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Anti-Apoptotic Proteins in Nerve Cell Survival and Neurodegeneration

BY

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ABSTRACT

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Apoptosis is a genetically regulated cell death program, which shows distinct morphological characteristics. It takes place during neuronal development and in some neurodegenerative diseases. During apoptosis, the intracellular proteins are degraded by various caspases, cysteine aspartases, which are regulated by pro- and anti-apoptotic signals. This thesis elucidates the role of anti-apoptotic proteins in nerve cell survival and neurodegeneration. Studies have focused on Bcl-2 family members and Inhibitor of Apoptosis Proteins (IAP).

XIAP and RIAP-2 are IAP proteins, which are expressed by neurons in the central nervous system. Kainic acid, a glutamate receptor agonist that induces seizures, increased XIAP immunoreactivity in rat hippocampus, whereas RIAP-2 expression in the same time decreased in degenerating neurons. Both XIAP and RIAP-2 were absent in dying neurons indicating that these proteins have a protective role in kainic acid induced neurodegeneration.

NAIP, another IAP family member, was shown to interact with the calcium binding protein Hippocalcin using the yeast two-hybrid system and immunoprecipitation experiments. Hippocalcin-NAIP interaction increased motoneuron survival in caspase-3 independent and dependent manners.

The anti-apoptotic Bcl-2 proteins, Bcl-2 and Bcl-x, were studied using cultured neurons and human neuronal progenitor cells. In the progenitor cells, Bcl-2 overexpression enhanced cell survival and induced downregulation of Caspase-2 (ICH-1) and caspase-3 (YAMA/CPP32). These results suggest a novel mechanism for the action of Bcl-2.

Estrogen was shown to inhibit death of cultured dorsal root ganglion neurons (DRG) after nerve growth factor withdrawal. The hormone increased the levels of Bcl-x, which may explain the known neuroprotective function of estrogen.

Key words: neuronal cell death, XIAP, RIAP-2, Hippocalcin, Bcl-2, Bcl-X, kainic acid, DRG, estrogen.

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To My Family

This thesis is based on the following original articles, which will be referred to in the text by their roman numbers:

I **Korhonen L.,** Hamner S., Olsson P.-A. and Lindholm D. (1997) Bcl-2 regulates the levels of cystein proteases ICH-1L and CPP32/Yama in human neuronal precursor cells. Eur. J. Neurosci. 9: 2489-2496.

II Patrone C., Andersson S., **Korhonen L.** and Lindholm D. (1999) Estrogen receptor-dependent regulation of sensory neuron survival in developing dorsal root ganglion. Proc. Natl. Acad. Sci. U. S. A. 96: 10905-10910.

III Mercer E. A., **Korhonen L**., Skoglösa Y., Olsson P.-A., Kukkonen J. P. and Lindholm D. (2000) NAIP interacts with hippocalcin and protects against calcium-induced cell death through caspase-3 dependent and independent pathways. EMBO J. 19: 3597-3607.

IV **Korhonen L**., Belluardo N. and Lindholm D. (2001) Regulation of X chromosome-linked inhibitor of apoptosis protein in kainic acid induced neuronal death in the rat hippocampus. Mol. Cell. Neurosci. 17: 364-372.

V Belluardo N. *, **Korhonen L**.*, Mudo G. and Lindholm D. (2002) Neuronal expression and seizure regulation of rat inhibitor of apoptosis protein-2 (RIAP-2) in the rat brain. Eur. J. Neurosci. (in press; * equal contribution)

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ABBREVIATIONS

AD	Alzheimer`s disease
BERKO	Estrogen receptor beta deficient mouse
BH	Bcl-2 homology domain
BIR	Baculoviral Inhibitory Repeat
bp	Base pair
CA	Cornu Ammon
CARD	Caspase recruitment domain
C. Elegans	Caenorhabditis Elegans
Caspase	Cystein-aspartase
CNS	Central nervous system
COS-7	Monkey kidney cell line
DED	Death effector domain
DEVD	Asp-Glu-Val-Asp, caspase-3 inhibitor
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
ER	Estrogen receptor
ERKO	Estrogen receptor alpha deficient mouse
FITC	Fluorescein isothiocyanate
GABA	Gamma aminobutyric acid
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GST	Glutation S-transferase
HA	Hemagglutinin
IAP	Inhibitor of Apoptosis Protein
ICE	Interleukin-1β converting enzyme
IRES	internal ribosome entry site
KA	Kainic acid
kb	kilo base pairs
kDa	kilo Dalton
LDH	Lactate dehydrogenase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAIP	Neuronal apoptosis inhibitor protein
hNTera	Neuroblastoma Tera-2 cell line
NeuN	Neuronal nuclei marker NeuN
NGF	Nerve Growth Factor
NMDA	N-methyl-D-aspartate receptor
NSC-34	Motoneuron cell line
N2a	Murine neuroblastoma cell line
PARP	Poly (ADP)-polymerase
PCR	Polymerase chain reaction
PD	Parkinson`s disease
ROS	Reactive oxygen radicals
RIAP-2	Rat inhibitor of apoptosis proein 2
RING	Really Interesting New Gene, structural domain in the IAPs
RNA	Ribonucleic acid
RPA	RNase protection assay
SDS	Sodium dodecylsulphate
SMA	Spinal muscular atrophy
SMN	Survival of motoneuron gene
TUNEL	dUTP mediated nick-end labelling
XIAP	X chromosome-linked inhibitor of apoptosis protein

INTRODUCTION

APOPTOSIS

General Features

Cell death can be roughly divided into two different forms: apoptosis and necrosis. Necrotic cell death is commonly observed for instance after toxic insults, in acute changes of osmolarity and after high intensity pathologic insults. It is characterised by organelle swelling, leakage of the cell contents to the extracellular space with following inflammatory response (Ankarcrona et al., 1995).

Apoptosis, or programmed cell death, was described in the early 1970` by Kerr et al. (Kerr et al., 1972). The term apoptosis comes from Greek and means "(leaves) falling off" referring to the fact that apoptosis/cell death is an essential part of life. Apoptosis is a genetically determined form of cell death with defined morphological characteristics. It is an active process, which needs ATP, and leads often, but not always, to caspase activation (Wyllie et al., 1984). During apoptosis, chromatin is condensated and DNA is cleaved into 180bp internucleosomal fragments. High molecular weight fragments (50-300kbp) are, however, also found (Wyllie, 1980; Wyllie et al., 1984; Oberhammer et al., 1993). The variety of DNA fragments produced is thought to depend on whether Apoptosis Inducing Factor (AIF) is released and related to activation or not of caspases (Walker et al., 1999; Susin et al., 1999; Bezvenyuk et al., 2000).

Table 1. Features of apoptosis, necrosis and paraptosis (mod. from Sperandio et al. 2000)

	Apoptosis	Necrosis	Paraptosis
Morphology			
Nuclear fragmentation	+	-	-
Chromatin condensation	+	-	+/-
Apoptotic bodies	+	-	-
Mitochondrial swelling	Sometimes	+	Late
Genomic effect			
DNA fragmentation	+	-	-
Caspase activity			
Caspase-3 processing	+	-	-
PARP cleavage	+ (85kDa)	+ (50-62kDa)	-
Inhibition by			
ZVAD.fmk	+	-	-
XIAP	+	-	-
Bcl-X	+	- (Usually)	-

Cells, which undergo apoptosis, are packed into small vesicles, so called apoptotic bodies, which are phagocyted by neighbouring cells, often macrophages or dendritic cells (Savill and Fadok, 2000). The recognition and engulfment of apoptotic cells seem to be conserved between nematodes, insects and mammals but the mechanisms are not yet fully (Savill and Fadok, 2000). Several surface receptors, among others scavenger receptors, integrins and phosphatidylserine receptor, mediate recognition, which depends on the phagocytic cells and their state of activation (Fadok et al., 1992; Martin et al., 1995; Fadok et al., 2000). Recent studies with nematode *C. Elegans* have revealed that certain genes are

required for correct engulfment of apoptotic bodies (Hengartner, 2001). Homologues genes have been found in mammals and they regulate both cytoskeletal rearrangement and migration during engulfment and correct linkage of outer signals to the inner machinery (Reddien and Horvitz, 2000; Gumienny et al., 2001). When apoptotic cells are phagocyted, anti-inflammatory cytokines, such as Transforming Growth Factor beta (TGF-β), are secreted (Savill and Fadok, 2000).

In some cases, a variant type of cell death exhibiting both signs of apoptosis and necrosis occurs. This is named paraptosis and it is characterised by cytoplasmic vacuolisation and a certain degree of chromatin condensation (Sperandio et al. 2000). A comparison between apoptosis, necrosis and paraptosis is presented in Table 1.

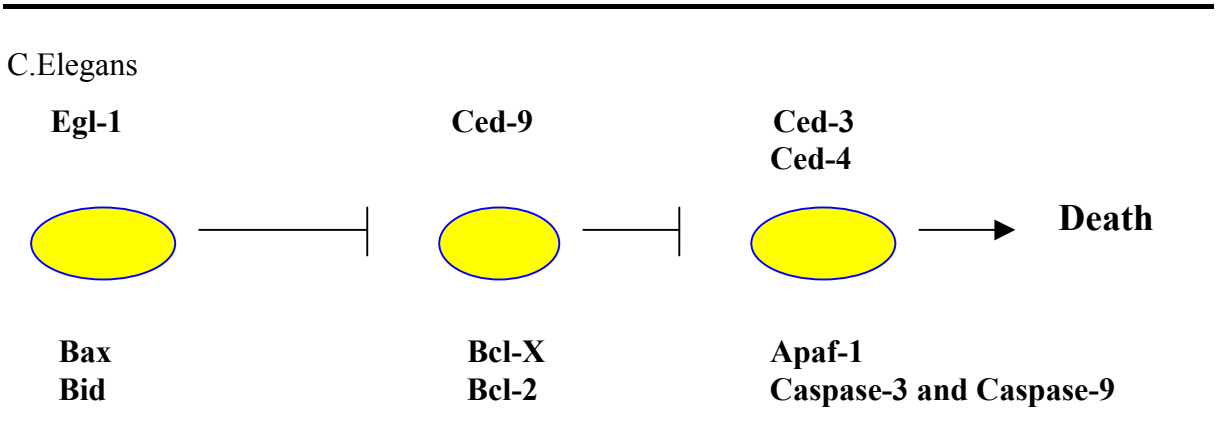
Signals for Apoptosis

The apoptotic programme can be activated by many different kinds of signals, such as trophic factor withdrawal, chemotherapeutic agents and developmental signals (LaCasse et al., 1998). Apoptosis can also become triggered via activation of death receptors of which Fas/APO-1/CD95 and TNF receptors are the best characterised (Schulze-Osthoff et al., 1998; Krammer, 1999). Ligand binding to these receptors, leads to recruitment of associated proteins and coupling of signals to intracellular mediators, such caspases as described below (Schmitz et al., 2000).

Caenorhabditis Elegans –an elegant genetic model for apoptosis

The nematode *Caenorhabditis Elegans (C. Elegans)* with its 1091 cells of which 131 die through apoptosis, has served as a simply genetical model for apoptosis (Ellis et al., 1991b). Studies have shown that there are at least 15 genes that function in programmed cell death (Metzstein et al., 1998). These 15 genes can be divided into four groups as shown in figure 1: (1) genes involved in decision making (*ces-1 and ces-2*); (2) in execution (*ced-3, ced-4, ced-9 and egl-1*); (3) in the engulfment of dying cells (*ced-1, ced-2, ced-5, ced-6, ced-7, ced-10, ced-12*); and (4) genes involved in degradation of cell corpses within engulfing cells (*nuc-1*).

Figure 1. Death genes in C. Elegans and the corresponding mammalian homologues



Genetical studies suggest that *ces-1 and ces-2* (ces, cell death specification) regulate a subset of cell death in *C. Elegans* since mutations in these genes block the death of certain neural cells (Ellis and Horvitz, 1991a). However, mutations in *ces-1 and ces-2* do not affect cell death in all cell types indicating that several parallel mechanisms control the decision making step of apoptosis (Ellis and Horvitz, 1991a). Indeed, *ces-2* encodes a member in a basic-leucine zipper family of transcription factors supporting the hypothesis that the cell death initiation is controlled at the level of gene expression (Metzstein et al., 1996).

Several studies have shown that *ced-3*, *ced-4* and *egl-1* are needed for cell death in the worm. Loss-of function mutations of the genes result in survival of almost all the cells in *C. Elegans*. *Egl-1* (egl, egg laying defective) was initially discovered as a gain-of-function mutation leading to ectopic expression of cell death in HSN (hermaphrodite specific neurons) resulting in egg-laying defect (Trent et al., 1983; Condradt and Horvitz, 1998). Egl-1 binds to Ced-9 and it is thought that it acts by releasing Ced-4 from Ced-9 (Conradt and Horvitz, 1998). Indeed, even the mammalian Egl-1 homologues, so called BH3-only proteins (see below), bind Bcl-2 family proteins is capable of inducing programmed cell death (Adams and Cory, 2001).

Ced-3 (ced, cell death abnormal) encodes a member of cysteine-aspartases (caspase, see below), which have a central role in apoptosis, whereas *ced-4* and its mammalian homologue APAF-1 (see below) regulate apoptosis at different levels (Yuan and Horvitz, 1992; Yuan et al., 1993; Zou et al., 1997; Chinnaiyan et al., 1997). Both of these genes are needed for cell death process since mutations in one or both of them blocks most of cell death (Ellis and Horvitz, 1986). Overexpression of Ced-3 can induce apoptosis independently of Ced-4 expression, whereas Ced-4 overexpression in Ced-3 negative cells results in very little cell death (Metzstein et al., 1998). Thus, Ced-4 seems to potentiate Ced-3 killing activity.

Contrary to *ced-3* and *ced-4*, *ced-9* has been shown to inhibit apoptosis and in loss of function mutation of this gene leads to ectopic expression of cell death (Hengartner and Horvitz, 1994). This can be inhibited if Ced-3 and Ced-4 are mutated indicating that Ced-9 works upstream of Ced-3 and Ced-4 (Hengartner et al., 1992). Mutations in *egl-1* however cannot rescue the phenotype suggesting that it functions upstream of Ced-9 (Conradt and Horvitz, 1998). The mammalian homologue of Ced-9, Bcl-2 protein also exhibits anti-apoptotic functions as explained below (Hengartner and Horvitz, 1994).

As pointed out earlier, the engulfment of dying cells is an important step during cell death. In *C. Elegans*, several genes have been identified to take part in this process, in particular a subset of genes important for cytoskeleton rearrangement (*ced-2*, *ced-5* and *ced-10*), *ced-1, ced-6* and *ced-7* whose functions are less understood (Ellis and Horvitz, 1991b; Hengartner, 2001).

When dying cells have been engulfed, the cell contents must be degradaded. In *C. Elegans* at least one gene, namely *nuc-1* is needed to perform part of this function. *Nuc-1* (nuc, nuclease) encodes or regulates the activity of nuclease needed for DNA degradation (Hedgecock et al., 1983; Ellis and Horvitz, 1991b). However, mutations in *nuc-1* or genes involved in engulfment do not prevent cell death indicating that the activities of these genes are not required for killing. Indeed, Nuc-1 is homologues to mammalian DNaseII, which is needed for DNA degradation during apoptosis (Lyon et al., 2000; Wu et al., 2000).

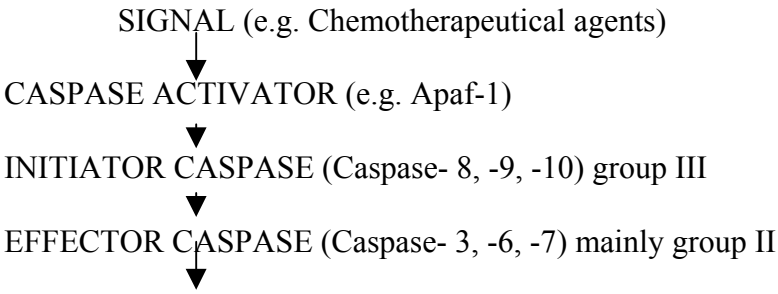
Mammalian Caspases

As mentioned above, the genetic analysis in *C. Elegans* revealed the presence of cysteine-aspartase ced-3, which is required for the execution phase of cell death (Yuan and Horvitz

1992; Yuan et al., 1993). Identification of ced-3 led to the discovery of the mammalian homologue interleukin-1β-converting enzyme (ICE), a cystein protease (Yuan et al., 1993). Today, 14 mammalian caspases have been cloned and characterised. Common to them all is that they are synthesised as inactive precursors composed of four distinct domains: an N-terminal pro-domain, a large subunit, a short domain and a linker region (Nicholson and Thorberry, 1997). The activation of caspases is induced by a proteolytic cleavage of the pro-domain and the linker region leading to a formation of a tetramer with two catalytic sites (Wilson et al., 1994; Rotonda et al., 1996).

The caspases can be classified in many different ways. Phylogenetically they are divided into two groups: one related to ICE (caspase-1) and the other to ced-3. These groups can be further divided into subdivisions depending on whether the pro-enzymes have a short (Caspase-3, -6, -7) or a large pro-domain (the rest of the caspases). Alternatively, the caspases can be arranged into different groups depending on their substrate specificity (Thorberry et al., 1997; Rano et al., 1997). Group I consists of caspases-1, -4, -5 and –13 and is characterised by the preference of a hydrophobic amino acids in the position P4 where as group II caspases (caspase-2, -3 and-7) require Asp in the same position. Group III caspases (caspase-6, -8, -9 and-10), on the other hand, prefer aliphatic amino acids in the position P4. These aliphatic amino acids are commonly found in the maturation sites of most group II and III caspases indicating that group III caspases can function as initiation caspases in the caspase cascade (Nicholson, 1999). In summary, the initiator caspases belonging to the group III cleave and activate the effector caspase (group II), which leads to an amplification of the cascade and destruction of the cell as presented in Figure 2.

Figure 2. Caspase activation



There are two different kinds of domains mediating the interaction between caspase activators and initiator caspases: the death effector domain (DED) and caspase recruitment domain (CARD). DED is found in FADD, which is an adapter protein linked to the Fas/APO-1/CD95 receptor upon Fas-ligand binding via its death domains (DD) (Boldin et al., 1996; Muzio et al., 1996). Caspase-8 has one DED motif in its pro-domain, and Caspase-10 contains two DED domains (Zheng et al., 2001; Medema et al., 1997). Interaction between the DED domains and FADD connects the apoptotic signals from Fas/APO-1/CD95 receptor to the caspases. CARD domain, which is present among others in Caspase-1, -2 and –9, is also thought to mediate the interaction between the adaptor and their respective caspase. For instance, CARD domains in APAF-1 binds CARD domains in Caspase-9 and in similar CARD domains of caspase-2 bind to RAIDD, which is an adapter

molecule in Tumour necrosis factor receptor 1 (TNF-R1) signalling (Duan and Dixit, 1997; Chou et al., 1998; Zhou et al., 1999).

Table 2. Mammalian caspases (Mod. from Chan and Mattson, 1999)

Caspase	**Structure**	**Subcellular localisation**	**Upstream caspases**	**KO phenotype and other biol. effects**
Caspase-1 (ICE)	pro/p20/link/p10	cytoplasm, nucleus external cell surface	Caspase-4, -8, -10 and –11	KO: Healthy, dev. Normal, resistant to endotoxic chock
Caspase-2 (Nedd2, ICH-1)	pro/p20/link/p12	cytoplasm, nucleus, mitochondria	Caspase-1, -3 and Granzyme B	KO: Resistance to apoptosis in B lymphoblasts, accel. cell death in motoneurons
Caspase-3 (CPP32, Yama, Apopain)	pro/p17/p12	cytoplasm, mitochondria	Caspase-6, -10 and Granzyme B	KO: perinatal death brain malformations
Caspase-4 (ICErel II, TX, ICH-2)	pro/p20/link/p10	cytoplasm		
Caspase-5 (ICErel III, TY)	pro/p20/link/p10	cytoplasm		
Caspase-6 (Mch2)	pro/p18/p13	cytoplasm	Caspase-3, -7 and Granzyme B	APP and lamin cleavage
Caspase-7 (Mch3, ICE-LAP3)	pro/p20/p12	cytoplasm, ER, mitochondria	Caspase-3, -8, -10 and Granzyme B	PARP cleavage
Caspase-8 (Mch5, MACH, FLICE)	pro/p18/link/p11		Caspase-3, -10 and Granzyme B	KO: Failures in heart dev., cell prol. in Hem. poet. system, death rec. med. apoptosis
Caspase-9 (Mch6, ICE-LAP6)	pro/p17/p12	cytoplasm, mitochondria	Caspase-3, -8 and Granzyme B	KO: Perinatal death, brain malformation, thymocytic resistance to apoptosis
Caspase-10 (Mch4)	pro/p17/p12	cytoplasm	Caspase-8, -10 and Granzyme B	Inherited mutation causes autoimmunoproliferative syndrome
Caspase-11 (mICH-3)	pro/p20/p10	cytoplasm	Granzyme B	Pro-inf. Cytokine processing
Caspase-12 (mICH-4)		cytoplasm		KO: Resistance to ER stress and in Cortical neurons to APP, but not to STS or trophic factor withdrawal
Caspase-13 (ERICE)		cytoplasm	Caspase-8	Activated by caspase-8
Caspase-14 (MICE)	p20/p10	cytoplasm	Caspase-8 and Granzyme B	Keratinocyte differentiation

APP= amyloid β protein; KO= knock-out mouse; link= linker; PARP= poly-ADP-ribose polymerase; pro= prodomain; STS= staurosporine

As indicated in Figure 2, the activation of the caspase cascade leads to the cellular destruction and appearance of the apoptotic characteristics described above. During this process, the caspases destruct various substrates of which more than 70 are characterised today (Chan and Mattson, 1999). One big group of caspase substrates is different kinds of structural genes, among others lamins, fodrin and gelsolin. PARP (poly-ADP-ribose polymerase) and DNA-PK, two proteins involved in the maintenance of genomic DNA integrity, become caspase victims, too. Many signalling proteins and apoptosis regulators have also been reported to become caspase targets. As an example of these are RasGAP, Raf1 and Akt1 in addition to Bcl-2, Bcl-XL and Bid. DNA itself is degraded by DNA degrading nuclease (CAD), which is activated after caspase cleavage of its inhbitor ICAD (Enari et al., 1998; Sakahira et al., 1998). Altogether, the cell destruction leads to impaired

cellular repair and homeostasis maintenance, to inactivation of anti-apoptotic proteins and to structural disassembly and ultimately engulfment and disposal of the dying cell.

Caspase activation can be inhibited by different means such as virus. Cowpox virus serves as an example of this: It produces a protein called Cytokine Response Modifier, which binds and inhibits caspase-1 (ICE) leading to the suppression of cell death in virally infected cells (Ray et al., 1992). Yet, another group of virally derived apoptosis inhibitors is the recently described Inhibitor of Apoptosis Protein (IAP) family, which was originally identified in baculoviruses due to its ability to suppress cell death (see below, Clem and Miller, 1994). Beside viral proteins, there are several synthetic peptides with the ability to inhibit caspases. These inhibitors have been shown to increase survival in both cultured cells and *in vivo* (Ekert et al., 1999). The selectivity of the inhibitors have been developed based on the specific substrate cleavage sites and they act as competitive pseudosubstrates. Depending on the chemical groups, they are either reversible or irreversible inhibitors. The former are often coupled to aldehyd (-CHO), ketone or nitrile groups and the latter are linked to fluoro- or chloro-methyl ketones (-CKM, -FMK).

Studies with gene deleted animals for caspase have substantiated the function of individual caspases *in vivo* (Zheng et al., 1999). Data from these studies is summarised in Table 2. Especially, caspase-3 and –9 deficient animals show severe phenotype indicating that they play a central role in cell death. Caspase-3 knockout mice show malformations in the brain suggesting that this caspase is needed for normal brain development (Kuida et al., 1996; Woo et al., 1998). Caspase-8 deficient mice have defects in the immune system indicating that caspase-8 is needed for proper function of immune cells (Varfolomeer et al., 1998).

The role of mitochondria in cell death

Although the role of mitochondria have been highlightened in several studies many details remains to be clarified. Mitochondria are needed for the apoptosis and it is thought that cytochrome c which is released from the mitochondria is essential for the activation of the caspase cascade (Liu et al., 1996). The release mechanism is not fully understood but several members in the Bcl-2 family are localised at the mitochondrial membrane. It has been shown that Bax promotes cytochrome c release whereas the anti-apoptotic proteins Bcl-2 and Bcl-X block the release (Kluck et al., 1997; Kharbanda et al., 1997; Yang et al., 1997; Jurgensmeier et al., 1998).

Another characteristic of apoptosis is the loss of mitochondrial membrane potential (Vayssiere et al., 1994). That itself could possibly cause cytochrome c release. One important structure in mitochondria is the permeability transition pore complex (PTPC), which consists of adenine nucleotide translocator (ANT) in the inner mitochondrial membrane, the cyclophilin D that binds to the ANT, and the voltage dependent channel (VDAC) (Marchetti et al., 1996; Loeffler and Kroemer, 2000). Several proteins have been described to bind to this large complex, among them Bax and Bcl-x, which can form and inhibit channel formation respectively (Narita et al., 1998).

Moreover, mitochondria play a role in cell death by their ability to bind proteins other than cytochrome c. Smac/DIABLO is a protein found normally attached to the mitochondria but that is released upon to induction of apoptosis (Du et al., 2000; Verhagen et al., 2000). Smac/DIABLO binds to IAPs in the cytosol and inhibits then anti-apoptotic function. In addition, apoptosis inducing factor (AIF) can be released from the

mitochondria (Susin et al., 1996; Susin et al., 1999). The release of AIF results in DNA fragmentation in the nucleus.

The apoptosome and Apaf-1

The cytochrome c release and mitochondrial dysfunction are inhibitable by cyclosporin A. In most cases, the apoptotic signals are coupled to mitochondria, which are essential for the cell death process (Newmeyer et al. 1994). During the mitochondrial phase of apoptosis, cytochrome c is released, which leads to the formation of the apoptosome complex (Liu et al., 1996).

The apoptosome consists of cytochrome c, ATP, Apaf-1 and pro-caspase-9, and its formation leads to the activation of caspase-9 and further amplification of the caspase cascade via activation of caspase-3 (Zou et al., 1997; Li et al., 1997; Srinivasula et al., 1998). The central molecule in this complex is Apaf-1 (apoptosis protease activating factor), which has a aminoterminal caspase recruitment domain (CARD), a central Ced-4 homology domain and a carboxyterminal WD40 repeats, which are thought to mediate protein-protein interactions and negatively regulates Apaf-1 activity.

Apaf-1 bound to cytochrome c hydrolyses ATP and undergoes oligodimerisation via its Ced-4 homology domain. At the same time, CARD domain recruits and facilitates procaspase-9 processing. In addition, caspase-3 and –7 have been reported to be recruited into the apoptosome complex where they are processed into smaller subunits (Cain et al., 2000). The size of the apoptosome complex has been reported to be approximately 1.4MDa using recombinant proteins, but has a size of 700kDa in native lysates (Cain et al., 2000; Bratton et al., 2001).

Apaf-1 deficient mice die during early development (between E16-P0) and exhibit reduced apoptosis in brain tissue show craniofacial and eye alterations (Yoshida et al., 1998). Similar changes have been also identified in caspase-9 deficient mice indicating that the apoptosome complex is important for the cell death process (Kuida et al., 1998 ; Hakem et al., 1998).

ANTI-APOPTOTIC MECHANISMS

The Bcl-2 family

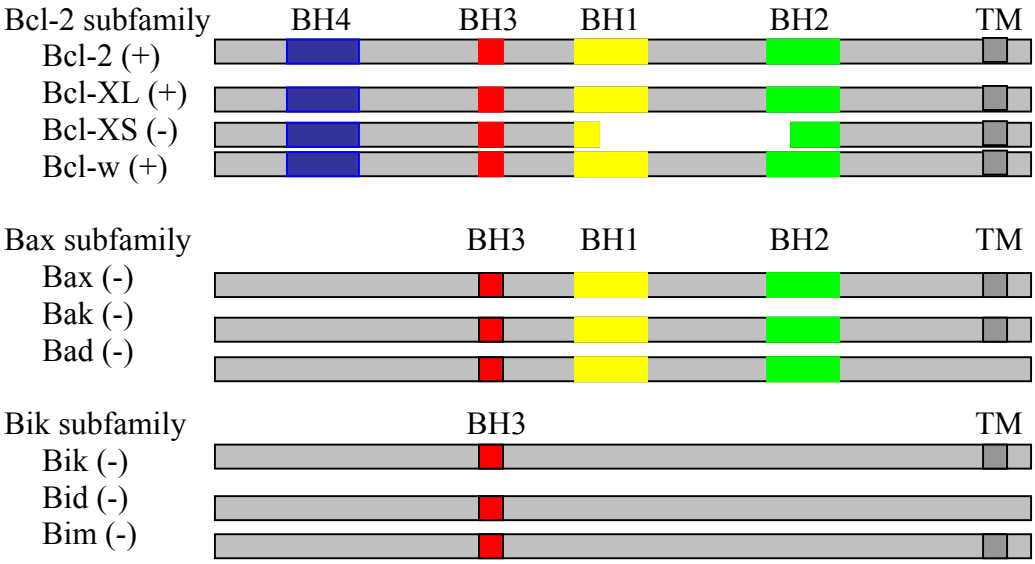
Bcl-2 is a mammalian homologue of ced-9, and belongs to a large gene family sharing structural similarity in the so called BH (Bcl-2 homology)-domain. As shown in Table 2, the Bcl-2 family can structurally be divided into three classes of proteins: Bcl-2, Bax and Bik subfamilies. The Bcl-2 subgroup includes proteins such as Bcl-2, Bcl-XL, BCL-xs and BCL-w, which all possess the four characterised BH-domains (BH1-4) and exhibit, with the exception of Bcl-Xs, anti-apoptotic properties (Tsujimoto et al., 1984; Boise et al., 1993; Reed, 1994; Gibson et al., 1996). The pro-apoptotic Bax-subgroup consists of Bax, Bak, Bad and Bok, which only have BH1-3 domains (Oltvai et al., 1993; Chittenden et al., 1995a, 1995b; Kiefer et al., 1995; Yang et al., 1995; Hsu et al., 1997). The third subfamily consists of"BH3 only" group of proteins and includes Bik, Bid and Bim (Boyd et al., 1995; Wang et al., 1996; O`Connor et al., 1998). Proteins belonging to this group have strong pro-apoptotic features indicating that the BH3 domain alone can induce apoptosis under certain conditions (Chittenden, 1995b; Cosulich et al., 1997; Hsu and Hsueh, 1998; Ray et al., 2000). Furthermore, when the BH3 region of Bcl-2 is changed to that of Bax, it looses its death antagonising effects suggesting that there are critical

differences between the BH3 domains found in pro- and anti-apoptotic members in the Bcl-2 family (Hunter and Parslow, 1996).

As indicated in Table 2, some of the members in the Bcl-2 family have in their carboxyterminal a hydrophobic transmembrane domain required for insertion to membranes (Hockenbery et al., 1990). Indeed, many family members can be found associated with the mitochondrial outer membrane, the nuclear membrane and endoplasmatic reticulum (Hockenbery et al., 1990; Gonzales-Garcia et al., 1994). The functional relevance of the membrane docking is still unclear but in the case of Bcl-2, membrane insertion seems to be associated with its capability to antagonize apoptosis in some systems but not in others (Hockenberry et al., 1990; Tanaka et al., 1993). In addition, deletion of the transmembrane domain in Bcl-x does not affect its anti-apoptotic properties (Borner et al., 1994; Gonzales-Garcia et al., 1995).

The Bcl-2 family proteins exhibit a variety of biological actions. Several Bcl-2 family members can form heterodimeric bindings, which are mediated by the insertion of BH3 of the pro-apoptotic protein into a hydrophobic pocked formed by BH1, BH2 and BH3 regions from the anti-apoptotic protein (Oltvai et al., 1993). Heterodimerization between anti- and pro-apoptotic proteins appears to inhibit the biological activity of their partners (Oltvai et al., 1993; Yang et al., 1995). It has also been reported that BH3-only proteins can function as cytoplasmic ligands for other pro-and anti-apoptotic family members thereby regulating their activity (Yang et al., 1995; Desagher et al., 1999).

Table3. Classification of proteins in the Bcl-2 family



(+)= anti-apoptotic protein; (-)= pro-apoptotic protein; TM= transmembrane domain

Some of the activities of Bcl-2 protein occur via binding to other proteins than Bcl-2 family proteins. Among the interactors reported for Bcl-2, and in certain cases for Bcl-X, are signalling molecules such as Raf-1, R-Ras p23 and 53BP-protein (Fernandez-Sarabia and Bischoff, 1993; Wang et al., 1996; Naumovski and Cleary, 1996). In addition, Bag-1, which regulates the Hsp70/Hsc70 chaperone system and to calcineurin, a calcium-activated

phosphatase, has been described to interact with Bcl-2 family proteins (Takayama et al., 1995 and 1997; Shibasaki et al., 1997).

The determination of the 3D-structure of Bcl-XL gave a hint as to the function of Bcl-2 family members as ion channels (Muchmore et al., 1996). The 3D-structure of Bcl-XL is very similar to that of some bacterial toxins e.g. diphtheria toxin, which contain a pore forming domain. Later studies have demonstrated that Bcl-2, Bcl-XL and Bax can form ion channels in synthetic lipid membranes (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997; Schlesinger et al., 1997). The pores formed by Bcl-2 and Bcl-XL are small and have a moderate cation preference and are formed only in non-physiological acidic conditions. In contrast, Bax forms larger pores, which prefer anions at physiological pH and in some studies, but not all, it has been showed that Bcl-2 can prevent *in vitro* channel formation by Bax (Antonsson et al., 1997; Schendel et al., 1998).

In addition, Bcl-2, Bcl-xL and Bcl-w have been reported to inhibit cytochrome c release, whereas Bax can promote it (Kluck et al., 1997; Kharbanda et al., 1997; Yan et al., 2000). Bak or BH3 oligopeptides added to isolated mitochondria induce cytochrome c release and lead to mitochondrial dysfunction (Jurgenmeister et al., 1998; Narita et al., 1998). It is thought that the Bcl-2 family members regulate cytochrome c release by associating and thereby affecting the mitochondrial PT pore (see above), which becomes opened by the loss of the electrochemical gradient ($\Delta\Psi$) during apoptosis (Petit et al., 1996). Bcl-2 and Bcl-X overexpression have been reported to protect against different agents, such as cyanide, that directly act on the mitochondria PT pore (Shimizu et al., 1996; Susin et al., 1996; Kluck et al., 1997). In addition, cytochrome c microinjection studies have shown that Bcl-2 and Bcl-x can block the cell death downstream of cytochrome c release (Li et al., 1997).

Bcl-2

Bcl-2 was first identified through its involvement in follicular B-cell lymphoma. In this cancer form, Bcl-2 expression is altered by a chromosomal translocation, t(14;18), which places the gene in a close proximity to enhancer elements of the immunoglobulin heavy chain locus (IgH) resulting in deregulated expression of Bcl-2 in B-cells (Tsujimoto et al., 1984). Expression of Bcl-2 in immature B-cells leads to growth independent of IL-3, which is normally needed for the survival (Vaux et al., 1988).

Bcl-2 gene codes for a 25kDa protein and can be found in two different splice variants: Bcl-2α and Bcl-2β. The β-form lacks the transmembrane domain, which appears to be dispensable for function as discussed above (Tanaka et al., 1993). In addition to splicing, phosphorylation of serine and threonine residues in the loop region between BH3 and BH4 regulates the Bcl-2 function. Several kinases, among others the Jun-N-terminal kinases, can phosphorylate Bcl-2 leading to the abolishment of the protective function of Bcl-2.

Bcl-2 is expressed in a variety of tissues, among others in the haematopoietic system, many inner organs and skin (Hockenbery et al., 1991; LeBrun et al., 1993; Novack and Korsmeyer, 1994). In the brain, Bcl-2 mRNA is expressed at higher levels during embryonic and late prenatal development than in postnatal and adult brain (Abe-Dohmae et al., 1993; Castren et al., 1994). In adult rat brain, Bcl-2 mRNA is expressed in the dentate gyrus in the hippocampus, in the olfactory bulb and cerebellar granule neurons (Castren et al., 1994). Studies on Bcl-2 protein expression have substantiated these findings and they indicate that Bcl-2 is scanty present in the adult CNS but retained in the peripheral nervous system (Merry et al., 1994).

Gene deletion studies have highlightened the role of Bcl-2 in neuronal survival. Bcl-2-deficient mice survive embryonic development but die in early adulthood due to polycystic kidney disease and thymic and splenic abnormalities (Veis et al., 1993; Nakayama et al., 1994; Kamada et al., 1995). Transgenic mice over-expressing bcl-2 gene in neural cells show resistance against naturally occurring cell death and experimental ischemia, facial, sciatic and optic nerve axotomy (Martinou et al., 1994; Dubois-Dauphin et al., 1994; Farlie et al., 1995; Michaelidis et al., 1996; Bonfanti et al., 1996). Bcl-2 has been reported also to induce differentiation of neural cells (Suzuki and Tsutomi, 1998; Middleton et al., 1998).

Bcl-2 proteins show a variety of biological activities as summarised in Table 4. Bcl-2 both decreases lipid peroxidation and prevents phosphatidylserine exposure at the plasma membrane (Hockenbery et al., 1993; Martin et al., 1995). Bcl-2`s effects on redox potential are mediated through decreased lipid peroxidation and effects on catalase, SOD and GSH levels leading to inhibition of ROS generation (Hockenbery et al., 1993; Kane et al., 1993). Bcl-2 also inhibits caspase-3 and -6 activation and has effects on ion balance (Lam et al., 1994; Chinnaiyan et al., 1996; Marin et al., 1996). On the mitochondria level, Bcl-2 can inhibit the transmembrane potential loss and calcium influx into mitochondria and cytochrome c outflow is also blocked (Baffy et al., 1993; Zamzami et al., 1996).

Table 4. Biological effects of Bcl-2

1. Plasmamembrane	Prevention of phosphatidylserine exposure, decreased lipid peroxidation
2. Redox potential	Decreased lipid peroxidation, inhibition of ROS generation, increase in catalase, SOD, GSH, elevated NAD/NADH
3. Proteases	Inhibition of Caspase-3 and -6 activation
4. Ion balance	Inhibition of hydroperoxide induced calcium outflow from ER, inhibition of nuclear calcium up-take
5. Mitochondria	Inhibition of transmembrane potential loss inhibition of calcium influx into mitochondria, prevention of cytochrome c outflow

ER= endoplasmatic reticulum; GSH= reduced gkutathione; ROS= Reactive oxygen species; SOD= sodium dismutase

Bcl-X

Bcl-X is another anti-apoptotic protein of the Bcl-2 family. Bcl-x gene encodes an approximately 20kDa protein, which can be found in three splice forms: Bcl-Xs, Bcl-XL and Bcl-Xβ (Boise et al., 1993). The long form (Bcl-XL) inhibits cell death upon growth factor depletion, but the short form (Bcl-Xs), which lacks the parts of the BH1 and BH2 domains, inhibits the ability of Bcl-2 to antagonize apoptosis (Boise et al., 1993; Gonzales-Garcia et al., 1995). Bcl-Xβ seems in some experimental systems to have an anti-apoptotic function, and in others it promotes apoptosis (Gonzales-Garcia et al., 1995).

Bcl-X mRNA is highly expressed in many tissues among others in the thymus, liver and bone marrow (Gonzales-Garcia et al., 1994; Krajewski et al., 1994). In the brain, Bcl-X mRNA is highly expressed during early development but the expression decreases towards adulthood (Frankowski et al., 1995; Hamner et al., 1999). On the protein level, Bcl-X expression has been reported to be low in the adult brain, but opposite reports also exist, too (Mizuguchi et al., 1996; Alonso et al., 1997; Shimohama et al., 1998; Vekrellis et al.,

1997). Bcl-Xs mRNA is present in the human thymus but the transcript is undectable in mouse tissues (Gonzales-Garcia et al., 1994). No expression studies have been published for Bcl-Xβ.

Bcl-X deficient mice die during embryonic development due mainly to the massive cell death in haematopoietic system and in brain neurons (Motoyama et al., 1995). The early occurring neuronal death can be prevented by Bax-deficiency indicating that Bax binding to Bcl-X critically regulates cell survival (Shindler et al., 1997). Similar kinds of results have been obtained using dorsal root ganglion neurons, which have high expression of Bcl-X during the developmental stages when most of the naturally occurring cell death takes place (Vogelbaum et al., 1998).

Transgenic mice over expressing Bcl-X in the immune system show thymocyte resistance against γ-irradiation and glucocorticoid treatment and overexpression under a neuronal promoter has been shown to rescue cells after hypoxia-ischemia and after facial axotomy (Chao et al., 1995; Parsadanian et al., 1998). On the contrary, naturally occurring cell death in the facial motor neurons is not inhibited in these animals (Parsadanian et al., 1998).

Bax

Bax is a pro-apoptotic family member in the Bcl-2 family and it exits in several alternatively spliced forms. Baxα consists of three BH domains and a carboxyterminal transmembrane domain, whereas baxβ and baxω lack the transmembrane domain. Baxγ on the other hand does not have the exon 4 and baxδ lacks the BH3 domain. By now, no functional significance has been demonstrated for baxβ, baxγ and baxδ. Baxω has a pro-apoptotic activity but it has been reported to also antagonise cell death under certain conditions (Zhou et al., 1998).

Bax mRNA is highly expressed in several tissues, among others in the brain, heart, lung and kidney. Bax protein is also found in these tissues but the expression of mRNA and protein do not always correlate with each other (Krajewski et al., 1994). The different splice forms are present in the brain. In general, Bax seems to be expressed more widely than Bcl-2 and the expression seems to decrease during development (Oltvai et al., 1993; Krajewski et al., 1994; Zhou et al., 1998; Shimohama et al., 1998). Bax is mainly present in the cytoplasma. After an apoptotic stimulus a conformational change occurs in Bax, which leads to its translocation to mitochondria. This can result in Bax oligomerisation, channel-forming activity and ultimately to cytochrome c release (Antonsson et al., 2000)

At the cellular level, Bax deficient mice show hyperplasia in several tissues among others in neurons (Knudson et al., 1995; Deckwerth et al., 1996). Bax deficient neurons survive better after facial nerve axotomy and cultured cortical neurons from these animals are more resistant against glutamate and DNA damage. Furthermore, symphathetic neurons lacking Bax are less dependent on NGF for their survival (Deckwerth et al., 1996).

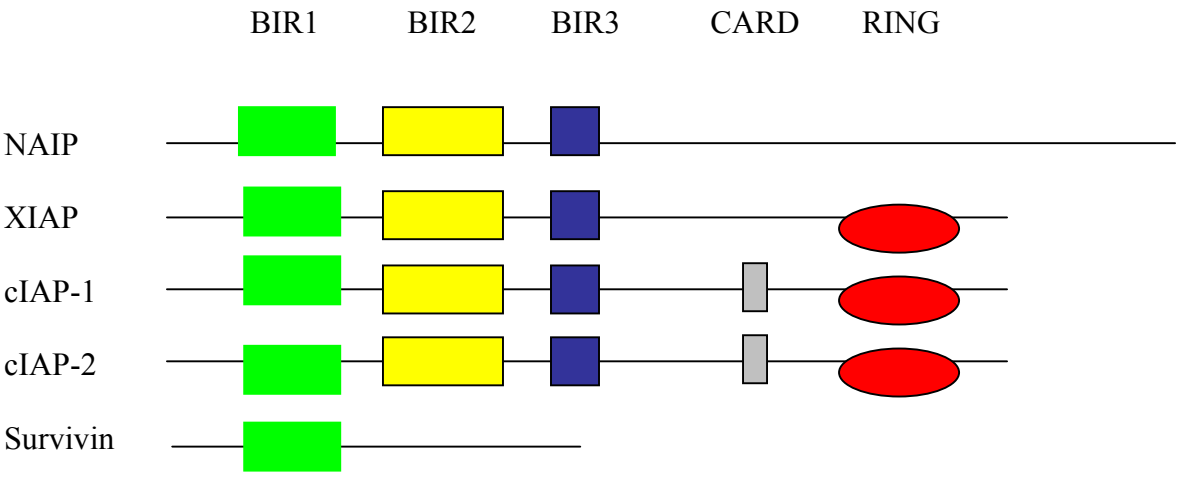
The Inhibitor of Apoptosis Protein Family

The IAPs were found in insect baculoviruses as proteins with the ability to promote survival of infected cells (Crook et al., 1993; Clem and Miller, 1994). The neuronal inhibitor of apoptosis protein (NAIP) was the first identified human homologue to insect IAPs (Roy et al., 1995). Subsequently, four other human IAPs (c-IAP-1/HIAP-2/hMIHB, c-IAP-2/HIAP-1/hMIHC, XIAP/hILP and Survivin) were characterised followed by identification of two Drosophila homologues, DIAP-1/dILP-1 and DIAP-2 (Hay et al., 1995: Rothe et

al., 1995; Duckett et al., 1996; Liston et al., 1996; Uren et al., 1996; Ambrosini et al., 1997). Today other homologues of IAP proteins have been characterised in different species (LaCasse et al., 1998).

As presented in Table 4, the IAP family proteins share a well-conserved motif of approximately 65 residues called the baculovirus IAP repeat (BIR). c-IAP-1 and -2 as well as NAIP and XIAP contain three BIR domains and Survivin has only one BIR region. This indicates that single BIR region can be sufficient for the anti-apoptotic action (Ambrosini et al., 1997). The individual BIR domains show both a structural and functional variation but common to them all is a highly hydrophobic centre (Hinds et al., 1999). The core structure has several conserved cystein and histidine residues, which have zinc coordinating properties needed for the IAPs`anti-apoptotic function (Vucic et al., 1998; Sun et al., 1999). In addition, sequences immediately next to the BIR have been demonstrated to be important for the interaction between IAPs and other proteins (Vucic et al., 1998; Sun et al., 1999).

Table 5. The structure of the mammalian IAP-family members



With the exception of NAIP and Survivin, all other IAP family proteins have a C-terminal RING motif, which can be found in over 200 different proteins. Typical for the RING domains are the histidine and cysteine rich regions with zinc binding capacity (Borden, 2000). The function of the RING-domains is still unclear, but the zinc ligation appears to be crucial for biological activity (Borden, 2000). The RING domain is found in other proteins involved in among others RNA processing, organelle transport and viral replication (Borden, 2000). The only unifying theme for RING domain function seems to be that they are involved in formation of large macromolar scaffolds. An example of this is the activity of RING domain for IAP autodegradation through ubiquitinylation in the thymocytes (Yang et al. 2000). There is also data suggesting that the RING-domain of IAPs may function as a negative regulator of apoptosis (Hay et al., 1995).

The IAP family exhibits a variety of biological activities. Different IAP family members antagonize cell death upon a variety of signals, among others against treatment with chemotherapeutic agents, Fas activation and trophic factor withdrawal (LaCasse et al., 1998). Part of this inhibition is mediated through direct binding and inactivation of caspases as described for XIAP, cIAP-1 and cIAP-2 (Roy et al., 1997; Deveraux et al., 1997;

Deveraux et al., 1998). Caspase inhibition appears to need only one BIR-region, which, in the case of XIAP, is the BIR2 domain (Takahashi et al., 1998). On the other hand, caspases can cut and thereby degradate some IAPs as shown for XIAP in Jurkat T cells after Fas stimulation (Deveraux et al., 1999). Likewise, survivin binds to active caspases-3 and –9 as well as to pro-caspase 9 but it remains to be proved whether this binding inhibits the caspases (Tamm et al., 1998).

As mentioned above, the action of XIAP, cIAP-1 and cIAP-2, can be modified by binding to Smac/DIABLO. Smac/DIABLO is a mitochondrial protein and that is released to the cytosol along with cytochrome c release in response to an apoptotic stimulus (Verhagen et al., 2000; Du et al., 2000). The release requires active caspases and is blocked by Bcl-2 (Adrien et al., 2001). In addition, it was recently shown that in TRAIL induced cell death, Smac/DIABLO release requires Bax (Deng et al., 2002). Smac/DIABLO mRNA is present at least in heart, kidney and testis. In the cytosol, Smac/DIABLO binds to the target IAP and thereby inhibits its binding to activated caspases. Smac/DIABLO can promote cell death both at the level of apoptosome complex and at the level of effector caspases (Srinivasula et al., 2001). Structural studies have revealed that the very aminoterminal of Smac/DIABLO (AVPI sequence) is critical for the XIAP BIR3 binding and thereby for the cytochrome c promoted apoptosis (Liu et al., 2000; Ekert et al., 2001; Srinivasula et al., 2001).

There is increasing evidence that IAPs also play a role in signal transduction. The cIAP-1 and cIAP-2 were first identified as central components in TNF-receptor signalling complex suggesting that they play a role in regulation of TNF dependent cell survival (Rothe et al., 1995; Chu et al., 1997; Stehlik et al., 1998b). In addition, XIAP interacts with TABI, an intracellular mediator of bone morphogenetic protein receptor signalling (Yamaguchi et al., 1999). As presented in Table 5, cIAP-1 and cIAP-2 have a CARD close to the RING domain (LaCasse et al., 1998). This domain mediates protein-protein interactions and is present in many pro-caspases, too. This suggests that CARD-mediated protein-protein interactions between IAPs and other proteins may play an important role in modulating the apoptotic process (Deveraux et al., 1999).

Neuronal apoptosis inhibitor protein (NAIP)

NAIP was originally identified as a gene deleted in many patients suffering from Spinal Muscular Atrophy type 1 (SMA1), a devastating human disease characterised by motor neuron degeneration in early life (Roy et al., 1995). Later studies have, however, shown that mutations in the SMN gene are more important as a cause of the disease but NAIP seems to have more a modulating role in the disease pathogenesis of SMA (Jablonka et al., 2000).

As mentioned above, NAIP consists of three BIR domains but lacks the carboxyterminal RING domain. *NAIP* encodes for an approximately 120kDa long protein. The BIR domains of NAIP have not been demonstrated to bind directly to activated caspases and the NAIP`s anti-apoptotic activity is mediated by other means (Roy et al., 1997).

Beside in motor neurons, NAIP appears to be widely expressed in the nervous system (Xu et al., 1997a). NAIP is present in hippocampus, in cortex, amygdala and thalamus. The NAIP deficient mice are more vulnerable to kainic acid induced hippocampal cell death than wild type controls (Holcik et al., 2000). Furthermore, it has been reported that NAIP delay motoneuron cell death in vivo and in vitro as well as suppresses neuronal differentiation and apoptosis in PC12 cells (Perrelet et al., 2000; Gotz et al., 2000). In

addition, adenovirally overexpressed NAIP also reduces ischemic damage in rat hippocampus and protects nigrostriatal pathway in a rat PD model suggesting an important role in neuronal degeneration (Xu et al., 1997b; Crocker et al., 2001).

X chromosome –linked inhibitor of apoptosis protein (XIAP)

XIAP is one of the first identified human homologues of viral IAPs. *XIAP* gene is found on the long arm of X chromosome (Xq25) and the mRNA encodes for a 57kDa protein found mainly in the cytosol. Structural studies of XIAP suggest that the BIR domains are classical zinc fingers but thay differ structurally from each other (Sun et al., 1999; Riedl et al., 2001; Huang et al., 2001; Chai et al., 2001). In addition, the function of the different BIR domains varies. The BIR2 domain can bind activated caspase-3 whereas caspase-9 binding is mediated via the BIR3 domain.

The RING domain of XIAP seems to have a role in XIAP degradation via ubiquitinylation as shown for glucocorticoid- and etoposide-treated thymocytes (Yang et al., 2000). The XIAP mutants lacking the carboxyterminal RING domain were more resistant to apoptosis-induced degradation and correspondingly, more effective at preventing the cell death process than wild type XIAP (Yang et al., 2000). In addition, the ubiquitin protein-ligase activity of XIAP seems to promote proteosomal degradation of active caspase-2 leading to increased anti-apoptotic effect (Suzuki et al., 2001).

XIAP mRNA can be found in many different tissues, for example in kidney, pancreas, thymus, skeletal muscle and brain (Uren et al., 1996). In several studies, various transcripts have been identified but details on potential splicing variants remains to be studied. Gene analysis has further shown that translation of XIAP is controlled by a 162-nucleotide internal ribosome entry site (IRES element), which is located in the 5`untranslated region of XIAP mRNA (Holcik and Korneluk, 2000). This IRES element enables an effective translation under physiological stress and it increases the protection against serum deprivation and gamma irradiation induced death in HeLa cells (Holcik and Korneluk, 2000). Similar IRES elements are also present in a limited number of other genes such as c-myc, VEGF and immunoglobulin heavy chain and it has been hypothesized that this mechanism might serve as a mechanism for guaranteed translation under compromised conditions like that after heat shock or growth arrest. In case of XIAP, it has been demonstrated that La autoantigen, which is a member in the RNA recognition motif group of general RNA-binding proteins, is required for translation through IRES element (Holcik and Korneluk, 2000).

The anti-apoptotic function of XIAP is established through direct binding and inhibition of active caspases (Deveraux et al., 1997, 1998; Takahashi et al., 1998). The binding of caspases is mediated through the BIR 2 and BIR3 domains as mentioned above (Riedl et al., 2000; Huang et al., 2000). BIR 2 binds active caspase-3 at a high affinity whereas caspase-9 binding is mediated through BIR3 domain. On the contrary, active caspase-3 has been shown to cleave and inactivate XIAP in the Jurkat T cells after irradiation (Deveraux et al., 1999). The cleavage site for caspase-3 is within or after the BIR2 and leads to the formation of two XIAP fragments: an aminoterminal fragment consisting of BIR1 and BIR2 and carboxyterminal fragment formed by BIR3 and RING. Overexpression of BIR1-BIR2 fragment has been shown to inhibit Fas-induced apoptosis in immune cells but this inhibition is significantly less potent that that of full-length XIAP. In contrast, overexpression of BIR3-RING fragment seems to slightly enhance Fas-mediated cell death, but protects against Bax induced cell death.

As mentioned above, Smac/DIABLO can inhibit the anti-apoptotic effects of XIAP. Recently, yet another mitochondrial protein has been reported to be released from mitochondria and to bind XIAP. This protein is a serine protease called Omi/Hrt2A contains a conserved IAP-binding motif (AVPS) at the aminoterminus (Suzuki et al., 2001; Verhagen et al., 2002; Martins et al., 2002; Hedge et al., 2002). This motif is exposed upon release into the cytosol leading to IAP binding via the BIR3 domain and caspase activation. Interestingly, Omi/Hrt2A can also induce apoptosis in a caspase-independent manner through its protease activity.

In addition to the above-mentioned interactors, XIAP has been shown to bind to a novel cellular protein called XAF1, XIAP associating factor 1 (Fong et al., 2000; Liston et al., 2001). XAF is produced as four distinct transcripts and it is expressed in many tissues and is present at low or undetectable levels in different cancer cell lines. Expression of XAF1 leads to a redistribution of XIAP from the cytosol to the nucleus followed by inability to inactivate active caspases. In addition, it has been reported that cells that express XAF1 at high levels are more resistant against cell death induced by chemotherapeutic agents indicating that XAF1 may be important in mediating the apoptosis resistance of cancer cells (Fong et al., 2000).

XIAP mRNA has been found in brain tissue but its precise localisation and function is not fully known. Overexpression of XIAP in the cultured cerebellar granule neurons (CGN) was shown to delay apoptotic cell death (Simons et al., 1999) and overexpression of XIAP by adenoviral vectors in rat hippocampus reduced cell damage and behavioural deficits (Xu et al., 1999). In addition, XIAP overexpression in the retinal ganglion delays the ganglion cell degeneration after axotomy and protect synergistically with GDNF against MPTP induced nigral cell death in a model of Parkinsons disease (Kugler et al., 2000; Ebenhardt et al., 2000).

XIAP has also been shown to suppress apoptosis in a Jun-c terminal kinase dependent pathway as well as Nuclear factor κB (NFκB) has been reported to regulate XIAP levels in different cell types and experimental paradigms (Sanna et al., 1998; Stehlik et al., 1998b; Tang et al., 2001). In addition, XIAP has been reported to be a cofactor in TGF-beta signalling and interact with BMP signalling elements (Yamaguchi et al., 1999; Birkey Reffey et al., 2001).

Recently, the first results on XIAP deficient mice were published (Harlin et al., 2001). These animals seem to be viable and lack major developmental disturbances. Neither Fas, UV irradiation nor oligomycin induced cell death were affected in XIAP deficient cells. Surprisingly, the levels of cIAP-1 and –2 were observed to be increased in the knock-out animals.

cIAP-1/RIAP-2

cIAP-1/HIAP2/MIAP2, was found through its binding to TNF (Tumor necrosis factor) receptor 2 associating proteins TRAF1 and TRAF2 (Rothe et al., 1995; Uren et al., 1996). The protein has three BIR domains, which bind and inhibit caspase-3, -7 and –9 and suppress cell death (Roy et al., 1997; see review LaCasse et al., 1998). However, the relative ability of IAP-2 to inhibit caspases is lower than that of XIAP (Roy et al., 1997).

IAP-2 has been cloned in different species including human, mouse, chicken and pig and it can be found in brain tissue (Rothe et al.,1995; Liston et al.,1996; Uren et al.,1996; Digby et al., 1996; Liston et al.,1997; You et al.,1997; Stehlik et al.,1998a). The gene encodes for an approximately 70kDa protein, which is found in the cytosol.

In general, little is known about cIAP-1 exact expression and function in different cell types. Various cytokines regulates cIAP-1 levels in endothelial cells and increase the survival of these cells (Stehlik et al., 1998b). There seems to be a reciprocal regulation of cIAP-1 and XIAP since the cIAP-1 expression level is increased in the XIAP deficient mice (Harlin et al., 2001). This indicates that these proteins can compensate for each other's functions.

NEURONAL CELL DEATH AND SURVIVAL

Cell death during the development

During brain development an excess of neurons are born and approximately 50% of them are later eliminated in order to ensure proper function (Oppenheim, 1991). The competition for neurotrophic factors directs the survival and cell death of early neurons (Levi-Montalcini, 1987; DiCicco-Bloom, 1993). The neurotrophic support is important during the development but neurotrophins functors also regulate maintenance and neuronal properties in the adulthood.

Nerve growth factor NGF was the first neurotrophic factor to be discovered (Levi-Montalcini, 1966). It is present in the nervous system and support the survival and maturation of sensory and sympathetic neurons in the PNS and cholinergic cells located in the forebrain and striatum (Bibel and Barde, 2000). NGF effects is mediated through the high affinity receptor trkA, which is a tyrosine kinase receptor leading to the activation of the MAP kinas and Akt pathways (Hempstead et al., 1991; Kaplan et al., 1991; Bibel and Barde, 2000). There is present yet another low affinity receptor for NGF, so called p75 receptor (Berg et al., 1991). Activation of this receptor can lead not only to cell survival but also to activation of the cell death in certain cells (Bredesen et al., 1998; Casademunt et al., 1999). Beside NGF there are other molecules such as BDNF and NT3 supporting survival and development of various classes of neurons (Conover and Yancopoulos, 1997).

As mentioned above, several important intracellular proteins are involved in regulation of cell death. With regard to the caspases, genetic studies have shown that certain caspases are essential for brain function. Caspase-3 knockout animals show brain abnormalities among others big ectopic cell masses in the cortex as well as hippocampal deformities (Kuida et al., 1996; Woo et al., 1998). Similar defect can be seen in caspase-9 deficient mice, not surprisingly since caspase-9 is thought to function upstream of caspase-3 (Kuida et al., 1998; Hakem et al., 1998). In addition, neurons from caspase-12 deficient mice show higher resistance against APP induced death in cultures than wild type neurons (Nakagawa et al., 2000). This is also observed using caspase-2 deficient mice indicating that cell death of cortical neurons is at least partially mediated through caspase-12 and caspase-2 (Bergeron et al., 1998). Caspase-1 and -11 deficient mice instead show resistance against ischemic cell death and caspase-1 deficient mice are resistant against cell death occurring after experimental autoimmune encephalitis (Kuida et al., 1995; Li et al., 1995; Wang et al., 1998).

Several members of the Bcl-2 family are expressed within the nervous system and show developmental regulation. Bcl-2 is developmentally downregulated in the CNS but the expression is retained in the PNS (Merry et al., 1994). Likewise, Bcl-X is down regulated in the CNS during the development (Frankowski et al., 1995; Hamner et al., 1999). On the contrary yet another members of the same family, Bcl-w is expressed at low levels in developing brain but is widely expressed in the mature brain (Hamner et al., 1999). These

results indicate a complex situation with changing levels of different family members, which provides an optimal survival for brain cells at different time points.

In addition, different family members in the IAP family have been shown to be expressed in the nervous system although they are not yet as well studied as the Bcl-2 family members. NAIP was the first IAP family members to be shown to be expressed in the nervous system and studies have shown that it is mutated in patients suffering from spinal muscular atrophy, a devastating genetical disease leading to muscular atrophy and death in childhood or young adulthood (Roy et al., 1995; Xu et al., 1997).

Neurodegeneration

As mentioned above naturally occurring cell death is an essential part of development of many organs including brain. There are, however, several disease processes affecting neurons with increased cell death. These disorders are characterised by degenerative changes leading to neuronal loss either after acute damages such as in brain trauma and stroke/ischemia or occurring during longer periods of times such as in human neurodegenerative diseases. Cell death in these disease is caused by different means which are still largely unknown.

In one common human disorder, Alzheimer's disease the brain pathology is related to by accumulation of beta amyloid peptide and formation of amyloid deposits (Yankner, 1996; Selkoe, 1997). In addition, hyperphosphorylation of Tau is part of the disease process leading to formation of senile placks and neurofibrillary tangles (Lee et al., 1999). Furthermore, mutations in a gene called presenilin have been associated with the disease (Sherrington et al., 1995; Levy-Lahad et al., 1995). These mutations have been seen especially in the early onset of AD and it has been shown that the mutated pesenilins increase the production of 42-APP, the APP form that mostly accounts for amyloid fibrid formation. Genetic studies have also shown that the apolipoprotein allele e4 serves as a common risk factor for AD and it has been reported to promote the polymerisation of APP to plack-forming fibrils (Yankner, 1996).

In PD, dopaminergic cells in the substantia nigra are primarily affected leading to rhypokinesia, tremor and at later stages often to dementia. Many reasons have been postulated to be causes of PD and exposure to various environmental factors seems to play a role in the pathogenesis (Jenner, 2001). Experimentally 1-methyl-4-phenyl-1,2,3,6tetrahydropyridine (MPTP), a drug that was abused in the USA in the 1970`s has been shown to be a potent drug to destroy nigral cells and induce symptoms similar to that seen in patients with PD (Tatton and Kish, 1997). Part of the cell death in substantia nigra is thought to be apoptotic (Tatton and Kish, 1997).

Neuronal cell death is also taking place after uncontrolled neuronal activity such as in epileptic seizures (Sperk et al., 1994). The mechanism behind this is partly glutamate receptor activation induced exitotoxicity with release of calcium into the cytosol. After prolonged activation of the glutamate receptors intracellular calcium levels can be elevated to such extend that downstream processes resulting in cell death are triggered.

ESTROGEN AND ESTROGEN RECEPTORS

Estrogen presents an important class of hormones with pleiotrophic effects. There is epidemiological evidence that estrogen replacement therapy for postmenopausal women is associated with improvement of cognitive performance, protection against cognitive deterioration and increased incidence of Alzheimers disease (Paganini-Hill and Henderson,

1994; Robinson et al., 1994). In addition, estrogen has been shown to be beneficial against cerebral stroke and cardiac infarct (Lafferty and Fiske 1994; Hurn and MacRae, 2000). Contrary to that estrogen failed to improve cognitive or functional outcome of patients with mild to moderate Alzheimers disease (Mulnard et al., 2000).

The beneficial effects of estrogen have been demonstrated in many animal models of neuronal degeneration. In ovarectomised rats, physiological concentrations of estrogen, attenuated extent of brain damage after cerebral ischemia (Dubal et al., 1998). In primary neurons, organotypic hippocampal cultures and the hippocampal cell clone HT22 estrogen attenuated neuronal injury caused by hypoxia, excitatory amino acids, superoxide anions and hydrogen peroxide (Goodman et al., 1996; Singer et al., 1996; Behl et al., 1997). Moreover, estrogen reduced the neurotoxic effects of β-amyloid and β-amyloid accumulation in the culture medium (Goodman et al., 1996; Keller et al., 1997).

Estrogen functions through estrogen receptors, namely the alpha receptor (ERα) and the beta receptor (ERβ) (for review see Pettersson and Gustafsson, 2001). These receptors belong to the steroid/thyroid hormone superfamily of nuclear receptors and share common structural characteristics. ERα and ERβ have an aminoterminal AF1 domain, a DNA binding domain and ligand binding domain. The aminoterminal AF1 domain has a ligand-independent function and is involved in transcriptional activation of target-gene expression. ERα and ERβ have some crucial differences in this domain; in ERα it is a strong stimulator of reporter-gene expression in cell lines whereas ERβ showed a lower activity under same conditions. In addition, studies with synthetic antiestrogens (Tamoxifen, ICI 164,384) have shown that these ligands function as partial estradiol agonists in the case of ERα but show antagonistic activity with ERβ indicating that there are differences in the AF1 domains between ERα and ERβ.

The DNA binding domain, which is very similar in ERα and ERβ, contains a zinc finger structure that mediates receptor dimerisation and binding to specific DNA sequences. The carboxyterminal ligand-binding domain mediates ligand binding leading to activation of target-gene expression. The ligand binding domains in ERα and ERβ are very similar even in the tertiary structure and thus many tested compounds bind to both the receptors with same affinity.

Estrogen receptors can be activated also independent of estrogen. It has been described that among others several kinases (eg PKC, PKA), growth factors (EGF, insulin, IGF-1), cytokines (IL-2) and neurotransmitters (Dopamine) as well as cell cycle regulators (CDKs) can lead to estrogen receptor activation and expression of target genes.

Estrogen receptors are expressed in various tissues among others in mammary glands, bone, uterus, central nervous system and cardiovascular system (Pettersson and Gustafsson, 2001). ERβ is expressed apart from the already mentioned tissues expressed in ovary, prostate and lungs. Knowledge about differences between these two receptors in various tissues has been gained from the studies with ERα (ERKO) and ERβ receptor deficient mice (BERKO).

BERKO mice show hypocellularity in the somotosensory cortex and an astroglial proliferation in the limbic system (Wang et al., 2001). In addition, these animals had signs of increased neuronal degeneration with age, suggesting that estrogen signalling through ERβ may be important for the pathogenesis of a various kinds of neurodegenerative disorders such as Alzheimers disease (Tang et al., 1996; Henderson et al., 1994). On the contrary, ERKO mice show loss of male-typical aggressive behaviour (Ogawa et al., 1997).

METHODS TO STUDY NEURONAL CELL DEATH AND SURVIVAL

Dorsal root ganglion neurons

Dorsal root ganglia are located along the spinal cord and contain a heterogeneous population of sensory neurons. These cells are derived from the neural crest cells and are distinguished by several characteristics including sensory modalities, neurotransmitter content, morphology and growth factor requirement (Verge et al., 1996). Morphologically they can be divided into big magnocellular neurons, which are associated with large-diameter myelinated axons, and smaller parvocellular neurons which give rise to small-diameter myelinated of unmyelinated primary afferent axons. Totally approximately 60-70% of the DRG neurons belong to the last category of cells. In addition, these DRG neurons can be differed by immunohistochemical method since they express several peptides like PACAP, substance P, somatostatin and calcitonin gene-related peptide (Lioudyno et al., 1998).

During the maturation process, DRG neurons are strongly dependent on neurotrophic support. Approximately 80% of the sensory and pain neurons in DRG are lacking in mice gene deleted for TrkA or its ligand NGF indicating that NGF is essential for the survival of these cells (Minichiello et al., 1995, Silos-Santiago et al., 1995). In addition, NT3 activation of TrkC has been reported to e essential for the survival of the relatively small population of DRG proprioceptive neurons (Ernfors et al., 1994a; Farinas et al., 1994; Klein et al., 1994). DRG neurons respond to several other trophic factors like BDNF, IGF-1 and GDNF (Ernfors et al., 1994b).

Several BCl-2 family members are expressed in the DRG neurons and it has been suggested that the relative levels of the anti- and pro-apoptotic proteins might regulated the cell death (Gillardon et al., 1996). Indeed, the ratio between Bcl-X and Bax plays a role in DRG neuron cell death (Vogelbaum et al., 1998). In addition, Bcl-x expression is high during the early postnatal development and downregulated with maturation (Vogelbaum et al., 1998). DRG neurons in Bax deficient mice, survive indefinitely *in vitro* in absence of NGF (Deckwerth et al., 1996). Furthermore, naturally occurring cell death in is eliminated in peripheral ganglia in Bax deficient mice during the embryonic life (White et al., 1998).

Motoneurons

Spinal motomeuron bodies are located in the ventral horn of the spinal cord and they are generated in excess during development. About 50 percent of them are eliminated through cell death and several neurotrophic factors regulate their survival during the naturally occurring cell death.

The neurotrophins BDNF and GDNF have been reported to support motoneuron survival in culture (Sendner et al., 1992; Henderson et al., 1994). Motoneurons are also supported by other neurotrophic factors such as CNTF and HGF (Sendner et al., 1990; Wong et al., 1997). Interestingly, there seems to be regional differences in trophic factor dependies between motoneurons derived from cervical, thoracal and lumbal regions as shown for HGF (Novak et al., 2000).

Motoneurons express receptors for NMDA and AMPA receptors, which mediate calcium influx in response to glutamate during the embryonic development. Thereby they are vulnerable for increased calcium levels as shown in several studies (Berridge et al., 1998; Rothstein et al., 1993).

Pathological motoneuron cell death can be seen among others in several neurodegenerative diseases, which affect spinal motoneurons. ALS and SMA can serve as an example for these. Furthermore, there are several experimental models, which mimic the damages seen in spinal cord injuries.

Little is known about downstream signals involved in motoneuron cell death. Several apoptosis genes have been identified in motoneurons and overexpression of Bcl-2 have been shown to significantly reduce motoneuron cell death during development (Martinou et al., 1994; Dubois-Dauphin et al., 1994). Interestingly, Bcl-2 deficient mice show only a small reduction of motoneuron numbers even though these mice show enhanced motoneuron loss postnatally suggesting that several parallel mechanisms exist (Michaelidis et al., 1996). Mutations in IAP family member NAIP have been identified in patient suffering from SMA even if NAIP is thought to have only a modulatory function in this disease (Roy et al., 1995). Furthermore, Bcl-2 and SMN protein seem to work synergistically in this disease in order to increase the anti-apoptotic function (Iwahashi et al., 1997). In addition, adenovirally administrated NAIP, HIAP1 and HIAP2 have been shown to rescue about 30-40% of motoneurons at one week after sciatic nerve axotomy (Perrelet et al., 2000).

Hippocampal neurons

Hippocampus is located as semicircles around thalamus and it plays a central role in spatial memory and learning. Hippocampus is structurally divided in different parts: the dentate gyrus, and the hippocampal area CA1-4 (CA, Cornu Ammon). Both the dentate gyrus and CA1-4 areas are three-layered structures. The CA1-4 areas consist of the polymorphic layer (stratum oriens), the pyramidal layer (stratum pyramidale) and the molecular layer (stratum radiatum/stratum lacunosum moleculare) whereas the layers in dentate gyrus are called hilus (polymorphic layer), granular layer and molecular layer.

Hippocamus has connections with regions such as entorhinal cortex, septum, brainstem, hypothalamus, thalamus and amygdala. The principal neurons in hippocampal CA1-4 areas are called pyramidal neurons and they present the majority of neurons in the hippocampus. The cell bodies of these neurons can be found in the pyramidal layer and the morphology varies depending on where the cells are located. These neurons mainly project via the fimbria-fornix formation to septum, thalamus and hypothalamus. Majority of the neurons in the dentate gyrus on the other hand are so called granule cells, which are smaller than the pyramidal neurons. These neurons send projections mainly to the CA3 area via mossy fiber axons.

In hippocampus there are interneurons, of which the GABAergic Basket cells in CA1 are the best characterised. These appear to mediate both feed forward and feedback inhibition of pyramidal neurons. In the hilus, the interneurons seem to have a slightly different subset of neurotrophic receptors and trophic factor requirements (Korhonen et al., 2000). Generally, hippocampal neurons express receptors for many neurotrophic factors and several neurotrophins are present in this tissue (Wetmore and Olson, 1993). In addition, many neurotrophic factors among others BDNF and HGF have been reported to increase the survival of the hippocampal neurons *in vitro* (Zafra et al., 1990; Korhonen et al., 2000).

Kainic acid models

Glutamate is the major excitatory transmitter in the brain and several studies have shown that different genes are regulated upon glutamate receptor activation. Kainic acid (KA),

which is an agonist for ionotropic non-NMDA glutamate receptors AMPA and KA receptors, is a widely used experimental tool to study activation of glutamate receptor.

Systemically administrated KA results in epileptoform seizures in the hippocampus with the propagation to other structures within the limbic system. KA is known to induce cell death in the brain (Olney, 1974). This effect on neuronal cell death is dependent on whether the drug is administrated intraperitoneally, intraventricularly or by intra-amygdaloid injections. Intraperitoneal injections result in neuronal death mainly in hippocampal CA1, CA3 and CA4 areas whereas cell death after intraventricular injections is mainly seen in CA3 subfield (Sperk et al., 1994; Nadler et al., 1978; Schwob et al., 1980). Similarly, intra-amygdaloid injections result in cell death in CA3 area (Ben-Ari et al., 1980; Pollard et al., 1994). Importantly, the dentate gyrus is consistently spared from KA caused neurodegeneration (Sperk et al., 1994).

Neurons in CA3 are highly vulnerable to KA and easily degenerate after recurrent seizures (reviewed by Ben-Ari and Cossart, 2000). Diazepam, a well-known anti-epileptic drug, can block the seizures and inhibit ensuring cell death indicating that normal degeneration in CA3 depends on seizure activity. This hypothesis is supported by the observations that repetitive high-frequency stimulation of CA3 neurons induces a selective cell loss suggesting that excessive activation of synaptic inputs to CA3 neurons is toxic.

Studies have shown that the epileptogenic activity of KA in CA3 is caused by the activation of high-affinity kainate receptors in the mossy fiber synapses (Frerking et al., 1998). Several kainate receptors have been cloned and the role of the different subunits in kainate receptor mediated activity has been studied using gene deleted animals. Granule cells and pyramidal cells in the CA3 area are enriched in GluR6-containing kainate receptors and electric activity upon mossy fiber stimulation and KA receptor activation is eliminated in GluR6 deficient mice (Mulle et al., 1998). In addition, higher concentrations of kainic acid are needed to provocate seizures in these animals. There are some studies where low expression of GluR5 is observed in the CA3 neurons but the role of this subunit is not fully understood (Vignes et al., 1998).

Previous studies have shown that nerve cell death mediated by KA is at least partially apoptotic although there are some reports suggesting that the cell death process after kainic acid is necrotic (Pollard et al., 1994; Simonian et al., 1996). Evidence for the apoptotic cell death comes from the studies, which have shown presence of TUNEL reactive cells and caspase-3 activation (Faherty et al., 1999). Several proteins, among them members of the Bcl-2 family, are regulated by KA suggesting an involvement of apoptosis (Lopez et al., 1999).

KA is also known to induce gene expression in an activity dependent manner. Some of these genes are regulated very rapidly (within minutes to some hours) whereas other genes need longer times before changes are observed in expression levels. Among genes regulated by KA, are neurotrophic factors, their receptors, synaptotagmin III, calbindin and NaClII (Zafra et al., 1990; Ernfors et al., 1991; Mudo et al., 1995; Vician et al., 1995; Lee et al., 1997; Gastaldi et al., 1997).

MATERIAL AND METHODS

EXPERIMENTAL ANIMALS

All animal experiments were approved by local ethical committees and carried out in accordance with the European Communities Council Directive (86/609/EEC).

Kainic acid injection (Paper IV, V)

Adult male rats (200-300g) were anaesthetised with ether or urethane and placed in stereotaxic apparatus. KA (0.35µg/ml/0.5µl, Sigma, St. Louis, MO, USA), or an equal amount of saline were injected into the lateral ventricles with following stereotaxic coordinates: AP= -0.85; L= 1.5; V= 4.3 from the Bregma (Paxinos and Watson, 1998). KA induced a tonic-clonic seizure in the rats that lasted for 30-60 minutes. Rats were killed by decapitation at 6h, 12h, 24h, 48h and 72h after injection, and brains were frozen and stored at -70°C until analysis.

CELL CULTURE

Cell lines (Paper I, III)

Cells (hN-tera2, N2a, NSC-34, COS-7) were cultured in high glucose Dulbecco`s modified Eagle`s medium (DMEM; Biochrom, Germany) supplemented with 10% foetal calf serum (Bottom, Germany). Human NT-2 cells were obtained from Stratagene (USA) and have the properties of neuronal precursor cells (Pleasure and Lee, 1993). Murine neuro 2a neuroblastoma cell line was a kind gift from Dr Metsis (Karolinska Institute, Stockholm, Sweden). NSC-34, a spinal cord motor neuron-like cell line was a kind gift from Dr Cashman (Cashman et al., 1992).

Primary cultures (Paper II, IV)

A. Dorsal root ganglia (DRG) Cultures

DRG neurons were prepared essentially as earlier described (Lioudyno et al., 1998). DRG were dissected from P3-P4 old Wistar rats (B&K, Sweden), and the ganglia were collected in phosphate buffered saline (PBS) buffer containing 0.45% glucose. The tissue was treated with 0.25% trypsin (Gibco, Sweden) for 30min at 37°C, and the digestion was terminated by the addition of DMEM (Gibco, Sweden), supplemented with 10% charcoal-stripped serum (Imperial Chemical Industries, U.K.). Cells were centrifuged for 3min at 900 rpm, the pellet was washed three times and a single cell suspension was obtained by passing the tissue through a Pasteur pipette. The cells were pre-plated for 2 hr on a 35 mm dish (Nunc, Denmark), followed by seeding of non-adherent cells (9,400 cells/cm^2) onto 24-well plates (Nunc, Denmark) coated with poly-DL-ornithine (0.1mg/ml; Sigma, Sweden) and laminin (6g/ml; Sigma, Sweden). NGF (50ng/ml) and cytosine β-D-arabino-furanoside (0.01mM, Sigma; Sweden) were added to the cultures. After one day fresh serum-free medium was added, together with different concentrations of 17β-estradiol (Sigma, Sweden), tamoxifen (Sigma, Sweden) or ICI 182,780 (kindly provided by A. Maggi, University of Milan, Italy).

B. Hippocampal cultures

Hippocampus was dissected from E 17 old Wistar rats (B&K, Sweden) as described (Zafra et al., 1990). Tissue was treated with papain (0.5µg/ml; Sigma, Sweden) and DL-cystine

(0.2 µg/ml; Sigma, Sweden) at +37°C 15 minutes and sequentially dissociated by adding DNaseI (Roche, Germany) and passing the tissue through a Pasteur pipette. Dissociated neurons were centrifuged for 3min at 900rpm. The pellet was suspended in DMEM (Biochrom, Germany) supplemented with 2% foetal calf serum (Biochrom, Germany) and neurons were plated onto a polyethyleneimine (Sigma, Sweden) coated plates at a density of $0.2\text{-}0.25 \times 10^6 \text{cells/cm}^2$.

Cell transfections (Paper I, III)
In order to establish stable cell lines, human NT-2 cells (paper I), NSC-32 cells (Paper III) and N2a cells (Paper III) were transfected using calcium phosphate with an expression plasmid for human bcl-2, NAIP and Hippocalcin. Several cell lines were selected for using G418 (Gibco, Sweden) and analysed by Northern and Western blotting.

Viability assays (Paper I, II, III, IV)
The viability of the cells was estimated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-assay (MTT, Sigma, Sweden), which is based upon the reduction of MTT by mitochondrial hydrogenases. Briefly, MTT (0.5mg/ml) was added to the cultures for 3h at +37°C. Formed crystals were dissolved in isopropanol-HCl and absorbance was measured at 570nm. There was a linear relationship between the absorbance and the survival of the cells.

An enzymatic assay for LDH (Roche, Germany) was also used to assay cell viability in Paper III. Briefly, cells were plated on a 96-well plate (Nunc, Denmark) at the density of $0.3 \times 10^6 \text{cells/cm}^2$ and after stimulation; supernatants were collected and proceeded for the analysis. Absorbance of enzymatically formed formazan dye was read at 500nm.

CELL BIOLOGY METHODS

Antibodies (Papers I, II, III, IV)
A specific anti-XIAP antibody was produced in rabbits by immunisation with a peptide, SSDRNFPNSTNSPRN, corresponding to residues 244-258 in mouse XIAP. The primary antiserum was affinity purified by gel-agarose columns, and the specificity of the antibody was tested by ELISA and Western blotting. For other primary and secondary antibodies, see table below.

Western blotting (Paper I, II, III, IV)
For western blotting, cells and tissue were lysed with lysis buffer (Paper I and III: 62mM Tris pH6.8, 6M Urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue, 5% 2-mercaptoethanol; Paper II: 1% NP-40, 20mM Tris pH8.0, 5mM EDTA; Paper III: 50mM HEPES pH 7.5; 250mM NaCl, 0.2% NP-40; Paper IV: 150mM Nacl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris pH 8.0) supplemented with protease-inhibitor cocktail (Roche, Germany). DNA was shared by sonication and lysates were pre-cleared subsequently by centrifugation. The protein concentrations were determined using BioRad DC Protein Assay (BioRad, Sweden) and equal amounts of total protein were loading onto each well. After separation on a SDS-gel, proteins were blotted onto a NC (BioRad, Sweden) or a PVDF-membrane (Amersham, U.K.). The membrane was blocked with 3-5% skim milk-TBS-T (10Mm Tris pH 8.0, 150mM NaCl, 0.1% Triton X-100) at RT for 1-2 hours. Incubation with primary antibodies was typically done over night at

+4°C. After extensive washing with TBS-T-buffer, horseradish peroxidase coupled secondary antibody (Dako, Denmark) was added and incubated at RT for 1 hour. After washing signal was visualised using ECL method (Pierce, USA).

Table 6. Used antibodies

Antibody	Company	Source	Dilution	Application	Used in study
CPP32	Transduction	mouse	1:1000	WB, IC	Paper I
ICH-1L	Transduction	mouse	1:1000	WB, IC	Paper I
Raf	Transduction	mouse	1:1000	WB	Paper I
PARP	Dr. Poirier	mouse	1:10000	WB	Paper I
Bcl-2	Santa Crux	rabbit	1:500	WB	Paper I
Bcl-X	Transduction	rabbit	1:1000	WB	Paper II
Bax	Santa Crux	rabbit	1:500	WB	Paper II
ERα	StressGen	mouse	7μg/ml	IC, IH	Paper II
ERβ	Affinity Bioreagents	rabbit	5μg/ml	IC, IH	Paper II
HA	oche	mouse	1:1000	WB, IP	Paper III
GFP	Clontech	rabbit	1:500	WB, IP	Paper III
Myc	Roche	mouse	1:1000	WB	Paper III
PARP	BioMol	mouse	1:10000	WB	Paper III
Caspase-3	Pharmingen	rabbit	1:100	IC, IH	Paper III, IV
GFAP	Roche	mouse	1:500	DS	Paper IV
NeuN	Chemicon	mouse	1:150	DS	Paper IV, V
OX42	Serotec	mouse	1:800	DS	Paper IV
cIAP-1	Santa Crux	goat	1:100	WB, IH	Paper V
Anti-mouse -HRP	Dako	rabbit	1:1000	WB	Paper I- IV
Anti-mouse-Biotin	Vector	horse	1:200	IC, IH	Paper I, II
Anti-mouse-FITC	Sigma	goat	1:200	DS	Paper IV, V
Anti-rabbit-HRP	Dako	goat	1:1000	WB	Paper II-IV
Anti-rabbit-Biotin.	Vector	horse	1:200	IC, IH	Paper III, IV
Anti-goat-HRP	Dako	rabbit	1:1000	WB	Paper V
Anti-goat-Biotin	Vector	horse	1:200	IH	Paper V

WB= Western blotting, IC= immunocytochemistry, IH= immunohistochemistry, IP= immunoprecipitation, DS= double staining

Immunocyto-and histochemistry (Paper I, II, III, IV, V)

For immunocytochemistry, cells were fixed with 2-4% paraformaldehyd (Paper I and II) or with acetone: methanol (1:1) (Paper III). For immunohistochemistry 13-15μm sections from P3-4 rats` DRG (Paper II) or adult rat brain (Paper III) were cut in a cryostat (Leitz Digital 1702, Wetslar, Germany) and mounted onto Superfrost slides (Metzel-Gläser, Germany). Slides were fixed in 4% PAF for 30min at +4°C (paper II) or with acetone: methanol (1:1) for 10min at -20°C. After fixation, endogenous peroxidases were quenched with 1% hydro peroxide. Cells and tissue sections were blocked (Paper I: PBS, 0.005% Triton X-100, 1% BSA; Paper II: PBS, 0.3% Triton X-100, 3% rat serum; Paper III and IV: TNB-blocking reagent (NEN, USA)) followed by incubation with primary antibodies. After extensive washing, secondary biotinylated antibody (1:200; Vector laboratories, USA) was added. Specific signal was visualised using DAB (Sigma, Sweden) as a chromogen. Cells were immersed in PBS-glycerol and tissue sections were dehydrated in an alcohol series and mounted with Entellan (Kebo, Sweden).

Double staining (Paper IV, V)
For double staining, brains were fixed and incubated with primary antibodies as above. After washing secondary biotinylated anti-rabbit antiserum (Vector laboratories, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse antiserum (Dako, Denmark) were added in dilution 1:200, and incubated for 2 hours at RT. Thereafter tissue sections were incubated with Texas Red™ coupled avidin (NEN, USA). Sections were dehydrated as above and mounted with Vectastain fluorescence mounting medium (Vector laboratories, USA).

TUNEL staining (Paper IV, V)
TUNEL labelling was performed as earlier described (Gavrieli et al., 1992). Briefly, frozen sections were fixed for 10 min with acetone: methanol (1:1) at -20°C, and permeabilised with 0.1% Tween-20/1% BSA/ PBS on ice. Fluorescein-conjugated 12-dUTP in a buffer containing TdT (Roche, Germany) was added and sections incubated for 1 h at +37°C, washed and analysed under fluorescence microscope (Zeiss, Germany).

Caspase-3 assay (Paper III, IV)
The caspase-3 assay was performed using a Caspase-3 Cellular Activity Assay kit (BioMol, USA). In brief, stimulated cells (Paper III, IV) or tissue (Paper IV) were lysed in cell lysis buffer supplemented with 0.1% NP-40, and centrifuged for 10 min at 10,000 x g. In a reaction volume of 100µl, 10µl of the lysate supernatant (between 1-3µg/µl) was added to assay buffer and the caspase-3/7 substrate Ac-DEVD-*p*Na (200µM) and incubated at 37°C. Release of *p*-nitroaniline was monitored by recording the optical density at 405 nm. Samples were performed in duplicate.

Calcium assay (Paper III)
The measurement of intracellular Ca^{2+} concentrations was performed using fura-2 essentially as previously described (Kukkonen et al., 1997), except that 1mM probenecid (*p*- (dipropylsulphamoyl) benzoic acid (Sigma, Sweden) was added to the medium during the fura-2 loading and measurement to inhibit the leak of fura-2.

XIAP recombinant protein (Paper IV)
This was produced in the baculovirus system using Sf9 cells according to the manufacturer´s instruction (InVitrogen, USA).

In vitro binding assays (Paper III)
GST-tagged hippocalcin was expressed, purified and immobilised onto glutathione beads according to standard methodology.

A. Reticulocyte lysates
^{35}S-labelled NAIP-BIR (aa 6-421), XIAP and luciferase proteins were generated from cDNAs by *in vitro* transcription and translation using the TNT T7-coupled reticulocyte lysate system (Promega, Sweden), but with the addition of 1mM $ZnCl_2$. ^{35}S-NAIP-BIR1-3 (15 µl), ^{35}S-XIAP (30 µl) or ^{35}S-luciferase (30 µl) were diluted in 700 µl of binding buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 1% NP-40) supplemented with either 1mM $CaCl_2$ or 1mM EGTA, and incubated for 1 hr with GST-hippocalcin immobilised onto

glutathione beads (50µl). The beads were recovered by centrifugation, washed 8 times in the appropriate binding buffer, boiled in SDS-PAGE sample buffer and the supernatant retrieved by centrifugation. Samples were resolved by SDS-PAGE and visualised following autoradiography.

B. Mammalian cells
COS-7 cells were transfected at 75% confluency using the calcium phosphate method. Cells were lysed 48 hr after transfection in 1 ml of ice cold lysis buffer (50mM HEPES, pH 7.5, 250mM NaCl, 0.2% NP-40, protease inhibitors (Roche, Germany), supplemented with either 1mM $CaCl_2$ or 1mM EGTA and incubated for 1 hr with GST-hippocalcin immobilised onto glutathione beads (50µl). The beads were recovered by centrifugation, washed 3 times in the appropriate binding buffer, boiled in SDS-PAGE sample buffer. Samples were resolved by SDS-PAGE and transferred to nitrocellulose membrane (BioRad, Sweden) for Western blot analysis. Anti-GFP (Clontech) and anti-Myc (Roche, Germany) were used as primary antibodies for the analysis followed by incubation with HRP-conjugated secondary antibodies (Dako, Denmark). Specific signals were detected by enhanced chemiluminescence.

Co-immunoprecipitation and Western blot analysis (Paper III)
COS-7 cells were transfected and lysed as described above in lysis buffer supplemented with either 1mM $CaCl_2$ or 1mM EGTA. Monoclonal anti-HA epitope antibody (3.5µg) (clone 12CA5) was incubated with the lysate for 2 hr at 4°C. Complexes were immunoprecipitated with protein G-sepharose (Pharmacia, Sweden) for 1 hr at 4°C, and washed 3 times with the lysis buffer. The sepharose beads were boiled in SDS-PAGE sample buffer and samples were resolved by SDS-PAGE followed by transfer to nitrocellulose membrane (BioRad, Sweden). Western blot analysis was performed as described above with the exception that anti-GFP (Clontech, USA) and anti-HA (Roche, Germany) were used as primary antibodies.

MOLECULAR BIOLOGY

RNA preparation (Paper I, III, IV, V)
Total RNA from cells (Paper I, III) or tissue (Paper IV) was extracted with the acid guanidine isothiocyanate: phenol-cloroform extraction method (Chomczynski and Sacchi, 1987). The concentration of extracted RNA was measured spectrophotometrically.

Northern blotting (Paper I, III, IV, V)
Total RNA was prepared and subjected to gel electrophoresis and transfected to a nylon filter (Amersham, U.K.). The filters were pre-hybridised with for 60min and there after hybridised overnight at 42°C with a [^{32}P]-dCTP (Amersham, U.K.) labelled (Rediprime; Amersham, U.K.) probe. In Paper IV [^{32}P]-UTP (NEN, USA) labelled cRNA probes were used instead of DNA probes and hybridisation was then carried out at 65°C. Filters were washed with 2XSSC, 0.1% SDS for 15 minutes and 0.1XSSC, 0.1% SDS for 30 minutes at 65 degrees. Mouse β-Actin was used as a control. Blot was exposed to PhosphoImager (Molecular Probes, USA) and specific transcript levels were compared with those of β-Actin.

RNase protection assay (Paper IV)
RNase protection assay was performed as earlier described (Palm et al., 1998). Shortly, specific riboprobe was produced through linearisation of the plasmid in the 5′end followed by transcription with T7 RNA polymerase as described in MAXIscript kit instructions (Ambion, USA). Anti-sense GAPDH-cRNA probe (a kind gift from Dr. K. Palm) was used as a control. The probes were hybridised with 10μg RNA and protected cRNA fragments were then separated on 4% polyacrylamide gels under denaturating conditions. Gels were exposed to PhosphoImager (Molecular Dynamics, USA) and amounts of rat XIAP-specific mRNA were analysed using ImageQuant software (Molecular Dynamics, USA). The sizes of the protected bands were determined with a RNA size marker (Ambion, USA).

In situ hybridisation (Paper III, IV, V)
In situ hybridisation for hippocalcin was performed as previously described using neonatal Wistar rats (B&K, Sweden) on spinal cord sections (Skoglösa et al., 1999). The oligonucleotide probe was 47 nucleotides in length, complementary to nucleotides 58-104 of the rat hippocalcin coding sequence (Scandinavian Gene Synthesis, Sweden). A 200-fold excess of cold probe was used as a negative control.

For RIAP-2 *in situ hybridisation* 14 μm serial coronal sections cut on a cryostat were thawed onto 3-aminopropyl ethoxysilane-coated slides. Following fixation in 4% paraformaldehyde for 15 min, slides were rinsed twice in PBS and once in distilled water. They were then incubated with 0.2M HCl for 10 min, deacetylated with 0.25% acetic anhydride in 0.1M ethanolamine for 20 min and dehydrated with increasing concentrations of ethanol. Slides were incubated for 16h in a humidified chamber at 52° C, with 8 $x10^5$ cpm of probe in 70μl hybridization cocktail (50% formamide, 20 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0), 0.3 M NaCl, 0.1 M dithiothreitol, 0.5 μg/ml yeast tRNA, 0.1 μg/ml poly-A-RNA, 1X Denhardt´s solution and 10% dextran sulfate). They were washed twice in 1X SSC at 62°C for 15 min, and then in formamide:SSC (1:1) at 62° for 30 min. After an additional wash in 1X SSC at 62°C, single-stranded RNA was digested by RNAse treatment (10 μg/ml) for 30 min. at 37°C in 0.5 M NaCl, 20 mM Tris-HCl pH 7.5, 2mM EDTA. After washing with SSC at 66 °C for 30 min the slides were dehydrated in ethanol and air-dried. The sections were exposed for three weeks to ß-Max Hyperfilm (Amersham), and subsequently coated with NTB-2 photoemulsion diluted 1:1 in water (Eastman-Kodak Co., Rochester, NY), and stored for 4 weeks in desiccated light-tight boxes at 4 C. Slides were developed with D19 (Eastman-Kodak Co.), fixed with Al-4 (Agfa Gevaert, Kista, Sweden) and counterstained with Cresyl Violet. Control hybridization for specificity of the cRNA probes was studied using sense ^{35}S-labeled riboprobes, which resulted in no signal above background.

When combining *in situ* hybridization for RIAP mRNA with the immunolabeling of cells with the neuronal marker NeuN, immunolabeling was performed after the in situ hybridization procedure, which was carried out exactly as detailed above. The sections were incubated for 15 min in blocking buffer consisting of 2.5% normal goat serum and 0.3% Triton X-100 in PBS and subsequently incubated overnight at 4°C in the presence of the primary antibody(NeuN 1:500; Chemicon, Temecula, CA, USA) Sections were washed three times for 5 min in PBS, and incubated at room temperature for 1 h with a biotinylated antimouse antiserum (Amersham, UK), diluted 1:200. After three 5 min washings with PBS, the sections were incubated for 1 h with a horseradish peroxidase–streptavidin complex (Vector, Burlingame, CA), diluted 1:100 in PBS. After

washing the peroxidase reaction was developed in the same buffer containing 0.05% 3,3-diaminobenzidine-4 HCl and 0.003% hydrogen peroxide. After a short wash with H_2O, sections were dehydrated in an ascending alcohol series, coated in NTB-2 emulsion and processed as described above.

CLONING

Cloning (Paper III, IV)
cDNA corresponding to amino acids (aa) 6-421 of NAIP was synthesised from human placenta cDNA using PCR primers and conditions as previously described (Roy et al., 1995), and cloned into pBluescript (Stratagene, USA). Full length XIAP cDNA was obtained by screening from a human foetal brain library (Clontech, USA). PCR was used to generate constructs with appropriate restriction sites for cloning into expression vectors. A 450 bp long rat XIAP DNA fragment between the BIR3 and RING domains, was cloned by polymerase chain reaction (PCR) using adult rat liver cDNA and the following primers: 5`(GAATTCAACATGCCAAGTGGTTTCC) and 3′(CCGGATCCTTAAGACATAAA AATTTTTTG). The sequences of all constructs were verified by automated sequencing.

Expression constructs (Paper III)
For expression in mammalian cells, NAIP cDNA (aa 6-421) was subcloned into pEGFPC1 (Clontech, USA) and pCDNA3.1HisC (Clontech, USA). Full-length hippocalcin was subcloned into pCDNA3.1HisC, pEGFPC3 (Clontech, USA), and pIRESneo behind an in-frame HA epitope tag. A tricistronic construct was generated in pIRESneo by inserting an IRES sequence downstream of HA-tagged hippocalcin, followed by EGFP NAIP-BIR1-3 (aa6-421), a synthetic intron, IRES sequence and neomycin resistance gene of the vector. Deletion constructs of NAIP's BIR domains were cloned into pYTH6 (BIR1aa46-157; BIR2aa161-268; BIR3aa264-362; BIR1+2aa46-233; BIR2+3aa161-344) and pEGFPC1 (BIR1+2aa46-233; BIR3aa264-362). Myc-tagged full-length human NAIP cDNA in pCDNA3 and human Bcl-2 cDNA in pSFFV were kindly provided by Alex MacKenzie and Stanley Korsmeyer, respectively.

For expression in yeast cells, cDNA encompassing the BIR domains of NAIP was subcloned into the GAL4 DNA-binding domain vector pYTH6 (aa46-344; Clontech, USA) and activation domain vector, pACT2 (aa46-421; Clontech, USA). Full length XIAP cDNA was subcloned into two-hybrid vectors pGBT9 (XX) and pGAD424 (XX), and hippocalcin into pYTH6 and into the bacterial GST expression vector pGEX-6P (XX).

Polymerase chain reaction (Paper I, III, IV)
Reverse transcription (RT)-PCR was carried out with extracted total RNA using *Taq* polymerase (Perkin Elmer, USA). Twenty-five to thirty cycles were usually performed with a PCR machine (PTC-100; MJ Research, USA) and the reaction steps were typically 94°C, 55°C and 72°C. The PCR products were subjected to gel electrophoresis and the bands were either sequenced or hybridised with specific probes.

Yeast two-hybrid system (Paper III)
A. Screening
pYTH6 NAIP-BIR (aa 46-344) was linearised and integrated into the yeast genome of yeast strain Y190 through a lithium acetate/PEG based transformation method and used to screen

a human foetal brain library (Clontech, USA) essentially as previously described (Aspenstrom and Olson, 1995). Transformants were grown on $Trp^{-}Leu^{-}His^{-}$ plates supplemented with 25mM 3-aminotriazole (Sigma, Sweden) for 4-8 days and streaked onto $Trp^{-}Leu^{-}His^{-}$ and $Trp^{-}Leu^{-}$ plates. A filter lift procedure was used to examine the colonies for β-galactosidase after 2 days (Aspenstrom and Olson, 1995).

B. ONPG assay

Double transformants were streaked onto $Trp^{-}Leu^{-}$ plates and grown for two days. Three individual colonies for each double transformant type were picked and grown overnight at 30°C in 5 ml $Trp^{-}Leu^{-}$ liquid media. Next day, culture volumes were increased and the yeast was grown to an OD_{600} of 1.0. The yeast was harvested by centrifugation; the pellet was resuspended in Z-buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$), and divided into triplicates. Following repeated freeze thawing in liquid nitrogen and a 30°C water bath, Z-buffer containing β-mercaptoethanol (2%, Kebo, Sweden) and the substrate ONPG (2.2mM; Sigma, Sweden) were added to the samples. The samples were incubated at 30°C until yellow colour developed at which time the reaction was terminated and cleared by centrifugation. The OD_{420} was determined for each sample and the β-galactosidase activity determined as described (Aspenström and Olson, 1995).

STATISTICAL METHODS AND IMAGE ANALYSIS

Image analysis (Paper I, II, IV, V)

Transcript levels were analysed by using PhosphoImager (Molecular Dynamichs, USA). Western blotting membranes were analysed with digitised image analysis system (JAVA; Corte Madera, USA). For analysis of immunostained cells in Paper IV a public domain NIH image program (http://rsb.info. nih.gov/nih-image) was used. Five non-overlapping standardised areas within the dentate gyrus, hilus and the CA1 and CA3 regions were analysed and more than 100 cells were counted for each section.

The brain region and nuclei distribution of RIAP-2 mRNA were evaluated from emulsion-dipped sections using the rat brain atlas of Paxinos and Watson. The relative levels of RIAP mRNA expression (intensity) per cells were evaluated from emulsion dipped slides by count of silver grains over the individual cells, using an image analysis system. A correction factor for overlapping grains was included. Labeled cells were defined on the basis of the presence of more than 8 silver grains compared to background, which was calculated by counting the grain in field lacking labeled cells, such as white matter. For each brain nucleus examined, the grains of at least 30 cells in three different parts of at least two sections were counted. The data are shown using an arbitrary semi-quantitative scale of labeling intensity described as low intensity (+), indicating an amount of silver grains between the fixed minimum level and 30 grains per cells; moderate intensity (++), indicating a number of grains between 30 and 60; and high intensity (+++), indicating a number of grains exceeding maximum level selected for moderate intensity.

In addition, the percentage of labeled cells within a particular structure was determined. For each brain area analysed, 200 cells were counted in three different fields of at least two sections. Data of percentage of labeled cells is shown as low density (<30%), moderate density (between 30 and 60%) and high density (>60%). The results were analysed by

ANOVA and Student´s t- test. Differences in p-value less than 0.05 were considered statistically significant. Values are shown as mean ±SEM.

Statistical methods (*Paper I, II, III, IV, V)*

Values are presented as mean ±SEM and analysed by ANOVA and Student´s *t*-test. P< 0.05 was considered statistically significant.

PRESENT INVESTIGATIONS

AIMS

General aim

The aim of the present investigations was to gain deeper insights into the apoptotic process in neuronal degeneration accompanying many devastating human diseases. This was accomplished by studying the expression, function and regulation of anti-apoptotic proteins belonging to the Bcl-2 and Inhibitor of Apoptosis Protein families.

Specific aims

- To study the role of Bcl-2 overexpression in human neuronal precursor cells with special reference to its effects on caspases.

- To study the distribution of estrogen receptors in dorsal root ganglion and to investigate the role and mechanism of estrogen as a survival factor for DRG neurons

- To study the mechanisms behind NAIP`s anti-apoptotic effect on motoneurons and search for novel interactors using the yeast two-hybrid system

- To characterise XIAP expression and function during neuronal development and in the kainic acid induced neuronal degeneration

- To study the expression of RIAP-2 mRNA and protein in the rat brain and their regulation after kainic acid

RESULTS AND COMMENTS

PAPER I: Bcl-2 over expression leads to a down regulation of caspases

In order to study cell death in progenitor neurons, human NT-2 cell line expressing many properties of neuronal precursor cells were used (Pleasure and Lee, 1993). These cells were stably transfected with bcl-2, a well-known apoptosis modulator that has been shown to play an important role in developmental cell death and neuronal survival (Tsujimoto et al., 1984; Reed et al., 1994; Garcia et al., 1992; Martinou et al., 1994; Michaelidis et al., 1996). Stably transfected cells showed resistance against cell death induced by serum withdrawal or neurotrophic factor deprivation. Bcl-2 over expressing cells also dramatically downregulated cystein proteases ICH-1L/caspase-2 and CPP32/Yama/caspase-3 at the post-translational level. Downregulation of CPP32/Yama/caspase-3 occurred at the transcriptional level.

Paper I describes a novel mechanism for Bcl-2 in cell death protection, namely the down regulation of caspases-2 and –3. The observed effect on these caspases suggests that expression of individual caspases are differentially regulated by Bcl-2 in these cells. On the contrary, caspase downregulation has not been seen in bcl-2 overexpressing mice suggesting that Bcl-2 may have different mechanisms for cell protection at different stages of development (Guarin et al., 1999). In our experiments, human neuronal progenitor cells were used as a model for early development.

PAPER II: Estrogen increases the survival of DRG neurons and up-regulates Bcl-X

Estrogens represent an important class of hormones that can promote development, maturation and function of CNS (McEwen et al., 1997; Pilgrim and Hutchison, 1994). Estrogen receptors α (ERα) and β (ERβ) are widely expressed in the central nervous system, but little is known about these receptors in the PNS (Kuiper et al., 1996). Therefore, the role of estrogen in survival of DRG neurons was examined in paper II.

ERα and ERβ were found in P3 DRG and in cultured postnatal DRG neurons. Studies with cultured DRG neurons revealed that 17β-estradiol inhibited death caused by NGF withdrawal. The anti-estrogen compounds ICI 182,780 and tamoxifen, blocked the 17β-estradiol induced increase in survival indicating that effect is receptor mediated. Effects of estrogen on neuronal survival have been observed in hippocampal neurons (Azcoitia et al., 1999). Recently, it was reported that ERβ deficient mice show hypocellularity in the somotosensory cortex, and an astroglial proliferation in the limbic system with signs of increased neuronal degeneration with age (Wang et al., 2001).

17-β-estradiol increased significantly the levels of the anti-apoptotic protein Bcl-x in DRG, whereas no change was observed for Bax. In hippocampal neurons and PC12 cells estrogen has been shown to modulate Bcl-X expression levels (Pike, 1999; Gollaputi and Oblinger, 1999). There is evidence that Bcl-X is developmentally regulated in DRG neurons (Vogelbaum et al., 1998). Interestingly, estrogen did not change the Bax expression level, which also is regulated in the DRG during the development (Vogelbaum et al., 1998). Estrogen receptor antagonist tamoxifen blocked the induction of Bcl-X suggesting an involvement of ERs. Whether the estrogen effect on Bcl-X is obtained through a direct genomic effect or is indirect remains to be studied.

PAPER III: NAIP binds hippocalcin and protects motor neurons against calcium induced cell death via BIR3 domain

Whilst XIAP, c-IAP1 & 2 and survivin (Deveraux et al., 1997; Roy et al., 1997; Tamm et al., 1998) inhibit specific caspases through direct interaction, this has not been observed for NAIP (Roy et al., 1997). This suggests that NAIP acts differently or requires additional molecules to protect cells against harmful stimuli. To study this, we used the yeast two-hybrid system to identify potential interactors for NAIP. The most potent interactor found using this system was Hippocalcin, which is a neuron-specific calcium binding protein. Employing *in situ* hybridisation, *hippocalcin* mRNA was localised to the neonatal rat spinal cord, and it was particularly present in the large motoneurons.

To understand the function of the NAIP–Hippocalcin interaction and its effects on motor neuron death, we studied the NSC-34 motor neuron-like cell line that exhibits properties of spinal cord motor neurons (Cashman et al., 1992). These cells were stably transfected with *NAIP* or *NAIP-Hippocalcin* together, and the cells were in both cases significantly more resistant to cell death induced by ionomycin than wild type cells.

No significant difference in the intracellular calcium levels between controls, *NAIP-BIR1-3* or *NAIP-BIR1-3/hippocalcin* transfected NSC-34 cells were observed with respect to basal Ca^{2+} levels. Nor was there any difference in peak and sustained Ca^{2+} responses to 1μM thapsigargin, in control and NAIP-BIR1-3/hippocalcin cells. This suggests that the protective effect of NAIP-BIR1-3 or NAIP-BIR1-3/hippocalcin against cell death is not caused by changes in the Ca^{2+} level regulation, as proposed for Bcl-2 induced protection (Lam et al., 1994). However, it is not possible here to rule out differences in long-term Ca^{2+} responses after NAIP overexpression.

To determine whether a single BIR domain or a combination of domains is required for NAIP to bind hippocalcin, deletion constructs of the BIR domains were analysed. BIR1 and 2 have no binding capacity to hippocalcin, whilst BIR3 domain bind hippocalcin. Expression of EGFP BIR3 itself had no protective effect in cells treated with thapsigargin, however, when co-expressed with hippocalcin, the protection was comparable to that afforded by NAIP-BIR1-3 and hippocalcin.

Caspase-3 has been shown to be a key caspase involved in apoptosis (Slee et al., 1999). Recent reports, however, indicate that cell death with the morphological characteristics of apoptosis can occur without caspase-3 activation. To study this, we analysed the NSC-34 and Neuro-2a cell lines for caspase-3 activation, at various time points after treatment with thapsigargin and staurosporine. In NSC-34 cells both staurosporine and thapsigargin readily activated caspase-3 which became apparent at 6 hr. In contrast, thapsigargin induced only a small increase in caspase-3 activity at 24 hr indicating that thapsigargin killed through a pathway not dependent on caspase-3. The same situation was also observed using immunocytochemistry, revealing few Neuro-2a cells positive for caspase-3 at 24 hr after thapsigargin treatment, but many more positive NSC-34 cells.

NAIP has been shown to be important in neuronal degeneration occurring in affected motor neurons in patients with SMA (Roy et al., 1995). Furthermore, elevated levels of NAIP can protect hippocampal neurons against ischemia (Xu et al., 1997). In Paper III, we have identified hippocalcin as a new interactor for NAIP. Hippocalcin belongs to larger family of calcium binding proteins as recoverin, which has been associated with cancer-associated retinopathy (Polans et al., 1995). This indicates that this protein family may play a significant role in neuronal survival. Using *in situ* hybridisation it was shown that

hippocalcin mRNA is present in the spinal cord motoneurons. These neurons are known to be vulnerable to elevated calcium levels, for instance after excessive glutamate receptor activation (Rothstein et al., 1993). Interestingly, cell lines overexpressing NAIP and hippocalcin were more resistant against calcium induced cell death and BIR3 region of NAIP together with hippocalcin was equally effective to protect cells as the whole molecule. In view of this, it is possibly that smaller peptides mimicking BIR3 could be neuroprotective which warrants more studies.

PAPER IV: XIAP is present in the rat brain and is regulated in the kainic acid induced neuronal degeneration in the rat hippocampus

Little is known about the expression of IAPs in the nervous system. In order to study the occurrence of *XIAP* mRNA in rat brain Northern blotting and RNase protection assay (RPA) were applied. Two transcripts of *XIAP* was found in rat brain corresponding to those earlier reported in human brain tissues using Northern blots (Duckett et al., 1996; Uren et al., 1996). Using a carboxyterminal *XIAP* probe spanning the linker region between BIR3 and RING in RPA, two protected transcripts of *XIAP* were detected (about 430 and 225bp), suggesting heterogeneity of *XIAP* in this region.

There was a widespread distribution of XIAP immunoreactive cells in the rat brain. XIAP immunoreactivity was found among others, in the hippocampus, in the cortex and in the substantia nigra. XIAP positive cells were present also in the amygdala, the thalamus, the hypothalamus and in the septum. In cerebellum XIAP, immunoreactivity was present in Purkinje cells, but absent in granule cells. Double labelling experiments showed that both neurons and glial cells express XIAP immunoreactivity.

KA, a glutamate receptor agonist, is known to induce seizures and cause neuronal degeneration in specific regions of rat brain such as in the hippocampus and in the amygdala (Olney at al., 1974; Nadler et al., 1978; Sperk, 1994; Pollard et al., 1994). To study whether XIAP is regulated by KA, immunohistochemistry was applied. The number of XIAP positive cells significantly increased in the CA3 region at 48 h after KA. The intensity of XIAP per cell, however, initially increased in the CA3 region at 12 h, and decreased thereafter. Double-staining experiments revealed that the majority of XIAP positive cells in CA3 region were neurons, but some glia cells were also positive at 48h. Compared with the CA3 region, the alterations in XIAP by KA were modest in the CA1 subregion and not present in other parts of the hippocampus. The immunohistochemical data was confirmed by Western blotting which showed that XIAP protein levels increased at 12 and 24 h after kainic acid, and decreased thereafter.

Using caspase-3 assay, an increased activity was observed in hippocampus at 24 and 48 h but not at 12 h after kainic acid. Similarly, immunocytochemistry for the active form of caspase-3 revealed that no cells were positive at 12h whereas some cells became immunoreactive for this antibody at 24 h in the CA3 area. However, few cells in this region were immunoreactive for both active caspase-3 and XIAP. Labelling of cells with the TUNEL method showed many positive cells in the CA3 region at 24h after kainic acid, but very few cells were positive for both TUNEL labelling and XIAP immunoreactivity. These results suggest that viable neurons express XIAP whilst TUNEL positive, dying cells, are largely devoid of this protein. This suggests that XIAP may undergo cleavage in a caspase-dependent manner in degenerating neurons.

To study this, we cultured embryonic hippocampal neurons in the presence of different concentrations of kainic acid. 50 μM or more kainic acid readily induced nerve cell death

in these cultures. At the same time, the activity of caspase-3 increased after kainic acid treatment could be blocked by the caspase-3 inhibitor, DEVD. XIAP protein was also up regulated by KA in the cultures as seen *in vivo*. Most significantly, a 25 kDa large XIAP protein band gradually appeared in the cultures that were inhibited by the addition of DEVD. These results suggest that XIAP becomes a target for caspase-3 after prolonged treatment with KA.

As shown in Paper IV, XIAP expression was relatively constant during the development and XIAP is widely expressed in the adult rat brain. Double labelling showed that XIAP protein was found in neurons and glial cells. The expression pattern of XIAP indicates an important role of this protein during development. However, preliminary data indicates a redundancy in the system since XIAP deficient mice are healthy and do not show any major phenotypic changes (Harlin et al., 2001). Interestingly, cIAP-1 and cIAP2-2 levels were increased in these animals suggesting overlapping functions for the IAP family members (Harlin et al. 2001).

KA known to induce cell damage in the limbic system regulated the number of XIAP positive cells and the XIAP expression levels. KA also activated caspase-3 and subsequently enhanced degradation of XIAP in the dying cells. Interestingly, the surviving neurons were XIAP immunoreactivity showing a biphasic regulation of the protein. As shown by others cell death induced by KA can be inhibited by p35 protein and by transgenic mice over expressing NAIP in the brain (Viswanath et al., 2000; Holcik et al., 2000). The fact that KA induced cell death is inhibited in both of the models suggests a fundamental role for the IAPs in exitotoxic neurodegeneration.

PAPER V: RIAP-2 expression in the nervous system and seizure regulation

Using a PCR approach the rat homolugue of human cIAP-1/HIAP-2, *RIAP-2*, was cloned. RIAP-2 was present in rat hippocampus in two transcripts with the size of about 4 and 5 kB. These transcripts sizes correspond to those found previously for mouse IAP-2 also using Northern blotting (Rothe et al., 1995; Uren et al., 1996; Liston et al., 1997).

The relative level of *RIAP-2* mRNA increased slightly during early postnatal development in rat hippocampus as determined and in adult rat brain *RIAP-2* was found in hippocampus, in cerebral cortex and in cerebellum. RIAP-2 was also expressed among others in olfactory bulb, in substantia nigra, cerebellum and in nuclei within thalamus. Both *RIAP-2* mRNA and protein RIAP-2 colocalised with NeuN, a marker for neurons, in different brain areas indicating that RIAP-2 is predominantly expressed by neurons in the adult rat brain.

KA transiently upregulated the expression of *RIAP-2* mRNA in cerebral cortex, and in the dentate gyrus and CA1 regions of the hippocampus. The highest increase in RIAP-2 in these regions occurred at 12 h after treatment, and the levels decreased thereafter. Interestingly, in rats with less severe seizures, the CA3b subfield showed an incomplete lesion with spared neurons among degenerating ones and an upregulation of RIAP-2 mRNA in these cells. This suggests that RIAP-2 in these neurons may contribute to neuronal survival occurring after less severe seizures. The number of RIAP-2 immunoreactive cells was reduced in the CA3 region after 6h following KA whereas many cells were positive in CA1 and dentate gyrus.

TUNEL positive cells were observed in the CA3 area at 12 hours after KA and the number of them reached the maximun at 24h declining thereafter. Using double staining, it

was shown that RIAP-2 protein was absent in dying TUNEL positive cells, suggesting that RIAP-2 may protect against neurodegeneration caused by KA.

Interestingly, both RIAP-2 and XIAP are expressed by neurons and are regulated by kainic acid. As mentioned earlier, XIAP and cIAP-1 have been shown to be reciprocally regulated but the mechanism for this regulation is still unclear. However, NF-κB has been shown to regulate cIAP-1 and XIAP levels in some cells (Chu et al., 1997; Stehlik et al.,1998; Wang et al., 1998). This remains to be studies further in neurons.

GENERAL DISCUSSION

The term apoptosis was introduced more than 30 years ago to describe a form of cell death exhibiting certain morphological characteristics in dying cells. It is now considered that the process of apoptosis occurs during development for the establishment of an optimised number of cells with correct morphology and function of most organs in the organism. In addition, apoptosis is needed in many disease processes for the elimination of unwanted cells infected for example with viruses or after DNA damage or in cancer.

In the beginning the cellular mechanisms behind apoptosis were poorly understood, but when more became known about the processes involved, the term programmed cell death was coined. This term has then often been used as a synonym to apoptosis. However, many processes, which are called apoptosis, do not fulfill the morphological criteria of apoptosis as originally described. For example, a subset of neurons can have apoptotic characteristics after various necrotic insults (Linnik et al., 1993; MacManus et al., 1993; Ray and Sapolsky, 1999). Moreover, both necrotic and apoptotic signs, such as ruptured membranes and DNA laddering, can be found in the same neuron (Sohn et al., 1998; Martin et al., 1998; Gwag et al., 1997). Furthermore, the same chemical compound, such as KA, also used in this study, can induce either apoptotic or necrotic cell death depending on time of exposure, concentration of agent and the age of animal or neurons treated (Martin et al., 1998). Neuronal cells are also likely to undergo an apoptotic form of death when greater amount of energy is available (Leist et al., 1997; Eguchi et al., 1997). Along this line, some authors have suggested that injured neurons first intend to undergo apoptosis but can be halted due to various factors such as the of availability of ATP and mitochondria function (Roy et al., 1999). In addition, the levels of free radicals (ROS) play an important part in regulation of apoptosis and paradoxical effects have been described in brain trauma after inhibition of ROS production (Lewen et al., 2001).

Some authors have suggested the name paraptosis for a type of cell death that does not exhibit all the morphological characteristics of apoptosis (Sperandio et al., 2000). Paraptosis was first described in 293T and the cell death was not affected by Bcl-X nor by treatment with caspase inhibitors albeit still being mediated by caspase-9 activation in an Apaf-1 dependent manner (Sperandio et al., 2000).

There are also reports that describe a caspase-independent cell death which shows apoptotic morphology. For example, death of retinal cells induced by oxidative stress can not be blocked by caspase inhibitors (Carmody et al., 2000). In addition, studies with caspase-3 or caspase-9 deficient mice have shown that cell death is occurring to a nearly normal extent within different populations of developing neurons (Oppenheim et al., 2001). However, the morphology of dying neurons differs from that seen in classical apoptosis and the kinetics of cell death is also delayed. There are now ample evidence that, beside caspases, other proteases, such as calpains, serine proteases and cathepsins become activated during cell death (Leist and Jäättelä, 2001).

As evident from the discussion above the current terminology on the concept of cell death is not completely satisfactory. Therefore, it has recently been suggested that the term active cell death (ACD) should be used for the type of cell death that requires activation of intracellular processes regardless of the strict morphology present in dying cells (Sloviter, 2002). In this respect the term passive cell death (PCD) should be used instead of necrosis, a word that does mean *bona fide* only death.

In the developing nervous system, depending on the neuronal subtype, about 20-80% of neurons born are eliminated during naturally occurring cell death which shows classical apoptotic morphology. This developmental cell death is needed for correct morphology and proper function of the nervous system. However, neuronal death and degeneration is seen also in several human diseases, such as in Alzheimer´s and Parkinson disease, in amyotrophic lateral sclerosis (ALS) and in spinal muscular atrophy (SMA) as well as after more acute injuries occurring in brain trauma and ischemia/stroke.

At the present time, mainly symptomatic therapy can be offered to patients afflicted by these conditions. In this repect a lot of effort has been put down to identify mechanisms and factors regulating neuronal death in these dirsordes and to find novel therapeutic methods. The role of apoptosis in the neurological disorders has been suggested, but data is still rather scarce and open to discussion.

Studies using synthetic caspase inhibitors have shown that cell death can be delayed in many types of brain injuries (Nicholson, 2000). Similar results have also been obtained studying caspase deficient mice and some mouse models for human disease suggesting that a possible improvement could be achieved in neurodegenerative diseases (Eldadah and Faden, 2000). These include ALS, neonatal hypoxic-ischemic brain damage, Parkinson´s disease, status epilepticus and ischemia/stroke. However, as discussed above the caspase deficient mice may have severe development disturbances, and they also exhibit a caspase-independent type of cell death (Oppenheim et al., 2001). This suggests that caution should be exerted in the interpretation of data especially for the human clinical setting and in designing attempts to use caspase inhibition as a therapeutic strategy to prevent neuronal cell death. The issue of safety and cancer promotion is another important aspect to consider here.

Studies using transgenic mice with overexpression of Bcl-2 in neurons have shown an improved outcome after various brain insults such as after trauma, axotomy and ischemia (Martinou et al., 1994; Michaelidis et al., 1996; Dubois-Dauphin et al., 1994; Farlie et al., 1995; Bonfanti et al., 1996). In addition, adenovirally administrated IAP family proteins were shown to protect neurons against neuronal cell death (Xu et al., 1999; Kugler et al., 2000; Ebenhardt et al., 2000; Xu et al., 1997). On the other hand, bcl-2 antisense approaches have been tested in some tumour models with good results. In these studies, bcl-2 antisense decreased tumor growth, which has been confirmed in some recent clinical studies (Schlagbauer-Wadl et al., 2000; Waters et al., 2000).

Neurotrophic factors are known to promote neuronal survival of various classes of neurons and they have also been studied for neuroprotection in different settings in experimental animals and even in man. For example, NGF was administrated intracerebroventricularly to patients suffering from Alzheimer´s disease with some improvement of nicotine binding in brain (Eriksdotter Jonhagen et al., 1998). However, by time it became impossible to carry out these studies further because of serious side effects due to severe pain. GDNF has also been administrated in the similar ways to a patient having Parkinson´s disease (Kordower at al., 1999). These patients have also been treated with embryonic nigral transplants, which have been shown to incorporate into the brain tissue and to release dopamine (Piccini et al., 1999). The recent concept of (neural) stem cell therapy will hopefully open new avenues for the treatment of these and other neurological diseases.

In conclusion, recent studies on the function and interaction of various antiapoptotic proteins have added considerable to our understanding about the molecular mechanisms regulating cell death and apoptosis. Many of these proteins and mechanism are also found in the nervous system and may contribute to the process of neurodegeneration. Increased knowledge about the antiapoptotic proteins and cell death in brain disorders will ultimately help and facilitate the design of novel drug targets and add to possible therapeutic approaches to treat and alleviate human neurological diseases.

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REFERENCES

Abe-Dohmae S, Harada N, Yamada K, Tanaka R. (1993) Bcl-2 gene is highly expressed during neurogenesis in the central nervous system. Biochem Biophys Res Commun.191:915-21.

Adams JM, Cory S. (2001) Life-or-death decisions by the Bcl-2 protein family. Trends Biochem Sci. 26:61-6.

Adrain C, Creagh EM, Martin SJ. (2001) Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. EMBO J.20:6627-36.

Alonso G, Guillemain I, Dumoulin A, Privat A, Patey G. (1997) Immunolocalization of Bcl-xL/S in the central nervous system of neonatal and adult rats. Cell Tissue Res. 288:59-68.

Ambrosini G, Adida C, Altieri DC. (1997) A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med. 3:917-921.

Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B, Orrenius S, Lipton SA, Nicotera P. (1995) Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron 15:961-973.

Antonsson B, Conti F, Ciavatta A, Montessuit S, Lewis S, Martinou I, Bernasconi L, Bernard A, Mermod J., Mazzei G, Maundrell K, Gambale F, Sadoul R, Martinou JC. (1997) Inhibition of Bax channel-forming activity by Bcl-2. Science 277:370-372.

Aspenstrom P and Olson MF. (1995) Yeast two-hybrid system to detect protein-protein interactions with Rho GTPases. Methods Enzymol, 256, 228-241.

Azcoitia I, Sierra A, Garcia-Segura LM. (1999) Neuroprotective effects of estradiol in the adult rat hippocampus: interaction with insulin-like growth factor-I signalling. J Neurosci Res. 58:815-22.

Baffy G, Miyashita T, Williamson JR, Reed JC. (1993) Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production. J Biol Chem.268:6511-9.

Behl C, Widmann M, Trapp T, Holsboer F. (1995) 17-beta estradiol protects neurons from oxidative stress-induced cell death in vitro. Biochem Biophys Res Commun.216:473-82.

Ben-Ari Y, Tremblay E, Ottersen OP. (1980) Injections of kainic acid into the amygdaloid complex of the rat: an electrographic, clinical and histological study in relation to the pathology of epilepsy. Neuroscience. 5:515-28.

Ben-Ari Y, Cossart R. (2000) Kainate, a double agent that generates seizures: two decades of progress. Trends Neurosci.23:580-7.

Berg MM, Sternberg DW, Hempstead BL, Chao MV. (1991) The low-affinity p75 nerve growth factor (NGF) receptor mediates NGF-induced tyrosine phosphorylation. Proc Natl Acad Sci U S A. 88:7106-10.

Bergeron L, Perez GI, Macdonald G, Shi L, Sun Y, Jurisicova A, Varmuza S, Latham KE, Flaws JA, Salter JC, Hara H, Moskowitz MA, Li E, Greenberg A, Tilly JL, Yuan J. (1998) Defects in regulation of apoptosis in caspase-2-deficient mice. Genes Dev.12:1304-14.

Berridge MJ, Bootman MD, Lipp P. (1998) Calcium--a life and death signal. Nature. 395:645-8.

Bezvenyuk Z, Salminen A, Solovyan V. (2000) Excision of DNA loop domains as a common step in caspase-dependent and -independent types of neuronal cell death. Brain Res Mol Brain Res.81:191-6.

Bibel M, Barde YA. (2000) Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. Genes Dev. 14:2919-37.

Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74:597-608.

Boldin MP, Goncharov TM, Goltsev YV, Wallach D. (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death.Cell.85:803-15.

Bonfanti L, Strettoi E, Chierzi S, Cenni MC, Liu XH, Martinou JC, Maffei L, Rabacchi SA. (1996) Protection of retinal ganglion cells from natural and axotomy-induced cell death in neonatal transgenic mice overexpressing bcl-2. J Neurosci. 16:4186-4194.

Borden KL. (2000) RING domains: master builders of molecular scaffolds? J. Mol. Biol. 295:1103-1112.

Borner C, Martinou I, Mattmann C, Irmler M, Schaerer E, Martinou JC, Tschopp J. (1994) The protein bcl-2 alpha does not require membrane attachment, but two conserved domains to suppress apoptosis. J Cell Biol.126:1059-1068.

Boyd JM, Gallo GJ, Elangovan B, Houghton AB, Malstrom S, Avery BJ, Ebb RG, Subramanian T, Chittenden T, Lutz RJ. (1995) Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. Oncogene 11:1921-1928.

Bratton SB, Walker G, Roberts DL, Cain K, Cohen GM. (2001) Caspase-3 cleaves Apaf-1 into an approximately 30 kDa fragment that associates with an inappropriately oligomerized andbiologically inactive approximately 1.4 MDa apoptosome complex. Cell Death Differ. 8:425-33.

Birkey Reffey S, Wurthner JU, Parks WT, Roberts AB, Duckett CS. (2001) X-linked inhibitor of apoptosis protein functions as a cofactor in transforming growth factor-beta signaling. J Biol Chem.276:26542-9.

Bredesen DE, Ye X, Tasinato A, Sperandio S, Wang JJ, Assa-Munt N, Rabizadeh S. (1998) p75NTR and the concept of cellular dependence: seeing how the other half die. Cell Death Differ. 5:365-71.

Cain K, Bratton SB, Langlais C, Walker G, Brown DG, Sun XM, Cohen GM. (2000) Apaf-1 oligomerizes into biologically active approximately 700-kDa and inactive approximately 1.4-MDa apoptosome complexes. J Biol Chem.275:6067-70.

Carmody RJ, Cotter TG. (2000) Oxidative stress induces caspase-independent retinal apoptosis in vitro. Cell Death Differ 7:282-91.

Casademunt E, Carter BD, Benzel I, Frade JM, Dechant G, Barde YA. (1999) The zinc finger protein NRIF interacts with the neurotrophin receptor p75(NTR) and participates in programmed cell death. EMBO J. 18:6050-61.

Cashman NR, Durham HD, Blusztajn JK, Oda K, Tabira T, Shaw IT, Dahrouge S, Antel JP. (1992) Neuroblastoma x spinal cord (NSC) hybrid cell lines resemble developing motor neurons. Dev Dyn 194: 209-221.

Castren E, Ohga Y, Berzaghi MP, Tzimagiorgis G, Thoenen H, Lindholm D. (1994) bcl-2 messenger RNA is localized in neurons of the developing and adult rat brain. Neuroscience. 61:165-77.

Chai J, Shiozaki E, Srinivasula SM, Wu Q, Datta P, Alnemri ES, Shi Y, Dataa P. (2001) Structural basis of caspase-7 inhibition by XIAP. Cell.104:769-80.

Chan SL, Mattson MP. (1999) Caspase and calpain substrates: roles in synaptic plasticity and cell death. J Neurosci Res. 58:167-190.

Chao DT, Linette GP, Boise LH, White LS, Thompson CB, Korsmeyer SJ. (1995) Bcl-XL and Bcl-2 repress a common pathway of cell death. J. Exp. Med. 182:821-828.

Chinnaiyan AM, Orth K, O'Rourke K, Duan H, Poirier GG, Dixit VM. (1996) Molecular ordering of the cell death pathway. Bcl-2 and Bcl-xL function upstream of the CED-3-like apoptotic proteases. J Biol Chem. 271:4573-6.

Chinnaiyan AM, Chaudhary D, O'Rourke K, Koonin EV, Dixit VM.(1997) Role of CED-4 in the activation of CED-3. Nature. 388:728-9.

Chittenden T, Harrington EA, O'Connor R, Flemington C, Lutz RJ, Evan GI, Guild BC. (1995a) Induction of apoptosis by the Bcl-2 homologue Bak. Nature 374:733-736.

Chittenden T, Flemington C, Houghton AB, Ebb RG, Gallo GJ, Elangovan B, Chinnadurai G, Lutz RJ. (1995b) A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. EMBO J. 14:5589-5596.

Chomczynski P, Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 162:156-9.

Chou JJ, Matsuo H, Duan H, Wagner G. (1998) Solution structure of the RAIDD CARD and model for CARD/CARD interaction in caspase-2 and caspase-9 recruitment. Cell.94:171-80.

Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW. (1997) Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-kappaB control. Proc Natl Acad Sci USA 94:10057-10062.

Clem RJ, Miller LK. (1994). Control of programmed cell death by the baculovirus genes p35 and iap. Mol Cell Biol. 14: 5212-5222.

Conover JC, Yancopoulos GD. (1997) Neurotrophin regulation of the developing nervous system: analyses of knockout mice. Rev Neurosci. 8:13-27.

Conradt B, Horvitz HR. (1998) The C. elegans protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. Cell.93:519-29.

Cosulich SC, Worrall V, Hedge PJ, Green S, Clarke PR. (1997) Regulation of apoptosis by BH3 domains in a cell-free system. Curr Biol. 7:913-920.

Crocker SJ, Wigle N, Liston P, Thompson CS, Lee CJ, Xu D, Roy S, Nicholson DW, Park DS, MacKenzie A, Korneluk RG, Robertson GS. (2001) NAIP protects the nigrostriatal dopamine pathway in an intrastriatal 6-OHDA rat model of Parkinson's disease. Eur J Neurosci.14:391-400.

Crook NE, Clem RJ, Miller LK. (1993) An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J Virol. 67:2168-2174.

Deckwerth TL, Elliott JL, Knudson CM, Johnson EM Jr, Snider WD, Korsmeyer SJ. (1996) BAX is required for neuronal death after trophic factor deprivation and during development. Neuron. 17:401-11.

Deng Y, Lin Y, Wu X. (2002) TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO. Genes Dev.16:33-45.

Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, Maundrell K, Antonsson B, Martinou JC. (1999) Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J Cell Biol. 144:891-901.

Deveraux QL, Takahashi R, Salvesen GS, Reed JC. (1997) X-linked IAP is a direct inhibitor of cell-death proteases. Nature 388: 300-304.

Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC. (1998). IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J. 15: 2215-2223.

Deveraux QL., Leo E, Stennicke HR, Welsh K, Salvesen GS, Reed JC. (1999) Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. EMBO J. 18: 5242-5251.

DiCicco-Bloom E, Friedman WJ, Black IB. (1993) NT-3 stimulates sympathetic neuroblast proliferation by promoting precursor survival. Neuron.11:1101-11.

Digby, MR, Kimpton, WG, York, JJ, Connick, TE, Lowenthal, JW. (1996) ITA, a vertebrate homologue of IAP that is expressed in T lymphocytes. DNA Cell. Biol.,15, 981-988

Du C, Fang M, Li Y, Li L, Wang X. (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell.102:33-42.

Duan H, Dixit VM. (1997) RAIDD is a new 'death' adaptor molecule. Nature. 385:86-9.

Dubal DB, Kashon ML, Pettigrew LC, Ren JM, Finklestein SP, Rau SW, Wise PM (1998) Estradiol protects against ischemic injury. J Cereb Blood Flow Metab.18:1253-8.

Dubois-Dauphin M, Frankowski H, Tsujimoto Y, Huarte J, Martinou JC. (1994) Neonatal motoneurons overexpressing the bcl-2 protooncogene in transgenic mice are protected from axotomy-induced cell death. Proc Natl Acad Sci USA. 91:3309-3313.

Duckett C, Nava V, Gedrich R, Clem R, Van Dongen J, Gilfillan M, Shields H, Hardwick JM, Thompson C. (1996) A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. EMBO J. 15: 2685-2694.

Eberhardt O, Coelln RV, Kugler S, Lindenau J, Rathke-Hartlieb S, Gerhardt E, Haid S, Isenmann S, Gravel C, Srinivasan A, Bahr M, Weller M, Dichgans J, Schulz JB. (2000) Protection by synergistic effects of adenovirus-mediated X-chromosome-linked inhibitor of apoptosis and glial cell line-derivedneurotrophic factor gene transfer in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. J Neurosci.20:9126-34.

Eguchi Y, Shimizu S, Tsujimoto Y. (1997) Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer Res. 57:1835-40.

Ekert PG, Silke J, Vaux DL. (1999) Caspase inhibitors. Cell Death Differ. 6:1081-1086.

Ekert PG, Silke J, Hawkins CJ, Verhagen AM, Vaux DL. (2001) DIABLO promotes apoptosis by removing MIHA/XIAP from processed caspase 9. J Cell Biol.52:483-90.

Eldadah BA, Faden AI. (2000) Caspase pathways, neuronal apoptosis, and CNS injury. J Neurotrauma.17:811-29.

Ellis HM, Horvitz HR. (1986) Genetic control of programmed cell death in the nematode C. elegans Cell. 44:817-29.

Ellis RE, Horvitz HR. (1991a) Two C. elegans genes control the programmed deaths of specific cells in the pharynx. Development.112:591-603.

Ellis RE, Jacobson DM, Horvitz HR. (1991b) Genes required for the engulfment of cell corpses during programmed cell death in Caenorhabditis elegans. Genetics. 129:79-94.

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature. 391:43-50.

Eriksdotter Jonhagen M, Nordberg A, Amberla K, Backman L, Ebendal T, Meyerson B, Olson L, Seiger, Shigeta M, Theodorsson E, Viitanen M, Winblad B, Wahlund LO. (1998) Intracerebroventricular infusion of nerve growth factor in three patients with Alzheimer's disease. Dement Geriatr Cogn Disord. 9:246-57

Ernfors P, Bengzon J, Kokaia Z, Persson H, Lindvall O. (1991) Increased levels of messenger RNAs for neurotrophic factors in the brain during kindling epileptogenesis. Neuron 7:165-176.

Ernfors P, Lee KF, Kucera J, Jaenisch R. (1994a) Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. Cell. 77:503-12.

Ernfors P, Lee KF, Jaenisch R. (1994b) Mice lacking brain-derived neurotrophic factor develop with sensory deficits. Nature. 368:147-50.

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol. 148:2207-2216.

Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM. (2000) A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature 405:85-90.

Faherty CJ, Xanthoudakis S, Smeyne R. (1999) Caspase-3-dependent neuronal death in the hippocampus following kainic acid treatment. Mol. Brain Res. 70:159-163.

Farinas I, Jones KR, Backus C, Wang XY, Reichardt LF. (1994) Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. Nature. 369:658-61.

Farlie PG, Dringen R, Rees SM, Kannourakis G, Bernard O. (1995) bcl-2 transgene expression can protect neurons against developmental and induced cell death. Proc. Natl. Acad. Sci. U. S. A. 92:4397-4401.

Frerking M, Malenka RC, Nicoll RA. (1998) Synaptic activation of kainate receptors on hippocampal interneurons. Nat Neurosci.1:479-86.

Fernandez-Sarabia MJ, Bischoff JR. (1993) Bcl-2 associates with the ras-related protein R-ras p23. Nature. 366:274-275.

Fong WG, Liston P, Rajcan-Separovic E, St Jean M, Craig C, Korneluk RG. (2000) Expression and genetic analysis of XIAP-associated factor 1 (XAF1) in cancer cell lines. Genomics 70:113-22.

Frankowski H, Missotten M, Fernandez PA, Martinou I, Michel P, Sadoul R, Martinou JC. (1995) Function and expression of the Bcl-x gene in the developing and adult nervous system. Neuroreport. 6:1917-1921.

Gastaldi M, Bartolomei F, Massacrier A, Planells R, Robaglia-Schlupp A, Cau P. (1997) Increase in mRNAs encoding neonatal II and III sodium channel αisoforms during kainate-induced seizures in adult rat hippocampus. Mol. Brain Res. 44:179-190.

Gavrieli Y, Sherman Y, Ben-Sasson SA. (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol.119:493-501.

Gibson L, Holmgreen S, Huang DC, Bernard O, Copeland NG, Jenkins NA, Sutherland GR, Baker E, Adams JM, Cory S. (1996) bcl-w, a novel member of the bcl-2 family, promotes cell survival. Oncogene 13:665-675.

Gillardon F, Klimaschewski L, Wickert H, Krajewski S, Reed JC, Zimmermann M. (1996) Expression pattern of candidate cell death effector proteins Bax, Bcl-2, Bcl-X, and c-Jun in sensory and motor neurons following sciatic nerve transection in the rat. Brain Res. 739:244-50.

Gollapudi L, Oblinger MM. (1999) Estrogen and NGF synergistically protect terminally differentiated, ERalpha-transfected PC12 cells from apoptosis. J. Neurosci. Res. 56:471-81.

Gonzalez-Garcia M, Perez-Ballestero R, Ding L, Duan L, Boise LH, Thompson CB, Nunez G. (1994) bcl-XL is the major bcl-x mRNA form expressed during murine development and its product localizes to mitochondria. Development. 120:3033-3042.

Gonzalez-Garcia M, Garcia I, Ding L, O'Shea S, Boise LH, Thompson CB, Nunez G. (1995) bcl-x is expressed in embryonic and postnatal neural tissues and functions to prevent neuronal cell death. Proc Natl Acad Sci USA. 92:4304-4308.

Goodman Y, Bruce AJ, Cheng B, Mattson MP. (1996) Estrogens attenuate and corticosterone exacerbates excitotoxicity, oxidative injury, and amyloid beta-peptide toxicity in hippocampal neurons. J Neurochem. 66:1836-44.

Gotz R, Karch C, Digby MR, Troppmair J, Rapp UR, Sendtner M. (2000) The neuronal apoptosis inhibitory protein suppresses neuronal differentiation and apoptosis in PC12 cells. Hum Mol Genet. 9:2479-89.

Guarin E, Seuret P, Nef S, de Bilbao F, Nef P, Dubois-Dauphin M. (1999) cpp32 messenger RNA neosynthesis is induced by fatal axotomy and is not regulated by athanatal Bcl-2 over-expression. Neuroscience. 90:653-664.

Gumienny TL, Brugnera E, Tosello-Trampont AC, Kinchen JM, Haney LB, Nishiwaki K, WalkSF, Nemergut ME, Macara IG, Francis R, Schedl T, Qin Y, Van Aelst L, Hengartner MO, Ravichandran KS. (2001) CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration. Cell. 107:27-41.

Gwag BJ, Koh JY, DeMaro JA, Ying HS, Jacquin M, Choi DW. (1997) Slowly triggered excitotoxicity occurs by necrosis in cortical cultures. Neuroscience 77:393-401.

Hakem R, Hakem A, Duncan GS, Henderson JT, Woo M., Sorengas MS, Elia A., de la Pompa JL., Kagi D., Khoo W., Potter J., Yoshida R., Kaufman SA., Lowe SW., Penninger JM., Mak TW. (1998) Differential requirement for caspase 9 in apoptotic pathways in vivo. Cell 94: 339-52

Hamner S, Skoglosa Y, Lindholm D. (1999) Differential expression of bcl-w and bcl-x messenger RNA in the developing and adult rat nervous system. Neuroscience. 91:673-684.

Hamner S, Arumae U, Li-Ying Y, Sun Y, Saarma M, Lindholm D. (2001) Functional characterization of two splice variants of rat bad and their interaction with bcl-w in sympathetic neurons. Mol. Cell. Neurosci. 17:97-106.

Harlin H, Reffey SB, Duckett CS, Lindsten T, Thompson CB. (2001) Characterization of XIAP-Deficient Mice. Mol Cell Biol. 21:3604-3608.

Hay BA, Wassarman DA, Rubin, GM. (1995) Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. Cell. 83: 1253-1262.

Hegde R, Srinivasula SM, Zhang Z, Wassell R, Mukattash R, Cilenti L, DuBois G, Lazebnik Y, Zervos AS, Fernandes-Alnemri T, Alnemri ES. (2001) Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. J Biol Chem.277:432-8.

Hedgecock EM, Sulston JE, Thomson JN. (1983) Mutations affecting programmed cell deaths in the nematode Caenorhabditis elegans. Science.220:1277-9.

Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV. (1991) High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature. 350:678-83.

Henderson CE, Phillips HS, Pollock RA, Davies AM, Lemeulle C, Armanini M, Simmons L, Moffet B, Vandlen RA, Simmons L, et al. (1994) GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science 266:1062-4.

Henderson VW, Paganini-Hill A, Emanuel CK, Dunn ME, Buckwalter JG. (1999) Estrogen replacement therapy in older women. Comparisons between Alzheimer's disease cases and nondemented control subjects.. Arch Neurol. 51:896-900.

Hengartner MO, Ellis RE, Horvitz HR (1992) Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. Nature. 356:494-9.

Hengartner MO, Horvitz HR. (1994) C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. Cell. 76:665-76.

Hengartner MO. (2001) Apoptosis: corralling the corpses. Cell.104:325-8

Hinds MG, Norton RS, Vaux DL, Day CL. (1999) Solution structure of a baculoviral inhibitor of apoptosis (IAP) repeat. Nat Struct Biol. 6:648-651.

Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348:334-336.

Hockenbery DM, Zutter M, Hickey W, Nahm M, Korsmeyer SJ. (1991) BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proc. Natl. Acad. Sci. U. S. A. 88:6961-6965.

Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ. (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell. 75:241-51.

Holcik M, Thompson CS, Yaraghi Z, Lefebvre CA, MacKenzie AE, Korneluk RG. (2000) The hippocampal neurons of neuronal apoptosis inhibitory protein 1 (NAIP1) -deleted mice display increased vulnerability to kainic acid-induced injury. Proc Natl Acad Sci USA. 97:2286-2290.

Holcik M, Korneluk RG. (2000) Functional characterization of the X-linked inhibitor of apoptosis (XIAP) internal ribosome entry site element: role of La autoantigen in XIAP translation. Mol Cell Biol. 20:4648-57.

Holcik M, Yeh C, Korneluk RG, Chow T.(2000) Translational upregulation of X-linked inhibitor of apoptosis (XIAP) increases resistance to radiation induced cell death. Oncogene.19:4174-7.

Hsu SY, Kaipia A, McGee E, Lomeli M, Hsueh AJ. (1997) Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. Proc Natl Acad Sci USA. 94:12401-1246.

Hsu SY, Hsueh AJ. (1998) A splicing variant of the Bcl-2 member Bok with a truncated BH3 domain induces apoptosis but does not dimerize with antiapoptotic Bcl-2 proteins in vitro. J. Biol. Chem. 273:30139-30146.

Huang Y, Park YC, Rich RL, Segal D, Myszka DG, Wu H. (2001) Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain. Cell.104:781-90.

Hunter JJ, Parslow TG. (1996) A peptide sequence from Bax that converts Bcl-2 into an activator of apoptosis. J. Biol. Chem. 271:8521-8524.

Hurn PD, Macrae IM. (2000) Estrogen as a neuroprotectant in stroke.J Cereb Blood Flow Metab.20:631-52

Iwahashi H, Eguchi Y, Yasuhara N, Hanafusa T, Matsuzawa Y, Tsujimoto Y. (1997) Synergistic anti-apoptotic activity between Bcl-2 and SMN implicated in spinal muscular atrophy. Nature 390:413-7.

Jablonka S, Rossoll W, Schrank B, Sendtner M. (2000) The role of SMN in spinal muscular atrophy. J Neurol.247 Suppl 1:I37-42.

Jansen B, Schlagbauer-Wadl H, Brown BD, Bryan RN, van Elsas A, Muller M, Wolff K, Eichler HG, Pehamberger H. (1998) bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. Nat Med. 4:232-4.

Jenner P. (2001) Parkinson's disease, pesticides and mitochondrial dysfunction. Trends Neurosci.24:245-7.

Jurgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC. (1998) Bax directly induces release of cytochrome c from isolated mitochondria. Proc Natl Acad Sci USA. 95:4997-5002.

Kamada S, Shimono A, Shinto Y, Tsujimura T, Takahashi T, Noda T, Kitamura Y, Kondoh H, Tsujimoto Y. (1995) bcl-2 deficiency in mice leads to pleiotropic abnormalities: accelerated lymphoid cell death in thymus and spleen, polycystic kidney, hair hypopigmentation, and distorted small intestine. Cancer Res. 55:354-359.

Kane DJ, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, Ord T, Bredesen DE. (1993) Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. Science 262:1274-7.

Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF. (1991) The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science. 252:554-8.

Keller JN, Germeyer A, Begley JG, Mattson MP. (1997) 17Beta-estradiol attenuates oxidative impairment of synaptic Na+/K+-ATPase activity, glucose transport, and glutamate transport induced by amyloid beta-peptide and iron. J Neurosci Res. 50:522-30.

Kerr JF, Wyllie AH, Currie AR. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 26:239-257.

Kharbanda S, Pandey P, Schofield L, Israels S, Roncinske R, Yoshida K, Bharti A, Yuan ZM, Saxena S, Weichselbaum R, Nalin C, Kufe D. (1997) Role for Bcl-xL as an inhibitor of cytosolic cytochrome C accumulation in DNA damage-induced apoptosis. Proc Natl Acad Sci U SA. 94:6939-6942.

Kiefer MC, Brauer MJ, Powers VC, Wu JJ, Umansky SR, Tomei LD, Barr PJ. (1995) Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. Nature. 374:736-739.

Klein R, Silos-Santiago I, Smeyne RJ, Lira SA, Brambilla R, Bryant S, Zhang L, Snider WD, Barbacid M. (1994) Disruption of the neurotrophin-3 receptor gene trkC eliminates la muscle afferents and results in abnormal movements. Nature. 368:249-51.

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275:1132-1136.

Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ. (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science. 270:96-9.

Kordower JH, Palfi S, Chen EY, Ma SY, Sendera T, Cochran EJ, Cochran EJ, Mufson EJ, Penn R, Goetz CG, Comella CD. (1999) Clinicopathological findings following intraventricular glial-derived neurotrophic factor treatment in a patient with Parkinson'sdisease. Ann Neurol.46:419-24.

Korhonen L, Sjoholm U, Takei N, Kern MA, Schirmacher P, Castren E, Lindholm D. (2000) Expression of c-Met in developing rat hippocampus: evidence for HGF as a neurotrophic factor for calbindin D-expressing neurons. Eur J Neurosci.12:3453-61.

Krajewski S, Krajewska M, Shabaik A, Wang HG, Irie S, Fong L, Reed JC. (1994) Immunohistochemical analysis of in vivo patterns of Bcl-X expression. Cancer Res. 54:5501-5507.

Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG, Reed JC. (1994) Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. Am J Pathol.145:1323-36.

Krammer P.H. (1999) CD95(APO-1/Fas)-mediated apoptosis: live and let die. Adv. Immunol. 71:163-210.

Kugler S, Straten G, Kreppel F, Isenmann S, Liston P, Bahr M. (2000) The X-linked inhibitor of apoptosis (XIAP) prevents cell death in axotomized CNS neurons in vivo. Cell Death Differ.7:815-24.

Kuida K, Lippke JA, Ku G, Harding MW, Livingston DJ, Su MS, Flavell RA. (1995) Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. Science. 267:2000-3.

Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic P, Flavell RA. (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature. 384:368-72.

Kuida K, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MSS., Rakic P, Flavell RA. (1998) Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase-9. Cell 94:325-37.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. (1996) Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA. 93:5925-5930.

Kukkonen JP, Shariatmadari R, Courtney MJ, Akerman KE. (1997) Localization of voltage-sensitive Ca2+ fluxes and neuropeptide Y immunoreactivity to varicosities in SH-SY5Y human neuroblastoma cells differentiated by treatment with the protein kinase inhibitor staurosporine. Eur J Neurosci.9:140-50.

LaCasse EC, Baird S, Korneluk RG, MacKenzie AE. (1998). The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene 17: 3247-3259.

Lafferty FW, Fiske ME. (1994) Postmenopausal estrogen replacement: a long-term cohort study. Am J Med.97:66-77

Lam M, Dubyak G, Chen L, Nunez G, Miesfeld RL, Distelhorst CW. (1994) Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca2+ fluxes. Proc Natl Acad Sci USA. 91:6569-6573.

LeBrun DP, Warnke RA, Cleary ML. (1993) Expression of bcl-2 in fetal tissues suggests a role in morphogenesis. Am J Pathol. 142:743-753.

Lee S, Williamson J, Lothman EW, Szele FG, Chesselet MF, Von Hagen S, Sapolsky RM, Mattson MP, Christakos S. (1997) Early induction of mRNA for calbindin-D 28k and BDNF but not NT-3 in rat hippocampus after kainic acid treatment. Mol. Brain Res. 47:183-194.

Lee VM, Trojanowski JQ. (1999) Neurodegenerative tauopathies: human disease and transgenic mouse models. Neuron. 24:507-10.

Leist M, Single B, Castoldi AF, Kuhnle S, Nicotera P. (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med.185:1481-6.

Leist M, Jaattela M. (2001) Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol.2:589-98.

Levi-Montalcini R. (1966) The nerve growth factor: Its mode of action on sensory and sympathetic nerve cells. Harvey Lect. 60: 217-259

Levi-Montalcini R. (1987) The nerve growth factor 35 years later. Science.237:1154-62.

Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettingell WH, Yu CE, Jondro PD, Schmidt SD, Wang K, et al. (1995) Candidate gene for the chromosome 1 familial Alzheimer's disease locus. Science. 269:973-7.

Lewen A, Skoglosa Y, Clausen F, Marklund N, Chan PH, Lindholm D, Hillered L. (2001) Paradoxical increase in neuronal DNA fragmentation after neuroprotective free radical scavenger treatment in experimental traumatic brain injury. J Cereb Blood Flow Metab.21:344-50.

Li F, Srinivasan A, Wang Y, Armstrong RC, Tomaselli KJ, Fritz LC. (1997) Cell-specific induction of apoptosis by microinjection of cytochrome c. Bcl-xL has activity independent of cytochrome c release. J Biol Chem. 272:30299-30305.

Li P, Allen H, Banerjee S, Franklin S, Herzog L, Johnston C, McDowell J, Paskind M, Rodman L, Salfeld J, et al. (1995) Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. Cell. 80:401-11.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479-489.

Linnik MD, Zobrist RH, Hatfield MD. (1993) Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. Stroke.24:2002-8

Lioudyno M, Skoglosa Y, Takei N, Lindholm D. (1998) Pituitary adenylate cyclase-activating polypeptide (PACAP) protects dorsal root ganglion neurons from death and induces calcitonin gene-related peptide (CGRP) immunoreactivity in vitro. J Neurosci Res. 51:243-56.

Liston P, Roy N, Tamai K, Lefebvre C, Baird S, Cherton-Horvat G, Farahani R, McLean M, Ikeda JE, MacKenzie A, Korneluk RG. (1996). Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature 379: 349-353.

Liston P, Lefebvre C, Fong WG, Xuan JY, Korneluk RG. (1997) Genomic characterization of the mouse inhibitor of apoptosis protein 1 and 2 genes. Genomics, **46**, 495-503.

Liston P, Fong WG, Kelly NL, Toji S, Miyazaki T, Conte D, Tamai K, Craig CG, McBurney MW, Korneluk RG. (2001) Identification of XAF1 as an antagonist of XIAP anti-Caspase activity. Nat Cell Biol.3:128-33.

Liu X, Kim CN, Yang J, Jemmerson R, Wang X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86:147-157.

Liu Z, Sun C, Olejniczak ET, Meadows RP, Betz SF, Oost T, Herrmann J, Wu JC, Fesik SW. (2000) Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. Nature.408:1004-8.

Loeffler M, Kroemer G. (2000) The mitochondrion in cell death control: certainties and incognita.Exp Cell Res.256:19-26.

Lopez E, Pozas E, Rivera R, Ferrer I. (1999) Bcl-2, Bax and Bcl-x expression following kainic acid administration at convulsant doses in the rat. Neuroscience 91:1461-1470.

Lyon CJ, Evans CJ, Bill BR, Otsuka AJ, Aguilera RJ. (2000) The C. elegans apoptotic nuclease NUC-1 is related in sequence and activity to mammalian DNase II. Gene.252:147-54.

MacManus JP, Buchan AM, Hill IE, Rasquinha I, Preston E. (1993) Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. Neurosci Lett.164:89-92.

Marchetti P, Castedo M, Susin SA, Zamzami N, Hirsch T, Macho A, Haeffner A, Hirsch F, Geuskens M, Kroemer G. (1996) Mitochondrial permeability transition is a central coordinating event of apoptosis. J Exp Med. 184:1155-1160.

Marin MC, Fernandez A, Bick RJ, Brisbay S, Buja LM, Snuggs M, McConkey DJ, von Eschenbach AC, Keating MJ, McDonnell TJ. (1996) Apoptosis suppression by bcl-2 is correlated with the regulation of nuclear and cytosolic Ca2+.Oncogene.12:2259-66.

Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM, Green DR. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med. 182:1545-1556.

Martin LJ, Al-Abdulla NA, Brambrink AM, Kirsch JR, Sieber FE, Portera-Cailliau C. (1998) Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: A perspective on the contributions of apoptosis and necrosis. Brain Res Bull.46:281-309.

Martins LM, Iaccarino I, Tenev T, Gschmeissner S, Totty NF, Lemoine NR, Savopoulos J, Gray CW, Creasy CL, Dingwall C, Downward J. (2002) The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. J Biol Chem.277:439-44.

Martinou JC, Dubois-Dauphin M, Staple JK, Rodriguez I, Frankowski H, Missotten M, Albertini P, Talabot D, Catsicas S, Pietra C, et al. (1994) Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron 13:1017-1030.

McEwen BS, Alves SE, Bulloch K, Weiland NG. (1997) Ovarian steroids and the brain: implications for cognition and aging. Neurology 48:S8-15.

Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH, Peter ME. (1997) FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J. 16:2794-804.

Merry DE, Veis DJ, Hickey WF, Korsmeyer SJ. (1994) bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. Development. 20:301-311.

Metzstein MM, Hengartner MO, Tsung N, Ellis RE, Horvitz HR. (1996) Transcriptional regulator of programmed cell death encoded by Caenorhabditis elegans gene ces-2. Nature. 382:545-7.

Metzstein MM, Stanfield GM, Horvitz HR. (1998) Genetics of programmed cell death in C. elegans: past, present and future.Trends Genet.14:410-6.

Michaelidis TM, Sendtner M, Cooper JD, Airaksinen MS, Holtmann B, Meyer M, Thoenen H. (1996) Inactivation of bcl-2 results in progressive degeneration of motoneurons, sympathetic and sensory neurons during early postnatal development. Neuron. 17:75-89.

Middleton G, Pinon LG, Wyatt S, Davies AM. (1998) Bcl-2 accelerates the maturation of early sensory neurons. J Neurosci.18:3344-50.

Minichiello L, Piehl F, Vazquez E, Schimmang T, Hokfelt T, Represa J, Klein R. (1995) Differential effects of combined trk receptor mutations on dorsal root ganglion and inner ear sensory neurons. Development. 121:4067-75.

Minn AJ, Velez P, Schendel SL, Liang H, Muchmore SW, Fesik SW, Fill M, Thompson CB. (1997) Bcl-x(L) forms an ion channel in synthetic lipid membranes. Nature 385:353-357.

Mizuguchi M, Sohma O, Takashima S, Ikeda K, Yamada M, Shiraiwa N, Ohta S. (1996) Immunochemical and immunohistochemical localization of Bcl-x protein in the rat central nervous system. Brain Res. 712:281-286.

Motoyama N, Kimura T, Takahashi T, Watanabe T, Nakano T. (1999) bcl-x prevents apoptotic cell death of both primitive and definitive erythrocytes at the end of maturation. J Exp Med. 189:1691-1698.

Muchmore SW, Sattler M, Liang H, Meadows RP, Harlan JE, Yoon HS, Nettesheim D, Chang BS, Thompson CB, Wong SL, Ng SL, Fesik SW. (1996) X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature. 381:335-341.

Mudo G, Salin T, Condorelli DF, Jiang XH, Dell'Albani P, Timmusk T, Metsis M, Funakoshi H, Belluardo N. (1995) Seizures increase trkC mRNA expression in the dentate gyrus of rat hippocampus. Role of glutamate receptor activation. J Mol Neurosci. 6: 11-22.

Mulle C, Sailer A, Perez-Otano I, Dickinson-Anson H, Castillo PE, Bureau I, Maron C, Gage FH, Mann JR, Bettler B, Heinemann SF. (1998) Altered synaptic physiology and reduced susceptibility to kainate-induced seizures in GluR6-deficient mice. Nature. 392:601-5.

Mulnard RA, Cotman CW, Kawas C, van Dyck CH, Sano M, Doody R, Koss E, Pfeiffer E, Jin S, Gamst A, Grundman M, Thomas R, Thal LJ. (2000) Estrogen replacement therapy for treatment of mild to moderate Alzheimer disease: a randomized controlled trial. Alzheimer's Disease Cooperative Study. JAMA. 283:1007-15.

Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM. (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death—inducing signaling complex. Cell.85:817-27.

Nadler JV, Perry BW, Cotman CW. (1978) Intraventricular kainic acid preferentially destroys hippocampal pyramidal cells. Nature 271: 676-677.

Nakayama K, Nakayama K, Negishi I, Kuida K, Sawa H, Loh DY. (1994) Targeted disruption of Bcl-2 alpha beta in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. Proc. Natl. Acad. Sci. U. S. A. 91:3700-3704.

Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J.(2000) Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature.403:98-103.

Narita M, Shimizu S, Ito T, Chittenden T, Lutz RJ, Matsuda H, Tsujimoto Y. (1998) Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. Proc. Natl. Acad. Sci. U. S. A. 95:14681-1466.

Naumovski L, Cleary ML. (1996) The p53-binding protein 53BP2 also interacts with Bc12 and impedes cell cycle progression at G2/M. Mol. Cell. Biol. 16:3884-3892.

Newmeyer DD, Farschon DM, Reed JC. (1994) Cell-free apoptosis in Xenopus egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. Cell. 79:353-364.

Nicholson DW, Thornberry NA. (1997) Caspases: killer proteases. Trends Biochem. Sci. 22:299-306.

Nicholson DW. (1999) Caspase structure, proteolytic substrates, and function during apoptotic cell death. Cell Death Differ. 6:1028-42.

Nicholson DW. (2000) From bench to clinic with apoptosis-based therapeutic agents. Nature.407:810-6.

Novack DV, Korsmeyer SJ. (1994) Bcl-2 protein expression during murine development. Am. J. Pathol. 145:61-73.

Novak KD, Prevette D, Wang S, Gould TW, Oppenheim RW (2000) growth factor/scatter factor is a neurotrophic survival factor for lumbar but not for other somatic motoneurons in the chick embryo. J Neurosci.20:326-37.

Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, Sikorska M. (1993) Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J.12:3679-3684.

O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, Huang DC. (1998) Bim: a novel member of the Bcl-2 family that promotes apoptosis. EMBO J. 17:384-395.

Ogawa S, Lubahn DB, Korach KS, Pfaff DW. (1997) Behavioral effects of estrogen receptor gene disruption in male mice. Proc Natl Acad Sci U S A. 94:1476-81.

Olney JW, Rhee V, Ho OL. (1974) Kainic acid: a powerful neurotoxic analogue of glutamate. Brain Res. 77, 507-512.

Oltvai ZN, Milliman CL, Korsmeyer SJ. (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74:609-619.

Oppenheim RW. (1991) Cell death during development of the nervous system. Annu. Rev. Neurosci. 14: 453-501

Oppenheim RW, Flavell RA, Vinsant S, Prevette D, Kuan CY, Rakic P. (2001) Programmed cell death of developing mammalian neurons after genetic deletion of caspases. J Neurosci.21:4752-60.

Paganini-Hill A, Henderson VW.(1994) Estrogen deficiency and risk of Alzheimer's disease in women. Am J Epidemiol.140:256-61.

Palm K, Belluardo N, Metsis M, Timmusk T.(1998) Neuronal expression of zinc finger transcription factor REST/NRSF/XBR gene. J Neurosci.18:1280-96.

Parsadanian AS, Cheng Y, Keller-Peck CR, Holtzman DM, Snider WD. (1998) Bcl-xL is an antiapoptotic regulator for postnatal CNS neurons. J Neurosci. 18:1009-1019.

Paxinos G, Watson C. (1998) The Rat Brain in Stereotaxic coordinates. Harbcourt Brace& Company, Academic Press,San Diego CA,U.S.A.

Perrelet D, Ferri A, MacKenzie AE, Smith GM, Korneluk RG, Liston P, Sagot Y, Terrado J, Monnier D, Kato AC. (2000) IAP family proteins delay motoneuron cell death in vivo. Eur J Neurosci.12:2059-67.

Petit PX, Susin SA, Zamzami N, Mignotte B, Kroemer G.(1996) Mitochondria and programmed cell death: back to the future. FEBS Lett. 396:7-13.

Pettersson K, Gustafsson JA. (2001) Role of estrogen receptor beta in estrogen action. Annu Rev Physiol. 63:165-92.

Piccini P, Brooks DJ, Bjorklund A, Gunn RN, Grasby PM, Rimoldi O, Brundin P, Hagell P, Rehncrona S, Widner H, Lindvall O.(1999) Dopamine release from nigral transplants visualized in vivo in a Parkinson's patient. Nat Neurosci. 2:1137-40.

Pike CJ. (1999) Estrogen modulates neuronal Bcl-xL expression and beta-amyloid-induced apoptosis: relevance to Alzheimer's disease. J. Neurochem. 72:1552-1563.

Pilgrim C, Hutchison JB. (1994) Developmental regulation of sex differences in the brain: can the role of gonadal steroids be redefined? Neuroscience 60:843-855.

Pleasure SJ, Lee VM. (1993) NTera 2 cells: a human cell line which displays characteristics expected of a human committed neuronal progenitor cell. J. Neurosci. Res. 35:585-602.

Polans AS, Witkowska D, Haley TL, Amundson D, Baizer L, Adamus G. (1995) Recoverin, a photoreceptor-specific calcium-binding protein, is expressed by the tumor of a patient with cancer-associated retinopathy. Proc Natl Acad Sci USA. 92:9176-9180.

Pollard H, Charriaut-Marlangue C, Cantagrel S, Represa A, Robain O, Moreau J, Ben-Ari Y. (1994) Kainate-induced apoptotic cell death in hippocampal neurons. Neuroscience 63: 7-18.

Rano T.A., Timkey T., Peterson E.P., Rotonda J., Nicholson D.W., Becker J.W., Chapman K.T., Thornberry N.A. (1997) A combinatorial approach for determining protease specificities: application to interleukin-1beta converting enzyme (ICE). Chem. Biol. 4:149-155.

Ray CA, Black RA, Kronheim SR, Greenstreet TA, Sleath PR, Salvesen GS, Pickup DJ.(1992) Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1 beta converting enzyme. Cell. 69:597-604.

Ray R, Chen G, Vande Velde C, Cizeau J, Park JH, Reed JC, Gietz RD, Greenberg AH. (2000) BNIP3 heterodimerizes with Bcl-2/Bcl-X(L) and induces cell death independent of a Bcl-2 homology 3 (BH3) domain at both mitochondrial and nonmitochondrial sites. J. Biol. Chem. 275:1439-1448.

Reddien PW, Horvitz HR. (2000) CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in Caenorhabditis elegans. Nat Cell Biol.2:131-6.

Reed JC. (1994) Bcl-2 and the regulation of programmed cell death. J Cell Biol. 124:1-6.

Riedl SJ, Renatus M, Schwarzenbacher R, Zhou Q, Sun C, Fesik SW, Liddington RC, Salvesen GS. (2001) Structural basis for the inhibition of caspase-3 by XIAP. Cell. 104:791-800.

Robinson D, Friedman L, Marcus R, Tinklenberg J, Yesavage J. (1994) Estrogen replacement therapy and memory in older women. J Am Geriatr Soc.42:919-22.

Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV. (1995) The TNFR2-TRAF signalling complex contains two novel proteins related to baculovirus inhibitor of apoptosis. Cell 83:1243-1252.

Rothstein JD, Jin L, Dykes-Hoberg M, Kuncl RW. (1993) Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity. Proc Natl Acad Sci USA. 90:6591-6595.

Rotonda J, Nicholson DW, Fazil KM, Gallant M, Gareau Y, Labelle M, Peterson EP, Rasper DM, Ruel R, Vaillancourt JP, Thornberry NA, Becker JW. (1996) The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. Nat Struct Biol. 3:619-625.

Roy N, Mahadevan MS, McLean M, Shutler G, Yaraghi Z, Farahani R, Baird S, Besner-Johnston A, Lefebvre C, Kang X. et al. (1995) The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. Cell 80: 167-178.

Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. (1997) The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J. 16: 6914-6925.

Roy M, Sapolsky R. (1999) Neuronal apoptosis in acute necrotic insults: why is this subject such a mess? Trends Neurosci.22:419-22.

Sakahira H, Enari M, Nagata S. (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis Nature.391:96-9.

Sanna MG, Duckett CS, Richter BW, Thompson CB, Ulevitch RJ. (1998) Selective activation of JNK1 is necessary for the anti-apoptotic activity of hILP. Proc Natl Acad Sci U S A.95:6015-20.

Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB, Fesik SW. (1997) Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science 275:983-986.

Savill J, Fadok V. (2000) Corpse clearance defines the meaning of cell death. Nature 407:784-788.

Schendel SL, Xie Z, Montal MO, Matsuyama S, Montal M, Reed JC. (1997) Channel formation by antiapoptotic protein Bcl-2. Proc Natl Acad Sci USA. 94:5113-5118.

Schendel SL, Montal M, Reed JC. (1998) Bcl-2 family proteins as ion-channels. Cell Death Differ. 5:372-380.

Schlesinger PH, Gross A, Yin XM, Yamamoto K, Saito M, Waksman G, Korsmeyer SJ. (1997) Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. Proc Natl Acad Sci USA. 94:11357-11362.

Schmitz I, Kirchhoff S, Krammer PH. (2000) Regulation of death receptor-mediated apoptosis pathways. Int. J. Biochem. Cell Biol. 32:1123-1136.

Schlagbauer-Wadl H, Klosner G, Heere-Ress E, Waltering S, Moll I, Wolff K, Pehamberger H, Jansen B. (2000) Bcl-2 antisense oligonucleotides (G3139) inhibit Merkel cell carcinoma growth in SCID mice.J Invest Dermatol. 114:725-30.

Schwob JE, Fuller T, Price JL, Olney JW. (1980) Widespread patterns of neuronal damage following systemic or intracerebral injections of kainic acid: a histological study. Neuroscience. 5:991-1014.

Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME. (1998) Apoptosis signaling by death receptors. Eur J Biochem. 254:439-459.

Selkoe DJ.(1997) Alzheimer's disease: genotypes, phenotypes, and treatments. Science 275:630-1.

Sendtner M, Kreutzberg GW, Thoenen H (1990) Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. Nature. 345:440-1.

Sendtner M, Holtmann B, Kolbeck R, Thoenen H, Barde YA. (1992) Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. Nature. 360:757-9

Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, et al. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 375:754-60.

Shibasaki F, Kondo E, Akagi T, McKeon F. (1997) Suppression of signalling through transcription factor NF-AT by interactions between calcineurin and Bcl-2. Nature. 386:728-731.

Shimizu S, Eguchi Y, Kamiike W, Waguri S, Uchiyama Y, Matsuda H, Tsujimoto Y. (1996) Bcl-2 blocks loss of mitochondrial membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory chain inhibitors. Oncogene 13:21-29.

Shimohama S, Fujimoto S, Sumida Y, Tanino H. (1998) Differential expression of rat brain bcl-2 family proteins in development and aging. Biochem Biophys Res Commun. 252:92-96.

Shindler KS, Latham CB, Roth KA. (1997) Bax deficiency prevents the increased cell death of immature neurons in bcl-x-deficient mice.J Neurosci.17:3112-9.

Silos-Santiago I, Molliver DC, Ozaki S, Smeyne RJ, Fagan AM, Barbacid M, Snider WD. (1995) Non-TrkA-expressing small DRG neurons are lost in TrkA deficient mice. J Neurosci.15:5929-42.

Simons M, Beinroth S, Gleichmann M, Liston P, Korneluk RG, MacKenzie AE, Bahr M, Klockgether T, Robertson GS, Weller M, Schulz JB. (1999) Adenovirus-mediated gene transfer of inhibitors of apoptosis protein delays apoptosis in cerebellar granule neurons. J. Neurochem. 72:292-301.

Simonian NA, Getz RL, Leveque JC, Konradi C, Coyle JT. (1996) Kainate induces apoptosis in neurons. Neuroscience 74:675683.

Singer CA, Rogers KL, Strickland TM, Dorsa DM. (1996) Estrogen protects primary cortical neurons from glutamate toxicity. Neurosci Lett. 212:13-6

Skoglosa Y, Takei N, Lindholm D. (1999) Distribution of pituitary adenylate cyclase activating polypeptide mRNA in the developing rat brain. Brain Res Mol Brain Res. 65:1-13.

Slee EA, Adrain C, Martin SJ. (1999) Serial killers: ordering caspase activation events in apoptosis. Cell Death Differ. 6:1067-1074.

Sloviter R. (2002) Apoptosis: a guide for the perplexed. Trends Pharm Sci 23: 19-24.

Sohn S, Kim EY, Gwag BJ. (1998) Glutamate neurotoxicity in mouse cortical neurons: atypical necrosis with DNA ladders and chromatin condensation. Neurosci Lett.240:147-50.

Sperandio S, de Belle I, Bredesen DE. (2000) An alternative, nonapoptotic form of programmed cell death. Proc Natl Acad Sci USA. 97:14376-14381.

Sperk G. (1994) Kainic acid seizures in the rat. Prog. Neurobiol. 42:1-32.

Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Alnemri ES. (1998) Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol Cell.1:949-957.

Srinivasula SM, Hegde R, Saleh A, Datta P, Shiozaki E, Chai J, Lee RA, Robbins PD, Fernandes-Alnemri T, Shi Y, Alnemri ES. (2001) A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. Nature. 410:112-6.

Stehlik C, de Martin R, Binder BR, Lipp J. (1998a) Cytokine indued expression of porcine inhibitor of apoptosis protein (iap) family member is regulated by NF-κB. Biochem.Biophys. Res.Commun., 243,827-832

Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR, Lipp J. (1998b) Nuclear factor (NF)-kappaB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. J Exp Med. 188:211-216.

Sun C, Cai M, Gunasekera AH, Meadows RP, Wang H, Chen J, Zhang H, Wu W, Xu N, Ng SC., Fesik SW. (1999) NMR structure and mutagenesis of the inhibitor-of-apoptosis protein XIAP. Nature 401:818-822.

Susin SA, Zamzami N, Castedo M, Hirsch T, Marchetti P, Macho A, Daugas E, Geuskens M, Kroemer G. (1996) Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J. Exp. Med. 184:1331-1341.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G. (1999) Nature 397:441-6.

Suzuki A, Tsutomi Y. (1998) Bcl-2 accelerates the neuronal differentiation: new evidence approaching to the biofunction of bcl-2 in the neuronal system. Brain Res.801:59-66.

Suzuki Y, Nakabayashi Y, Takahashi R. (2001) Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 andenhances its anti-apoptotic effect in Fas-induced cell death. Proc Natl Acad Sci U S A. 98:8662-7.

Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. (2001) A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol Cell. 8:613-21.

Takahashi R, Deveraux Q, Tamm I, Welsh K, Assa-Munt N, Salvesen GS, Reed JC. (1998) A single BIR domain of XIAP sufficient for inhibiting caspases. J Biol Chem. 273: 7787-7790.

Takayama S, Sato T, Krajewski S, Kochel K, Irie S, Millan JA, Reed JC. (1995) Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. Cell 80:279-284.

Takayama S., Bimston D.N., Matsuzawa S., Freeman B.C., Aime-Sempe C., Xie Z., Morimoto R.I., Reed J.C. (1997) BAG-1 modulates the chaperone activity of Hsp70/Hsc70. EMBO J. 16:4887-4896

Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T, Reed JC. (1998) IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res. 58:5315-5320.

Tanaka S, Saito K, Reed JC. (1993) Structure-function analysis of the Bcl-2 oncoprotein. Addition of a heterologous transmembrane domain to portions of the Bcl-2beta protein restores function as a regulator of cell survival. J. Biol. Chem. 268:10920-10926.

Tang MX, Jacobs D, Stern Y, Marder K, Schofield P, Gurland B, Andrews H, Mayeux R. (1996) Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease. Lancet 348:429-432.

Tang G, Minemoto Y, Dibling B, Purcell NH, Li Z, Karin M, Lin A. (2001) Inhibition of JNK activation through NF-kappaB target genes. Nature. 414:313-7.

Tatton NA, Kish SJ. (1997) In situ detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. Neuroscience. 77:1037-48.

Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW. (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem. 272:17907-17911.

Trent C, Tsung N, Horvitz HR. (1983) Egg-laying defective mutants of the nematode Caenorhabditis elegans. Genetics 104:619-649.

Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM. (1984) Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science 226:1097-1099.

Uren A, Pakusch M, Hawkins CJ, Puls KL, Vaux DL. (1996) Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. Proc Natl Acad Sci USA. 93: 4974-4978.

Walker PR, Leblanc J, Carson C, Ribecco M, Sikorska M. (1999) Neither caspase-3 nor DNA fragmentation factor is required for high molecular weight DNA degradation in apoptosis. Ann N Y Acad Sci.887:48-59.

Varfolomeev EE, Schuchmann M, Luria V, Chiannilkulchai N, Beckmann JS, Mett IL, Rebrikov D, Brodianski VM, Kemper OC, Kollet O, Lapidot T, Soffer D, Sobe T, Avraham KB, Goncharov T, Holtmann H, Lonai P, Wallach D. (1998) Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 andis lethal prenatally. Immunity.9:267-76.

Vayssiere JL, Petit PX, Risler Y, Mignotte B. (1994) Commitment to apoptosis is associated with changes in mitochondrial biogenesis and activity in cell lines conditionally immortalized with simian virus 40. Proc Natl Acad Sci U S A. 91:11752-6.

Vaux DL, Cory S, Adams JM. (1998) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature. 335:440-442.

Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ. (1993) Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell. 75:229-240.

Vekrellis K, McCarthy MJ, Watson A, Whitfield J, Rubin LL, Ham J. (1997) Bax promotes neuronal cell death and is downregulated during the development of the nervous system. Development 124:1239-1249.

Verge VM, Gratto KA, Karchewski LA, Richardson PM (1996) Neurotrophins and nerve injury in the adult. Philos Trans R Soc Lond B Biol Sci 351:423-430

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL. (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins Cell.102:43-53.

Verhagen AM, Silke J, Ekert PG, Pakusch M, Kaufmann H, Connolly LM, Day CL, Tikoo A, Burke R, Wrobel C, Moritz RL, Simpson RJ, Vaux DL (2002) HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. J Biol Chem.277:445-54.

Vignes M, Clarke VR, Parry MJ, Bleakman D, Lodge D, Ornstein PL, Collingridge GL.(1998) The GluR5 subtype of kainate receptor regulates excitatory synaptic transmission in areas CA1 and CA3 of the rat hippocampus. Neuropharmacology. 37:1269-77.

Vician L, Lim IK, Ferguson G, Tocco G, Baudry M, Herschman HR. (1995) Synaptotagmin IV is an immediate early gene induced by de- polarization in PC12 cells and in brain. Proc Natl Acad Sci USA. 92:2164-2168.

Viswanath V, Wu Z, Fonck C, Wei Q, Boonplueang R, Andersen JK. (2000) Transgenic mice neuronally expressing baculoviral p35 are resistant to diverse types of induced apoptosis, including seizure-associated neurodegeneration. Proc. Natl. Acad. Sci. U. S. A. 97:2270-2275.

Vogelbaum MA, Tong JX, Rich KM (1998) Developmental regulation of apoptosis in dorsal root ganglion neurons. J Neurosci. 18:8928-8935.

Vucic D, Kaiser WJ, Miller LK. (1998) A mutational analysis of the baculovirus inhibitor of apoptosis Op-IAP. J. Biol. Chem. 273:33915-33921.

Wang HG, Rapp UR, Reed JC. (1996) Bcl-2 targets the protein kinase Raf-1 to mitochondria. Cell 87:629-638.

Wang K, Yin XM, Chao DT, Milliman CL, Korsmeyer SJ. (1996) BID: a novel BH3 domain-only death agonist. Genes Dev. 10:2859-2869.

Wang S, Miura M, Jung YK, Zhu H, Li E, Yuan J. (1998) Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. Cell.92:501-9.

Wang L, Andersson S, Warner M, Gustafsson JA. (2001) Morphological abnormalities in the brains of estrogen receptor beta knockout mice. Proc. Natl. Acad. Sci. U. S. A. 98:2792-2796.

Waters JS, Webb A, Cunningham D, Clarke PA, Raynaud F, di Stefano F, Cotter FE. (2000) Phase I clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. J Clin Oncol. 18:1812-23.

Wetmore C, Olson L. (1993) Expression and regulation of neurotrophins and their receptors in hippocampal systems. Hippocampus. Spec No:171-81.

Wilson KP, Black JA, Thomson JA, Kim EE, Griffith JP, Navia MA, Murcko MA, Chambers SP, Aldape RA, Raybuck SA et al. (1994) Structure and mechanism of interleukin-1 beta converting enzyme. Nature. 370:270-275.

White FA, Keller-Peck CR, Knudson CM, Korsmeyer SJ, Snider WD. (1998) Widespread elimination of naturally occurring neuronal death in Bax-deficient mice. J Neurosci.18:1428-39.

Wong V, Glass DJ, Arriaga R, Yancopoulos GD, Lindsay RM, Conn G. (1997) Hepatocyte growth factor promotes motor neuron survival and synergizes with ciliary neurotrophic factor. J Biol Chem. 272:5187-91.

Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW, Mak TW .(1998) Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev. 12:806-19.

Wyllie AH. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature. 284:555-556.

Wyllie AH., Morris RG, Smith AL, Dunlop D. (1984) Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J. Pathol. 142:67-77.

Wu YC, Stanfield GM, Horvitz HR. (2000) NUC-1, a caenorhabditis elegans DNase II homolog, functions in an intermediate step of DNA degradation during apoptosis. Genes Dev.14:536-48.

Xu DG, Korneluk RG, Tamai K, Wigle N, Hakim A, Mackenzie A, Robertson GS. (1997) Distribution of neuronal apoptosis inhibitory protein-like immunoreactivity in the rat central nervous system. J Comp Neurol. 382:247-59.

Xu DG, Crocker SJ, Doucet JP, St-Jean M, Tamai K, Hakim AM, Ikeda JE, Liston P, Thompson CS, Korneluk RG, MacKenzie A, Robertson GS. (1997). Elevation of neuronal expression of NAIP reduces ischemic damage in the rat hippocampus. Nature Med. 3: 997-1004.

Xu D, Bureau Y, McIntyre DC, Nicholson DW, Liston P, Zhu Y, Fong WG, Crocker SJ, Korneluk RG, Robertson GS. (1999) Attenuation of ischemia-induced cellular and behavioral deficits by X chromosome-linked inhibitor of apoptosis protein overexpression in the rat hippocampus. J. Neurosci. 19:5026-5033.

Yamaguchi K, Nagai S, Ninomiya-Tsuji J, Nishita M, Tamai K, Irie K, Ueno N, Nishida E, Shibuya H, Matsumoto K. (1999) XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. EMBO J. 18:179-187.

Yan C, Chen J, Chen D, Minami M, Pei W, Yin XM, Simon RP. (2000) Overexpression of the cell death suppressor Bcl-w in ischemic brain: implications for a neuroprotective role via the mitochondrial pathway. J Cereb Blood Flow Metab. 20:620-630.

Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. (1995) Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell. 80:285-291.

Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X .(1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science.275:1129-32.

Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD. (2000) Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science 288:874-877.

Yankner BA. (1996) Mechanisms of neuronal degeneration in Alzheimer's disease. Neuron.16:921-32.

Yoshida H, Kong YY, Yoshida R, Elia AJ, Hakem A, Hakem R, Penninger JM, Mak TW. (1998) Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell.94:739-50.

You M, Ku PT, Hrdlickova R, Bose HR. (1997) ch-IAP1, a member of the inhibitor-of-apoptosis protein family, is a mediator of the antiapoptotic activity of the v-Rel oncoprotein. Mol. Cell Biol., 17, 7328-7341.

Yuan J, Horvitz HR. (1992) The Caenorhabditis elegans cell death gene ced-4 encodes a novel protein and is expressed during the period of extensive programmed cell death. Development. 116:309-320.

Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR. (1993) The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell 75:641-652.

Zafra F, Hengerer B, Leibrock J, Thoenen H, Lindholm D.(1990) Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. EMBO J. 9:3545-50.

Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M, Kroemer G.(1996) Mitochondrial control of nuclear apoptosis. J Exp Med.183:1533-44.

Zheng TS, Hunot S, Kuida K, Flavell RA. (1999) Caspase knockouts: matters of life and death. Cell Death Differ. 6:1043-53.

Zheng L, Schickling O, Peter ME, Lenardo MJ. (2001) The death effector domain-associated factor plays distinct regulatory roles in the nucleus and cytoplasm. J Biol Chem. 276:31945-52

Zhou M, Demo SD, McClure TN, Crea R, Bitler CM. (1998) A novel splice variant of the cell death-promoting protein BAX. J Biol Chem.273:11930-6.

Zhou P, Chou J, Olea RS, Yuan J, Wagner G. (1999) Solution structure of Apaf-1 CARD and its interaction with caspase-9 CARD: a structural basis for specific adaptor/caspase interaction. Proc Natl Acad Sci U S A.96:11265-70.

Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. (1997)Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell.90:405-13.