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Studies on the Bcl-2 Family of Apoptosis Regulators in the Nervous System

BY

SUSANNE HAMNÉR

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ABSTRACT

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Apoptosis is a type of cell death with a specific morphology and molecular program, which is essential for the development of the nervous system. However, inappropriate cell death has been implicated in several neurodegenerative diseases. The Bcl-2 protein family is a class of proteins, which can regulate the cell death program in either a positive (pro-apoptotic family members) or a negative (anti-apoptotic family members) way.

This thesis further elucidates the role of Bcl-2 family members in the nervous system. Special focus has been put on the anti-apoptotic family member Bcl-w, whose function in the nervous system was previously unknown, and the pro-apoptotic family member Bad which serves as a link between growth factor signalling and apoptosis.

Bcl-w mRNA was found to be upregulated during rat brain development suggesting increasing importance of Bcl-w with age in the nervous system. In contrast, mRNA levels encoding the anti-apoptotic protein Bcl-x were downregulated during development. Bcl-w was also found to have an anti-apoptotic function in neurons, rescuing sympathetic neurons from cell death after nerve growth factor deprivation.

To further elucidate the mechanism by which Bcl-w exerts its function, we screened a yeast two-hybrid library for proteins interacting with Bcl-w. Two of the isolated positive clones encoded the pro-apoptotic protein Bad and a novel splice variant of Bad with a different carboxyterminal sequence. Both isoforms of Bad induced cell death in sympathetic neurons, which could be counteracted by Bcl-w, indicating that Bcl-w and Bad can interact both physically and functionally.

Further studies on the genomic structure of the Bad gene suggested the presence of an additional splice variant, not expressing the first exon. Immunohistochemical analysis indicates that the isoform(s) not expressing the first exon is more widely expressed in adult rat brain than the known forms.

Finally, we show that high cell density can enhance survival of cerebellar granule neurons and that bcl-2 and bcl-x mRNA levels are upregulated in high density cultures.

Key words: Bcl-2, Bcl-w, Bad, Apoptosis, Nervous system, Cerebellar granule cells, Sympathetic neurons.

Susanne Hamnér, Department of Neuroscience, Unit of Neurobiology, Box 587, BMC, SE-751 23 Uppsala, Sweden

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The only thing you *have*
to do is to die once

This thesis is based on the following papers referred to in the text by their Roman numerals:

I: Ogha, Y., Zirrigiebel, U., **Hamnér, S.**, Michaelidis, T.M., Cooper, J., Thoenen, H. and Lindholm, D. (1996) Cell density increases bcl-2 and bcl-x expression in addition to survival of cultured cerebellar granule neurons. *Neuroscience* 73: 913-917

II: Hamnér, S., Skoglösa, Y. and Lindholm, D.(1999) Differential expression of bcl-w and bcl-x messenger RNA in the developing and adult rat brain. *Neuroscience* 91: 673-684.

III: Hamnér, **S.**, Arumäe, U., Li-Ying Y., Sun, Y-F., Saarma, M. and Lindholm, D. (2000) Functional characterization of two splice variants of rat Bad and their interaction with Bcl-w in sympathetic neurons. *Molecular and Cellular Neuroscience* In press

IV: Hamnér, S. and Lindholm, D. Immunohistochemical localisation of Bad in the neonatal and adult rat brain. Manuscript

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ABBREVIATIONS

aa: Amino acids
ALS: Amyotrophic lateral sclerosis
ANT: adenine nucleotide translocator
Bcl-2: B-cell lymphoma/leukaemia 2
BDNF: Brain derived neurotrophic factor
bp: Basepairs
CAD: Caspase activated DNase
CARD: Caspase recruitment domain
ced: cell death abnormal
ces: cell death specific
C. elegans: Caenorhabditis elegans
CNS: Central nervous system
CrmA: Cytokine response modifier A
DD: Death domain
DED: Death effector domain
DRG: Dorsal root ganglion
$\Delta\Psi m$: Mitochondrial membrane potential
E: Embryonic day
ECM: Extracellular matrix
egl: egg laying defective
ERK: Extracellular signal-regulated kinase
FAK: Focal adhesion kinase
gf: gain-of-function
GSHPx: Gluthatione peroxidase
HMW: High molecular weight
HSN: Hermaphrodite specific neurons
IAP: Inhibitor of apoptosis protein
ICAD: Inhibitor of CAD
IGF-1: Insulin-like growth factor
IKK: Inhibitor of NFκB kinase
lf: loss-of-function
MAPK: Mitogen activated protein kinase
MEK: MAPK/ERK kinase
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NFκB: Nuclear factor κB
NGF: Nerve growth factor
nt: nucleotide(s)
P: Postnatal day
PCR: Polymerase chain reaction
PI3-K: Phosphatidylinositol 3-kinase
PKA: cAMP-dependent protein kinase
PKB: Protein kinase B
PKC: Ca^{2+} dependent protein kinase
PNS: Peripheral nervous system
PTPC: Permebility transition pore complex
ROS: Reactive oxygen species
SCG: Superior cervical ganglion
SMA: Spinal muscular atrophy
SMN: Survival motor neurons
TM: Transmembrane
VDAC: Voltage dependent anion chan

INTRODUCTION

Apoptotic mechanisms

Types of cell death

Multicellular organisms have a requirement to adjust their cell number in different tissues to establish and maintain proper function and morphology. This is accomplished by mitosis to increase the cell number and cell death to decrease the number. Many diseases are caused by either excess of cells, such as cancer and autoimmune diseases or by inappropriate cell loss in for example neurodegenerative diseases and AIDS. The controlled type of cell death that occurs during development but also in some pathological events, is in many cases performed by a distinct molecular mechanism and characterised by a particular morphology first described by (Kerr *et al*., 1972) who mounted the term apoptosis. Αποπτωσισ in Greek means "falling off" in terms of leaves from trees, and this natural phenomenon can be seen as a symbol for the controlled clearance of cells during apoptosis. To avoid inflammation, apoptotic cells shrink and are neatly divided into small membrane-enclosed vesicles called apoptotic bodies which can be readily phagocytosed by neighbouring cells. The more chaotic type of cell death, which in many cases is involved after external injury to the cell, is called necrosis. Contrary to apoptosis, necrosis causes cell swelling and ultimately bursting, resulting in increased membrane permeability, release of intracellular components and ultimately to inflammation.

In apoptotic cell death, not only the cytoplasm, but also the nucleus is condensed and is later fragmented, while other organelles remain intact (Kerr *et al*., 1972). Another hallmark for apoptosis is the chromatin condensation and DNA fragmentation into internucleosomal fragments of multiples of approximately 180 basepairs (Wyllie, 1980; Wyllie *et al*., 1984) and/or high molecular weight fragments (HMW) of 50 or 300 kilobasepairs (Oberhammer *et al*., 1993). Yet another characteristic is the externalisation of the membrane lipid phosphatidylserine, a process that appears to be crucial for the phagocytosis of the apoptotic cell (Fadok *et al*., 1992; Martin *et al*., 1995). Apoptosis is usually an active process that requires ATP and is in many cases dependent on mRNA and protein synthesis (Wyllie *et al*., 1984), whereas necrosis is passive. The differences between apoptosis and necrosis is summarised in Table 1. In addition to these morphological and biochemical characteristics, apoptosis utilises a special molecular program, which is described below. However, some of the proteins involved in this program are also involved in necrosis. It should be noted that the border between apoptosis and necrosis is not always clear and in some cases the dying cells do not exhibit all features characteristic of one or the other.

Apoptotic genes in *Caenorhabditis elegans*

The nematode *Caenorhabditis (C.) elegans* is an excellent model organism for studies of cell death, since exactly 1090 cells are produced, of which 131 undergo programmed cell death during development in an apoptotic manner (Sulston *et al*., 1983). Screening for *C. elegans* mutants with abnormal cell death lead to the identification of four main genes that directly regulate the death pathway. These genes were named ced-3, ced-4, ced-9 and egl-1 for cell death abnormal and egg-laying defective respectively (Ellis and Horvitz, 1986;

Apoptosis	Necrosis
Physiological or pathological	Pathological
Cells shrinkage and formation of apoptotic bodies	Cell swelling and bursting
No change in plasma membrane permeability	Increased plasma membrane permeability
Organelles intact	Organelle destruction
Nuclear condensation and fragmentation	
DNA fragmentation in intranucleosomal fragments	Random DNA fragmentation
Externalisation of phosphatidylserine	
Phagocytosis, no inflammation	Release of intracellular content, inflammation response
Active process, often requires RNA and protein synthesis	Passive process

Table 1: Characteristic features for apoptosis and necrosis

Hengartner *et al*., 1992; Trent *et al*., 1983). Ced-3 and Ced-4 are essential for the programmed cell death to occur since loss-of-function (lf) of these genes resulted in an almost complete block of developmental cell death (Ellis and Horvitz, 1986). Ced-9 on the other hand inhibits cell death, since lf mutations of ced-9 exhibit ectopic death of cells normally not undergoing programmed cell death and result in embryonic lethality (Hengartner *et al*., 1992). Gain-of-function (gf) mutations of egl-1 result in a defect in egglaying, caused by the ectopic cell death of hermaphrodite specific neurons (HSN), whereas lf mutations, in analogy with Ced-3 and Ced-4, blocked the developmental cell death of most somatic cells (Conradt and Horvitz, 1998; Trent *et al*., 1983).

The ectopic cell death induced in ced-9 (lf) mutants can be completely blocked by the additional (lf) mutations of ced-3 and ced-4 (Hengartner *et al*., 1992). This suggests that Ced-9 acts upstream of Ced-3 and Ced-4. Mutations in egl-1, however, cannot rescue the cell death seen in Ced-9 mutants and therefore appear to act upstream of Ced-9 (Conradt and Horvitz, 1998). Overexpression of Ced-3 can induce cell death in the absence of Ced-4, although to a lesser extent, whereas overexpression of Ced-4 can induce very little cell death in ced-3 loss-of-function mutants, suggesting that Ced-4 potentiates the killing activity of Ced-3 and is upstream of Ced-3. Furthermore, Ced-9 can inhibit Ced-4 induced cell death directly, but can only inhibit Ced-3 if a functional Ced-4 is present (Shaham and Horvitz, 1996). In summary, the order of the cell death program in *C. elegans* is delineated in Fig. 1.

An indication of how the ced genes function came with the discovery that Ced-9 interacts physically with Ced-4 (Chinnaiyan *et al*., 1997; Spector *et al*., 1997; Wu *et al*., 1997). Ced-4 was also found to interact with Ced-3, thereby acting as a biochemical linker between Ced-9 and Ced-3 (Chinnaiyan *et al*., 1997). Egl-1 also binds to Ced-9, thereby displacing Ced-4 and releasing Ced-4 to perform its apoptotic function (Conradt and Horvitz, 1998; del Peso *et al*.,

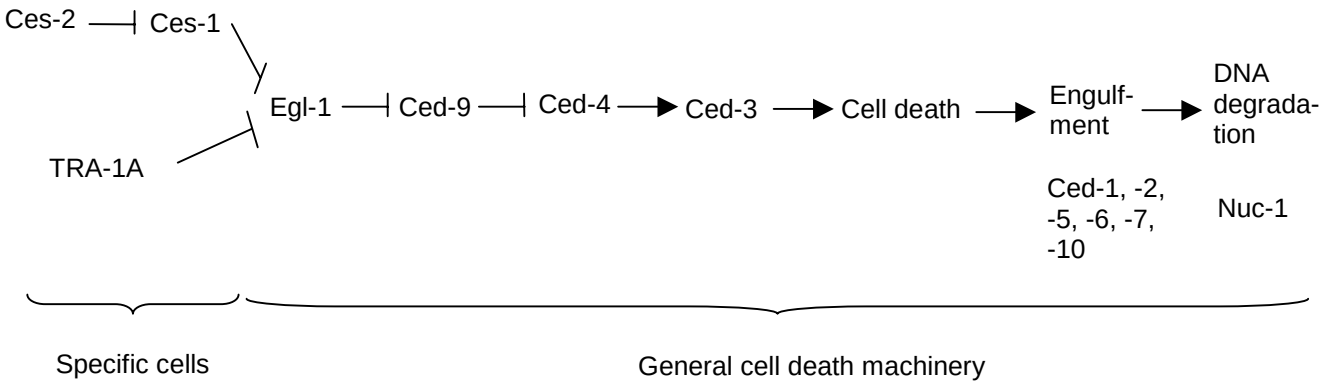

Figure 1: The molecular order of the cell death program in *C.elegans*

1998). Ced-4 was found to induce the processing of Ced-3 into an active molecule probably by inducing Ced-3 oligomerisation (Yang *et al*., 1998b).

Cell-specific transcription factors appear to regulate the onset of the apoptotic program. This was shown for NSM sister cells, where cell death is controlled by the genes ces-1 and ces-2 (Ellis and Horvitz, 1991) and in HSN neurons where TRA-1A represses egl-1 transcription (Conradt and Horvitz, 1999). In addition, several genes required for the engulfment of cell corpses, (ced-1, -2, -5, -6, -7 and -10) and for DNA degradation (Nuc-1) have been identified (Ellis and Horvitz, 1991; Hedgecock *et al*., 1983).

Mammalian homologues

Programmed cell death is an evolutionary conserved mechanism and all essential genes in the executionary phase of C. elegans cell death have homologues in mammalian cells.

Ced-3 was found to be homologous to the interleukin-1β-converting enzyme (ICE) (Yuan *et al*., 1993) and subsequently several other homologous proteases have been discovered (see below) and now constitute a family of at least 14 so-called caspases, for cysteine proteases cleaving at aspartate residues (Alnemri *et al*., 1996; Wolf and Green, 1999). The caspase can be subdivided into initiating caspases (e.g. caspase-9), which can cleave and activate more downstream executionary caspases (e.g caspase-3).

Ced-4 is homologous to the middle part of the mammalian protein Apaf-1, which like Ced-4 can activate caspases [Zou, 1997 #1005].

The Ced-9 protein was also revealed to have a whole family of mammalian homologues, the founding member being the proto-oncogene Bcl-2 (Hengartner and Horvitz, 1994). The Bcl-2 family consists of both anti- and pro-apoptotic members and Egl-1 was found to be homologous to the so-called BH3-only pro-apoptotic members (see below)(Conradt and Horvitz, 1998).

The mechanisms and interactions between the cell death genes appears to be conserved in mammals with the exception that the anti-apoptotic Bcl-2 family members do not seem to bind and inhibit Apaf-1 directly (Moriishi *et al.*, 1999; Hausmann *et al.* 2000; Newmeyer *et al.*, 2000). Instead, at least part of their function is to inhibit the release of the mitochondrial intermembrane space protein cytochrome *c* into the cytoplasm. Once in the cytoplasm, cytochrome *c* interacts with Apaf-1 and induces its pro-apoptotic activity.

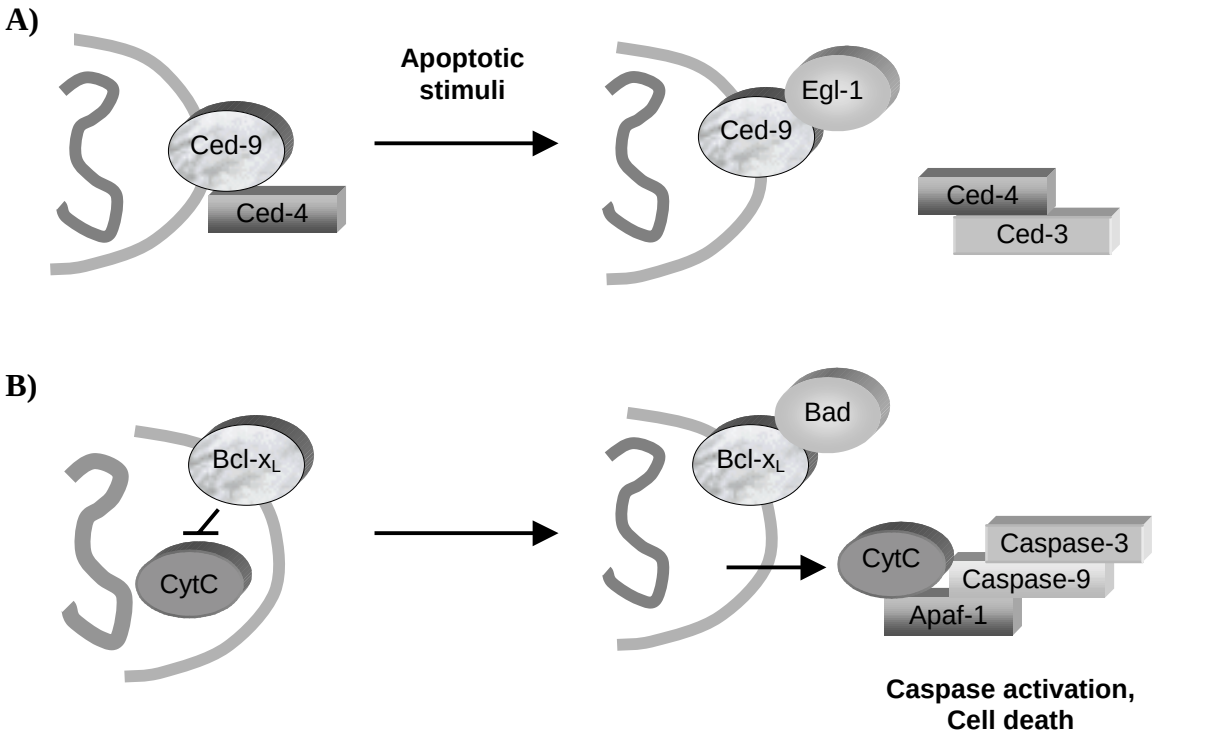

Figure 2: Apoptotic pathways in C.elegans (A) and mammals (B)

The Bcl-2 family

The Bcl-2 family members can be divided into three major groups, the anti-apoptotic members, the pro-apoptotic members and the so-called BH3-only members that also are pro-apoptotic. The sequence similarities between the members are confined to four Bcl-2 homology (BH) domains (Chittenden *et al*., 1995a; Yin *et al*., 1994) (Fig. 3). The anti-apoptotic subfamily, including Bcl-2 (Tsujimoto *et al*., 1984), Bcl-x_L (Boise *et al*., 1993) and Bcl-w (Gibson *et al*., 1996), contain all four of the BH domains. The pro-apoptotic Bcl-2 family members include Bax (Oltvai *et al*., 1993), Bak (Chittenden *et al*., 1995b; Farrow *et al*., 1995; Kiefer *et al*., 1995) and Bok (Hsu *et al*., 1997) and consist of the BH1, BH2 and BH3 domains (Fig. 3).

The region most important for the pro-apoptotic activity seems to be the BH3 domain since this region alone can induce apoptosis (Chittenden *et al*., 1995a; Cosulich *et al*., 1997; Holinger *et al*., 1999). This is also supported by the fact that the BH3-only family members, e.g. Bad (Yang *et al*., 1995), Bid (Wang *et al*., 1996b) and Bim (O'Connor *et al*., 1998), are pro-apoptotic despite their lack of homology to the other Bcl-2 family members outside the BH3 domain. However, recent studies have shown that the BH3 domain is not always essential for inducing cell death (Hsu and Hsueh, 1998; Ray *et al*., 2000).

The BH1, BH2 and BH4 domains are important for the protective function of the antiapoptotic members (Borner *et al*., 1994b; Huang *et al*., 1998; Yin *et al*., 1994). These are also the regions required for heterodimerising with the pro-apoptotic protein Bax (Hirotani *et al*., 1999; Yin *et al*., 1994). The N-terminal part of Bcl-2 and Bcl-x_L, including the BH4 domain can be cleaved off by caspases, whereby they are converted to pro-apoptotic molecules, showing the importance of BH4 in anti-apoptotic function (Cheng *et al*., 1997; Clem *et al*., 1998; Kirsch *et al*., 1999). The importance of the BH1 and BH2 for cell death

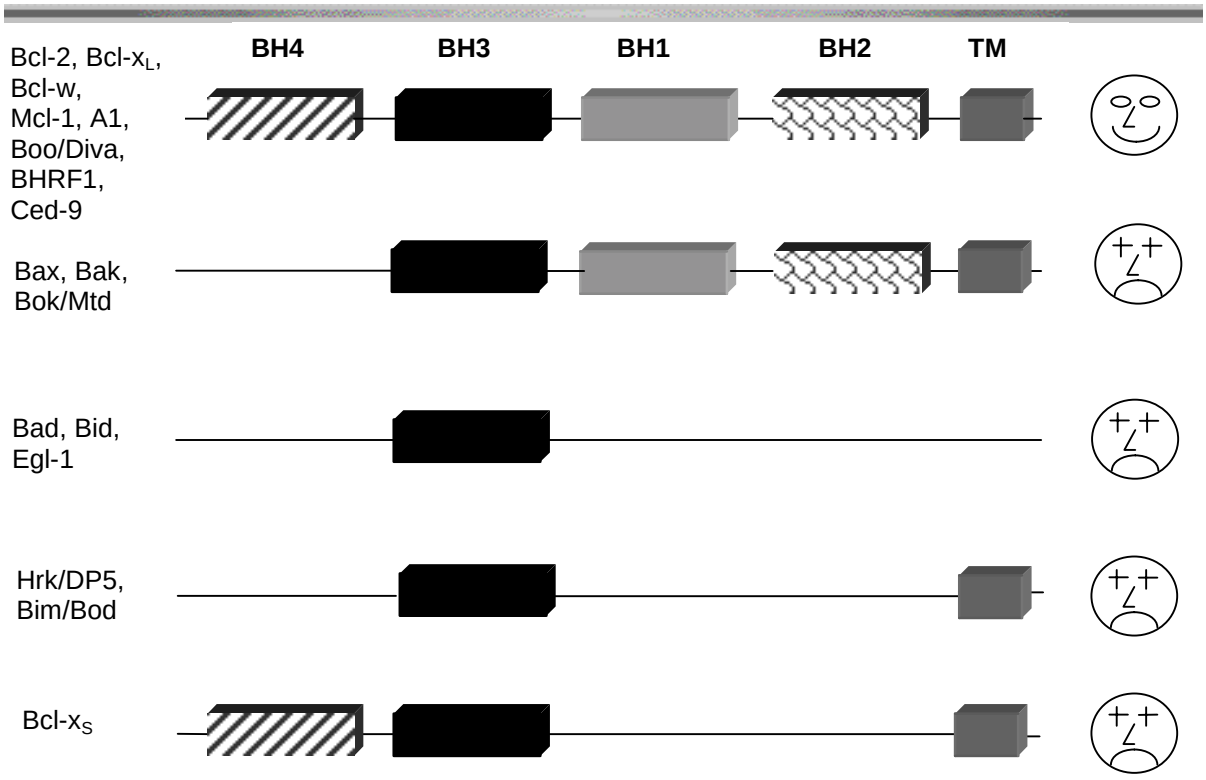

Figure 3: The structure of the Bcl-2 family members

inhibition is shown for example by the fact that one of the splice variants of Bcl-x, Bcl-x_S, which lacks the BH1 and BH2 domains, show a pro-apoptotic effect, perhaps acting as a competitor for Bcl-x_L and Bcl-2 (Boise *et al*., 1993). In addition, mutations of conserved residues in BH1 or BH2 abrogates the protective function of Bcl-2 (Yin *et al*., 1994).

Many of the Bcl-2 family members possess a carboxyterminal transmembrane domain that enables them to insert into membranes. Accordingly, these family members often localise to the mitochondria or to other intracellular membranes (Gonzalez *et al*., 1995; Hockenberry *et al*., 1990). The transmembrane domain is however not essential for the protective effect of Bcl-2 or Bcl-x, since deletion of this domain from Bcl-2 does not alter Bcl-2's protective capacity considerably (Borner *et al*., 1994a; Hockenberry *et al*., 1993). In addition, the splice variant Bcl-xβ that lacks the transmembrane domain is still able to confer protection in some models (Gonzalez *et al*., 1995).

The Bcl-2 family members can form homo- or hetero-dimers with each other and the ratio between pro-apoptotic and anti-apoptotic family members appear to be important for the regulation of the death process (Oltvai *et al*., 1993; Yang *et al*., 1995). The solution structure of the Bcl-x_L-Bak heterodimer revealed that this dimerisation is accomplished by the BH1, BH2 and BH3 of the anti-apoptotic family member and the BH3 domain of the pro-apoptotic member (Sattler *et al*., 1997). Although the ability for the anti-apoptotic members to heterodimerise with pro-apoptotic members in many cases correlates with their protective function (Chittenden *et al*., 1995a; Yin *et al*., 1994), this is not always the case. Bcl-x_L mutants that are unable to bind Bax can still inhibit cell death (Cheng *et al*., 1996) and A1 can protect against Bad- or Bax-induced cell death even if it can't bind these proteins. In addition, Bcl-w and A1 cannot protect against apoptosis induced by Bak or Bik even if they can heterodimerise with these proteins (Holmgreen *et al*., 1999). Recent data also suggest that dimerisations might not be as abundant as earlier studies have suggested. For example Bcl-2

does not appear to form homodimers, whereas Bax-Bcl-2 heterodimers and Bax homodimers or even larger oligomers form readily (Conus *et al*., 2000).

Apart from their binding to other Bcl-2 family members, Bcl-2 and/or Bcl-x_L has been shown to interact with a number of proteins. These include the protein kinase Raf1 that can be targeted to the mitochondria by Bcl-2 (Wang *et al*., 1994; Wang *et al*., 1996a); the heatshock protein regulator Bag-1 (Takayama *et al*., 1997; Takayama *et al*., 1995); the death domain containing MRIT (FLIP/Casper) which also associate with the death receptor complex (Han *et al*., 1997); the permeability transition pore complex (PTPC) component adenine nucleotide translocator (ANT) (Marzo *et al*., 1998a); SMN, a gene mutated in spinal muscular atrophy (Iwahashi *et al*., 1997); and the presenilins, two proteins involved in Alzheimers disease (Alberici *et al*., 1999; Passer *et al*., 1999). Many of these interactions are dependent on the BH4 domain and consequently pro-apoptotic family members rarely bind these proteins.

The Bcl-2 family members appear to perform their function by several mechanisms. Bcl-2 has an anti-oxidant function, reducing the damage from reactive oxygen species (ROS) formed after many apoptotic stimuli (Hockenberry *et al*., 1993; Kane *et al*., 1993). However, Bcl-2 can also protect against apoptosis in hypoxia where no ROSs are produced (Jacobson and Raff, 1995; Tsujimoto *et al*., 1997). Another perhaps more important function, is the inhibition of cytochrome *c* release from mitochondria, shown for Bcl-2, Bcl-x and Bcl-w (Kluck et al., 1997; Yang et al., 1997; Kharbanda *et al*., 1997; Yan *et al*., 2000). In contrast, the pro-apoptotic family member Bax, promotes release of cytochrome *c* in the absence of other death stimuli and this release can be blocked by Bcl-x_L (Myers *et al*.1998, Jürgensmeier *et al*., 1998).

Bcl-2 and Bcl-x_L can, however, also block cell death downstream of cytochrome *c* release since it also protects cells against injection of cytochrome *c* (Brustugun *et al*., 1998) (Li *et al*., 1997a; Zhivotovsky *et al*., 1998), and after Bax-induced cytochrome *c* release (Rosse *et al*., 1998). How this rescuing effect is accomplished is not known, but in *C. elegans* Ced-9 can directly inhibit Ced-3 by acting as a substrate for this protease (Xue and Horvitz, 1997), raising the possibility that Bcl-2 and Bcl-x may act in a similar fashion. Pro-apoptotic family members, or at least BH3 peptides, can also induce caspase activation and apoptosis independent of cytochrome *c* release (Holinger *et al*., 1999).

Apart from their effect on cytochrome c release, Bcl-2, Bcl-x_L and Bcl-w have been shown to protect against loss of mitochondrial membrane potential ($\Delta\Psi m$) (Vander Heiden and Thompson, 1999; Yan *et al*., 2000; Yang *et al*., 1997; Zamzami *et al*., 1996). Bax, on the other hand, can promote reduction of membrane potential in Jurkat cells (Pastorino *et al*., 1998; Xiang *et al*., 1996), although other investigators using a cell-free system have shown that this is not a direct effect (Myers *et al*. 1998, Jürgensmeier *et al*, 1998). The mechanism of how the Bcl-2 family members regulate mitochondrial membrane potential is not clear but one possibility is that they act as ion channels. Indeed, the determination of Bcl-x_L's three-dimensional structure revealed a structure similar to that of the membrane translocation domain of diphteria toxin, which works as a pore (Muchmore *et al*., 1996). Furthermore, Bcl-x_L, Bcl-2 and Bax can form cation-specific channels in synthetic membranes (Antonsson *et al*., 1997; Minn *et al*., 1997; Schendel *et al*., 1997). While the Bcl-2 channel only conduct at acidic pH, the Bax channel can also function at physiological pH and this conductance can be inhibited by Bcl-2 (Antonsson *et al*., 1997). The Bax channel appears to consist of an oligomer of at least six Bax molecules, whereas the monomeric Bax does not exhibit any channel activity (Antonsson *et al*., 2000).

The BH3-only family members are usually cytoplasmic and appear to act like "ligands", binding to other anti- or pro-apoptotic family members and thereby regulating their activity (Desagher *et al*., 1999; Yang *et al*., 1995). Surprisingly however, the solution structure of the BH3-only member Bid is remarkably similar to that of Bcl-x, despite their overall lack of

primary structure homology (Chou *et al*., 1999) and Bid was also shown to have an ion channel activity (Schendel *et al*., 1999).

Bcl-2

Bcl-2 (B-cell lymphoma/leukaemia 2) was originally cloned from the chromosomal breakpoint in the t(14;18) translocation in pre-B-cell leukaemia cells. The bcl-2 gene is, in these cells and in additional B-cell malignancies, translocated from its normal position on chromosome 14 to the immunoglobulin heavy chain locus on chromosome 18, resulting in dysregulated expression (Cleary *et al*., 1986; Tsujimoto *et al*., 1984). Bcl-2 was found to be a proto-oncogene that inhibited cell death rather than promoted cell proliferation (Hockenberry *et al*., 1990; Vaux *et al*., 1988). The Bcl-2 protein possesses all four BH domains and has a transmembrane (TM) domain in its carboxyterminal end. This TM domain renders Bcl-2 to localise primarily to the mitochondrial membrane but also to the nuclear envelope and endoplasmic reticulum (Hockenberry *et al*., 1990; Krajewski *et al*., 1993). Between the BH4 and BH3, a loop domain is present containing a number of serine and threonine residues that can be phosophorylated by several kinases including members of the Jun-N-terminal kinase (JNK) class of kinases. Phosphorylation of these residues inactivates Bcl-2's protective function (Haldar *et al*., 1995; Maundrell *et al*., 1997; Yamamoto *et al*., 1999).

Bcl-2 has been shown to be alternatively spliced into Bcl-2α and Bcl-2β (Tsujimoto and Croce, 1986). The Bcl-2β form lacks the carboxyterminal part including the transmembrane domain and does not exhibit any protective function (Tanaka *et al*., 1993). As discussed above however, it is not the transmembrane domain of Bcl-2 that is essential for protection since deleting only the transmembrane domain do not alter the protective function considerably (Borner *et al*., 1994a; Borner *et al*., 1994b; Hockenberry *et al*., 1993).

Bcl-2 protein is expressed in many haematopoietic cells, but also in other tissues, like kidney, prostate, pancreas, intestine, lung, peripheral and central nervous system, hair follicles and skin (Hockenbery *et al*., 1991; LeBrun *et al*., 1993; Novack and Korsmeyer, 1994). In the nervous system, bcl-2 mRNA and protein are expressed in many structures during embryonic and postnatal development, including the cortex, cerebellum, hippocampus, spinal cord and olfactory bulb. Most of these structures show decreased expression of bcl-2 in adult, but the expression is retained at high levels in peripheral ganglia such as superior cervical ganglia (SCG) and dorsal root ganglia (DRG)(Castrén *et al*., 1994; Merry *et al*., 1994).

Bcl-2 protects cells from a wide range of apoptotic stimuli including growth factor or serum withdrawal (Garcia *et al*., 1992; Vaux *et al*., 1988; Zhong *et al*., 1993), radiation, glucocorticoid treatment (Sentman *et al*., 1991; Strasser *et al*., 1991), several chemotherapeutic drugs (Miyashita and Reed, 1993), excitotoxicity (Behl *et al*., 1993), oxidative stress (Hockenberry *et al*., 1993; Kane *et al*., 1993; Zhong *et al*., 1993) and Ca^{2+} ionophores (Lam *et al*., 1994; Strasser *et al*., 1991; Zhong *et al*., 1993). In addition to apoptosis, necrotic cell death can in some cases be blocked by Bcl-2 (Kane *et al*., 1995; Tsujimoto *et al*., 1997). Bcl-2 is not able to block all cell death, however, since its for example do not block the negative selection of thymocytes (Sentman *et al*., 1991) and does not inhibit cell death of ciliary neurotrophic factor-deprived ciliary neurons (Allsopp *et al*., 1993) or Fas-induced cell death in lymphocytes (Strasser *et al*., 1995). Studies on transgenic mice overexpressing Bcl-2 show that Bcl-2 also can inhibit naturally occurring cell death in the nervous system, resulting in a larger brain and an excess of cells in the facial nucleus and the retina (Martinou *et al*., 1994). These animals are also less sensitive to ischaemia (Martinou *et al*., 1994) and to facial, sciatic and optic nerve axotomy (Dubois-Dauphin et al., 1994; Farlie et al., 1995; Bonfanti *et al*., 1996).

Mice deficient in bcl-2 appear normal at birth but their postnatal growth is retarded and they die at around 2-3 weeks. Other characteristics are the appearance of polycystic kidneys, small ears, hypopigmented hair and excessive apoptosis in the thymus and spleen (Veis *et al*., 1993). There is also excessive loss of trigeminal and nodose sensory neurons during the period of physiological cell death in bcl-2 deficient mice (Pinon *et al*., 1997). In contrast, this period passes normally in the facial nucleus, SCG and DRG, but later in postnatal development also these neurons degenerate in the Bcl-2 knock-out (Michaelidis *et al*., 1996).

Apart from its anti-apoptotic function, Bcl-2 appears to play a role in differentiation and maturation. Bcl-2 can induce differentiation of a neural cell line (Zhang *et al*., 1996) and can promote regenerations of retinal axons (Chen *et al*., 1997). In addition, Bcl-2 deficient mice show delayed maturation of trigeminal sensory neurons (Middleton *et al*., 1998), whereas Bcl-2 transgenic mice show increased thymocyte maturation (Sentman *et al*., 1991) Bcl-2 also blocks cell cycle entry, reflecting the fact that failed attempts to entry the cell cycle often results in cell death (Mazel *et al*., 1996; O'Reilly *et al*., 1996).

Interestingly, Bcl-2 can also potentiate cell death in certain circumstances. This was shown for enediyne-induced apoptosis, which require a high reducing potential for maximal activity (Cortazzo and Schor, 1996) and in retinal glial Müller cells in Bcl-2 transgenic mice (Dubois-Dauphin *et al*., 2000).

Bcl-x

Bcl-x was cloned by a low stringency hybridisation using bcl-2 as a probe and was found to be alternatively spliced into Bcl-x_L (for long), Bcl-x_S (for short) (Boise *et al*., 1993) and Bcl-xβ (Gonzalez *et al*., 1994). Bcl-x_S lacks the BH1 and BH2 domains while Bcl-xβ lacks the carboxyterminal transmembrane domain. Like Bcl-2, Bcl-x_L protects cells from cell death after various apoptotic stimuli, whereas Bcl-x_S exhibits a pro-apoptotic function, inhibiting the survival promoting activity of Bcl-2 (Boise *et al*., 1993; Gonzalez *et al*., 1995). Bcl-xβ appears to have an anti-apoptotic function in some systems and pro-apoptotic in others (Gonzalez *et al*., 1995; Shiraiwa *et al*., 1996). Consistent with the similar primary structure between Bcl-2 and Bcl-x_L the latter also localise to mitochondria (Gonzalez *et al*., 1995) and to nuclear and microsomal fractions (Frankowski *et al*., 1995).

Transgenic mice overexpressing Bcl-x_L under the lck promotor in the immune system exhibit a phenotype nearly indistinguishable from that of the Bcl-2 transgene under the same promotor, including protection of thymocytes against γ-irradiation and glucocorticoid treatment and an increase in thymocyte maturation (Chao *et al*., 1995). When overexpressed under a neuronspecific promotor, Bcl-x_L rescues cells after facial nerve axotomy and hypoxia-ischaemia, but did not block the naturally occurring cell death in for example the facial nucleus (Parsadanian *et al*., 1998). In contrast to Bcl-2, Bcl-x deficient mice die during embryonic development, due to massive apoptosis of postmitotic neurons and haematopoietic cells in the liver (Motoyama *et al*., 1995). The excessive embryonic neuronal cell death, but not the embryonic lethality, can be blocked if these mice are also deficient in Bax (Schindler *et al*., 1997).

Bcl-x_L mRNA and protein are highly expressed in many embryonic and adult tissues including brain, liver, thymus and bone marrow (Gonzalez *et al*., 1994; Krajewski *et al*., 1994b). In the brain, mRNA levels of Bcl-x have been shown by some investigators to be maintained at a high level in the adult, in contrast to Bcl-2 (Frankowski *et al*., 1995; Gonzalez *et al*., 1995), but see also result section). On the protein level, there is a discrepancy between different studies of the Bcl-x expression pattern in the brain, with some investigators showing decreased Bcl-x protein levels (Alonso *et al*., 1997; Mizuguchi *et al*., 1996), while others

show unchanged levels (Shimohama *et al*., 1998; Vekrellis *et al*., 1997). Whereas human Bcl-x_S mRNA can be readily detected in human thymus (Boise *et al*., 1993) it's not detectable in mouse tissues (Gonzalez *et al*., 1994).

Bcl-w

Bcl-w was cloned using polymerase chain reaction (PCR), based on its homology with Bcl-2. The bcl-w gene can be alternatively spliced to join an exon of an adjacent gene, homologous to the Drosophila rox2 gene, but the consequence of this splicing event has not been elucidated. Bcl-w possesses all the BH domains, but the loop region between BH4 and BH3 is substantially shorter than in Bcl-2 and Bcl-x. Bcl-w can protect haematopoietic cells from cell death induced by growth factor withdrawal, glucocorticoid treatment and γ-irradiation, but like Bcl-2 and Bcl-$x_{L,}$ it is ineffective against Fas-induced cell death (Gibson *et al*., 1996). Bcl-w can also protect against cell death induced by Bax and Bad in 293T cells (Holmgreen *et al*., 1999). Mutation of the highly conserved glycine 94 in the BH1 region abolished Bcl-w's protective function but not it's ability to heterodimerise with Bak, Bad and Bid, suggesting Bcl-w exerts its function not solely by binding to pro-apoptotic members (Holmgreen *et al*., 1999).

Bcl-w mRNA is expressed in many tissues with the highest levels in brain, colon and salivary gland but, in contrast to Bcl-x and Bcl-2, it is not highly expressed in the lymphoid system (Gibson *et al*., 1996).

Mice deficient in bcl-w appear normal, although slightly smaller than wildtype mice, with the marked exception of degenerated testes with increased apoptosis of sertoli cells and germ cells, resulting in male sterility (Print *et al*., 1998; Ross *et al*., 1998). In some mouse backgrounds, loss of Bcl-w also affects the oocyte survival in females. The death of sertoli cells and the oocyte depletion can be blocked if the mice are simultaneously deficient in Bax (MacGregor *et al*., 1999).

Bcl-w protein levels increase in surviving neurons in the caudate putamen and parietal cortex after focal cerebral ischemia in the adult rat (Minami *et al*., 2000; Yan *et al*., 2000) but apart from that, Bcl-w's role in the nervous system has not been studied.

Bax

Bax was identified by coimmunoprecipitation with Bcl-2. In contrast to Bcl-2 and Bcl-x, Bax accelerates cell death and inhibits Bcl-2's protective function in a concentration-dependent manner (Oltvai *et al*., 1993). In addition, Bax can induce cell death without additional death stimuli (Vekrellis *et al*., 1997; Xiang *et al*., 1996). Bax-induced caspase activation is dependent on mitochondria and involves release of cytochrome *c* from mitochondria (Myers *et al*., 1998; Jürgensmeier *et al*., 1998), but Bax can also induce caspase-independent cell death in certain systems (Lindenboim *et al*., 2000; Pastorino *et al*., 1998; Xiang *et al*., 1996).

Bax is alternatively spliced into baxα, which possess the BH1, BH2 and BH3 domains as well as a putative carboxyterminal transmembrane domain; baxβ, which lacks the transmembrane domain; baxγ, which lacks exon 2, and is truncated due to frameshift (Oltvai *et al*., 1993); baxδ, which lacks the BH3 domain (Apte *et al*., 1995); and baxω, which also lacks the transmembrane domain (Zhou *et al*., 1998). So far, no functional studies have been performed for Baxβ, Baxγ or Baxδ. Baxω has an intrinsic pro-apoptotic activity but can also protect against cell death induced by other agents, such as TNF (Zhou *et al*., 1998).

Contrary to Bcl-2, the putative transmembrane domain of Bax does not seem to function to insert Bax into membranes in healthy cells, where most Bax resides in the cytoplasm (Wolter *et al*., 1997). Instead a conformation change of Bax takes place after many apoptotic stimuli, which facilitates Bax' insertion into the membrane and translocates Bax from the cytosol to the mitochondria. This conformational change can be induced by a number of apoptotic stimuli including staurosporin, growth factor withdrawal and Fas ligation (Khaled *et al*., 1999; Murphy *et al*., 1999; Nechushtan *et al*., 1999; Putcha *et al*., 1999). In addition, the BH3-only pro-apoptotic protein Bid has been shown to bind Bax and induce this conformational change (Desagher *et al*., 1999). The conformational change might also result in Bax oligomer formation, which acquires a channel activity, inducing cytochrome c release (Antonsson *et al*., 2000).

Mice deficient in bax show hyperplasia of many cell types, including lymphocytes and neurons, but curiously enough, also loss of male germ cells (Deckwerth *et al*., 1996; Knudson *et al*., 1995; White *et al*., 1998). In addition, facial motoneurons in Bax deficient mice survive after axotomy, sympathetic neurons are resistant to growth factor withdrawal (Deckwerth *et al*., 1996), and cortical neurons are resistant to glutamate toxicity and DNA damage (Xiang *et al*., 1998).

In analogy with the unexpected finding that inactivation of Bax cause increased cell death in certain tissues, Bax has been shown to increase sensory neuron survival after withdrawal of NGF or BDNF (Middleton *et al*., 1996). Bax can also rescue CNTF-deprived ciliary neurons, which are not rescued by Bcl-2 or Bcl-x_L. In addition, Bax protects newborn mice from death after Sindbis virus infection and Bax-deficient adult mice are less resistant to Sindbis infections (Lewis *et al*., 1999). Thus it appears that Bax, although in most cases is pro-apoptotic, can have an anti-apoptotic role under certain circumstances.

Bax mRNA is highly expressed in brain, stomach, heart, lung, kidney and pancreas and the protein levels show a similar but not identical distribution (Krajewski *et al*., 1994a). Bax expression in general, is more widespread than Bcl-2. The different splice variants appear to be differentially expressed but the there is a discrepancy between different studies on which variant is dominating in certain tissues. For example, Oltvai *et al*., (1993) reported Bax-β (and an additional unidentified band) to be the predominant form in brain, whereas Zhou *et al*., (1998) showed mRNA and protein levels of Bax-α and Bax-ω to be highly expressed in brain, and Krajewski *et al*., (1994a) only showed expression of Bax-α in this tissue. The overall protein levels of Bax are decreased during development (Shimohama *et al*., 1998; Vekrellis *et al*., 1997) and decreased Bax levels are implicated in loss of trophic factor dependence in peripheral neurons (see below) (Easton *et al*., 1997).

Bad

Bad (Bcl-x_L/Bcl-2 associated death promoter) is a pro-apoptotic "BH3-only" Bcl-2 family member that was cloned as an interacting partner for Bcl-2. It was found to bind Bcl-x_L even more strongly and to antagonise the survival-promoting activity of Bcl-x_L by displacing Bax in Bcl-x_L/Bax heterodimers (Yang *et al*., 1995). Bad can inhibit protection by Bcl-x_L after IL-3 deprivation from haematopoietic cell lines and after staurosporine treatment and can also induce cell death in absence of additional apoptotic stimuli in several cell lines as well as primary cerebellar granule cells (Datta *et al*., 1997; Ottilie *et al*., 1997; Yang *et al*., 1995; Zha *et al*., 1997). Overexpressing Bad in vivo under a haematopoetic promoter results in a profound loss of thymocytes and these cells are more susceptible to apoptotic stimuli than wildtype thymocytes (Mok *et al*., 1999). Bad does not have a transmembrane domain and only shares homology to other Bcl-2 family members in the BH3-region. The BH3 region is

essential for heterodimerisation with Bcl-2 or Bcl-x_L and for Bad's death-inducing ability (Kelekar *et al*., 1997; Ottilie *et al*., 1997; Zha *et al*., 1997).

Bad is phosphorylated on at least three different serine residues resulting in the disruption of the binding of Bad to Bcl-x_L at the mitochondria, and the sequestering of Bad to the cytosol by the protein 14-3-3 (Zha *et al*., 1996). This results in more free Bcl-x_L and more Bcl-x_L bound to Bax, thus in turn resulting in less free Bax. This disruption of the balance between pro- and anti-apoptotic Bcl-2 family members inhibits the release of cytochrome *c* and the activation of the apoptotic program (Fig. 4). So far phosphorylation of Bad has been detected serine residues, Ser-112, Ser-136 and Ser-155 and the phosphorylations can be accomplished by many kinases involved in signal transduction, thus providing a link between survival signals from growth factors and the apoptotic program. Insulin like growth factor (IGF-1) can induce the phosphorylation of Ser-136 of Bad via phosphatidylinositol 3-kinase (PI3-K) and protein kinase B (PKB)/Akt in cerebellar granule cells, thereby promote the survival of these neurons. Inhibition of this pathway blocks IGF-1 mediated survival (Dudek *et al*., 1997). The same pathway seems to mediate IL-3 induced survival of immune cells (del Peso *et al*., 1997). IL-3 can also induce the phosphorylation on Ser-112, which can be mediated by cAMP dependent protein kinase (PKA) (Harada *et al*., 1999) or by a MAPK/ERK kinase (MEK) dependent pathway through an Rsk kinase (Bonni *et al*., 1999; Scheid *et al*., 1999). PKA needs to be anchored to the mitochondria to exert phosphorylation of Bad which is in agreement with this being the primary localization of unphosphorylated Bad bound to Bcl-x_L(Harada *et al*., 1999). PKA can also phosphorylate the third known phosphorylation site of Bad, Ser-155 (Lizcano *et al*., 2000; Zhou *et al*., 2000).

Bad can also be dephosphorylated in response to apoptotic stimuli. Glutamate was shown to induce dephosphorylation of Bad through the influx of Ca^{2+} and the activation of the phosphatase calcineurin (Wang *et al*., 1999), which induces the binding of Bad to Bcl-x_L and results in an opposite switch in the Bcl-x_L-Bax balance and activation of the apoptotic program (Fig 4). Ceramide can also indirectly induce dephosphorylation of Bad by inactivating the PI3-K-Akt pathway (Basu *et al*., 1998; Zundel and Giaccia, 1998).

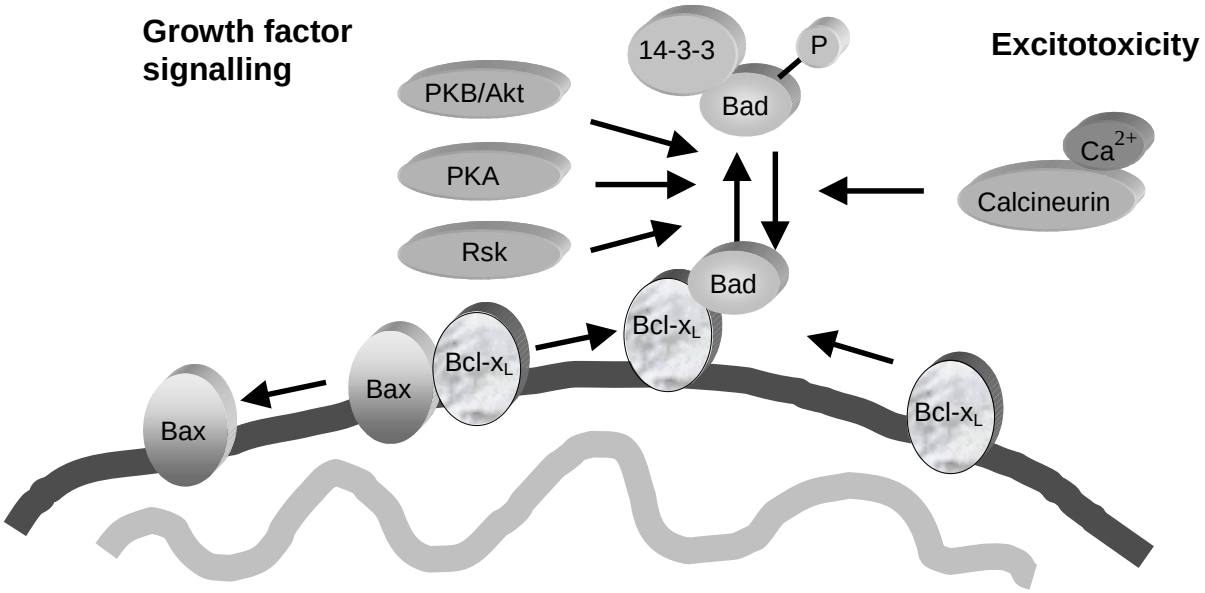

Figure 4: Bad phosphorylation. Bad can be phosphorylated or dephosphorylated by several kinases/phosphatases. Unphosphorylated Bad binds to Bcl-x_L, which results in less free Bcl-x_L and more free Bax

Bad protein is expressed in many tissues, including testis, breast, colon and spleen (Kitada *et al*., 1998). In the brain, the levels of Bad protein decreases with age (Shimohama *et al*., 1998) and at least some forms of Bad are restricted to the choroids plexus in the adult brain (D'Agata *et al*., 1998; Rickman *et al*., 1999), but see also result section). Bad has also been shown to be upregulated after several apoptotic stimuli, including withdrawal of nerve growth factor from sympathetic neurons and γ-irradiation and glucocorticoid treatment in thymocytes (Aloyz *et al*., 1998; Mok *et al*., 1999).

Other Bcl-2 family members

Although this thesis focuses only on the Bcl-2 family-members Bcl-2, Bcl-x, Bcl-w, Bax and Bad, this is an overview on some of the other Bcl-2 family members (see also Fig. 3).

Mcl-1 is an anti-apoptotic family member that is induced during differentiation of myeloid cells (Kozopas *et al*., 1993). However, Mcl-1 can be alternatively spliced and then resembles a BH3-only family member with pro-apoptotic activity (Bingle *et al*., 2000).

A1 is also an anti-apoptotic family member induced in haematopoietic cells upon differentiation and proliferation stimuli (Lin *et al*., 1993).

BHRF-1 is a viral anti-apoptotic protein encoded by Epstein Barr virus, which may ensure that virus-infected cells do not undergo apoptosis (Henderson *et al*., 1993; White *et al*., 1992).

Boo/Diva is a family member possessing BH4, BH1, BH2 and TM domains but with poor homology in the BH3 domain. Boo/Diva is anti-apoptotic in many circumstances but can also exhibit a pro-apoptotic function. The expression of Boo/Diva is restricted to the ovary in adult mouse (Inohara *et al*., 1998b; Song *et al*., 1999).

Bak is a pro-apoptotic family member, consisting of BH3, BH1, BH2 and a transmembrane domain. Bak can accelerate cell death after several apoptotic stimuli and antagonise protection by Bcl-2 but can also inhibit cell death under certain conditions (Chittenden *et al*., 1995b; Farrow *et al*., 1995; Kiefer *et al*., 1995).

Bok/Mtd, in similarity with Bax and Bak, have BH3, BH1, BH2 and TM domains and induce apoptosis in mammalian cell lines as well as in primary neurons. Bok/Mtd cannot interact with Bcl-2, Bcl-x_L or Bcl-w and none of these proteins can inhibit Bok/Mtd induced cell death. Instead, other anti-apoptotic family members, including Mcl-1 and BHRF-1 appear to fulfil this function (Hsu *et al*., 1997; Inohara *et al*., 1998a). Interestingly, a novel splice form of Bok, lacking part of its BH3 domain can still induce apoptosis but does not bind Mcl-1 and BHRF-1 (Hsu and Hsueh, 1998).

Bid is a pro-apoptotic BH-3-only pro-apoptotic family member that can heterodimerise to both Bcl-2 and Bax (Wang *et al*., 1996b). Binding to Bax results in a conformational change in Bax that seems to be important for its pro-apoptotic activity (Desagher *et al*., 1999). Bid is an important link in cell death induced by tumour necrosis factor (TNF) or Fas since these stimuli induce the cleavage of Bid by Caspase-8 (Li *et al*., 1998). Cleaved Bid translocates to mitochondria and has a stronger death-inducing activity than full-length Bid, either by modulating other Bcl-2 family members or by itself acting as an ion channel (Schendel *et al*., 1999).

Noxa is a BH3-only family member induced after x-ray irradiation in a p53-dependent manner. p53- or irradiation-induced apoptosis appears to be partially dependent on the expression of Noxa since cell death induced by these stimuli can be partially blocked by antisense oligonucleotides to Noxa (Oda *et al*., 2000).

Hrk/DP5 is a BH3-only family member but it also possesses a transmembrane domain. Hrk/DP5 induces cell death in several cell lines and primary sympathetic neurons and its mRNA expression is induced upon deprivation of nerve growth factor (NGF) from sympathetic neurons and after treatment with amyloid β (Aβ), a toxic protein secreted in Alzheimers disease (Imaizumi *et al*., 1999; Imaizumi *et al*., 1997; Inohara *et al*., 1997).

Bim/Bod is another pro-apoptotic family member with a BH3 and a TM domain(Hsu *et al*., 1998; O'Connor *et al*., 1998). Bim is normally sequestered to the microtubule-associated dynein complex but it is released upon apoptotic stimuli and can thereby interact with anti-apoptotic family members, thus playing a similar role to Bad and Bid (Puthalakath *et al*., 1999). Mice deficient in Bim/Bod show an excess of lymphoid cells and these cells are less sensitive to different apoptotic stimuli than wild type cells (Bouillet *et al*., 1999).

Caspases

Caspases are cysteine proteases, homologous to the *C. elegans* Ced-3 protein and cleave substrates with an aspartate residue in the P_1 position. The preferred P_2-P_4 substrate residues vary between various caspases and contribute to different substrate specificities (Thornberry *et al*., 1997). All caspases are translated as procapases, containing an aminoterminal prodomain, a large subunit and a small subunit. These are activated by cleavage to form a heterotetramer, consisting of two large and two small subunits (Wilson *et al*., 1994). Once activated the caspases can cleave their substrates, including other caspases, which are thereby activated, and so on. Procaspases have a low protease activity but can activate each other if brought into close proximity (reviewed by Wolf and Green, 1999).

There are at least 14 known mammalian caspases, which can be divided into three major groups, the cytokine processing caspases, the initiator caspases and the excecutioner caspases (Wolf and Green, 1999). The founding member of the caspase family, caspase-1, or interleukin-1β-convering enzyme (ICE), is important for processing and activation of the cytokine interlekin-1β (Thornberry *et al*., 1992), but appears not to play a major role in apoptosis (Kuida *et al*., 1995; Li *et al*., 1995). Other caspases belonging to this subfamily are caspase-11 (Wang *et al*., 1998) and possibly caspase-4, -5, -12, -13 and -14 (Wolf and Green, 1999).

The initiating caspases include caspase-2, -8, -9 and -10 and have large prodomains including specific sequence motifs. These motifs are shared with adapter proteins and through homophilic interactions between these motifs, the adapter protein activate the caspase, often by enforced oligomerisation (Salvesen and Dixut, 1999; Yang *et al*., 1998c). One such motif is the caspase recruitment domain (CARD, (Hofmann *et al*., 1997) found in e.g. caspase-9 and the adapter protein Apaf-1, which is homologous to the *C. elegans* Ced-4 protein. Caspase-9 (Mch6/ICE-LAP6, (Duan *et al*., 1996; Srinivasula *et al*., 1996) and Apaf-1 (Zou *et al*., 1997) are part of the apoptosome and will be dealt with below, but it should be noted here that after activation by Apaf-1, caspase-9 can cleave and activate the downstream executioner caspases.

Another domain acting in the same way as CARD is the death effector domain (DED) found in caspase-8 and the adapter FADD, which also binds to the death receptors (e.g. Fas and

TNF-R) via a similar death domain (DD). Binding of the ligands Fas-L or TNF to their receptors results in trimerisation of the receptors and aggregation of a death inducing signalling complex (DISC) where caspase-8 (FLICE/MACH/Mch5, Muzio *et al*., 1996) is included (Walczak and Krammer, 2000). This aggregation brings several caspase-8 molecules together resulting in their autoactivation and subsequent cleavage of executioner caspases, such as capase-3 (Muzio *et al*., 1997) (Fig. 5). Alternatively, caspase-8 can cleave the pro-apoptotic Bcl-2 family member Bid and thereby induce the translocation of Bid to mitochondria, where it activates the mitochondrial pathway to apoptosis (see below) (Luo *et al*., 1998).

The excecutioner subgroup consists of caspase-3, -6 and -7, which have short prodomains without known function. Caspase-3 (CPP32/Yama/Apopain, Fernandes-Alnemri *et al*., 1994) is perhaps the most studied caspase so far and appears to play a crucial role in many apoptotic models. It is also the mammalian caspase with the substrate specificity most similar to the *C. elegans* Ced-3 protease (Xue *et al*., 1996). Caspase-3 activation is a common detection method for apoptotic cell death and can be measured either by fluorescent substrates or with antibodies detecting only the activated form of caspase-3. Caspase-3 deficient mice have severe malformations of the brain and die within 1-3 weeks of postnatal life. However, other organs appear more or less normal and thymocytes from caspase-3 deficient mice respond normally to apoptosis induction by Fas, glucocorticoids and staurosporine, arguing against caspase-3 as a universal executioner (Kuida *et al*., 1996).

In addition to cleaving other caspases, caspases can cleave a large number of other cellular proteins (reviewed by (Cryns and Yuan, 1998). Some of the morphological and biochemical hallmarks of apoptosis can at least partially be explained by caspase cleaved substrates. One of these substrates is the nuclear structural protein lamin, which correlates with chromatin condensation (Rao *et al*., 1996). Another apoptotic characteristic correlated with caspase activation is DNA fragmentation. The caspase activated DNase (CAD/DFF40) is normally inhibited by an inhibitory subunit (ICAD/DFF45). However, ICAD can be cleaved by caspases and CAD is thereby activated (Enari *et al*., 1998; Liu *et al*., 1997). Several enzymes involved in DNA repair are also cleaved by caspases including the first caspase substrate to be identified; poly(ADP-ribose) polymerase (PARP) (Lazebnik *et al*., 1994), which is often used as a marker for caspase activation. In addition, caspase cleavage can result in a positive feed-back loop, inactivating anti-apoptotic proteins or activating pro-apoptotic proteins. As already mentioned, Bcl-2 and Bcl-x_L are converted into pro-apoptotic molecules after cleavage (Cheng *et al*., 1997; Clem *et al*., 1998; Kirsch *et al*., 1999), whereas Bid is activated (Luo *et al*., 1998). Several signalling molecules are cleaved by caspases, including inactivation of the survival kinases Raf-1 and Akt (Widmann *et al*., 1998) as well as activation of the JNK pathway activator MEKK1 (Cardone *et al*., 1997).

Caspases can be directly inhibited by a number of viral or cellular inhibitors. Viruses have a requirement to block the cellular apoptotic response following infection and the first identified caspase inhibitor was the cowpox cytokine response modifier A (CrmA, Ray *et al*., 1992). Another potent viral caspase inhibitor is the baculovirus protein p35 (Clem *et al*., 1991). Both of these viral inhibitors act as pseudosubstrates for caspases and by inhibiting caspases they also inhibit cell death in many circumstances (Gagliardini *et al*., 1994; Xue and Horvitz, 1995), reviewed by (Ekert *et al*., 1999). Another baculoviral caspase inhibitor is the *Cydia pomonella* inhibitor of apoptosis (CpIAP, Crook *et al*., 1993). Subsequently, a number of mammalian IAPs have been identified (reviewed by (Deveraux and Reed, 1999), which can protect cells from a variety of apoptotic stimuli (Duckett *et al*., 1998; Liston *et al*., 1996). In contrast to the anti-apoptotic Bcl-2 family members, the IAP family inhibit cell death by directly binding to and inactivating caspases (Deveraux *et al*., 1997; Roy *et al*., 1997).

The therapeutic potentials of inhibiting caspase activity and thereby apoptosis, has lead to the development of a number of synthetic peptide caspase inhibitors (reviewed by Ekert *et al*., 1999). These peptides work as pseudosubstrates like CrmA and p35, and can inhibit cell death in several disease models including ischaemia (Hara *et al*., 1997), in addition to naturally occurring neuronal death in chick embryos (Milligan *et al*., 1995).

Although caspases are central in many cell death pathways, they might not always be required for cell death to occur. Broad range caspase inhibitors fail to inhibit cell death after several apoptotic stimuli even though the death process was significantly delayed. In addition, it seems like caspases are required for nuclear changes in apoptosis such as chromatin condensation and fragmentation, whereas for example cell shrinkage and membrane blebbing are in many cases caspase independent (reviewed by Borner and Monney, 1999).

Other proteases can also be activated by apoptotic stimuli and execute the death commitment. These include calpains, another cysteine protease family activated by Ca^{2+}. Calpains are mainly activated during necrosis, when the intracellular Ca^{2+} levels are dramatically elevated, but have recently been implicated also in apoptotic cell death (reviewed by Wang, 2000).

Role of mitochondria in apoptosis

The requirement for mitochondria in apoptosis was first shown by (Newmeyer *et al*., 1994), using a cell-free system. In the same system a number of factors were isolated that were essential for caspase activation and DNA fragmention. Surprisingly, one of these factors was identified as the respiratory chain component cytochrome *c*. It was further observed that cytochrome *c* is released from mitochondrial intermembrane space to the cytosol upon apoptotic stimuli (Liu *et al*., 1996).

The mechanism by which cytochrome *c* is released has been the subject of much debate and there is still no clear answer to the question. Many of the Bcl-2 family members are localised to mitochondria and Bcl-2, Bcl-x_L and Bcl-w has been shown to inhibit the release of cytochrome *c* (Kluck et al., 1997; Yang et al., 1997; Kharbanda *et al*., 1997; Yan *et al*., 2000), while Bax promote this release (Myers *et al*., 1998; Jürgensmeier *et al*., 1998). Although Bax can form an oligomeric channel in liposomes that theoretically is large enough for releasing cytochrome *c* (Antonsson *et al*., 2000), the endogenous existence of such a channel in mitochondrial membranes remains to be confirmed.

Another feature often accompanying the cell death program, is the loss of mitochondrial membrane potential ($\Delta\Psi m$) (Vander *et al*., 1997; Vayssiere *et al*., 1994). The change in $\Delta\Psi m$ causes matrix swelling and ultimately outer mitochondrial membrane rupture. Again, the responsible factor for this loss is unknown, but one candidate is the permeability transition pore complex (PTPC). The main three components of the PCPT are the adenine nucleotide translocator (ANT) of the inner mitochondrial membrane, the mitochondrial matrix protein cyclophilin-D which binds ANT, and the voltage dependent anion channel (VDAC) of the outer membrane (reviewed by Crompton, 1999). The complex is formed at contact sites between the two membranes, thus bringing ANT and VDAC together. A number of other proteins have been shown to bind to the PTPC and interestingly enough these include Bcl-x_L and Bax. ANT and Bax can form a channel together in artifical liposomes and Bcl-2 could inhibit this channel (Brenner *et al*., 2000; Marzo *et al*., 1998a). Bax and Bcl-x_L can also interact with and regulate VDAC (Narita *et al*., 1998; Shimizu *et al*., 1999).

In summary, three channels responsible for the cytochrome c release and/or disruption of $\Delta\Psi m$ have been proposed so far: A multimeric Bax channel, the PTPC or a combination of Bax and PTPC.

Since loss of ΔΨm results in rupture of the outer mitochondrial membrane, it could theoretically account for the release of cytochrome *c*. However, the release of cytochrome c and the subsequent caspase activation has been shown to precede loss of ΔΨm in several systems (Krohn *et al*., 1999; Vander *et al*., 1997; Yang *et al*., 1997). Furthermore Apaf-1 deficient cells do not exhibit disruption of ΔΨm, suggesting it to occur downstream of Apaf-1 induced caspase activation (Yoshida *et al*., 1998). Indeed, caspases have been shown to disrupt the ΔΨm and to activate the PTPC (Marzo *et al*., 1998b). Thus, cytochrome *c* and loss of ΔΨm might not always go hand in hand. Instead, an early specific release of limited amounts of cytochrome *c* and the resulting Apaf-1 and caspase activation could result in a positive feed-back loop where caspases activate the PTPC, causing outer membrane rupture and release of the remaining cytochrome *c*. However, in some systems the release of cytochrome *c* might occur after loss of ΔΨm.

Cytochrome *c* is not the only apoptotic regulator released from mitochondria upon apoptotic stimulation. Apoptosis inducing factor (AIF) is another factor that translocates to the nucleus where it causes chromatin condensation and DNA HMW fragmentation (Susin *et al*., 1996; Susin *et al*., 1999b). Caspases can also be localised to mitochondria and released in apoptotic cells (Mancini *et al*., 1998; Susin *et al*., 1999a; Zhivotovsky *et al*., 1999). In addition, it was very recently shown, that an additional apoptotic factor, Smac/DIABLO was released from mitochondria during apoptosis. This factor binds to IAPs and thereby counteracts their inhibition of caspases (Du *et al*., 2000; Verhagen *et al*., 2000).

The apoptosome

Once in the cytoplasm, cytochrome *c* can activate a caspase cascade in the presence of dATP (Liu *et al*., 1996). Both these factors bind to and activate the apoptosome complex. One of the major components of this complex is the mammalian homologue to Ced-4, apoptotic protease activating factor-1 (Apaf-1). Apaf-1 consists of an N-terminal CARD domain, a central Ced-4 homology domain and a C-terminal WD40 repeat region (Zou *et al*., 1997). The WD40 repeats appears to negatively regulate the activity of Apaf-1 and this region makes Apaf-1 different from Ced-4, which is constitutively active in absence of Ced-9. Accordingly, when the WD40 region is deleted from Apaf-1, it acquires a constitutively active state (Srinivasula *et al*., 1998). Binding of cytochrome *c* and dATP to Apaf-1 results in a conformational change which enables Apaf-1 to oligomerise and bind to caspase-9 via a homophilic CARD-CARD interaction (see above) (Li *et al*., 1997b; Srinivasula *et al*., 1998). When Apaf-1 is oligomerised, several caspase-9 molecules are brought into proximity and can be autoproteolysed (Srinivasula *et al*., 1998), followed by processing of more downstream caspases, including caspase-3 (Li *et al*., 1997b). Recent studies show that caspase-3 also gets recruited to the apoptosome complex and that the active apoptosome consists of multimers of Apaf-1, caspase-9 and caspase-3, summing up to 700-1300 kD (Cain *et al*., 1999; Zou *et al*., 1999). Early studies implicated that Bcl-x_L can bind to the apoptosome complex in analogy with the Ced-9 binding to Ced-4 (Hu *et al*., 1998; Pan *et al*., 1998). Recent findings challenge the existence of such an interaction, however (Hausmann *et al*., 2000; Moriishi *et al*., 1999; Newmeyer *et al*., 2000), indicating that the inhibition of the apoptosome by Bcl-x_L occur solely on the level of cytochrome *c* release from the mitochondria.

The importance of the apoptosome complex in physiological cell death is demonstrated in Apaf-1 or caspase-9 deficient mice. Both these mice show marked malformations of the brain due to ectopic cell masses (Cecconi *et al*., 1998; Hakem *et al*., 1998; Kuida *et al*., 1998; Yoshida *et al*., 1998), resembling the phenotype of caspase-3 deficient mice (see above). However, the Apaf-1 mutant phenotype is more severe than those of caspase-9 and capase-3

deficient mice, implying that other caspases might be activated by Apaf-1. All cell death is not blocked in Apaf-1 deficient cells, however, raising the possibility that other Apaf-1 like molecules exist. Indeed, two Apaf-1 homologous proteins have recently been identified; FLASH which functions in death receptor signalling (Imai *et al*., 1999) and CARD4/Nod which can activate NFκB (Bertin *et al*., 1999; Inohara *et al*., 1999).

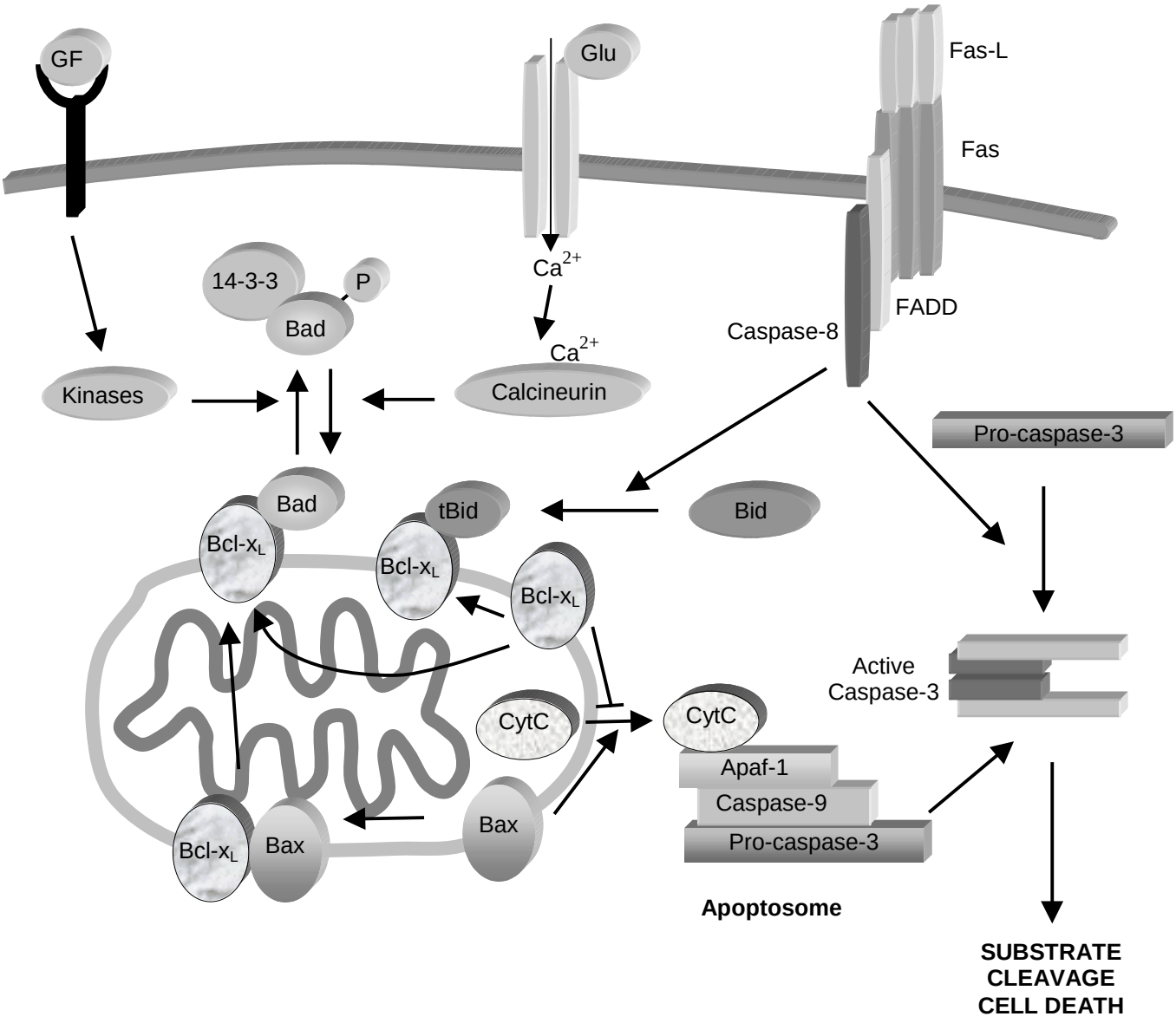

Figure 5: Summary of the mammalian apoptotic pathways

Neuronal survival and cell death

Neural development

During the development of the nervous system typically 20-80% of the produced neurons in each population undergo programmed cell death. A major part of this cell death take part relatively late during development in post-mitotic neurons that have already projected their axons to their targets (reviewed by Oppenheim, 1991). Early studies showed that the presence of a target was essential for the survival of the projecting neurons (Hamburger and Levi-Montacini, 1949). The subsequent findings that trophic factors was released from these targets lead to the formulation of the neurotrophic theory, which states that trophic factors are produced by the target cells in limited amount and thus only a limited number of projecting neurons can survive (Purves, 1980). Target cells are, however, not the only source of neurotrophic factors. Surrounding glia cells and afferent inputs might also play an important role for the life-or-death decision for a given neuron (Oppenheim, 1991). In addition, depolarising conditions as well as cell contacts with other cells and with the extracellular matrix may enhance survival (see below).

Bcl-2 transgenic mice and Bax-deficient mice show increased cell numbers in several peripheral and central nervous system (PNS and CNS) structures (Deckwerth *et al*., 1996; Martinou *et al*., 1994; White *et al*., 1998). Although these animals are viable, their brain function may not be unaffected. Accordingly it was recently shown that Bcl-2 transgenic mice exhibit impaired motor coordination (Rondi-Reig *et al*., 1999).

In addition to the relatively late post-mitotic cell death, recent evidence implies that neuronal cell death takes place during the closure of the neural tube (Weil *et al*., 1997) and in mitotic cells in the proliferating neuroepithelium (Thomaidou *et al*., 1997). Failure to complete this early cell death phase may account for the big morphological malformations of the brain seen in caspase-3, caspase-9 and Apaf-1 deficient mice (Cecconi *et al*., 1998; Hakem *et al*., 1998; Kuida *et al*., 1998; Kuida *et al*., 1996). Bcl-x deficiency cannot compensate for the lack of cell death or the malformations in caspase-3 deficient mice (Roth *et al*., 2000). This finding, together with the relatively mild neuronal phenotype seen in Bax-deficient mice and Bcl-2 transgenes indicate that this early cell death is not regulated by the Bcl-2 family.

Neurodegenerative diseases and brain damage

Although neuronal cell death is an absolute requirement during the development of the nervous system, ectopic cell death in the adult brain can be deleterious. This is the case in several neurodegenerative diseases as well as after ischaemic and traumatic damage to the brain. In many cases this cell death is associated with apoptotic morphology and/or activation of the apoptotic program. A few examples are given below:

Apoptotic death might play a role in Alzheimers disease even though other kinds of cell death are probably also involved (Kuziak *et al*., 1996). A major contributor to the neurotoxicity in Alzheimers is the amyloid-β protein that is secreted and aggregated in abnormal amounts in Alzheimer brains. The mechanism by which amyloid-β causes cell death is not clear, but in neuronal cultures amyloid-β causes caspase and calpain dependent cell death with apoptotic morphology (Jordan *et al*., 1997; Loo *et al*., 1993). Interestingly, neurons from caspase-12 or -2 deficient mice show resistance to amyloid-β, indicating that these caspases play an important role in amyloid-β toxicity (Nakagawa *et al*., 2000; Troy *et al*., 2000). In addition, caspases can cleave the amyloid precursor protein (APP), perhaps

resulting in a positive feed-back loop where more amyloid-β is produced (Gervais *et al.*, 1999).

The most common mutations of familiar Alzheimer are those of the presenilin proteins. Mutations of these proteins potentiate production of amyloid-β. Both presenilin-1 and -2 interacts with Bcl-2 and Bcl-x_L and can be cleaved by caspases, linking the presenilins to the apoptotic program (Alberici *et al.*, 1999; Kim *et al.*, 1997; Passer *et al.*, 1999).

In addition to these *in vitro* studies, postmortem studies on Alzheimer brains reveals an upregulation of Bax protein in amyloid plaques, whereas Bcl-2 is upregulated in surrounding neurons and glia cells (MacGibbon *et al.*, 1997; Satou *et al.*, 1995; Tortosa *et al.*, 1997; Tortosa *et al.*, 1998). Caspase-3 activity is also elevated in Alzheimer hippocampi (Gervais *et al.*, 1999).

Parkinsons is another neurodegenerative disease in which there is a specific cell loss of dopaminergic neurons of the substantia nigra. A commonly used animal model for Parkinsons is the administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or its metabolite MPP^+, which causes apoptotic cell death of nigral neurons and activation of caspase-2 (Tatton and Kish, 1997; Yang *et al.*, 1998a). Interestingly, transgenic mice overexpressing Bcl-2 show decreased susceptibility to MPTP and blocks caspase-2 activation (Yang *et al.*, 1998a).

Amyotrophic lateral sclerosis (ALS) is a disorder associated with loss of motor neurons both in the spinal cord and in the motor cortex. The most common cause for familiar ALS is a mutation in superoxid dismutase (SOD-1). Transgenic mice expressing mutant SOD-1 is a commonly used animal model for familiar ALS. Overexpression of Bcl-2 or caspase inhibitors delays disease onset and mortality in this model (Kostic *et al.*, 1997; Li *et al.*, 2000).

Spinal muscular atrophy (SMA) is another motor neuron disease affecting young children and is one of the most common autosomal recessive genetic disorders. Interestingly, one of the genes affected in SMA is the neuronal apoptosis inhibitory protein (NAIP) that belongs to the IAP family of caspase inhibitors (Roy *et al.*, 1995). Another gene often mutated or deleted in SMA is survival motor neurons (SMN), which has been shown to bind to and potentiate the anti-apoptotic function of Bcl-2 (Iwahashi *et al.*, 1997).

In ischaemic and traumatic brain injury, the primary damage site is usually correlated with necrotic death. However, apoptotic cell death can be seen in the prenumbra surrounding the damage site and during reperfusion (Rink *et al.*, 1995; Skoglosa *et al.*, 1999; Yuan and Yankner, 2000). Caspases are activated in animal models of ischaemic and traumatic brain injury (Kang *et al.*, 2000; Namura *et al.*, 1998; Yakovlev *et al.*, 1997). Overexpression of NAIP or deficiency in caspase-11 reduces cell death in ischaemia models (Kang *et al.*, 2000; Xu *et al.*, 1997), and inhibition of caspase-3-like proteases reduced apoptosis and improved neurological recovery after experimental traumatic brain injury (Yakovlev *et al.*, 1997).

In summary, apoptosis proteins are involved in many neurological diseases and interfering with the apoptotic program might be a potential therapeutic strategy for these diseases.

Neurotrophic factors

As stated above, neurons are dependent on tropic factors for survival. The early work by Rita Levi-Montalcini and others lead to the purification of the first neurotrophic factor which was named nerve growth factor (NGF) (Bueker, 1948; Cohen *et al.*, 1954; Levi-Montalcini and Hamburger, 1953). In the eighties and early nineties, three more neurotrophic factors of the same family were cloned and named brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4 (reviewed by Chao, 1992). An additional family member

has been found in fish, called NT-6. Collectively, this family of trophic factors are called neurotrophins and they bind to tyrosine kinase receptors called TrkA (binds NGF), TrkB (binds BDNF and NT-4) and TrkC (binds NT-3). NT-3 can also bind TrkA and TrkB in some circumstances. In addition all neurotrophins can bind to a low-affinity receptor, p75 (Chao, 1992) (Fig. 6).

Different neurotrophins appear to act on different neuronal populations and at different time-points during development of the peripheral nervous system (PNS). For example superior cervical ganglia (SCG) sympathetic neurons are dependent on NGF whereas neurons of the vestibular ganglia are dependent on BDNF. In the central nervous system (CNS) the picture appears to be more complex and the CNS defects in neurotrohin deficient mice are limited (Snider, 1994). Other neurotrophic factors may serve a more important role in the CNS, such as insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF) and growth factors of the glia derived neurotrophic factor (GDNF) family (Baloh *et al*., 2000; Dore *et al*., 1997; Korsching, 1993). Alternatively a redundancy might exist, so that a given population of neurons can respond to more than one trophic factor.

Ligand binding to Trk receptors (as well as other tyrosine receptor kinases) induces dimerisation and autophosphorylation of the receptor and triggers a number of signal transduction pathways that ultimately results in survival, differentiation or proliferation of the cell. Phosphorylated Trk receptors recruit the adaptor protein Shc, which in turn activates Grb2, Sos, p21-Ras, Raf-1, MEK and extracellular signal regulated kinase (ERK). ERK is then translocated to the nucleus where it can regulate gene transcription (reviewed by Friedman and Greene, 1999) (Fig. 6). In addition, other kinases can be activated by ERK, such as Rsk, which can phosphorylate the pro-apoptotic protein Bad (see above) and the transcription factor CREB which results in transcription of survival genes (Bonni *et al*., 1999). Removal of growth factors (or other forms of cellular stress) results in inactivation of the ERK pathway and activation of two other pathways of the MAPK superfamily; the c-Jun N-terminal kinase (JNK) pathway and the p38 pathway (Xia *et al*., 1995). The JNK pathway results in activation of the transcription factor c-Jun and might enhance transcription of apoptotic genes.

Another important pathway for tyrosine kinase receptors is that activated by PI3-K. PI3-K phosporylates phosphatidylinositol lipids which can target Akt/PKB to the plasmamembrane where it is phosphorylated and activated by phospatidylinositol-3-phosphate-dependent kinase (PDK) (reviewed by (Coffer *et al*., 1998). Apart from Bad (see above), Akt can phosphorylate: caspase-9 and thereby cause its inactivation (Cardone *et al*., 1998); the forkhead transcription factor FKHRL1 and thereby inhibiting the transcription of e.g. Fas ligand (Brunet *et al*., 1999); the inhibitor of NFκB kinase (IKK), resulting in activation of nuclear factor κB (NFκB) (Ozes *et al*., 1999; Romashkova and Makarov, 1999); the transcription factor CREB (Du and Montminy, 1998), and many other proteins including several enzymes involved in glucose metabolism (Coffer *et al*., 1998).

In addition to these major pathways, tyrosine receptors can also activate other signalling molecules including PLCγ that results in release of Ca^{2+} from intracellular stores and activation of PKC (Friedman and Greene, 1999).

Interestingly, the p75 receptor elicits a totally different signalling cascade. This receptor belongs to the TNFR/Fas superfamily and possesses a death domain in its intracellular part. p75 appears to increase the sensitivity of Trks to the neurotrophins but in the absence of their respective Trk receptor, neurotrophins can induce caspase dependent cell death via the p75 receptor. The mechanism for p75 death signalling is not clear but it appears to involve the JNK pathway (reviewed by (Casaccia-Bonnefil *et al*., 1998). The similarity between p75 and TNFR/Fas suggests a similar cell death mechanism for these receptors. Caspase inhibitors can

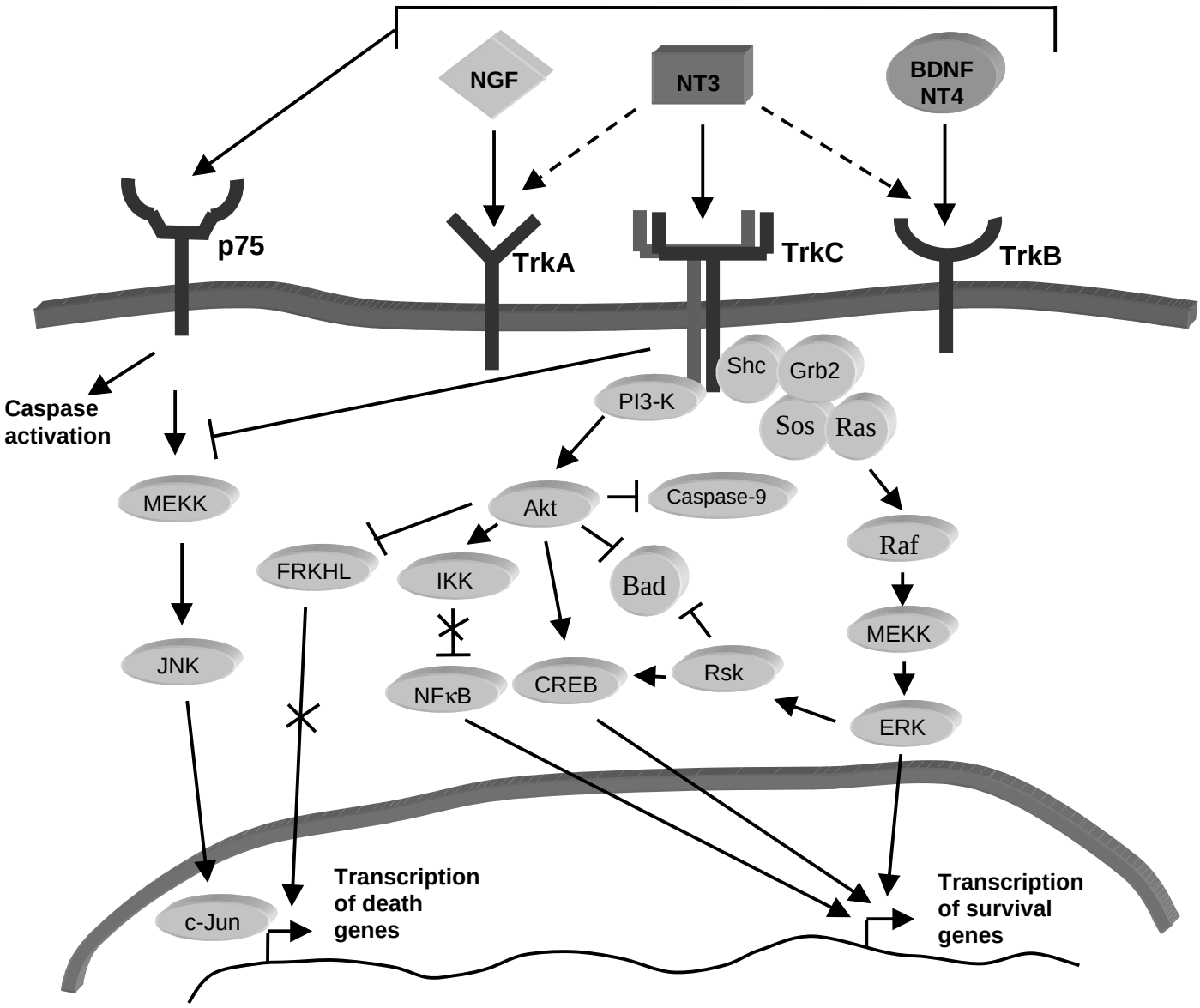

Figure 6: The neurotrophins, their receptors and some of their signal pathways

block p75 induced cell death, but caspase 8 appears not to be activated, suggesting involvement of other caspases such as caspase-1 or -2 (Gu *et al*., 1999).

Cell adhesion

Apart from soluble trophic factors, other parameters are also important for cell survival. One such parameter is cell adhesion to the extracellular matrix (ECM) or to other cells. Cells that lose contact with the extracellular matrix undergo apoptosis, a phenomenon called anoikis (for homelessness) (Frisch and Francis, 1994; Meredith *et al*., 1993). The signalling pathways for this cell death are not elucidated but the stressregulated kinases MEKK1 and JNK are activated after cell detachment (Cardone *et al*., 1997; Frisch *et al*., 1996a). In addition, caspases are activated and caspase inhibitors or overexpression of Bcl-2 can attenuate cell death (Boudreau *et al*., 1995; Frisch and Francis, 1994).

Integrins are important receptors for ECM molecules and can elicit signal transduction pathways through the focal adhesion kinase (FAK) (Clark and Brugge, 1995). Constitutively active FAK can indeed protect cells from anoikis (Frisch *et al*., 1996b). Overexpression of integrin can also enhance survival and upregulate the expression levels of Bcl-2 (Zhang *et al*., 1995).

Cell-cell contact-mediated cell survival is less studied but may also involve integrins or alternatively other cell adhesion molecules, such as N-CAMs or cadherins.

Model systems for neuronal cell death

In my attempt to summarise the apoptotic pathways above, data from different model systems have been put together. This involves a potential risk since the apoptotic pathways in different cell types and after different stimuli is probably not identical. Moreover, much of the results are obtained from transformed cell lines whose response to apoptotic stimuli is unlikely to represent the cell death program in normal, untransformed cells. Therefore, I will here present two well-studied models of neuronal cell death involving primary neuronal cultures, which have been used in this thesis: superior cervical ganglia sympathetic neurons and cerebellar granule cells.

Superior Cervical Ganglia (SCG) sympathetic neurons

Sympathetic neurons are part of the autonomic nervous system and regulate visceral functions to a "fight and flight" status by for example increasing heart rate and reducing the digestive activity. The superior cervical ganglia innervate blood vessels in the brain and in the skin of the head as well as the muscles in the eye and several cranial glands (Haines, 1997).

NGF-deprivation from SCG sympathetic neurons is one of the best-established model systems for neuronal cell death. The requirement of NGF for SCG maintenance was shown already in 1960 by Levi-Montalcini and Booker (1960). *In vivo*, rat SCG neurons undergo naturally occurring cell death between postnatal days 3 and 7 (Wright *et al*., 1983), and after this period the SCG neurons gradually lose their trophic factor dependence (Goedert *et al*., 1978).

When cultured without NGF, most SCG sympathetic neurons die within 48 h in an apoptotic, mRNA- and protein-synthesis dependent manner (Deckwerth and Johnson, 1993; Martin *et al*., 1988). The molecular and biochemical events taking place after NGF deprivation have been carefully studied, but are still not fully elucidated.

One of the first events to occur is a general drop in mRNA and protein synthesis and a decrease in glucose uptake (Deckwerth and Johnson, 1993). Opposite to this general decrease in macromolecular synthesis, the mRNA and protein levels of the transcription factor c-Jun, as well as its phosphorylation by Jun kinase, is increased. The inactivation of c-Jun with antibodies or dominant-negative mutants protects against cell death (Eilers *et al*., 1998; Estus *et al*., 1994; Ham *et al*., 1995). c-Jun can in turn induce other genes necessary for the apoptotic program. One such candidate gene is the tumour suppressor gene p53, which in turn can induce the transcription of Bax. Accordingly, NGF deprivation induced cell death is dependent on both p53 and Bax expression (Aloyz *et al*., 1998; Deckwerth *et al*., 1996). In addition, c-Jun might induce genes involved in cell proliferation and a failed attempt to enter the cell cycle could result in apoptosis. One gene possibly acting in this way is CyclinD1 that accordingly is induced after NGF deprivation (Freeman *et al*., 1994).

Cell death after NGF deprivation is caspase-dependent (Martinou *et al*., 1995; Deshmukh *et al*., 1996) and the caspase involved appear to be caspase-2, since anti-sense oligonucleotides directed against caspase-2, but not those directed against caspase-3, inhibits cell death (Stefanis *et al*., 1998; Troy *et al*., 1997). The release of cytochrome c from the mitochondria is another essential feature of cell death induced by NGF deprivation (Neame *et al*., 1998). This release is a reversible effect and the level of mitochondrial cytochrome c is restored upon readdition of NGF, suggesting a preserved mitochondrial ultrastructure and argues against the suggestion that release of cytochrome *c* is a consequence of mitochondrial swelling and membrane rupture. Indeed, mitochondria appear to shrink rather than swell after NGF deprivation (Martinou *et al*., 1999). Release of cytochrome *c* is per se not sufficient for induction of cell death, since cytoplasmic microinjection of cytochrome *c* cannot kill cells in the presence of NGF (Deshmukh and Johnson, 1998). Thus, NGF deprivation must activate other cytosolic factors that help to induce cell death downstream of cytochrome *c* release.

The signal transduction pathways utilised by NGF for promotion of survival is not clear. The PI3-K/Akt pathway has been implicated (Crowder and Freeman, 1998), but inhibition of PI3-K only partially inhibits the cell survival signals from NGF and do not inhibit all Akt/PKB activation, suggesting additional activators upstream of Akt/PKB (Tsui-Pierchala *et al*., 2000; Virdee *et al*., 1999). The pro-apoptotic Bcl-2 family member Bad is phosphorylated on serine-112 in response to NGF (Roberts *et al*., 2000). This residue has been shown in other systems to be phosphorylated by ERK and the cAMP activated protein kinase (PKA) (Bonni *et al*., 1999; Harada *et al*., 1999; Scheid *et al*., 1999), implicating that these kinases might play a role also in NGF-mediated survival signalling. Accordingly, ERK is activated by NGF (Virdee and Tolkovsky, 1995), and cAMP can rescue SCG neurons from cell death after NGF deprivation (Edwards *et al*., 1991).

The Bcl-2 family members appear to play an important role in regulating cell death in sympathetic neurons. The pro-apoptotic members Bax, Bak and DP5 induces cell death in the presence of NGF and this cell death could be blocked by co-expression of Bcl-2 or Bcl-x_L and/or by caspase inhibitors (Imaizumi *et al*., 1997; Martinou *et al*., 1998; Vekrellis *et al*., 1997). In addition, mature neurons that normally are independent of trophic factors, become susceptible to NGF deprivation if they overexpress Bax (Easton *et al*., 1997). On the contrary, the anti-apoptotic members Bcl-2, Bcl-x_L and Bcl-xβ can rescue neurons from cell death after NGF deprivation (Garcia *et al*., 1992; Gonzalez *et al*., 1995). Bcl-x_L and Bcl-xβ have an additative effect in this system, indicating that they perform their function by different mechanisms (Gonzalez *et al*., 1995).

The importance of Bax in the cell death program after NGF deprivation is shown by the fact that sympathetic neurons from Bax deficient mice survive also in the absence of NGF. This survival is not altered if the mice are also deficient in Bcl-2, indicating that Bcl-2 and Bax function independently in this system or that Bax function downstream of Bcl-2 (Deckwerth *et al*., 1998). The early changes after NGF deprivation, such as decreased protein synthesis and glucose uptake, increased c-Jun expression and phosphorylation, reduction of soma size, and retraction of neurites, still occur in SCG neurons from Bax deficient mice (Deckwerth *et al*., 1998; Deckwerth *et al*., 1996). A similar phenotype is seen in NGF deprived neurons overexpressing Bcl-2 or in mature neurons independent of NGF (Easton *et al*., 1997; Greenlund *et al*., 1995). In addition to the effects of Bax *in vitro*, Bax is also essential for the physiological cell death of SCG neurons *in vivo*, since the number of neurons in these ganglia is increased in Bax deficient mice when compared to wild-type mice (Deckwerth *et al*., 1996). SCG of Bcl-2 deficient mice, on the other hand, are substantially decreased in size due to excessive cell death of these neurons occurring after the normal period of physiological cell death (Michaelidis *et al*., 1996). Sympathetic neurons from Bcl-2 deficient mice also show

decreased viability when cultured in presence of NGF and accelerated cell death after NGF withdrawal (Greenlund *et al*., 1995).

A summary of the cell death pathway in sympathetic neurons is given in figure 7.

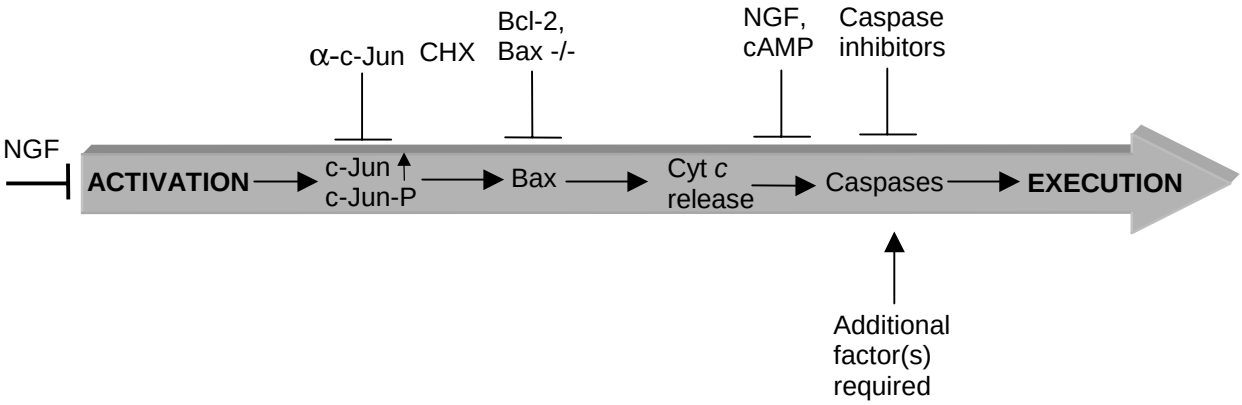

Figure 7: Temporal sequence of the cell death pathway of NGF-deprived SCG sympathetic neurons and the point of action for death inhibitors (adapted from (Deshmukh and Johnson, 1998)

Cerebellar granule cells

The cerebellum is one of the largest brain structures and function to coordinate and fine-tune posture, balance and movements. It receives sensory input from many areas of the brain and spinal cord and projects for example via thalamus to the motor cortex, where signals from cerebellum influence the motor output. The most numerous cell type in the entire brain are the cerebellar granule cells which receives signals from mossy fibre afferents and projects to the efferent purkinje neurons. During development cerebellar granule cells are formed in the external granular layer (EGL) and migrate inwards to the granule cell layer (Haines, 1997). The large number of granule cells that can be cultured from one rat or mouse cerebellum makes it suitable for *in vitro* studies of cell death in the central nervous system.

BDNF is an important neurotrophic factor for cerebellar granule cells and can promote survival of these cells in vitro (Segal *et al*., 1992). It can also protect cerebellar granule cells from various apoptotic stimuli such as glutamate excitotoxicity and low K^+ (see below) and promote fibre outgrowth (Lindholm *et al*., 1993). However, BDNF deficient mice show grossly normal cerebellar development, indicating that other factors can promote survival of cerebellar granule cells (Jones *et al*., 1994). Another factor that can enhance cerebellar granule cell survival is IGF-1 (D'Mello *et al*., 1993) but the signalling pathways appears to differ between BDNF and IGF-1. BDNF-induced but not IGF-1-induced survival can be blocked by MEK inhibitors (Bonni *et al*., 1999), whereas IGF-1 mediated survival could be blocked by PI3-K inhibitors (D'Mello *et al*., 1997; Dudek *et al*., 1997). BDNF can also activate PLCγ, elevate intracellular Ca^{2+} levels and thereby activate PKC and BDNF-mediated survival is blocked by PKC inhibitors (Zirrigiebel *et al*., 1995).

The most common model for apoptosis in cerebellar granule cells is switching from serum containing medium with high (25 mM) potassium (K^+) concentration to a serumfree medium with low (5 mM) K^+ concentration, after which the neurons die in an apoptotic, macromolecule synthesis dependent manner (D'Mello *et al*., 1993). This cell death can be prevented by IGF-1, BDNF and NT-4 (D'Mello *et al*., 1993; Kubo *et al*., 1995). 25 mM K^+ mediates survival by keeping the plasmamembrane depolarised, but the signal transduction pathways for this response is unknown. Elevations of intracellular Ca^{2+} appears to be involved

(Galli *et al*., 1995), whereas it is not dependent on PI3-K (D'Mello *et al*., 1997; Dudek *et al*., 1997).

Like NGF-deprived sympathetic neurons, K^+/serum deprived cerebellar granule cells acquire decreased glucose uptake, protein and RNA synthesis. In addition c-Jun mRNA and protein levels, as well as c-Jun phosphorylation, are increased (Miller and Johnson, 1996; Watson *et al*., 1998).

As in many other cell death models, cerebellar granule cells deprived of depolarising concentrations of K^+ show dephosphorylation of Bad (Gleichmann *et al*., 2000), translocation of cytochrome c to the cytoplasm (Gleichmann *et al*., 1998) and caspase activation (Miller *et al*., 1997). Caspase and calpain inhibitors as well as expression of inhibitor of apoptosis proteins (IAPs) block or delay cell death (D'Mello *et al*., 1998; Nath *et al*., 1996; Simons *et al*., 1999). Furthermore, cerebellar granule cells from Bax deficient mice do not undergo apoptosis following K^+ deprivation (Miller *et al*., 1997).

THE PRESENT STUDY

Aims of the study

General aim

The aim of this thesis is to gain deeper insight into how the Bcl-2 family members act to regulate cell death in the nervous system. This is accomplished by studying the function of Bcl-2 family members within neurons and by studying their mutual interactions. In addition the expression levels of the Bcl-2 family members in the nervous system and their regulation during as well as after survival or death stimuli are studied.

Specific aims

- To study the expression levels and patterns of Bcl-x and Bcl-w mRNA in the developing and adult rat brain using RNA blotting and *in situ* hybridisation.
- To study the protein expression patterns of Bcl-w and Bad using immunohistochemistry.
- To study the regulation of Bcl-2, Bcl-x and Bax mRNA expression in cerebellar granule cells in response to BDNF and IGF-1 and at different cell densities.
- To search for novel interactors of Bcl-w using the yeast two-hybrid system and to confirm these interactions with co-immunoprecipitations.
- To study the function of Bcl-w, Bad and a novel splice variant of Bad in superior cervical ganglia (SCG) sympathetic neurons in the presence and absence of nerve growth factor (NGF)

Material and methods

Experimental animals

Wistar rats (Max-Planck-Institute or B&K, Sweden) were used in all experiments. Rats were killed by CO_2 asphyxiation or decapitation. The study was approved by the local ethical committee.

Primary neuronal cell culture (Paper I, II and III)

Two types of neuronal cell cultures have been used in this thesis: Cerebellar granule cells and superior cervical ganglia (SCG) sympathetic neurons.

Cerebellar granular cells were prepared from postnatal day 6 (P6)-P7 Wistar rats as described previously (Lindholm *et al*., 1993). Briefly, the tissue was treated with papain and DNAse at 37˚C 15 min and was sequentially dissociated. For the growth factor studies in paper I and for paper II, cells were plated at a density of $2.5x10^5$ cells/cm^2 on polypolyethylenimine-coated plates. In the cell density study in paper I, cells were plated in various cell densities ranging from 1.1 to 8.8 x 10^5. The cells were grown overnight in DMEM with 10% FCS, before changing to a defined serum-free medium (Brewer and Cotman, 1989). The medium was in some cultures supplemented by insulin (4 μg/ml), IGF-1 (20 ng/ml), and/or brain-derived neurotrophic factor (BDNF, 20 ng/ml) as survival promoting factors. In paper I, cell number was determined or cells were harvested for RNA preparation at various time points. In paper II, insulin and BDNF was withdrawn three days after plating and cells were harvested 24 h later.

Sympathetic neurons were prepared from P1-P2 mice by digesting the SCG with collagenase, dispase and trypsin, and triturating the ganglia mechanically with siliconized glass Pasteur pipette. Nonneuronal cells were removed by extensive preplating. The neurons were cultured in polyornithine-laminin-coated dishes in a 1:1 ratio of F12:DMEM media (Life Technologies AB, Sweden) containing 3% of fetal calf serum (HyClone, Cramlington, UK), SATO serum substitute (Davies, 1995), and 30 ng/ml of mouse 2.5 S NGF (Promega, Madison, WI, USA) at 37°C in humid atmosphere containing 5% CO_2. Neither antibiotics nor antimitotic drugs were included in the culture medium.

Determination of cell number (paper I and III)

In paper I, cell numbers were determined by trypan blue exclusion or by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT)-assay (Mosmann, 1983). For trypan blue exclusion, 0.2 % trypan blue were added to the cells which were detached with trypsin, collected by centrifugation and counted. For MTT assay, 0.5mg/ml of MTT was added to the cells and incubated for 3h at 37˚C. The purple precipitate formed by the living cells was solubilised and measured spectrophotometrically.

In paper III, the injected neurons, identified by their fluorescence of green fluorescent protein, (GFP), that was alive 72 h after injection were counted. Healthy fluorescent neurons with intact nucleus, phase-bright cytoplasm and intact neuritic tree were considered as living.

Vectors and cloning

A partial cDNA for rat bcl-w was isolated by screening a P14-P16 rat brain Uni-ZAP library (Stratagene, CA, USA) with a 240 fragment obtained by degenerate polymerase chain reaction (PCR). The remaining part of the coding sequence for rat Bcl-w was cloned by PCR. Rat bad-α and bad-β was isolated by a yeast-two-hybrid screen (see below) and the 5' ends were cloned by PCR.

The following constructs have been used in this thesis:

pBluescript vector for cRNA-probes for RNA blotting, RPA and ribo-*in situ*: pBKS-Bcl-2(503 bp, (Castrén *et al*., 1994)), pBKS-Bcl-x(650 bp, exon I), pBKS-Bcl-x_L(aa 112-216), pBKS-Bax (nt 43-3), pBKS-Bcl-w (nt 138-381), pBKS-Bad (nt 1-306), pBKS-Bad(nt 306-615) and pBKS-18S rRNA
Bcl-w deletion constructs cloned into the GAL-4 DNA-binding domain vector pYTH for yeast two-hybrid: aa 1-172 (full-lengthΔTM), aa 1-42 (BH4), aa 41-78 (BH3), aa 79-172 (BH1+BH2), aa 1-78 (BH4+BH3) and aa 41-172 (BH3+BH1+BH2).
Mammalian expression vectors for microinjection and co-immunoprecipitation: pEGFP-Bcl-w, pCDNA-Badα and pCDNA-Badβ.

RNA preparation, RNA blotting and Ribonuclease protection assay (RPA) (paper I-IV)

RNA was prepared from rat tissues using the guanidine isothiocyanate /acidic phenol method (Chomczynski and Sacchi, 1987).
RNA blotting: 20 μg of total RNA was loaded on a 1% agarose formaldehyde containing gel and blotted to nylon filters (Amersham, UK) in 10x SSC. For paper I cRNA probes for rat bcl-2, bcl-x_L, bax and 18S rRNA was used. For study II, cDNA probes for rat bcl-w and bcl-x were used. A 850 PstI fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) generously provided by Dr. C. Bark.
The cRNA probes were transcribed with T7 polymerase from pBKS and the DNA probes were labelled by random priming (RPN1633, Amersham, UK). The filters were hybridised in 50% formaldehyde overnight at 65˚ for cRNA probes and at 42˚C for DNA probes, and washed in 2x SSC, 0.1 % SDS at successively higher temperatures.
RPA: cDNA corresponding to nucleotides 1-306 or 306-615 of bad-α was cloned into pBKS and transcribed with T7 RNA polymerase (Ambion, TX, USA), using ^{32}P-labelled UTP (NEN, MA, USA). A molecular weight marker (#7780, Ambion) was also transcribed. RNA was prepared as above and 5 μg of RNA was hybridised with the probe at 42°C overnight. Unhybridized probe was digested and doublestranded RNA was precipitated according to the protocol for the RPAIII^{TM} kit (Ambion, TX, USA) and loaded on a 6% polyacrylamide gel together with the marker.
For both RNA blotting and RPA bands were detected and quantified by a PhosphorImager (Molecular Dynamics, CA, USA).

In situ hybridization (paper II)

Rats of embryonic day 13 (E13), E17 and E20 were sacrificed and frozen in isopentan on dry ice. Brains from postnatal day 4 (P4), P10, P14 and adult rats were dissected and frozen in the same way. 14 μm sections were prepared on a cryostat (Leitz Digital 1702, Wetslar, Germany) and mounted on slides coated with 50 μg/ml poly-L-lysine (Sigma, Missouri). The antisense oligonucleotides were 5'- CCT TGG AAA AGT TCG TCG GAA ACC TGG GTG AAG CGT TGC TGG GCT GAG-3' for Bcl-w and 5'-CCG TAG AGA TCC ACA AAA GTG TCC CAG CCG TTC TCC TGG ATC CAA GG-3' for Bcl-x_L. 50 ng oligo was 3' labelled with 100 mCi ^{35}S-dATP (NEN, Massachusetts) using terminal deoxynucleotidyl transferase (USB, Ohio). 1x10^7 cpm/ml of the labelled oligo was added to the hybridisation solution (50% formamide (Kodak), 4x SSC, 1x Denhardt's, 1% N-lauroyl-sarcosine (Sigma), 20 mM phosphate buffer pH 7.0, 100 mg/ml dextran sulphate (Pharmacia, Sweden), 275 μg/ml tRNA (Sigma), 490 μg/ml salmon testes DNA (Sigma), 125 mM DTT (Sigma)). To the control sections, a 200x excess of cold oligo was added. The mixture was added to the sections, which were incubated at 42˚C overnight. After hybridisation, the sections were washed four times with 1x SSC, rinsed in cold water and dehydrated in an ethanol series. The slides were exposed to X-ray film (Hyperfilm, β-max, Amersham) followed by dipping in emulsion (NTB2, Kodak).

Immunohistochemistry (paper IV and unpublished results)

Wistar Rats were perfused with 0.4% paraformaldehyde and the brains were post-fixed in paraformaldehyde over night and cryoprotected in 30% sucrose in PBS for 2 days, or brains from CO_2-sufficated rats were frozen in isopentane on dry ice. 14 μm sections were prepared on a chryostat (Leitz Digital 1702, Wetslar, Germany) and mounted on Superfrost slides (Menzel-Gläser, Germany). The slides were fixed in 4% paraformaldehyde for 30 min on ice and washed for 3 x 5 min in PBS. Endogenous peroxidase was inhibited by treatment with 0.3% H_2O_2 in PBS for 15 min at RT, the slides were washed for an additional 3 x 5 min and blocked in TNB buffer (TSA-Kit, NEN, MA, USA) over night at +4ºC. The slides were incubated with the primary antibody (polyclonal anti-Bad, sc-943-G, Santa Cruz, CA, USA or polyclonal anti-Bcl-w (AAP-050, Stressgen, Canada) in dilution 1: 500 at +4ºC over night. The slides were washed in PBS, 0.3% Triton X-100, 5x15 min, incubated with secondary antibody (biotinylated anti-goat IgG or anti-rabbit IgG, Vector, CA, USA) for 2 h at RT, washed for an additional 5x15 min, incubated in ABC solution (Vectastain ABC kit, Vector, CA, USA) for 1 h at RT, washed 3 x 5 min in PBS and 2 x 5 min in 50 mM Tris-HCl, pH 7.4, and developed by Sigma Fast diaminobenzidine tetrahydrochloride (DAB) and Urea-H_2O_2 tablets (Sigma, MO, USA).

Double labeling; *in situ* hybridisation together with immunohistochemistry (paper II)

Rats were perfused as above and 30 μm sections were cut on a cryostat (Leitz Digital 1702, Wetslar, Germany) and collected in PBS. Sections were treated with 1 mg/ml proteinase K (Promega) in 100 mM Tris-HCl pH 8.0, 50 mM EDTA for 10 min at RT, washed in proteinase buffer for 5 min, acetylated in 0.25% acetic anhydride in 0.1 M Triethanolamine, pH 8.0 for 10 min and washed in PBS 2x5 min. They were then pre-hybridised in 50% formamid, 0.3 M NaCl, 20 mM Tris-HCl pH 8.0, 5 mM EDTA, 10% Dextran Sulphate, 1x Denhardts, 500 mg/ml tRNA, 20 mM DTT for 2h at 60˚C. cRNA probes for sense and anti-sense bcl-w was transcribed using T3 or T7 RNA polymerase. Fresh hybridisation solution containing 5 milj. cpm/ml probe was added to the sections which were hybridised over night at 60˚C. After hybridisation the sections were RNase-treated and washed. After in situ hybridisation, immunohistochemistry was performed as above with the antibodies: anti-calbindin D (Sigma), diluted 1:200 and anti-tyrosine hydroxylase (kindly provided by Dr. Hatanaka, Osaka University, Japan), diluted 1:2000. The sections were mounted on gelatin-coated slides, air-dried overnight, dehydrated in an ascending ethanol series containing 0.3% ammonium acetate, dipped in NTB2 emulsion (Kodak) and exposed for 7 weeks.

Yeast two-hybrid system (paper III)

Linearised pYTH-Bcl-w full-length or deletion constructs were integrated into the genome of Y190 yeast cells with lithium acetate/PEG based transformation method as described (Aspenström and Olson, 1995; Mercer *et al*., 2000) and selected on tyrosine deficient plates. A yeast clone expressing the full-length bcl-w was used to screen a library from adult rat brain fused to the Gal-4 activation domain (Clontech, CA, USA). Positive clones were selected on histidine deficient plates and assayed for β-galactosidase activity.

Co-immunoprecipitation (paper III)

pEGFP-Bcl-w or empty pEGFP vector together with pcDNA-Bad-α or pcDNA-Bad-β were co-transfected into COS-7 cells using the calcium phosphate method. After 16 h, cells were collected and lysed in 50 mM HEPES, pH 7.3, 250 mM NaCl, 0.2% NP40 with protease inhibitors for 30 min on ice. Lysates were incubated with monoclonal anti-GFP-antibodies (Clontech, CA, USA) under rotation for 2 h at +4°C followed by a 1h incubation with Protein

G-Sepharose (Amersham Pharmacia Biotech, Sweden). The precipitates were washed three times with lysis buffer, resuspended in SDS loading buffer, boiled and loaded on a SDS-polyacrylamide gel. The proteins were blotted to PVDF filter and detected with polyclonal antibodies against Bad (#9292, New England Biolabs, MA, USA), goat-anti-rabbit secondary antibody conjugated to horseradish peroxidase (DAKO, Denmark) and enhanced chemiluminesence.

Microinjection (paper III)

SCG sympathetic neurons (see above) maintained 5-6 days in vitro with NGF were used for injection. The medium was changed to L-15 (Life Technologies AB, Sweden) containing 10% of FCS and standard antibiotics. pEGFP-Bcl-w (50 ng/µl), pCDNA-Bad-α (100ng/µl), pCDNA-Bad-β (100ng/µl) or combinations of these were pressure-injected into the nuclei using manual micromanipulator MMO-220 (Narishige International Ltd, London, UK) and Transjector 5246 (Eppendorf, Germany) under direct visual control. After injection, L-15 medium was changed to culture medium and neurons were grown further with NGF, or NGF was neutralized with function-blocking antibodies (Boehringer, Mannheim, Germany). In addition, 10 ng/µl of plasmid (pGreen Lantern-1) (Life Technologies AB, Sweden), encoding green fluorescent protein (GFP) was included in the injection mixture. Bcl-w was expressed as a GFP fusion protein. Neurons surviving the injection procedure were counted 3-4 h after injection (initial neurons). In every experiment, between 50-100 neurons were successfully injected for every plasmid combination. 72-75 h after injection, the number of surviving injected neurons was determined as described above. All treatment groups of every NGF-deprivation or NGF-maintenance experiment were injected on the same day on the same culture. Experiments were repeated three times on independent cultures. Uninjected control neurons were counted from one dish in every experiment. As introduced DNA had some unspecific toxic effect, control neurons were mock-injected with empty pcDNA3.1 or pEGFP-C1 vectors. Significance of differences between the means was estimated by one-way ANOVA followed by Tuckey´s post hoc test at the significance level of $\alpha=0.05$. Bad-α and Bad-β proteins produced from injected plasmids were demonstrated by staining the injected neurons with anti-Bad antibodies (#9292, New England Biolabs, MA, USA) after counting the experimental results. For that, neurons were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS. Staining of NGF-deprived neurons was not possible due to their loosened attachment.

Results

Bcl-2, bcl-x, bcl-w and bax mRNA levels in response to neurotrophic factors (paper I & II).

Since the growth factors BDNF and IGF-1 can promote survival of cerebellar granule cells (D'Mello *et al*., 1993; Lindholm *et al*., 1993; Segal *et al*., 1992), we explored whether regulation of the mRNA levels of the Bcl-2 family members could account for part of this survival response. This was however, found not to be the case. Neither bcl-2, bcl-x nor bax mRNA levels were changed after addition of these neurotrophic factors. The levels of GAPDH mRNA and 18S rRNA were also unchanged. When cerebellar granule cells were first grown with insulin and BDNF and then deprived of these factors, we obtained a slightly different picture. Bcl-x and bcl-w mRNA levels were downregulated in this case, but the mRNA levels of GAPDH also decreased, suggesting a more general decrease in mRNA

production which is a common feature in apoptotic cells. However, β-Actin mRNA levels and the total rRNA levels did not change (unpublished results).

Bcl-2 and bcl-x mRNA levels in high cell density (paper I)

In vivo, cerebellar granule cells are densely packed. We therefore investigated if high cell density influenced the survival of these neurons in vitro. Indeed, high cell densities promoted survival of neurons both in the presence and absence of IGF-1 (Fig. 8A). In addition, bcl-2 and bcl-x mRNA levels were increased at higher cell densities (Fig. 8B). Thus, neurotrophic factors and cell-cell contact promotes survival by at least partially distinct pathways.

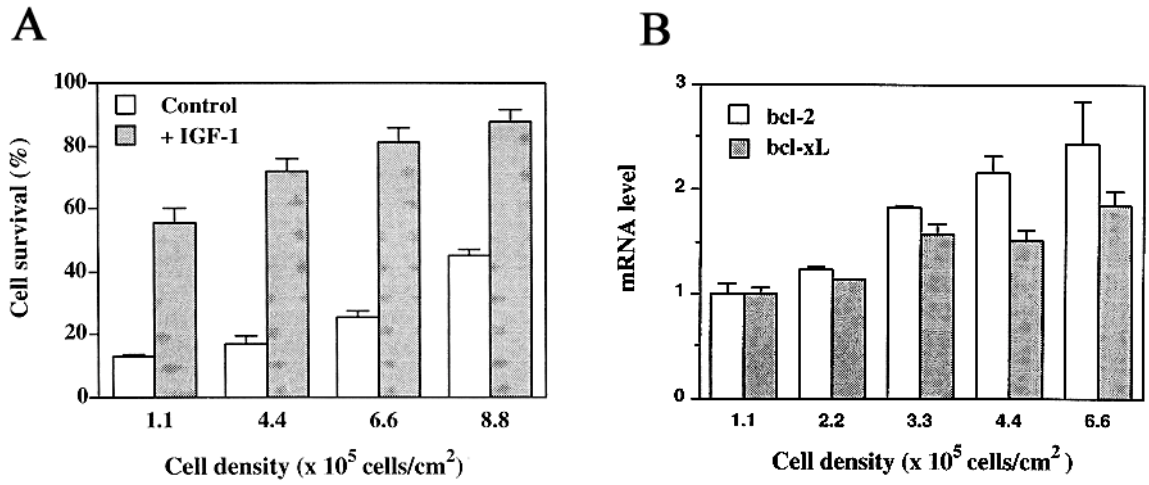

Figure 8: A) Cell survival of cerebellar granule cells at different cell densities, in the absence and presence of IGF-1. B) mRNA levels of bcl-2 and bcl-x_L at different cell densities in the presence of IGF-1.

Isolation of rat bcl-w cDNA (paper II)

To isolate the rat bcl-w cDNA, a polymerase chain reaction (PCR) fragment (obtained with degenerate primers of the mouse bcl-w sequence) was used to screen a postnatal rat brain library. The resulting sequence showed high homology to the mouse and human counterparts, diverging only at one and two amino acids, respectively.

Bcl-w, bcl-x and bad mRNA levels during rat brain development (paper II-IV).

We examined the mRNA levels of bcl-w, bcl-x and bad during rat brain development. Whereas bcl-x mRNA levels decreased during the first postnatal weeks, bcl-w mRNA levels were dramatically increased (Fig. 9A). The two bands in the bcl-x blot do not represent bcl-x_L and bcl-x_S, since both are recognised by a bcl-x_L specific probe. Neither bad-α, nor the newly identified bad-β mRNA levels changed between P0 and adult brain (Fig. 9B). However,

preliminary data suggests that additional forms of Bad might be differentially regulated during development.

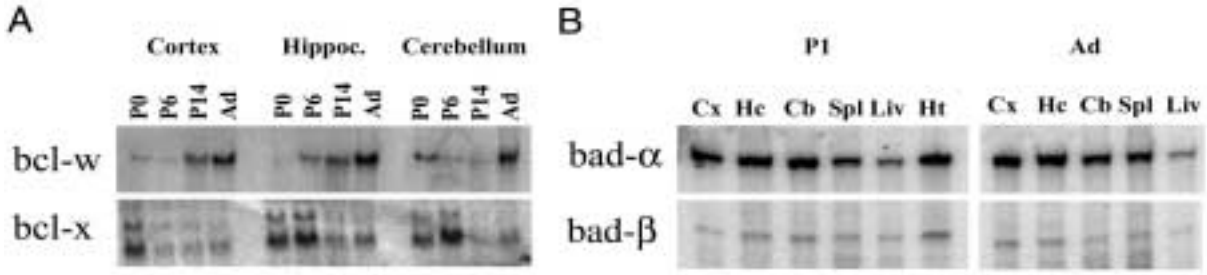

Figure 9: mRNA expression of bcl-w, bcl-x (A), bad-α and bad-β (B) during development of the brain and other tissues

Abbrevations used in figures:

5Gn: Trigeminal ganglion
5n: Trigeminal nerve
7: Facial nucleus
9/10Gn: Glossopharyngeal/vagus ganglia
Ad: Adult
Am: Amygdala
CA1-4: CA regions of the hippocampus
Cb: Cerebellum
Cx: Cerebral cortex
CxP: Cortical plate
Coll: Colliculi
DG: Dentate gyrus
DRG: Dorsal root ganglia
EGL: External granular layer
Hb: Habenular nucleus
Hc: Hippocampus
HT: Hypothalamus
Ht: Heart
Liv: Liver
NE: Neuroepithelium
P: Postnatal
Pir: Piriform cortex
Retic: Ret
SC: Spinal cord
Spl: Spleen
SN: Substantia nigra
Sp5: Spinal trigeminal nucleus
Str: Striatum
Thal: Thalamus
Ver: Vermis

Bcl-w mRNA expression pattern in rat brain (paper II).

To further characterise the expression patterns of bcl-w mRNA expression in the developing and adult rat brain, we performed *in situ* hybridisation. Bcl-w was expressed in many structures of the embryonic brain with high levels in the early ventricular neuroepithelium and later in hippocampus, the cortical plate, cerebellum and trigeminal ganglia (Fig. 10A). In the adult brain the labelling is more pronounced and structures with particularly high expression levels are hippocampus, cerebellum, cortex, locus coeruleus, substantia nigra and the facial nucleus (Fig. 10B). Preliminary data show a similar pattern of Bcl-w protein expression in the adult brain (Fig. 11)

Bcl-x_L mRNA expression pattern in rat brain (paper II).

Bcl-x_L mRNA was widely expressed in embryonic brain with high levels in the entire neuroepithelium at E13. In contrast to bcl-w, bcl-x was also present in spinal cord and in dorsal root ganglia. Later in embryonic development, bcl-x is highly expressed in structures like cortical plate, hippocampus, thalamus, cerebellum and colliculi, as well as trigeminal and

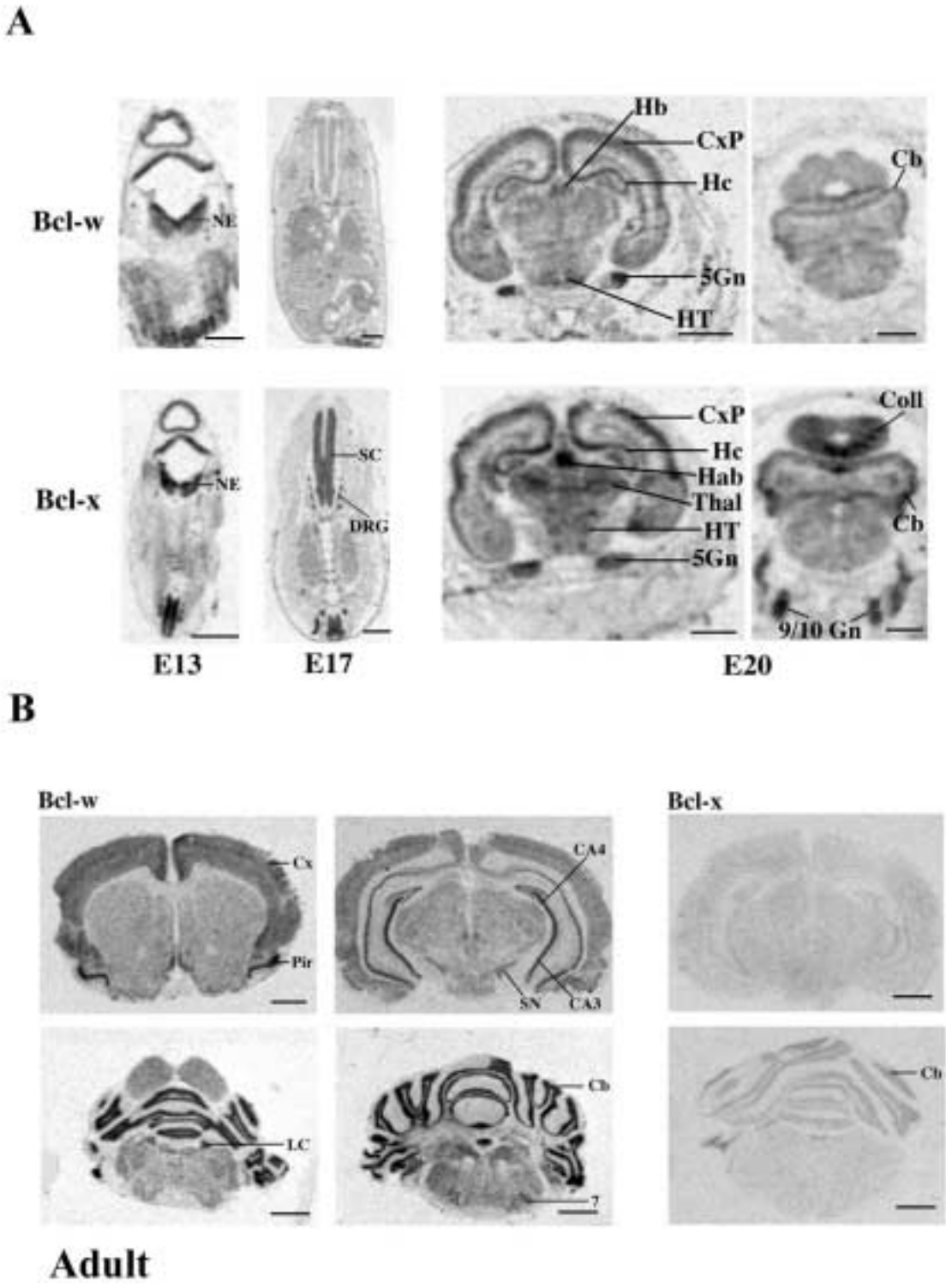

Figure 10: Bcl-w and Bcl-x mRNA expression in embryonic (A) and adult (B) nervous system. Scale bar 2 mm

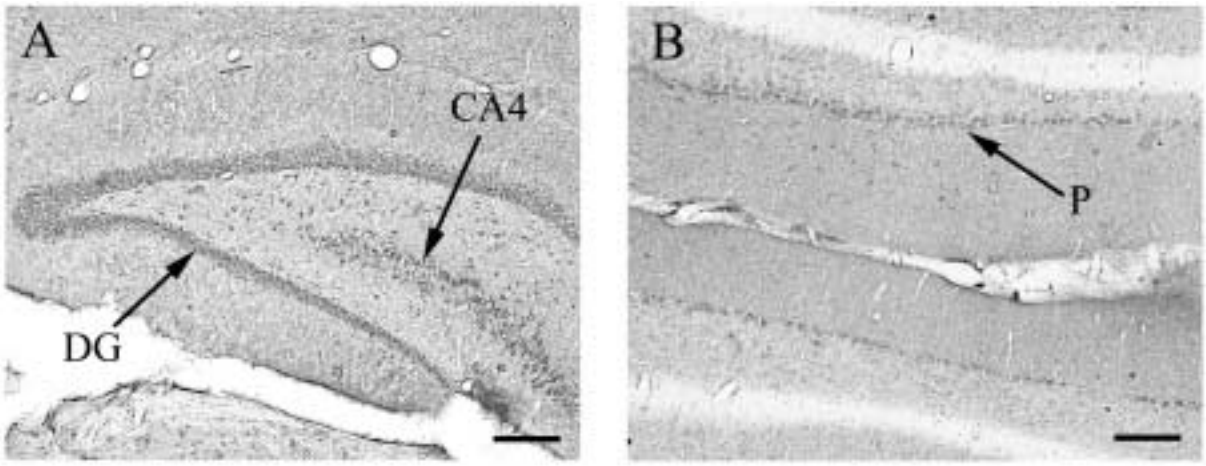

Figure 11: Expression of Bcl-w protein in the adult hippocampus (A) and cerebellum (B). Scale bar 0.2 mm

glossopharyngeal/vagus ganglia (Fig. 10A). During the first postnatal weeks, bcl-x mRNA decreased and was only expressed at low levels in the hippocampus and cerebellum (Fig. 10B)

Proteins interacting with Bcl-w (paper II and unpublished results)

In an attempt to elucidate the mechanism by which Bcl-w performs its anti-apoptotic function, we screened a yeast two hybrid library for potential interactors for Bcl-w. Out of 4 million transformants, we obtained approximately 3000 positive colonies, growing on histidine deficient plates for selection. Approximately 200 of these were verified with β-galoctosidase activity, another selective marker for interaction, and the 30 clones possessing the strongest activity were sequenced. Two of these clones corresponded to Bad, a pro-apoptotic Bcl-2 family member. In addition, one clone corresponded a novel splice variant of Bad, which we named Bad-β (see below). These interactions were confirmed in mammalian cells, using co-immunoprecipitation. Using different deletion constructs of Bcl-w we discovered that all of the BH domains of Bcl-w were needed for binding to Bad-α and Bad-β. Other potential interactors, which were not confirmed and whose function we did not study, include:
Gtl-2: A mRNA important for embryonic development but suggested not to be translated based on its short open reading frames and lack of Kozack site (Schuster-Gossler *et al*., 1998). The enforced expression of Gtl-2 protein in the yeast two-hybrid system and its interaction with Bcl-w, may represent a condition never occurring *in vivo*.
Cytochrome c oxidase (COX), subunit I: Given the Bcl-2 family members function at mitochondria, this finding was of course of great interest. However, COX-I is normally encoded by mitochondrial DNA that uses slightly different codons for translation. If encoded by nuclear DNA, which is the case in the two-hybrid system, the translation is truncated at an more upstream stop codon, given rise to a very short, hydrophobic peptide not likely to be present *in vivo*.
Hsp70: This heatshock protein has earlier been shown to interact with Bag-1 (Takayama *et al*., 1997), which in turn binds Bcl-2 (Takayama *et al*., 1995). It's possible that a similar ternary complex forms with Bcl-w. Another possibility is of course a direct interaction between Bcl-w and Hsp70. It should be noted, however, that heatshock proteins have often turned out to be false positives (www.fccc.edu/research/labs/golemis/main_false.html).

γ-Actin: Binding of Bcl-w to actin could possibly sequester Bcl-w to the cytoskeleton, like the pro-apoptotic family member Bim is sequestered to microtubules. However, our preliminary results show that Bcl-w is localised to the mitochondria.
ArrestinD: Arrestins are regulatory proteins that function to desensitise G protein-coupled receptors (Lefkowitz, 1998). Bcl-w's potential role in G protein signalling remains to be elucidated.
Intersectin: An adaptor protein with SH3 domains possibly involved in endocytosis (Yamabhai *et al*., 1998).
Snapin: A recently identified synaptic protein important for neurotransmittor release (Ilardi *et al*., 1999).

Identification of (a) novel splice variant(s) of rat bad (paper III and IV).

One of the positive clones identified as an interactor of Bcl-w, was found to corrspond to a novel splice variant of rat Bad, Bad-β. This variant differs from Bad-α in its carboxyterminus. The stop codon in Bad-α is spliced out and Bad-β utilises the 3' untranslated region to encode a unique carboxyterminal aa sequence (Fig. 12). To map the exon-intron boundaries of the bad gene, we performed PCR on genomic DNA and found that the splice donor site of Bad-β correlated with an exon-intron boundary whereas the splice acceptor site was situated within an exon.

We also observed an exon-intron boundary immediately upstream of an ATG which is utilised as a start codon by the human variant of Bad (Ottilie *et al*., 1997), but is 42 aa downstream of the start codon utilised by the mouse and rat homologues. We wanted to explore the possibility that rat bad is alternatively spliced at this splice site and that rat bad could also use the more downstream ATG as a start codon. Indeed, using a RPA probe spanning this splice site we obtained two bands, one corresponding to both the first and the second coding exon of bad and one band corresponding only to the second coding exon. Thus,

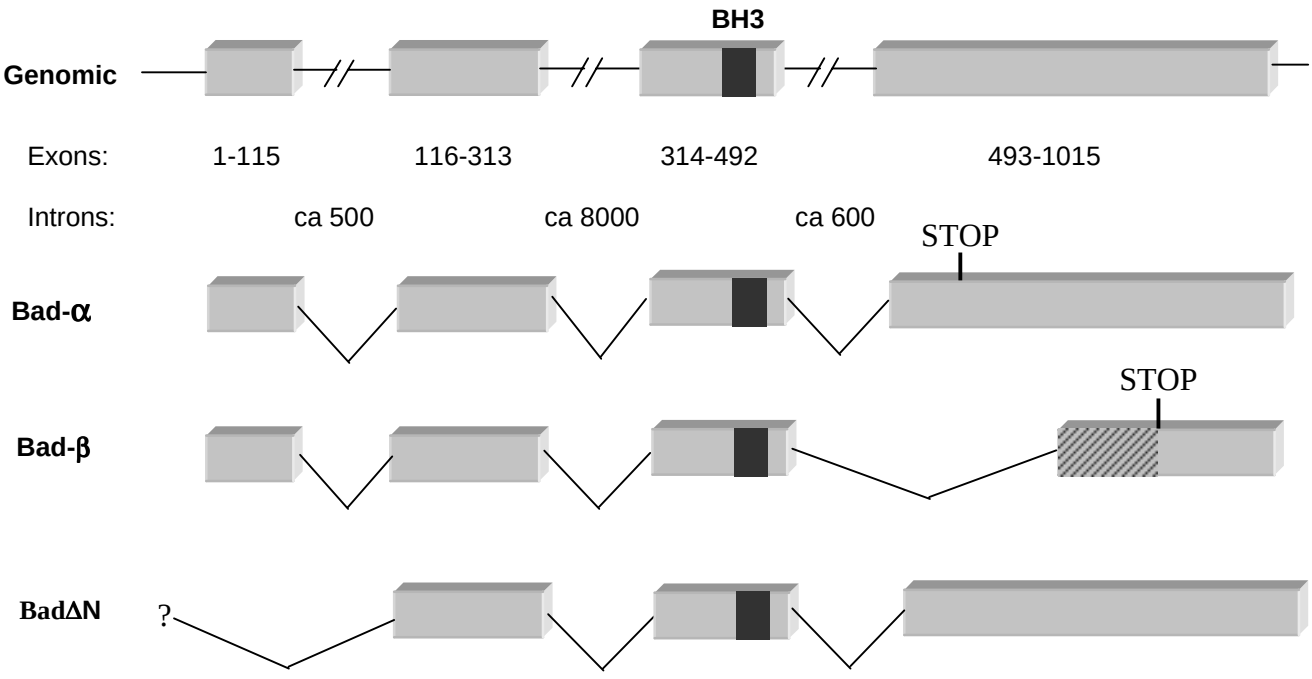

Figure 12: Genomic organisation of the bad gene and the structure of the splice variants of bad.

an additional isoform of bad appears to exist, which does not express the first coding exon of the known forms (Fig. 12). This variant could either be spliced to a more upstream exon, possibly being untranslated, or it could be transcribed from an alternative promoter within the first intron.

Bad mRNA and protein expression in rat brain (paper IV)

As noted above, neither bad-α, nor bad-β mRNA levels appear to change between P0 and adult tissues (Fig 9B). However, preliminary data suggest that the novel isoform, differing in the aminoterminus might be differentially regulated, although more thorough quantitative studies are needed.

To investigate the expression pattern of Bad protein in neonatal and adult brain, we performed immunohistochemistry on slices from these tissues, using an antibody against the carboxyterminus of Bad-α. In agreement with earlier studies (Rickman *et al*., 1999), we detected widespread Bad labelling in the neonatal brain, including hippocampus, cortex, amygdala, thalamus and cerebellum (Fig. 13A-B). In the adult, the labelling was more restricted, but we still detected many Bad-immunoreactive neurons in structures like hippocampus, cortex and thalamus (Fig. 13C-D). Other investigators have found Bad protein expression to be restricted to choroid plexus in adult brain (D'Agata *et al*., 1998; Rickman *et al*., 1999) using an antibody against the most aminoterminal part of Bad. This antibody would not detect the putative novel isoform lacking the first exon. Thus, this isoform of Bad may be the predominate form in adult rat brain.

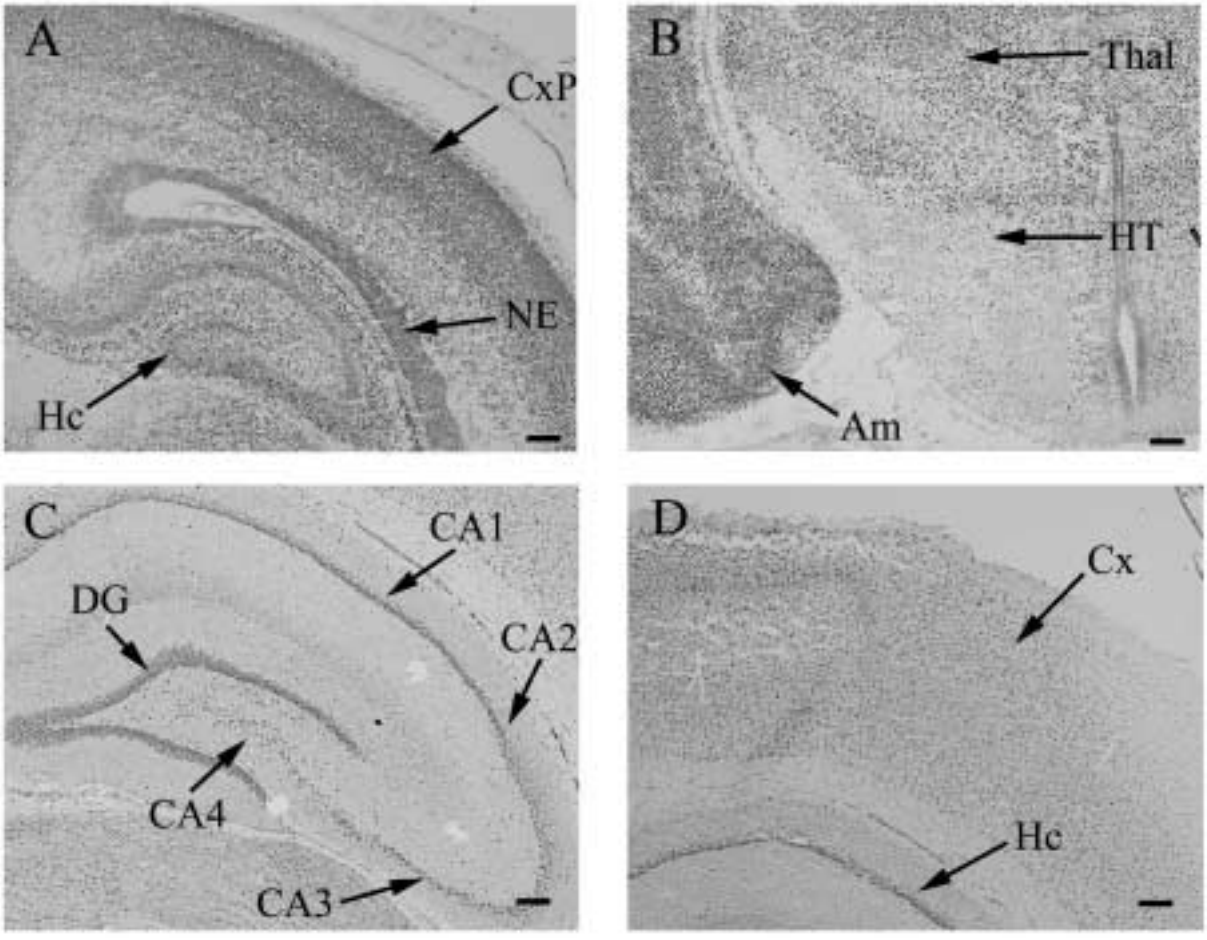

Figure 13: Expression of Bad protein in neonatal (A-B) and adult (C-D). Scale bar 0.2 mm

Bad-α and Bad-β induce cell death in sympathetic neurons (paper III).

To study the function of Bad-α and Bad-β in neurons, we microinjected expression plasmids encoding these proteins into SCG sympathetic neurons. We found that both Bad-α and Bad-β could induce cell death of SCG neurons in the presence of NGF (Fig. 14A). More dead neurons were found in neurons injected with plasmids for Bad-β than for Bad-α. However, immunocytochemistry of injected neurons showed higher expression levels of Bad-β than Bad-α. Thus, we cannot conclude that Bad-β is a more potent killer.

We also injected SCG neurons deprived of NGF with expression plasmids encoding Bad-α and Bad-β to investigate whether they could accelerate cell death after NGF deprivation. This was not the case however. Instead, a small, although not significant, increase in survival was observed.

Bcl-w protects sympathetic neurons from cell death (paper III).

In addition to Bad-α and Bad-β, we also explored the function of Bcl-w in SCG neurons. Neurons injected with expression plasmids for Bcl-w were almost completely rescued after NGF deprivation (Fig. 14B). In addition, Bcl-w counteracted the death-inducing activity of Bad-α and Bad-β in NGF-maintained neurons (Fig. 14A), probably through the interaction between Bcl-w and Bad detected by the two-hybrid system and co-immunoprecipitation. Bad-α and Bad-β could not, however, inhibit the rescuing effect by Bcl-w in NGF-deprived neurons (Fig. 14B)

Discussion

This thesis gives an insight into how some of the Bcl-2 family members may act to regulate apoptosis in the nervous system. In particular, it spreads some light on the previously sparsely studied family member, Bcl-w. Using RNA blotting and *in situ* hybridisation, we show that Bcl-w is highly expressed in the adult nervous system, suggesting an important function for Bcl-w to maintain survival of mature neurons. This could be important from a clinical point of view, in particular since bcl-w mRNA is highly expressed in some structures that degenerate in neurodegenerative diseases, such as the substantia nigra. We have also shown for the first time that Bcl-w has a protective function in neurons, protecting SCG neurons from dying after neurotrophic factor withdrawal. Bcl-w also protects from cell death induced by the pro-apoptotic family member Bad. In accordance with this, we detected a physical interaction between Bcl-w and Bad in the yeast two-hybrid system and in co-immunoprecepitation studies. Although we do not show direct evidence for interaction between Bcl-w and Bad within sympathetic neurons, this is a possible explanation for protective function of Bcl-w. Bad did not, however, counteract the survival effect of Bcl-w in the absence of Bad, suggesting that Bad functions upstream of Bcl-w.

In contrast to Bcl-w, Bcl-x mRNA was downregulated during brain development. Other studies have shown a retained mRNA expression of Bcl-x in the adult brain (Frankowski *et al*., 1995; Gonzalez *et al*., 1995). Different techniques and species might account for these discrepancies. Similarly, there are contradictory results concerning the protein levels of Bcl-x during brain development. Whereas Alonso *et al*. (1997) and Mizuguchi *et al*. (1996) showed

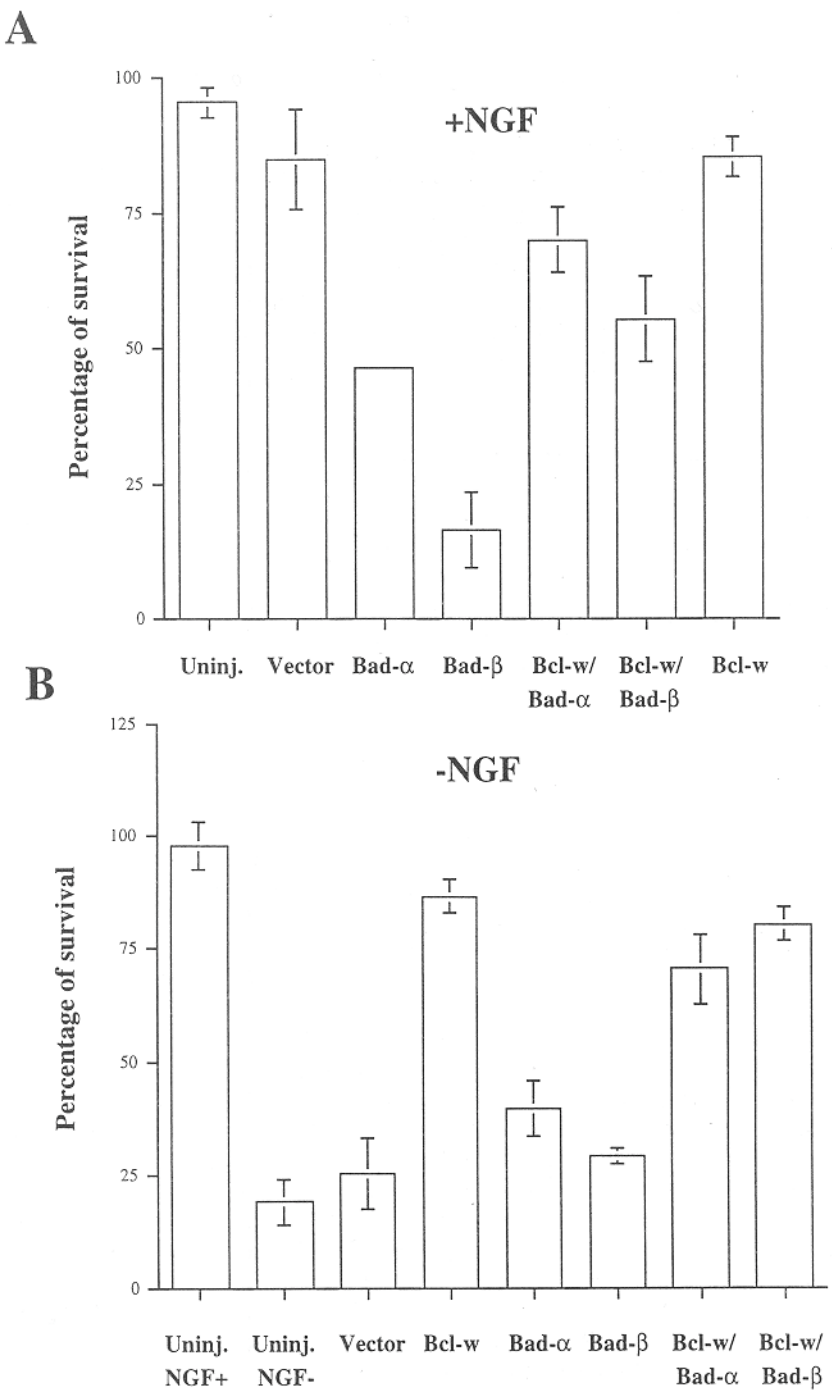

Figure 14: Survival of SCG sympathetic neurons in the presence (A) and absence (B) of NGF and injected with expression plasmids for Bcl-w, Bad-α and Bad-β.

decreased expression of Bcl-x protein in the adult brain compared to the neonatal, Vekrellis *et al*. (1997) detected unchanged levels of Bcl-x during development.

A novel splice variant of Bad was identified in a yeast two-hybrid screen using Bcl-w as a bait. This splice variant was termed Bad-β and possesses a unique carboxyterminal sequence. Further studies on the structure of the Bad gene indicated the existence of an additional variant, which does not express the first coding exon of the known forms. Many other Bcl-2 family members and other apoptotic regulators like caspase-9 and Apaf-1 are alternatively spliced (reviewed by Jiang and Wu, 1999). Although in some cases there are clear functional differences between the different splice variants, in other cases, the consequence of alternative splicing is not clear.

Both the known Bad-α and the Bad-β form induced cell death in sympathetic neurons in the presence of NGF. Independent of our study, Bad-α was found to have a pro-apoptotic function in SCG neurons by (Roberts *et al*., 2000). These authors also showed that Bcl-2, in analogy with Bcl-w, could protect against Bad induced cell death possibly by inducing the specific degradation of the Bad protein. In contrast to its death inducing effect in the presence of NGF, neither isoform of Bad could accelerate cell death after NGF deprivation. Instead both isoforms showed a small, although not significant protective effect. This was surprising but a similar finding was observed for Bax in sensory neurons (Middleton *et al*., 1996). Neither bad-α nor bad-β mRNAs were found to be regulated during development and the ratios between the two forms were not altered. Thus, for the few parameters studied here, we did not observe any difference between Bad-α and Bad-β.

In contrast, the expression pattern of the third isoform, lacking the first exon of the known form, appears to differ from the form(s) expressing the first exon. Particularly, this new isoform appears to be more abundant in adult brain. Further studies are required to elucidate the function of this new isoform.

Several studies have shown that cell contact with extracellular matrix proteins is important for survival. We show here that also cell-cell contacts enhance survival of cerebellar granule cells. Bcl-2 and bcl-x mRNAs were upregulated in high cell density cultures, indicating a role for these proteins in the survival response to cell-cell contacts. In contrast, neither bcl-2 nor bcl-x mRNAs were regulated by the neurotrophic factors IGF-1 and BDNF. In addition, bcl-x and bcl-w mRNA levels were not regulated after growth factor deprivation in these cells. Earlier studies have also shown that Bcl-2, Bax, Bcl-x mRNA or protein levels do not change in cerebellar granule cells exposed to low K^+ concentrations or the glutamate agonist NMDA (Gleichmann *et al*., 1998). Thus, the mechanism by which cell-cell contact promotes survival appears to differ from that utilised by other survival factors.

In summary this thesis has resulted in increased knowledge about the Bcl-2 family members in the nervous system, in particular about the relatively new family member Bcl-w that might be an important regulator of cell death in the adult nervous system. It has also revealed the existence of several isoforms of the pro-apoptotic protein Bad although further studies are required to clarify the functional importance of these isoforms. Finally, it has shown the importance of cell-cell contacts as a survival factor in neuronal cultures. As shown here and in other studies the function and mutual interactions of the Bcl-2 family members is complex, but the increased knowledge about these molecules provided by these thesis add to our understanding of the cell death machinery, which is important during brain development and in neurodegeneration.

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