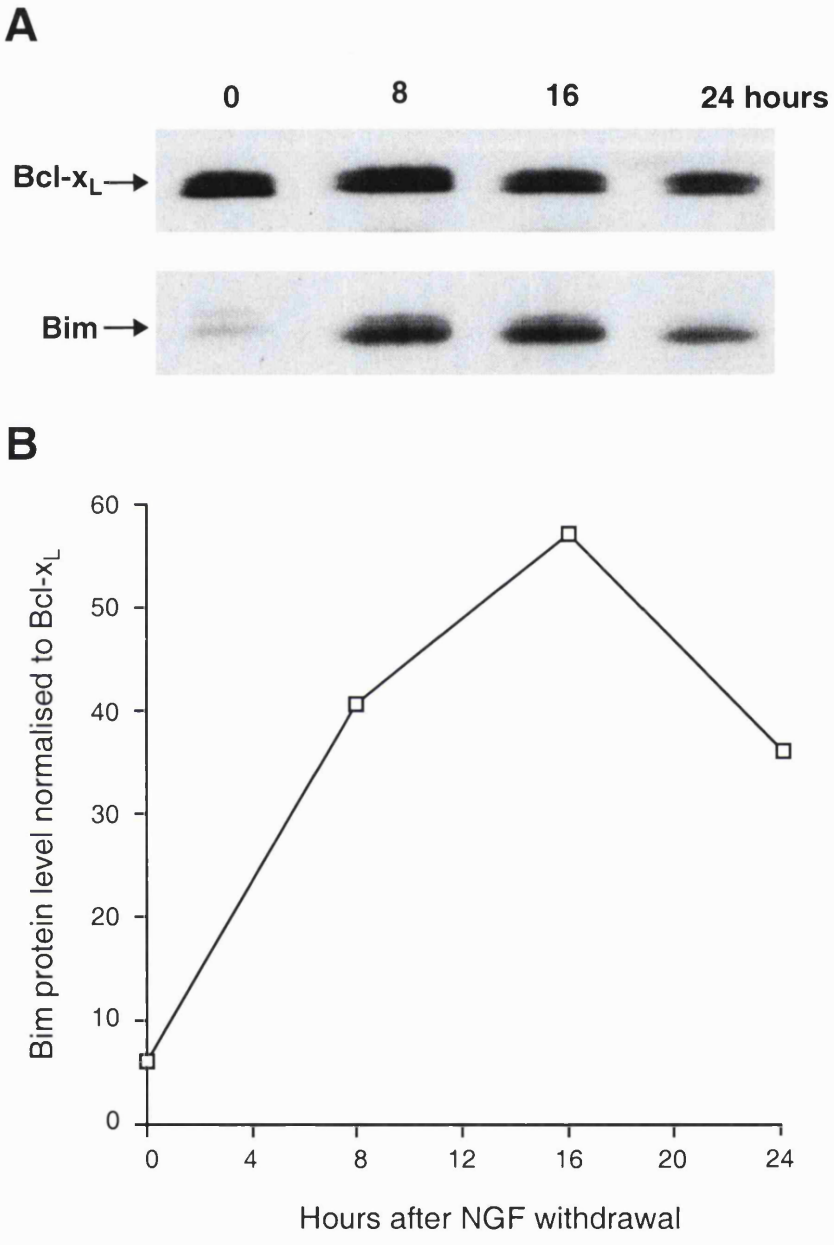
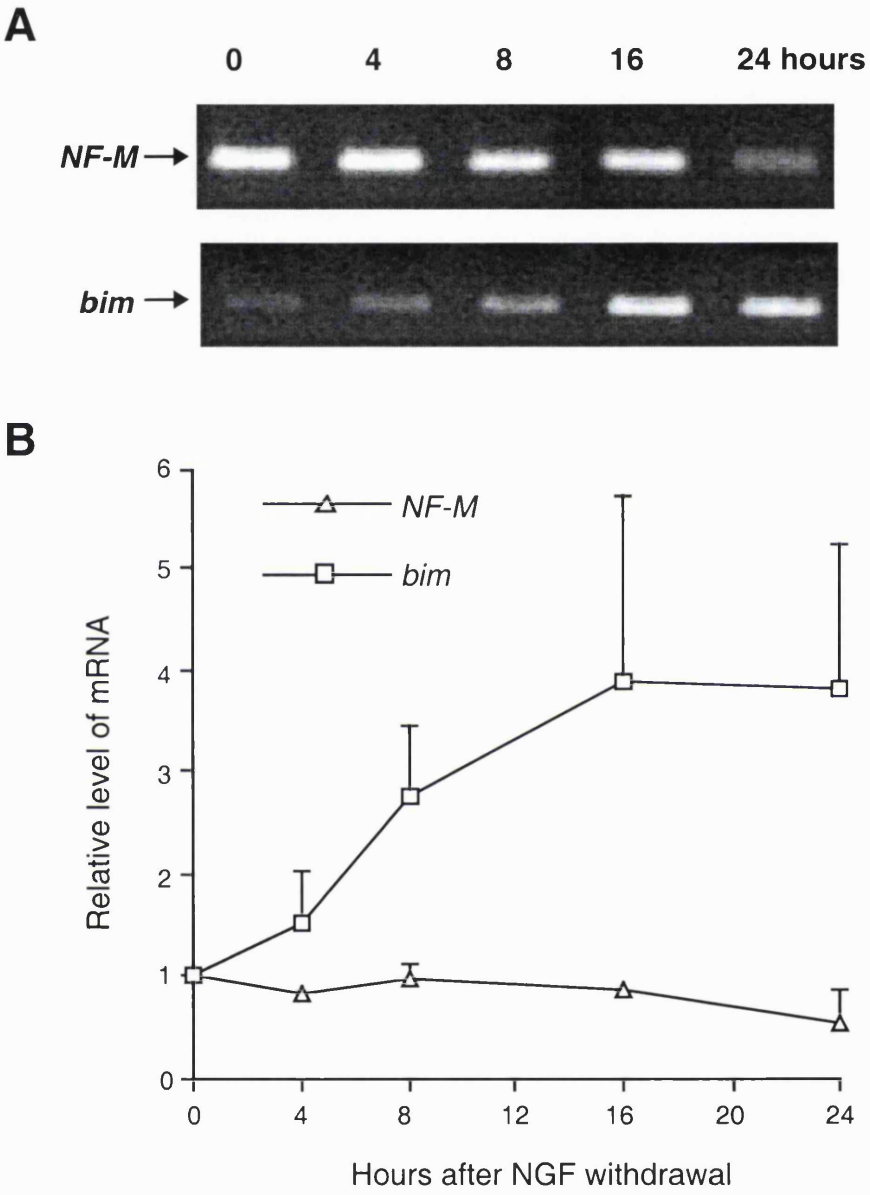

A
0
8
16
24 hours
Bcl-x$_L$
Bim
B
Bim protein level normalised to Bcl-x$_L$
60
50
40
30
20
10
0
0
4
8
12
16
20
24
Hours after NGF withdrawal

Figure 6.6 *bim* mRNA levels increase in sympathetic neurons after NGF deprivation

Sympathetic neurons were withdrawn from NGF and then RNA isolated at several timepoints over the course of 24 hours.

(A) RT-PCR was performed using *bim*-specific primers. As a control, *neurofilament-M (NF-M)* primers were used. The PCR products were separated on 2.5% agarose gels and visualised by staining with ethidium bromide. A typical PCR result is shown. *bim* mRNA increases after NGF withdrawal, whereas *neurofilament-M* is mostly constant but declines slightly at 24 hours.

(B) The RT-PCR described in Figure 6.6A was repeated using three different sets of neuronal RNA. The intensity of the bands was determined using a Kodak imaging system. The average result with the standard error is shown. *bim* mRNA levels increase approximately four-fold, with the maximal level being reached by 16 hours. The increases in *bim* level at 8, 16, and 24 hours are significant ($p<0.05$, Mann-Whitney *U* test).


A
0
4
8
16
24 hours
NF-M
bim
B
NF-M
bim
Relative level of mRNA
0
1
2
3
4
5
6
0
4
8
12
16
20
24
Hours after NGF withdrawal

Thus the increase in Bim protein levels is due, at least in part, to an increase in the level of *bim* mRNA.

6.2.3 Induction of *bim* mRNA is inhibited by dominant negative c-Jun

To determine whether upregulation of *bim* mRNA requires AP-1 activity, neurons were infected with adenoviruses expressing FLAGΔ169 or β-galactosidase and then were maintained in NGF-containing medium or withdrawn from NGF. After 16 hours, RNA was isolated and RT-PCR experiments were performed using primers specific for *bim* or *NF-M*. Five independent experiments were carried out and the gels were scanned on a densitometer to determine the relative levels of *bim* and *NF-M* under the different conditions tested. The average results and standard errors are shown in Figure 6.7. NGF withdrawal led to a significant increase in *bim* mRNA level in cells infected with the adenovirus expressing β-galactosidase. This increase was similar to that obtained during the previous experiment with uninfected neurons and therefore shows that adenoviral infection does not affect the increase in *bim*. The increase in the level of *bim* following NGF withdrawal was significantly reduced in cells infected with the FLAGΔ169 adenovirus ($p<0.01$, Figure 6.7). In contrast, *NF-M* mRNA levels did not increase after NGF withdrawal. It appeared that there was some decrease in *NF-M* levels due to the expression of FLAGΔ169, which could be due to a putative AP-1 site in the *NF-M* promoter (Elder *et al.*, 1992). However, this decrease was not significant ($p>0.05$). These results suggest that c-Jun/AP-1 activity is required for the increase in *bim* mRNA levels that occurs after NGF deprivation.

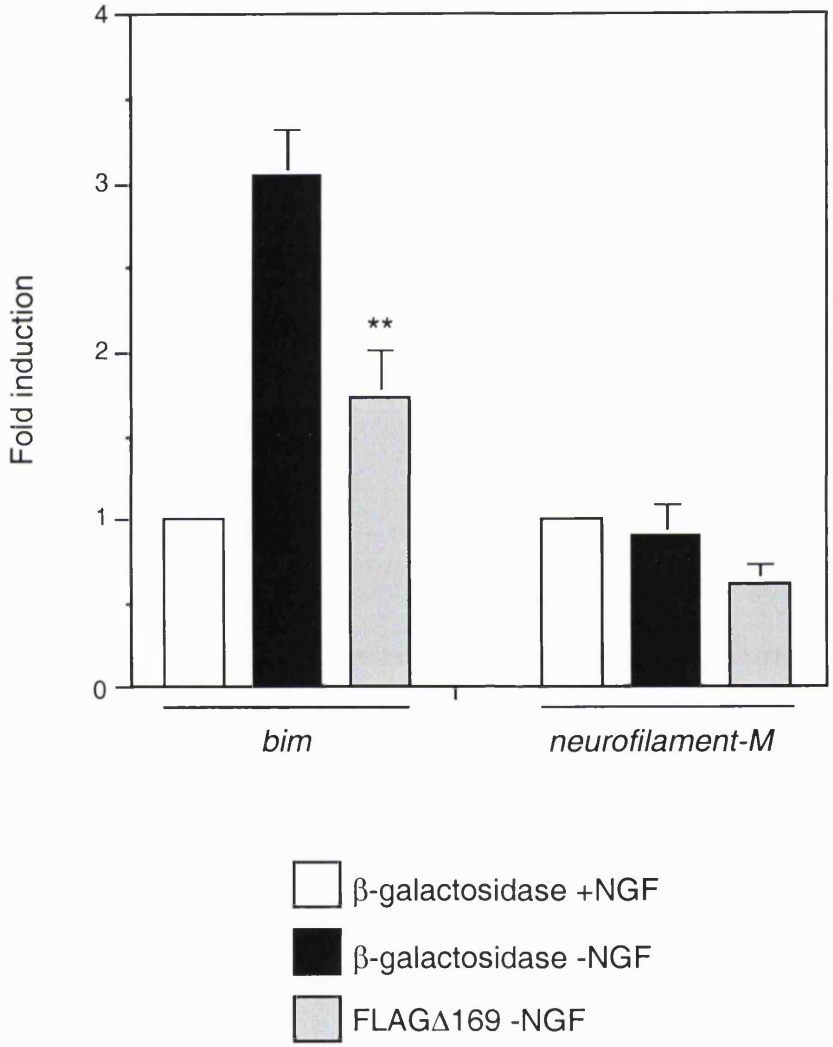
It is important to note that in these experiments, RNA was isolated from the whole cell population, but at the MOI used (100) only 45% of the infected cells express detectable FLAGΔ169 protein (Figure 5.5). It is therefore possible that in the infected sub-population of neurons expressing high levels of FLAGΔ169, the reduction in the level of *bim* would be greater than that observed when RNA is isolated from the whole cell population.

6.2.4 Overexpression of Bim_{EL} causes mitochondrial cytochrome c release and apoptosis

The results described so far suggest that upregulation of *bim* mRNA following NGF withdrawal requires AP-1 activity and *bim* is therefore a potential target of c-Jun transactivation. However, the function of Bim in sympathetic neurons is unknown. To investigate this a CMV expression vector for FLAG-tagged Bim_{EL} was constructed. The

Figure 6.7 The increase in *bim* mRNA level that occurs after NGF deprivation is prevented by the expression of FLAGΔ169

Sympathetic neurons were infected with adenoviruses expressing either FLAGΔ169 or β-galactosidase. After NGF withdrawal for 16 hours, RNA was isolated, reverse transcribed, and PCR performed using primers specific for *bim* or *neurofilament-M*. The PCR products were seperated on 2.5% agarose gels and visualised by ethidium bromide staining. The experiment was repeated with 5 different sets of RNA, and the intensity of the bands was determined using a Kodak imaging system. The average result is shown with the standard error. The increase in *bim* mRNA that occurs after NGF deprivation was significantly inhibited by the expression of FLAGΔ169 (** $p<0.01$, Student's t-test)


4
3
2
1
0
Fold induction
**
bim
neurofilament-M
β-galactosidase +NGF
β-galactosidase -NGF
FLAGΔ169 -NGF

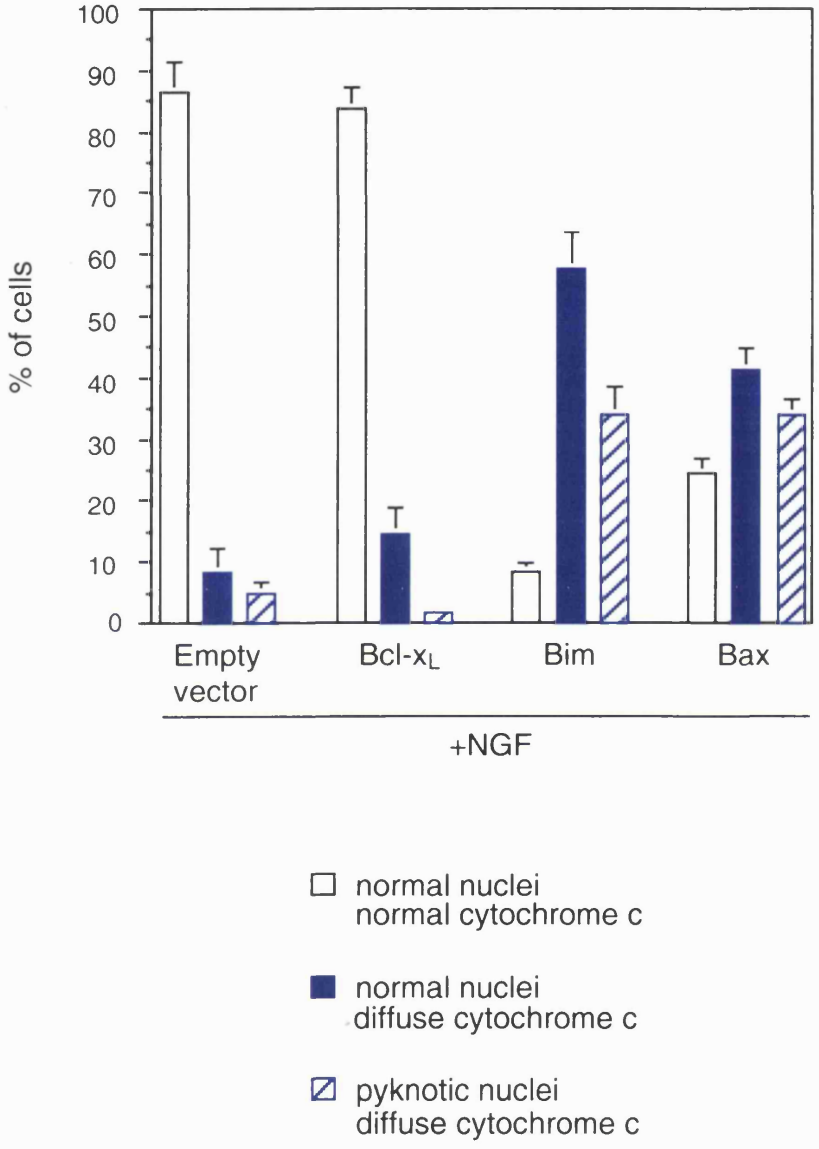
full length Bim_{EL} coding region was cloned from sympathetic neuron cDNA by PCR and inserted into pcDNA1, with the FLAG epitope at the N-terminus (this construct was a gift from Stephen Neame). To determine whether overexpression of Bim_{EL} could promote cytochrome c release and neuronal death, microinjection experiments were carried out with the Bim_{EL} expression vector. Sympathetic neurons cultured for 7 days in the presence of NGF were injected either with a Bim_{EL} expression plasmid, or with vectors encoding Bcl-x_L or Bax, or with the empty vector, together with guinea-pig IgG as an injection marker. 24 hours later, neurons were fixed and stained with antibodies specific for cytochrome c and guinea pig IgG (to identify the injected cells). The cells were also stained with Hoechst dye to visualise nuclear morphology. All of the neurons injected with pcDFLAG-Bim expressed the recombinant protein (detected with an anti-FLAG antibody, data not shown). The number of neurons with a diffuse cytochrome c staining pattern and pyknotic nuclei was scored. Figure 6.8 shows the average result for 5 experiments with the standard error. Overexpression of Bim_{EL} caused a significant increase in the percentage of cells with a diffuse cytochrome c staining pattern as well as an increase in the percentage of cells with pyknotic nuclei, compared to Bcl-x_L or the empty vector. Bax expression also induced the release of cytochrome c and led to apoptosis in a substantial number of the neurons. These results demonstrate that Bim_{EL} (and Bax) can promote apoptosis in sympathetic neurons and do so by inducing cytochrome c release.

6.3 Discussion

To identify the step in the neuronal cell death pathway at which c-Jun functions, an adenoviral gene delivery system has been used to transduce sympathetic neurons. Expression of either FLAGΔ169 or Bcl-2 prevented the release of cytochrome c after NGF deprivation, whereas expression of β-galactosidase or MEKK1 did not. The redistribution of mitochondrial cytochrome c to the cytosol which occurs after NGF withdrawal is critical for the death of these neurons (Neame *et al.*, 1998). If FLAGΔ169 had not prevented the release of cytochrome c (but had blocked cell death as expected) there would have been an increase in neurons with a diffuse cytochrome c staining pattern but which lacked the nuclear changes typical of apoptosis, as is the case when neurons are treated with the caspase inhibitor ZVADfmk (Neame *et al.*, 1998). In the presence of NGF, adenoviral-mediated expression of activated MEKK1 induced the redistribution of cytochrome c into the cytosol. Thus, stimulation of the JNK/c-Jun

Figure 6.8 Overexpression of Bim_{EL} in sympathetic neurons induces cytochrome c release and causes cell death

The full length Bim_{EL} coding region was cloned by PCR from sympathetic neurons, and inserted into the CMV expression vector pcDNA1, with an N-terminal FLAG epitope tag. Sympathetic neurons were cultured for 7 days and then injected either with the Bim_{EL} expression plasmid at 0.2 mg/ml, or with vectors encoding Bax or Bcl-x_L or the empty vector pcDNA1, together with guinea-pig IgG as an injection marker. 24 hours later, neurons were fixed and stained with antibodies specific for cytochrome c and guinea pig IgG (to identify the injected cells). The cells were also stained with Hoechst dye to visualise nuclear morphology. The number of neurons with a diffuse cytochrome c staining pattern and pyknotic nuclei was scored. Figure 6.8 shows the average result from 5 experiments with the standard error.


100
90
80
70
60
50
40
30
20
10
0
% of cells
Empty vector
Bcl-xL
Bim
Bax
+NGF
normal nuclei
normal cytochrome c
normal nuclei
diffuse cytochrome c
pyknotic nuclei
diffuse cytochrome c

pathway by MEKK1, which is known to kill sympathetic neurons in the presence of NGF, also causes the release of cytochrome c (Figure 6.2). It is worth noting that this contrasts with simply microinjecting cytochrome c into the cytosol, which does not kill these neurons (Deshmukh and Johnson, 1998; Neame *et al.*, 1998). On the other hand, injection of cytochrome c into NGF-deprived cycloheximide-treated sympathetic neurons causes rapid apoptosis (Deshmukh and Johnson, 1998). Thus, it has been suggested that neurons, in contrast to other cells, must develop "competence-to-die" after NGF withdrawal to become susceptible to the effect of cytochrome c present in the cytosol (Deshmukh and Johnson, 1998). Overexpression of MEKK1 apparently overcomes this mechanism suggesting that "competence-to-die" may be controlled by an event downstream of MEKK1. One possibility is that MEKK1 induces the release of additional pro-apoptotic factors from the mitochondria, such as Smac/DIABLO, which has been shown to antagonise the IAP family of caspase inhibitor proteins (Du *et al.*, 2000; Verhagen *et al.*, 2000). It has been postulated that neuronal IAP proteins may inhibit pro-caspase-9 and form part of the "competence-to-die" mechanism that prevents cytochrome c from activating the apoptosome complex when sympathetic neurons are cultured in the presence of NGF (Wiese *et al.*, 1999).

This is the first demonstration that c-Jun functions upstream of cytochrome c release in the death pathway in neurons. While this thesis was in preparation, work from Roger Davis showed that cytochrome c release and apoptosis are prevented in fibroblasts prepared from mutant embryos in which the *Jnk* genes were disrupted (Tournier *et al.*, 2000) which is consistent with the results presented here. Other transcription factors that promote apoptosis have recently been shown to induce mitochondrial cytochrome c release. For example, c-Myc induced sensitisation to apoptosis in fibroblasts is mediated by cytochrome c redistribution to the cytosol (Juin *et al.*, 1999). Also, p53-induced apoptosis proceeds via cytochrome c release and subsequent activation of caspases in Saos-2 cells (Schuler *et al.*, 2000).

Bax, as well as other proteins of the Bcl-2 family, is strongly implicated in the regulation of cytochrome c release. Bax and Bak can cause cytochrome c release from mitochondria, whereas Bcl-2 and Bcl-x_L block these effects (Jurgensmeier *et al.*, 1998; Narita *et al.*, 1998). Bid translocates to mitochondria during apoptosis where it appears to act on Bax to induce the release of cytochrome c (Li *et al.*, 1998; X. Luo *et al.*, 1998; Desagher *et al.*, 1999). Studies with *bax* (–/–) mice have shown that Bax plays a critical

role during apoptosis in sympathetic neurons (Deckwerth *et al.*, 1996). Experiments with neurons cultured from these mice have demonstrated that c-Jun phosphorylation functions upstream (or independently) of Bax since c-Jun N-terminal phosphorylation was not inhibited after NGF withdrawal even though the neurons were protected from apoptosis (Deckwerth *et al.*, 1998). c-Jun also seems to function upstream of Bcl-2 since apoptosis induced by overexpression of c-Jun in fibroblasts can be inhibited by Bcl-2 (Bossy-Wetzel *et al.*, 1997), while Bcl-2 does not block c-Jun induction during the death of sympathetic neurons (Ham *et al.*, 1995). Bad may also function in the death of sympathetic neurons since overexpression of Akt, which phosphorylates and inactivates Bad (Datta *et al.*, 1997), promotes survival after NGF withdrawal (Dudek *et al.*, 1997; Philpott *et al.*, 1997).

To determine whether any of the pro-apoptotic members of the Bcl-2 family are targets of c-Jun that promote the release of cytochrome c in neurons, western blotting was performed to look at Bax, Bad, Bid, and Bak. No increase in the level of these proteins was observed after NGF deprivation. Bid is regulated by cleavage (Li *et al.*, 1998; X. Luo *et al.*, 1998) but in the case of NGF-deprived sympathetic neurons no loss of full-length Bid or appearance of cleavage products was detected, suggesting that Bid is not involved in apoptosis in these cells. In contrast, Bid has recently been suggested to be a downstream target of the JNK pathway because cleavage of Bid, which occurs in response to UV, was prevented in *Jnk1* (–/–) *Jnk2* (–/–) fibroblasts (Tournier *et al.*, 2000). However, UV-induced apoptosis in these cells is transcription-independent arguing against a strong role for c-Jun. There are conflicting reports about Bax expression in sympathetic neurons. Greenlund *et al.* (1995b) showed that the *bax* mRNA did not increase in level in sympathetic neurons withdrawn from NGF, whereas Aloyz *et al.* (1998) showed that Bax protein levels increased two-fold. Bax appears to mediate cytochrome c release in sympathetic neurons by translocating from the cytosol to the mitochondrial membrane (Putcha *et al.*, 1999). It has also been suggested that in HeLa cells and cerebellar granule neurons, Bax is permanently present in the mitochondrial membrane but undergoes a conformational change when the cells are induced to die (Desagher *et al.*, 1999). In sympathetic neurons, I did not observe an increase in the level of Bax after NGF deprivation, suggesting that Bax promotes cytochrome c release by a mechanism involving translocation and/or conformational changes. In addition, Bad levels do not appear to be transcriptionally regulated, and

thus Bad may be regulated by phosphorylation as previous reports suggest (Datta *et al.*, 1997; Dudek *et al.*, 1997; Philpott *et al.*, 1997).

One member of the Bcl-2 family which substantially increased in level after NGF withdrawal was Bim. Expression of this protein increased within 8 hours and was maximal by approximately 16 hours. In addition, an increase in *bim* mRNA was detected within 8 hours. c-Jun N-terminal phosphorylation starts to increase between 4-8 hours after NGF deprivation (Ham *et al.*, 1995) and thus one would expect an increase in target gene mRNA soon after this time. Also, it is worth noting that the timepoints at which maximal levels of the *bim* mRNA and protein were observed preceded the cycloheximide and NGF commitment points (16 and 22 hours respectively, Martin *et al.*, 1988; Deckwerth and Johnson, 1993). When neurons were infected with viruses expressing FLAGΔ169, there was a significant decrease in the level of *bim* mRNA, indicating that AP-1 activity is required for the induction of the *bim*. Although *bim* upregulation was not completely inhibited, it is important to note that at the MOI used, only 45% of the neurons visibly expressed dominant negative c-Jun (as determined by immunofluorescence), and therefore presumably a proportion of the neurons did not express sufficient FLAGΔ169 to prevent activation of the *bim* promoter.

In a similar manner to the regulation of *bim* by c-Jun, the BH3-only protein Noxa was recently shown to be transcriptionally upregulated by p53 (Oda *et al.*, 2000). Expression of the Noxa was increased in mouse fibroblasts exposed to irradiation, and this depended on p53. On the other hand, antisense oligonucleotides targetted to *Noxa* inhibited p53-induced apoptosis.

To begin to address the question of the importance of Bim for death in sympathetic neurons, a Bim_{EL} expression plasmid was microinjected into neurons maintained in the presence of NGF. Expression of Bim_{EL} caused the rapid loss of mitochondrial cytochrome c, and induced apoptosis. To demonstrate that Bim is required for the death of sympathetic neurons, it would be necessary to inhibit its function, possibly by microinjection of anti-Bim antibodies, or by culturing neurons from *bim* (–/–) mice.

Bim was isolated in a yeast two-hybrid screen for proteins that bound to Bcl-2 in a human cDNA expression library (O'Connor *et al.*, 1998), and shortly after the rat homologue (termed BOD) was discovered in a screen to identify proteins that bound to Mcl-1 (a Bcl-2 homologue) in an ovarian fusion cDNA library (Hsu *et al.*, 1998). Bim

is not able to interact with Bax, but rather seems to exert its effect by dimerising with anti-apoptotic Bcl-2 proteins, and thus inhibiting their effect on survival. In *bim* (–/–) mice, effects on lymphoid and myeloid tissues have been reported, and cells show reduced apoptosis in response to a variety of stimuli, resembling cells from transgenic *bcl-2* mice, suggesting that the main function of Bim is to antagonise Bcl-2 (Bouillet *et al.*, 1999). Phenotypic effects in the nervous system have not yet been reported. One could hypothesise that upregulation of Bim increases the formation of Bim/Bcl-2 (or Bim/Bcl-x_L) heterodimers. Bax (and perhaps Bak) is therefore freed from Bcl-2/Bclx_L inhibition and homodimerises in the mitochondrial membrane and/or interacts with pore proteins within the mitochondrial membrane to cause cytochrome c release. Interestingly, the transcriptional regulation of Bim reported here contrasts with a paper which suggests that Bim is inactivated by binding to the LC8 protein in the dynein motor complex (Puthalakath *et al.*, 1999). It will be interesting to determine whether this inhibition by sequestration to the motor complex might also function in neurons. Perhaps this transcriptional upregulation of Bim is neuron specific. Future work could be directed toward further examination of the interaction between c-Jun and Bim. In particular, the *bim* promoter needs to be cloned. If a c-Jun binding site was present, one could determine whether c-Jun actually binds to the promoter during NGF withdrawal-induced death, and look at activation of the promoter in response to NGF withdrawal or MEKK1 expression, as well as inhibition by FLAGΔ169. Furthermore, one could investigate the generality of this mechanism of cell death, since c-Jun has been implicated in apoptosis in other types of neurons, such as granule neurons (Watson *et al.*, 1998) and hippocampal neurons (Schlingensiepen *et al.*, 1994; Behrens *et al.*, 1999), and also in the death of NIH3T3 fibroblasts (Bossy-Wetzel *et al.*, 1997).

In conclusion, these results suggest that dominant negative c-Jun prevents neuronal apoptosis by inhibiting the mitochondrial release of cytochrome c. Furthermore, Bim is implicated as one target of c-Jun that mediates this release.

Chapter 7: General discussion

Cell death by apoptosis plays a critical role during the development of the nervous system and there is increasing evidence that neurons die by apoptosis in neurodegenerative diseases. Developing sympathetic neurons deprived of their survival factor (NGF) die by apoptosis in a transcription-dependent manner, and these neurons have proved to be a useful model system for studying the mechanisms of apoptosis. The transcription factor c-Jun has previously been strongly implicated in the death of these neurons. There is a large increase in the levels of both *c-jun* mRNA and c-Jun protein after NGF withdrawal (Estus *et al.*, 1994; Ham *et al.*, 1995), in contrast to a general decrease in macromolecular synthesis. Microinjection of an anti-c-Jun antibody or expression of a c-Jun dominant negative mutant (FLAGΔ169) promotes survival after NGF deprivation, while overexpression of wild-type c-Jun induces apoptosis in the presence of NGF (Estus *et al.*, 1994; Ham *et al.*, 1995). To study the regulation of c-Jun expression during the death of sympathetic neurons I performed microinjection studies using a *c-jun* promoter reporter gene (*c-jun* CAT). After sympathetic neurons were deprived of NGF, there was a large increase in CAT immunoreactivity in neurons that were injected with the reporter gene. This increase was dependent on the jun1 and jun2 TRE sites within the promoter, which can preferentially bind c-Jun/ATF-2 heterodimers. This demonstrates that NGF withdrawal stimulates the *c-jun* promoter, and that this may depend on binding of c-Jun protein to the TRE elements in the *c-jun* promoter. In further support of this autoregulation hypothesis (first suggested by Angel *et al.* in 1988), microinjection of dominant negative c-Jun completely blocked the increase in endogenous c-Jun protein levels that normally occurs after NGF deprivation. Also, multiple copies of the jun2 TRE alone can function as a transcriptional enhancer that is activated by NGF withdrawal, when positioned upstream of the SV40 promoter. Since ATF-2 has been implicated in the activation of the *c-jun* promoter, and it can be phosphorylated by JNK, it would be interesting to determine the pattern of expression and the phosphorylation state of ATF-2 in sympathetic neurons undergoing apoptosis. Indeed, experiments in this laboratory have shown that ATF-2 becomes more phosphorylated after NGF withdrawal from sympathetic neurons, with maximal activation at approximately 8 hours (Eilers *et al.*, manuscript submitted).

Having investigated how the *c-jun* promoter and c-Jun expression were regulated after NGF deprivation, I next studied the role of the JNK/c-Jun pathway in the initiation of cell death. Activation of the pathway would be predicted to trigger apoptosis, since wild-type c-Jun induced cell death when overexpressed in the presence of NGF (Ham *et al.*, 1995). Indeed, I showed that expression of a constitutively active form of MEKK1 by microinjection of an expression plasmid into sympathetic neurons led to increases in the levels of c-Jun protein and c-Jun phosphorylation, and also triggered apoptosis. These effects could be completely blocked by co-expression of dominant negative SEK1 (SEK*AL*). Since this mutant form of SEK1 effectively inhibited activation of the JNK/c-Jun pathway in response to MEKK1, it might be expected that its expression in the absence of NGF would promote survival. However, dominant negative SEK1 did not prevent the increases in c-Jun protein level and c-Jun phosphorylation, or the cell death that occurred after NGF deprivation. The mechanism of action of SEK*AL* is not clear. It has been suggested that sequential activation of each kinase occurs in this pathway, such that MEKK1 binds to SEK1, and then once SEK1 is activated it dissociates and binds to JNK (Xia *et al.*, 1998). This particular SEK1 mutant, in which the phosphorylation (activation) sites have been altered, should therefore bind to MEKK1 but not be activated, sequestering MEKK1 and therefore suggesting that MEKK1 is not involved in the death of sympathetic neurons. The role of SEK1 itself is therefore unclear. It would be interesting to directly inhibit SEK1, or to inhibit MKK7, the only other JNK kinasé cloned to date. There are many proteins in addition to MEKK1 that have been suggested to function as JNK kinase kinases. Recent data indicates that ASK1 may be the critical regulator of the JNK pathway during sympathetic neuron death (Kanamoto *et al.*, 2000). Dominant negative ASK1 promotes survival in the absence of NGF, and also prevents the induction of endogenous c-Jun. Furthermore, overexpression of wild-type ASK1 induces c-Jun expression and apoptosis in the presence of survival factor. Cdc42/Rac1 may function upstream, since activated forms of these Rho-like GTPases induce cell death, while dominant negative forms promote survival after NGF deprivation (Bazenet *et al.*, 1998).

That JNK itself plays a role in sympathetic neuron death has not been clearly established until recently. SB203580, a chemical inhibitor of JNK and p38 activity, promotes survival after NGF deprivation (Eilers *et al.*, manuscript submitted). Importantly, there was no increase in p38 activity or its phosphorylation in sympathetic

neurons during apoptosis, suggesting that the anti-apoptotic effect of SB203580 is due to inhibition of JNK (Eilers *et al.*, 1998). In addition, overexpression of the JNK binding domain of JIP-1 blocked cell death as effectively as Bcl-2 (Eilers *et al.*, manuscript submitted).

It is not yet known what functions further upstream in the pathway, in particular, what connects the NGF receptor to the JNK cascade. At the cell surface, the response to NGF (or lack of it) appears to be mediated by the TrkA tyrosine kinase receptor (Johnson *et al.*, 1986; Kaplan *et al.*, 1991a; Kaplan *et al.*, 1991b) and the p75 neurotrophin receptor (Klein *et al.*, 1991). The presence of NGF activates a survival signal from TrkA (Riccio *et al.*, 1997; Senger and Campenot, 1997), while its absence triggers activation of the death pathway, through lack of signalling from the TrkA receptor and/or pro-apoptotic signalling from p75 (Bamji *et al.*, 1998). Ligand-mediated activation of p75 is sufficient to cause apoptosis in the presence of NGF, while in p75-deficient sympathetic neurons apoptosis is delayed both in culture and *in vivo* (Bamji *et al.*, 1998). It has been suggested that the opposing effects of the two receptors on sympathetic neuron survival provide a mechanism whereby neurons are able to recognise not only whether there is a sufficient NGF, but also whether they are receiving inappropriate neurotrophins, presumably because of incorrect target innervation (Miller and Kaplan, 1998).

How might TrkA and p75 be linked to the c-Jun kinase cascade? Currently, this is unclear. A protein that binds to the p75 intracellular domain and appears to participate in the death response, at least in retinal cells, was recently isolated and termed NRIF, although it was suggested that this protein may translocate directly to the nucleus upon activation rather than activating a signalling cascade (Casademunt *et al.*, 1999). Interestingly, p75 also binds TRAF6, a docking protein which is able to activate ASK1 and JNK in fibroblast and Schwann cells (Khursigara *et al.*, 1999). Intracellular messengers, such as ceramide and reactive oxygen species (ROS) may also be involved. p75 can elevate intracellular ceramide levels through increased sphingomyelin hydrolysis (Dobrowsky *et al.*, 1994) and p75-mediated apoptosis in a neuroblastoma cell line was shown to be dependent on ceramide (Lievremont *et al.*, 1999). Ceramide has been shown to activate JNK and cause apoptosis in other systems (Westwick *et al.*, 1995; Verheij *et al.*, 1996). Interestingly, in these non-neuronal cells, ceramide-induced cell death could be blocked by dominant negative mutants of SEK1 and c-Jun (Verheij *et al.*, 1996). However, in contrast with these results, ceramide was actually shown to

be neuroprotective in sympathetic neurons, preventing both *c-jun* induction and cell death after NGF withdrawal (Ito and Horigome, 1995; Nair *et al.*, 2000). Thus, in sympathetic neurons ceramide may transduce the NGF survival signal via the p75 neurotrophin receptor. Reactive oxygen species (ROS) may also play a role in neuronal cell death. Levels of ROS peak at three hours after NGF withdrawal and expression of copper/zinc superoxide dismutase (Cu/ZnSOD) delays death in sympathetic neurons (Greenlund *et al.*, 1995a). Inhibition of NADPH oxidase, an enzyme that generates ROS, also promotes survival in NGF-deprived sympathetic neurons (Tammariello *et al.*, 2000). Cu/ZnSOD protein does not, however, delay apoptosis if injected after 8 hours, suggesting that ROS are involved as an early signalling event rather than just directly causing cellular damage (Greenlund *et al.*, 1995a). Furthermore, in other mammalian cells ROS have been shown to activate JNK (Lo *et al.*, 1996). How ROS are connected, if at all, to the downstream kinase cascade or gene expression remains to be elucidated.

Since the components of the upstream kinase pathway leading to the activation of c-Jun have been studied, I next investigated the possible targets of c-Jun transactivation. Several potential c-Jun target genes have been identified to date. The most likely candidate for a target death gene so far is *fas ligand.* Recent evidence suggests that the *fas ligand* gene, which contains an AP-1 site within its promoter (Faris *et al.*, 1998b; Kasibhatla *et al.*, 1998), may be a target of c-Jun transactivation in differentiated PC12 cells undergoing apoptosis induced by NGF withdrawal (Le-Niculescu *et al.*, 1999). However, I did not find that FasL protein was induced in sympathetic neurons after NGF withdrawal in immunoblotting experiments (data not shown). Determining how c-Jun is connected to the general cell death machinery, such as members of the Bcl-2 family, cytochrome c release from the mitochondria, and caspase activity, is crucial to understanding the role of c-Jun during cell death.

To investigate the function of c-Jun in neurons undergoing apoptosis, an adenoviral gene delivery system was developed. Previously, I had performed microinjection experiments with sympathetic neurons, but experiments were limited to working at the single cell level. Adenoviruses had previously been used successfully to transduce whole populations of cells, including sympathetic neurons both *in vitro* and *in vivo* (Le Gal La Salle *et al.*, 1993; Slack *et al.*, 1996). Thus, recombinant adenoviruses that expressed dominant negative c-Jun and the constitutively active form of MEKK1 were generated. To characterise the viruses, I demonstrated that infection of

neurons with viruses that expressed dominant negative c-Jun or Bcl-2 could promote survival in the absence of NGF, compared to a β-galactosidase-expressing control virus. In addition, expression of MEKK1 by viral infection induced apoptosis, while viruses expressing FLAGΔ169, Bcl-2, or β-galactosidase had no significant toxicity in the presence of NGF. To examine whether c-Jun functions upstream of cytochrome c release, a critical event during the death of sympathetic neurons, cells were infected with the dominant negative c-Jun adenovirus and then withdrawn from NGF. Expression of FLAGΔ169 or Bcl-2 blocked the release of cytochrome c from mitochondria and thereby prevented cell death. Adenoviral-mediated expression of MEKK1 induced the redistribution of cytochrome c into the cytoplasm in the presence of NGF. These results indicate that c-Jun functions upstream of cytochrome c release in the death pathway in sympathetic neurons. Consistent with this, it has recently been shown that cytochrome c release is prevented in *Jnk1* (–/–) *Jnk2* (–/–) murine embryo fibroblasts in response to UV radiation (Tournier *et al.*, 2000).

Bcl-2 family proteins regulate the release of cytochrome c. Thus, to identify a more direct transcriptional target of c-Jun, I examined the pattern of expression of pro-apoptotic Bcl-2 proteins in sympathetic neurons deprived of NGF. The levels of Bid, Bak, Bax, and Bad did not alter after NGF withdrawal. In contrast, the level of Bim protein increased significantly after NGF deprivation, with a maximum induction at approximately 16 hours. In addition, the level of *bim* mRNA increased after NGF deprivation, with a peak at 16 hours. Interestingly, expression of FLAGΔ169 by infection with a recombinant adenovirus substantially inhibited the induction of *bim* mRNA after NGF withdrawal, suggesting that AP-1 activity is important for the increase in *bim*. Therefore, a possible target of c-Jun transactivation that can induce the release of cytochrome c has been identified. This provides a link between the JNK/c-Jun pathway and the general death machinery of cytochrome c/Apaf-1 and the caspases. To further support this hypothesis, it would be interesting to determine whether MEKK1 expression could induce expression of the *bim* gene. The discovery of a putative target gene, such as *bim*, suggests many experiments to confirm its activation by c-Jun. These include cloning of the promoter and looking for possible c-Jun binding sites, and then determining whether c-Jun, and perhaps ATF-2 or other AP-1 proteins, actually bind to these sites during NGF withdrawal-induced death. Regulation of a *bim* reporter gene in response to NGF withdrawal or overexpression of MEKK1 could also be examined. To confirm the role of Bim in sympathetic neurons, anti-Bim antibodies

could be microinjected or neurons from *bim* (–/–) mice cultured (or their numbers determined *in vivo* during development). Bim is a relatively new Bcl-2 family member, and its involvement in any form of neuronal cell death has not yet been reported.

Of course it is still possible that there are other c-Jun target genes that promote cell death, and it would be interesting either to investigate other pro-apoptotic members of the Bcl-2 family, or perhaps search for unknown genes using differential display or cDNA arrays. Indeed, the pro-apoptotic Bcl-2 family member DP5 was identified because its mRNA was transcriptionally upregulated in sympathetic neurons deprived of NGF (Imaizumi *et al.*, 1997), and this is therefore a potential candidate for a c-Jun target gene.

Much is now known about the regulation and role of c-Jun in sympathetic neurons undergoing apoptosis. c-Jun phosphorylation also plays a critical role in cerebellar granule neurons induced to undergo apoptosis by withdrawing the survival signal (Watson *et al.*, 1998). In addition, antisense oligonucleotides that inhibited c-Jun expression promoted survival in cultured hippocampal neurons (Schlingensiepen *et al.*, 1994). Interestingly, there is an accumulating body of *in vivo* evidence showing that c-Jun immunoreactivity colocalises with degenerating neurons in ischaemic areas, in neurons with neurofibrillary tangles (a characteristic of Alzheimer's disease), in the rat cerebellum after exposure to radiation, and in the spinal cord from patients with amyotrophic lateral sclerosis (Herdegen and Leah, 1998). Moreover, a transgenic mouse expressing a mutant form of c-Jun that cannot be phosphorylated was protected from kainate-induced apoptotic cell death in the hippocampus (Behrens *et al.*, 1999). These results suggest that c-Jun may have a causal role in the initiation of apoptosis in neurodegenerative diseases. It would be interesting to investigate the expression of Bim in such cases. The recombinant adenovirus expressing FLAGΔ169 could be used both *in vitro* and *in vivo* to directly examine the role of c-Jun in neurodegenerations. Manipulation of c-Jun activity and the function of target genes, such as Bim, may have therapeutic benefit.

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Publications associated with this thesis

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