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APOPTOSIS-ASSOCIATED CHANGES IN NEURONAL GENE EXPRESSION
With special emphasis on the insulin-like growth factor system

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Doctoral dissertation

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ABSTRACT

Active cellular suicide, apoptosis, plays an important role in the correct formation of the nervous system during development. Furthermore, during neurodegenerative conditions, such as Alzheimer's disease, apoptosis may have a major impact on the outcome. In neuronal cells, the constant interaction between pro-apoptotic and pro-survival molecules and signaling pathways is considered to determine whether the cell will survive or not. If the pro-apoptotic signaling exceeds the strength of the signal signifying pro-survival, the cell is likely to undergo apoptosis. However, to be able to undergo apoptosis, the cell should complete a highly regulated process that requires new RNA and protein synthesis and the activation of specific, normally dormant cellular enzymatic machinery.

Until recently, the majority of studies on apoptotic "death genes" and signaling cascades have been performed in non-neuronal systems. Consequently, there is a gap in the knowledge of the molecular mechanisms underlying neuronal apoptosis. The aim of this study was to shed light on changes in gene expression occurring during neuronal apoptosis. Neuronal apoptosis was induced in rat cerebellar granule cells (CGCs) using potassium deprivation and a PCR-based differential display (DD) technique was used to detect changes in gene expression. Two candidate genes were originally identified from the DD-analysis, and their expression, in combination with other related genes, was examined. The first candidate gene, PTZ-17 (pentylenetetrazol-related cDNA 17) was markedly upregulated specifically by potassium deprivation-induced apoptosis. On the contrary, the second candidate gene, insulin-like growth factor binding protein -5 (IGFBP-5) was downregulated. Also IGFBP-2, and -3 the type 1 IGF receptor (IGF1R), and PTEN phosphatase expression was shown be altered during neuronal apoptosis. Interestingly, IGFBP-2 and -3 showed opposite expression pattern in potassium deprivation- and okadaic acid-induced apoptosis. IGF1R showed a transient upregulation at the mRNA level in potassium-deprived CGCs. The PTEN expression was markedly downregulated specifically in CGCs during apoptosis induced by withdrawal of both, potassium and serum, whereas deprivation of only potassium did not affect the expression. This may indicate that PTEN is downregulated in order to balance lack of survival signaling.

The IGF system and the related signaling pathways have been previously found to be critical for neuronal survival and apoptosis. The IGFs have been considered as a potential drug for several neurodegenerative diseases. However, little is known about the regulation of the IGFBPs during apoptosis. This thesis provides insight into the expression of IGF system during neuronal apoptosis. The results indicate that the IGFBPs are expressed in distinct ways with each of them having a characteristic expression pattern. Furthermore, the changes in expression vary depending on the apoptosis model used, which may demonstrate the presence of several apoptotic mechanisms in neuronal cells.

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Medical Subject Headings: apoptosis; neurons; cerebellum/cytology; gene expression profiling; gene expression regulation; gene expression; polymerase chain reaction; blotting, Northern; blotting, Western; insulin-like growth factors; insulin-like growth-factor-binding proteins; animal; rats

Life is good, a horse makes it better.

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Kuopio, December 2001

Maaria Ikonen
(formerly known as Roschier-Teivaanmäki)

ABBREVIATIONS

AD	Alzheimer's disease
AIF	apoptosis inducing factor
ALS	acid labile subunit
APAF-1	Apoptotic protease-activating factor-1
Ara-C	cytosine-β-D-arabinofuranoside
BDNF	brain-derived neurotrophic factor
BH	Bcl-2 homology domain
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
C. Elegans	*Caernorhabditis elegans*
CaMKK	calsium / calmodulin dependent kinase kinase
cAMP	cyclic adenosine monophosphate
CARD	caspase-activating recruitment domain
Caspases	cysteinyl aspartate-specific proteinases
cDNA	complementary DNA
CGCs	cerebellar granule cells
CNS	central nervous system
CREB	cAMP response element-binding protein
crmA	cowpox viral cytokine-response modifier A
CSF	cerebrospinal fluid
cyt c	cytohrome c
DD	differential display
DED	death effector domain
DEPC	diethylpyrocarbonate
DISC	death-inducing signaling complex
DIV	day *in vitro*
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E	embryonic
ECL	enhanced chemiluminiscence
ECM	extracellular matrix
EDTA	ethyldiamine tetraacetic acid
ERK	extracellular signal regulated protein kinase
EST	expressed sequence tag
EtdBr	ethidium bromide
FADD	Fas-associated death-domain
Fas L	Fas ligand
FBS	fetal bovine serum
FGF	fibroblast growth factor
FKHR	forkhead transcription factor
FLIP	FADD-like ICE inhibitory protein
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
gDNA	genomic DNA
GRB	growth factor receptor-bound protein
GSK	glycogen synthase kinase
GTP	guanine
HRP	horseradish peroxidase
I^{CAD}	inhibitor of caspase activated deoxyribonuclease
ICE	interleukin-1β-converting enzyme
IDE	insulin degrading enzyme
IGF	insulin-like growth factor
IGF1R	type 1 insulin-like growth factor receptor
IGF2R	type 2 IGF receptor / mannose-6-phosphate receptor
IGFBP	insulin-like growth factor binding protein
IGFBPrP	IGFBP related protein
IPTG	isopropyl-thiogalactopyranoside
IR	insulin receptor
IRS	insulin receptor substrate
JNK	c-Jun N-terminal kinase
KCl	potassium chloride
kDa	kilodalton
KO	knock-out
LDH	lactate dehydrogenase
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
MEK	MAP/ERK kinases
MEKK	mitogen-activated protein kinase / ERK kinase kinase -1
$MgSO_4$	magnesium sulfate
MOPS	3-[*N*-morpholino] propanesulfonic acid
M_w	molecular weight

NaCl	sodium chloride
NaOH	sodium hydroxide
NF-κB	nuclear factor kappa B
NGF	nerve growth factor
OE	overexpressing
P	postnatal
PAPP-A	pregnancy-associated plasma protein
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	plateled-derived growth factor
PDK	3-phosphoinositide dependent kinase
PDK	3-phosphoinositide dependent kinase
PH	pleckstrin homology
PI-3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PKB	protein kinase B /Akt protein kinase
PKC	protein kinase C
PP	protein phosphatase
PTB	phosphotyrosine binding
PTEN	phosphatase and tensin homolog deleted on chromosome ten
PTK	protein tyrosine kinase
PTZ-17	pentylenetetrazol-related cDNA-17
RNA	ribonucle ic acid
RT-PCR	reverse transcription PCR
RXR	retinoid X-receptor
SAPK	stress-activated protein kinase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH	src homology
SSCP	single-stranded conformational polymorphism
STI	soybean trypsin inhibitor
TBE	tris-borate-EDTA
TE	tris-EDTA
TGF	transforming growth factor
TM	transmembrane domain
TNF	tumor necrosis factor
TopoII	topoisomerase II
TRADD	TNF-receptor associated death domain
UV	ultraviolet
X-gal	bromo-chloro-indolyl-galactopyranoside
XIA	X-gal-IPTG-ampicillin

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by the Roman numerals **I-V**.

I Roschier M, Kuusisto E and Salminen A. A differential display protocol to identify differentially expressed mRNAs in potassium-deprived cerebellar granule cells. Brain Res Brain Res Protoc 2000;5:121-131.

II Roschier M, Kuusisto E, Kyrylenko S and Salminen A. Expression of seizure-related PTZ-17 is induced by potassium deprivation in cerebellar granule cells. Biochem Biophys Res Commun 1998;252:10-13.

III Roschier M, Kuusisto E, Suuronen T, Korhonen P, Kyrylenko S and Salminen A. Insulin-like growth factor binding protein 5 and type-1 insulin-like growth factor receptor are differentially regulated during apoptosis in cerebellar granule cells. J Neurochem 2001;76:11-20.

IV Ikonen M, Suuronen T, Kyrylenko S and Salminen A. Insulin-like growth factor binding protein -2, -3 and -5 are differentially regulated during neuronal apoptosis. (manuscript)

V Kyrylenko S, Roschier M, Korhonen P and Salminen A. Regulation of PTEN expression in neuronal apoptosis. Brain Res Mol Brain Res 1999;73:198-202.

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1. INTRODUCTION

Death belongs to life as well as birth; whenever there is a new life born, another life ends somewhere. There is a time and place for all things and appropriate death brings the scales into balance, not only on the scale of the global ecosystem, but also within a single living organism. If there is too much or not enough death, the life of the whole organism is endangered. Actually, the presence of “meaningful” death is crucial to the very existence of multicellular organisms; the organism as a whole benefits from the controlled removal of certain individual cells that are unnecessary or faulty.

The cellular process in which the cell commits suicide using its own execution machinery is called apoptosis. It is accomplished through normally dormant signals and pathways, which are activated when a cell begins the suicide process. Thus, apoptosis is an active process; to proceed it needs activation and synthesis of many signaling and effector molecules. Although the final outcome of the apoptotic process is always the same, the actual molecular mechanisms are diverse and many of them still remain to be elucidated. The traditionally described characteristics of apoptotic cell death include chromatin condensation, fragmentation of DNA, blebbing of the plasma membrane, and formation of “apoptotic bodies”, which are phagocytozed by neighboring cells.

The term apoptosis was coined in the early 1970, although the phenomenon of apoptosis was described already in the late 1700s. The word, apoptosis, is derived from Greek roots meaning, “dropping off” e.g. falling of leaves. Since its original morphological characterization as a distinct form of cell death, apoptosis has been widely studied for its incidence, biochemistry and genetics.

The ability of cells to die by apoptosis is a fundamental property of animal cells, it is an invariable part of animal development, and it often continues into adulthood. During development, the role of apoptosis is often very clear. For example, the cells that are needed only for the formation, not the final function of a certain organ or part of a body, can be carefully cleaned away. In particular in the nervous system, the cells that are “in the wrong place at the wrong time” are terminated before they cost too much, for example, in terms of energy consumption. Although the apoptotic destruction itself is an “expensive” process that consumes much energy and building materials to no constructive purpose, it is a sound investment in terms of the organism as an entity. When compared to the life of the whole organism, cells are apparently cheap and expendable.

Although apoptosis has a fundamental role in normal development, it is also considered to play a significant role in a plethora of human diseases. Hence, at the moment it is one of the hottest topics in the field of biology, representing a possible target for pharmacological intervention in a wide variety of critical and global diseases, such as some neurodegenerative diseases and cancers (Fadeel et al. 1999a). Indeed, one vital organ where incorrect regulation of apoptosis at adult age can have fatal consequences,

is the brain. In fact, neuronal apoptosis is suggested to play a significant role in several neuropathological conditions, such as stroke, Alzheimer's disease and Parkinson's disease (Bredesen 1995). Hence, there is an intriguing possibility to treat some neurodegenerative diseases by inhibiting apoptosis in neuronal cells. Despite the massive amount of research conducted into the neurodegenerative diseases, the molecular mechanisms underlying neuronal apoptosis still remain poorly understood. One could state that, understanding apoptosis in general, including understanding the basic molecular mechanisms, will hopefully help unravel some of the fundamental secrets of life.

2. REVIEW OF THE LITERATURE

2.1. Biology of apoptosis

Almost all cells, regardless of their phylogenetic origin or physiological specialization, ultimately senesce and die. There are several ways of dying, depending on the nature and severity of the death stimulus, type of the cell affected, and homeostatic conditions of the cell and its surroundings. The two major forms of cell death recognized today are apoptosis and necrosis (Kerr et al. 1972). The main difference is thought to be the requirement for energy: apoptosis is an active process consuming energy and requiring macromolecular synthesis (Tata 1966), while necrosis occurs passively. In spite the fact that there are several features that can distinguish these processes from each other, also common and overlapping characteristics appear, and there are cases where no clear cut distinction between apoptosis and necrosis can be found (Farber 1994, Columbano 1995). While the characterization of differences between apoptotic and necrotic cell death remains incomplete, recent findings suggest that apoptotic cell death differs even from cell to cell, and each induction strategy is likely to involve a unique set of genes (Schwartz and Osborne 1993).

Apoptosis is a way of dying which is inconspicuous and still results from active processes in the cell that require energy and gene expression (Tata 1966, Schwartz et al. 1990). The morphological characteristics of apoptosis include: plasma membrane and nuclear membrane blebbing, cell shrinkage, and chromatin condensation and fragmentation (Kerr et al. 1972). The molecular mechanisms underlying the morphological changes consist of a large network of signaling pathways in which the key players often are the same molecules that most cells express also constitutively. This means that the components of the basic enzymatic machinery that mediate apoptotic cell death exist also in a normal living cell, but it seems that their potentially lethal activities must be suppressed in order the cell to survive (Raff 1992, Raff et al. 1993).

Apoptosis is a highly regulated process, being controlled by various ligands and signaling pathways. However, some pathways and events of the apoptotic program have been conserved among species and are considered as mediators of fundamental events in apoptotic signaling and cell death process itself, and therefore, they appear to be of particular significance (Steller 1995). These include for example, the action and regulation of the bcl-family of proteins, cytochrome c release from mitochondria which is accompanied with mitochondrial dysfunction, and activation of caspases (Dickson 1998). The molecular genetic studies in the nematode *Caernorhabditis elegans (C. elegans)* have revealed the existence of conserved genes and gene families, such as *ced-3* (homologs of mammalian caspases), *ced-4* (Apaf-1), and *ced-9* (the bcl-family of proteins), which are involved in apoptosis (Ellis and Horvitz 1986).

2.1.1. Molecular mechanisms of apoptosis.

One common way to characterize apoptosis is to divide it into different phases according to the types of the sequential events. First, there has to be some kind of causative factor, which activates the apoptotic pathway. This activation is followed by induction of the actual apoptotic signaling, and an initiator phase, including changes in the mitochondrial permeability, and activation of upstream caspases. The apoptotic process is then further spread to an effector phase, which ultimately leads to the actual degradation of the cell, including cytoplasmic blebbing, endonuclease activity and nuclear fragmentation and cell death (Honig and Rosenberg 2000).

The diversity of stimuli that can activate and regulate apoptosis is extensive, and so will be the diversity of subsequent signaling pathways. A trigger for apoptosis can be some complex external signal e.g. resulting from depletion of growth factors or nutrients, accumulation of toxic metabolites, oxygen deprivation or excessive formation of reactive oxygen species. It can also be a more specific signal mediated through precise signaling, e.g. Fas (APO-1, CD95) and its corresponding receptor (Fas/TNF receptor 1). The process leading to apoptosis is regulated through various kinases and second messengers, such as protein tyrosine kinases (PTKs), MAPK and Ras signaling pathways, protein kinase C, and cAMP (cyclic AMP) and Ca^{2+} related pathways (Hale et al. 1996). Also loss of cell attachment in a normal healthy cell can initiate apoptotic death (Kinloch et al. 1999, Song and Steller 1999). This literature review does not attempt to cover all of the possible pathways in apoptosis, but only to illustrate some examples of those which are well characterized or thought to be important in apoptosis. The following two examples, the cell surface death receptor pathway and the mitochondria-initiated pathway, are the major systems known to activate the executioners of apoptosis, caspases.

The Fas-ligand (FasL) activated pathway is one of the best-understood initiators of apoptosis, and the corresponding receptors are widely distributed throughout the body. It is often active in cells from the immune system, and it is important in the downregulation of the immune response and elimination of self-reactive lymphocytes (Hale et al. 1996, Kinloch et al. 1999). The first stage of apoptosis signaling principally occurs through the interaction between extracellular ligands, FasL, TNF-α, or TRAIL, and their cognate receptors such as members of the TNF receptor family, Fas/APO-I/CD95, TNFR1, and TRAIL receptors, respectively (Aravind et al. 1999). This is followed by the receptor trimerization and activation of the death-domain containing intracellular part, and recruitment of TRADD (TNF-receptor associated death domain) and/or FADD (Fas-associated death-domain/TNFR-associated death domain protein). These components form the death-inducing signaling complex (DISC) that recruits and activates procaspase -8, which subsequently activates downstream caspases, such as caspase-3/ICE, and finally results in apoptotic cell death (Song and Steller 1999). However, even if the death signal is propagated from the death receptor, it can be suppressed at several points along the pathway. There are proteins that directly inhibit the procaspase recruitment and /or activation at the level of DISC. These include death effector domain containing proteins, FADD-like ICE inhibitory proteins (FLIP) that compete with procaspases of FADD binding (Thome et al. 1997). The apoptotic signal can be also silenced even before the actual receptor binding, by “decoy-receptors”

(Golstein 1997), or even just prior to the activation of initiator caspases, by e.g. crmA (Ray et al. 1992).

Some evidently important turning points in the course of apoptotic cell death process take place in mitochondria. The precise and detailed role of mitochondria in apoptosis remains still unresolved, but it is recognized that many of the key events in vertebrate cell apoptosis seem to focus on mitochondria (Green and Reed 1998). These include the mitochondrial membrane permeability transition and disruption of essential mitochondrial functions and release of soluble mitochondrial intramembrane proteins (SIMPs), such as cytochrome c (cyt c), procaspase-3 and apoptosis-inducing factor (AIF) (Green and Reed 1998, Vieira and Kroemer 1999). The suggested role of the mitochondria as the "sensor of cellular stress" might derive from their ancient endosymbiotic bacterial origin (Margulis 1996). Mitochondria can trigger apoptotic cell death by at least three general mechanisms: 1) disruption of electron transport and energy metabolism, 2) release of pro-apoptotic caspase activators, or 3) alteration of cellular redox potential (Green and Reed 1998). When cyt c is released to cytoplasm, it can activate caspases and induce apoptosis (Liu et al. 1996). Another consequence of the release may also be disruption of the electron transport chain and energy production of mitochondria (Scaife 1966, Garcia-Ruiz et al. 1997). This in turn depends on the cell type and the amount of cyt c in the mitochondria; if there is excess of cyt c, the mitochondria may tolerate this release, and the electron transport chain will remain functional. Also caspase activation by cyt c depends on the amount of endogenous caspase inhibitors, which can prevent consequences of cyt c release, even when it is released in large quantities (Green and Reed 1998).

The release of cyt c from mitochondria may not be a universal phenomenon, since it seems to happen frequently in vertebrates; there is no clear evidence of its function in non-vertebrates (Green and Reed 1998). Nevertheless, the ability of the mitochondria to control the cell survival and to sensor the "stress-status" makes them important players when it comes to the decision of whether to commit suicide or not (Green and Reed 1998). Support for the important role comes also from the findings from several neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, in which the mitochondrial function is often disturbed, contributing to the improper regulation of apoptosis (Cassarino and Bennett 1999, de la Monte and Wands 2001).

What happens when destructive changes in the mitochondria take place and cyt c is released? One major following event is the subsequent binding of cyt c to apaf-1, which is the mammalian homologue of the *C. elegans* death gene product CED-4 (Li et al. 1997b). Apaf-1, cyt c and procaspase-9 form a complex that activates caspase-9 (Zou et al. 1999). Subsequently, caspase-9 is released from the complex and transmits the apoptotic signals to downstream caspase activation, including caspase-3 (Zou et al. 1999). This complex formation might exist to ensure that a minor leakage of cyt c from mitochondria will not cause cells to undergo apoptosis, and sets a relatively high threshold for subsequent caspase activation (Budihardjo et al. 1999).

Since the mitochondria play a major role in cellular survival and apoptosis, also proteins that are involved in mitochondrial functions are of special interest. One family of proteins that is critical to both processes, regulation of apoptosis and regulation of

mitochondrial function in apoptosis is the Bcl-2 family (Adams and Cory 1998). Bcl-2 was shown to be the mammalian homolog of CED-9, recognized for its anti-apoptotic function in *C. elegans* (Vaux et al. 1992). All members of the Bcl-2 family contain at least one of the four Bcl-2 homology domains (BH1 to BH4) that are important to the homo- and heterodimerization and thus to their function on cell survival (Adams and Cory 1998). The anti-apoptotic proteins of the family include e.g. Bcl-x_L, Bcl-w and Mcl-1, in addition to Bcl-2. Pro-apoptotic members of the family include the more closely related proteins that contain more than one BH-domain such as Bax and Bak, and also more distantly related proteins that contain only the BH3 domain, such as Bik, Blk, Bad and Bid (Adams and Cory 1998, Fadeel et al. 1999b). Heterodimerization is not required for anti-apoptotic function but it is essential for the pro-apoptotic function in the group of proteins that contain only the BH3 domain (Chittenden et al. 1995).

The pro-survival Bcl-2 is a 26-kDa protein that is localized in the mitochondrial, endoplasmic reticulum and perinuclear membranes (Korsmeyer 1995). Bcl-2 belongs to the family of proto-oncogenes, but the ability of bcl-2 to block apoptosis is different from other oncogenes; it can support cells that are destined to die without affecting the proliferation rate of the cell (Kinloch et al. 1999). It is possible that the location of Bcl-2 might be a key feature of its function. Since Bcl-2 is involved in maintaining the homeostatic status, including the mitochondrial membrane status and balance of the interactions between the members of the Bcl-2 protein family (e.g. Bax) and interactions between Bcl-2 and other proteins (e.g. direct interaction with cyt c or apaf-1), it may be one of the crucial players in the mitochondria-based apoptosis sensing system (Green and Reed 1998). In addition, bcl-2 has a role in the regulation of calcium homeostasis in the cell (McConkey and Orrenius 1997). Overall, the level of Ca^{2+} has a major effect on the signaling, growth and survival of cells, both in normal and stress situations.

Although the initiating events in apoptosis are diverse and might involve cell specific characteristics, it is likely that all signals converge into a common final pathway (King and Cidlowski 1995). These final execution events in apoptosis are performed through a cascade of proteases, which proteolytically cleave proteins involved in the maintenance of cellular integrity and homeostasis (Patel et al. 1996). These proteases, named caspases, belong to the family of cysteine proteases. Caspases were implicated in apoptosis together with the finding that *ced-3* gene product is required in cell death in *C. elegans* (Thornberry et al. 1992, Yuan et al. 1993). In mammalian cells, the related protease, interleukin-1β-converting enzyme (ICE or caspase-1) was the first member of a large family of proteases discovered, which are now recognized to be involved in inflammation and apoptosis (Thornberry et al. 1992, Yuan et al. 1993). The caspases can be divided into two categories according to their role in apoptosis: the initiator caspases, for example caspase-8, and executor caspases, for example, caspase-3, and -6 (Patel et al. 1996). Another type of classification is according to the sequence motifs in their pro-domains: caspases with death effector domain (DED), such as caspase-8, and -10, are activated directly by interacting with the intracellular domains of death receptors, such as the Fas-receptor. Caspases with a caspase-activating recruitment domain (CARD), that include caspase-1, -2, -4, -5, -9, -12, are most probably activated by the apaf-1/cyt c complex. Finally, there are caspases with short pro-domains, such as caspase-3, which are thought to be activated by most of the other caspase pathways (Yuan and Yankner 2000).

Caspases are synthesized as proenzymes and remain inactive in most healthy cells. Upon activation by different death signals, the single-chain procaspases are cleaved, and the resulting activated subunits assemble into a heterotetramer to form the active protease (Thornberry and Lazebnik 1998, Song and Steller 1999). Caspases are very specific proteases cleaving only after a specific aspartic acid residue (Thornberry and Lazebnik 1998). The targets of apoptotic proteolysis include for example poly (ADPribose) polymerase (PARP), laminin B1, α-fodrin, β-actin, I^{CAD} (inhibitor of caspase-activated deoxyribonuclease), and proteins involved in DNA repair, mRNA splicing and DNA replication. Their proteolysis contribute to the characteristic apoptotic morphology and DNA fragmentation, which is further culminated in the formation and engulfment of apoptotic bodies containing the remainders of the apoptotic cell (Thornberry and Lazebnik 1998, Kinloch et al. 1999). The major apoptotic pathways are summarized in figure 1.

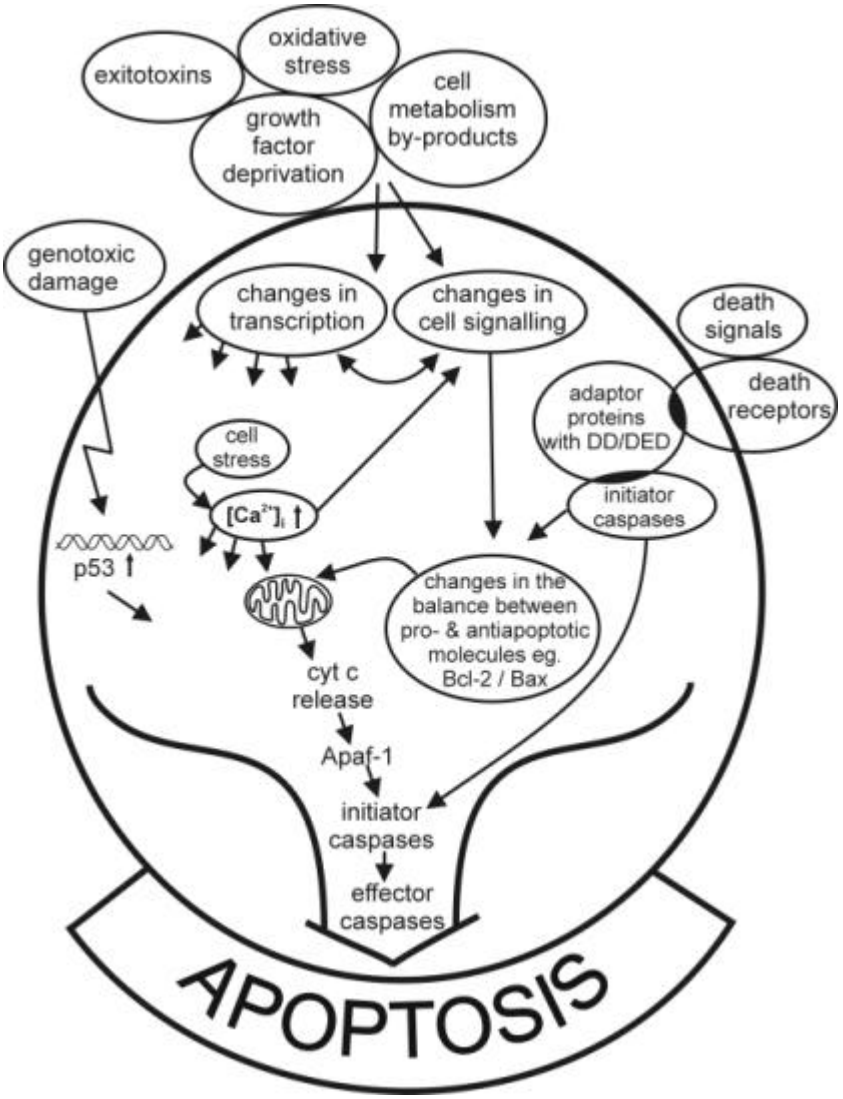

Fig. 1. Schematic representation of apoptotic pathways.

2.1.2. Neuronal apoptosis and survival signaling

In the vertebrate nervous system up to 50-80% or more of different types of neurons die before the embryonic development is complete. Despite massive death during development, mature neurons are among the most long-lived cell types in mammals. Therefore, since neurons are post-mitotic cells, i.e. they are not able to divide, a constant survival support is considered to be necessary for the maintenance of neuronal cells and nervous system. According to the fundamental discovery of Viktor Hamburger and Rita Levi-Montalcini, the survival of immature neurons during development largely depends on the availability of their appropriate innervating targets (Hamburger and Levi-Montalcini 1949). This theory led to the discovery of neurotrophic factors, but did not yet fully explain the mechanism of how the neurons are excluded from the developing brain, and it was assumed that the neurons die "simply by starvation" (Yuan and Yankner 2000). However, later it was shown in a cell culture model, that the death after NGF (nerve growth factor) deprivation is an active process and requires new RNA and protein synthesis (Martin et al. 1988). Furthermore, apoptosis is suggested to be a default pathway for those neurons that do not receive enough neurotrophic support from their target cells, and are not functionally active (Raff et al. 1993). Indeed, all the required elements for apoptosis are present in normal cells, only their apoptotic activity seems to be suppressed by means of various kinds of survival and trophic signaling (Raff et al. 1993). Apparently, there exists a "constant battle between good and bad fellows", which includes the "good" survival factors that constantly reduce and inactivate the amount or activity of "bad" cell death effector proteins to harmless levels (Raff 1992, Raff et al. 1993). Eventually, at a so-called cell death and survival checkpoint, the ratio between anti-apoptotic Bcl-2 and pro-apoptotic Bax determines whether the cell will survive or die by apoptosis (Gross et al. 1999). If the survival factors (e.g. growth factors) are inadequate, the amount of anti-apoptotic restraining force might be insufficient, and consequently the cell may proceed into apoptosis.

Important neurotrophic factors include neurotrophic growth factors (NGFs), neurotrophins -3 and -4, neuropoietins, insulin-like growth factors (IGFs), transforming growth factors (TGFβ), brain-derived neurotrophic factor (BDNF) and fibroblast growth factors (FGFs). These factors, as well as others, regulate the growth and survival of neurons, both during development and adult life, by signaling through specific cell surface receptors and several downstream messengers and pathways (Korsching 1993, Arumae et al. 1997). The major survival pathways that are activated by these factors, and that have been repeatedly implicated in neuronal survival are the phosphatidylinositol-3-kinase (PI-3K) and mitogen activated protein (MAP) kinase or extracellular signal regulated protein kinase (ERK) pathways (Pettmann and Henderson 1998). These signaling pathways have a variety of targets. They include several other signaling components and multiple transcription factors. Therefore, there are complex tangles of signaling pathways, which in post-mitotic cells establish a security system with multiple regulation and check points. Both pathways, with special emphasis on the PI-3K pathway, are described in more detail in later sections highlighting the insulin-like growth factor system.

2.1.3. Role of apoptosis in neurodegenerative diseases.

Neuronal apoptosis is a normal event during developement when massive numbers of cells die: it enables the correct formation of the central nervous system (Bredesen 1995). On the contrary, many neurodegenerative diseases involve abnormal cell death that leads to damage of the nervous system. As an immediate response to acute disorders, such as brain trauma or stroke, neurons are considered to die by necrotic mechanisms. Subsequent damage to the system may occur also through apoptotic mechanisms, especially in the area innervated by or surrounding the injury site (Honig and Rosenberg 2000). Cell loss may be involved also in many chronic diseases, such as amyotrophic lateral sclerosis, Alzheimer's disease and Parkinson's disease (Mattson 2000). Definite evidence of apoptosis in these kinds of diseases is difficult to obtain because of several complicating features such as the high morphological specialization of neuronal cells, the long time course of the disease, and methodological difficulties (Honig and Rosenberg 2000, Yuan and Yankner 2000). Neurons are evidently the most complicated cells in the body, not only do they have soma and normal cell organelles, but also synapses, dendritic arbor and axon complemented with several exquisitely detailed structures within these cellular compartments. Therefore, it cannot be assumed that it does not matter whether the injury to a particular cell happens at the level of the cell soma or at the axon hillock, for example. This morphological specialization and spatial segregation of different parts of the cell may also enable the cell to respond only "partially" to injury or to undergo apoptosis in several different ways depending on the site of onset. It may also be the astrocytes, microglial, or other supporting cells that are injured primarily, which later induces a secondary injury to the neuronal cells (Honig and Rosenberg 2000). Furthermore, according to mathematical estimates of the probability to find an apoptotic cell in a sample obtained from autopsy specimen, it is most unlikely that one will find remnants or characteristics of an apoptotic cell. For example, if the duration of a given chronic disease is ten years, during which 40% of the cells die by apoptosis, which preserves its characteristic signs for 24 hours; the probability to find an apoptotic cell in a given histological specimen is 0.01% (Honig and Rosenberg 2000). Despite the small probability and high complexity of the system, some clear evidence of apoptotic markers have been found for example in samples from Alzheimer's disease patients (Smale et al. 1995, Anderson et al. 1996, Shimohama 2000, Raina et al. 2001).

Currently used drugs to treat Alzheimer's disease patients do not cure the disease or efficiently inhibit the loss of neuronal cells. In contrast, their effects rely on intensifying the actions of the remaining cells without stopping the cell loss (Shvaloff et al. 1996, Cutler and Sramek 2001). One way to inhibit the cell loss is to identify and eliminate the direct causes of apoptotic cell death during the disease. While the elimination of death inducing signals and processes might not be enough to cure the patient, especially if some damage has already occurred, introducing some survival promoting signals to the remaining cells, might stop neuronal loss. Consequently, there has been great interest in use of neurotrophic factors as therapeutical agents to inhibit the cell loss involved in several neurodegenerative diseases (Shvaloff et al. 1996, Dore et al. 1997). One of the most promising candidates have been the insulin-like growth factors (IGFs), known for their pleiotrophic roles in promoting cellular survival, proliferation and differentiation (Dore et al. 1997). The recent advances in understanding the significance

of the biological actions of the IGFs in the nervous system have revived interest in the potential of IGFs in treating patients with neurodegenerative diseases (Dore et al. 1997). There are several pathological processes related to Alzheimer's disease that are influenced by the IGFs. For example, it is known that IGFs support the survival of cholinergic neurons and upregulate the expression of choline-acetyltransferase, needed for the production of acetylcholine (Konishi et al. 1994). Furthermore, it is known that IGFs regulate the release of acetylcholine in hippocampal cells and interestingly also facilitate memory related functions (Saatman et al. 1997, Markowska et al. 1998, van Dam et al. 2000). Additionally, IGFs can protect against β-amyloid induced toxicity (Niikura et al. 2001).

2.2. IGF SYSTEM

2.2.1. IGF system members

The insulin-like growth factor (IGF) system is composed of a large number of widely expressed proteins that regulate cellular growth and differentiation. The IGFs were first identified in 1957, and called *sulfation factors* (Salmon and Dauhaday 1957). Subsequently they were called *non-suppressible insulin-like activity* (Froesch et al. 1966), and *multiplication-stimulating activity* (Dulak and Temin 1973). Later they were named again as somatomedins (Daughaday et al. 1972), followed by the name IGFs (Daughaday and Rotwein 1989). The period of molecular IGF-I studies began in 1978, when the human IGF-I and IGF-II were purified and the amino acid sequence was determined (Rinderknecht and Humbel 1978a, Rinderknecht and Humbel 1978b). The actual growth factors, IGFs, are ubiquitous peptide growth factors that share structural homology with proinsulin (Cohick and Clemmons 1993). They regulate proliferation and differentiation of various cell types, and moreover, have overlapping activities with insulin in the regulation of metabolism. However, unlike insulin, IGFs are produced locally in several tissues, and in addition, are abundant in the circulation (Cohick and Clemmons 1993). Growth hormone is the major regulator of IGF expression (Salmon and Dauhaday 1957). In addition, several other factors including hormones (such as estrogen and glucagon), growth factors (such as FGF and PDGF) and cytokines (such as interferon-γ and interleukin-1β) are also known to regulate their expression (Rosenfeld and Roberts 1999).

Members of the IGF system include: *1)* the actual growth factors IGF-I, IGF-II and insulin; *2)* their corresponding receptors, type 1 IGF receptor (IGF1R), type 2 IGF receptor/mannose-6-phosphate receptor (IGF2R/M-6-P receptor) and insulin receptor; *3)* six IGF binding proteins (IGFBPs 1-6); *4)* nine additional IGFBP related proteins (IGFBP-rPs 1-9); *5)* IGFBP cleaving proteases; *6)* and a glycoprotein known as ALS (acid-labile subunit) (Clemmons 1993, Cohick and Clemmons 1993, LeRoith et al. 1995, Collett-Solberg and Cohen 2000).

IGF-I and IGF-II are single chain polypeptides of 67 and 70 amino acids, respectively, and they consist of four domains, named A, B, C, and D. The D domain is unique to IGFs (Daughaday and Rotwein 1989). The human IGF-I gene is located in chromosome

12, whereas IGF-II is in chromosome 11, just next to the insulin gene (Brissenden et al. 1984).

The main effects of IGFs on cell proliferation, survival and differentiation are mediated through type 1 insulin-like growth factor receptor (IGF1R), which is structurally similar to insulin receptor (IR) (LeRoith et al. 1995). The IGF1R is a heterotetramer consisting of two extracellular IGF binding α-subunits and two membrane-spanning β-subunits linked by disulfide bonds. The IGF1R β-subunit contains a hydrophobic TM region and a highly conserved tyrosine kinase domain (LeRoith et al. 1995). The structural homology of the IGF1R to the insulin receptor (IR) is greatest (84%) in the β subunit tyrosine kinase domain (Rosenfeld and Roberts 1999). Both insulin and IGF receptors undergo ligand-induced autophosphorylation, which is a key event in the signal transduction events initiated by the IGF1R (LeRoith et al. 1995). The structurally distinct IGF-IIR is a monomeric protein lacking tyrosine kinase activity, which is also known as the cation-independent mannose-6-phosphate receptor. The signal transduction events initiated by the IGF-IIR still remain largely unresolved. In addition to IR and IGF1R, there are IR-IGF1R hybrid receptors, which consist of one α-β IGF1R and one α-β insulin hemireceptor. The details of the function and role of these hybrid receptors remain unknown (LeRoith et al. 1995, Rother and Accili 2000).

The biological functions of the IGFs are regulated by a structurally homologous group of IGF binding proteins (IGFBPs), which share no homology with the IGF receptors (Collett-Solberg and Cohen 2000). The IGFBP superfamily consists of IGFBPs 1-6 that are the classical, “high-affinity” binding proteins, and additional, “low-affinity” IGFBPs 7-10 (preferentially referred as IGFBPrPs 1-4) (Baxter et al. 1998). The chromosomal locations of the IGFBPs are in close proximity to the homeobox gene clusters (Hox A through Hox D), and it has been speculated that IGFBP and Hox genes have evolved together from single genes by duplications and translocations (Allander et al. 1993, Hong et al. 1995). The genes for human IGFBPs -1 and -3 are located in chromosome 7 and lie next to Hox-A gene. IGFBPs -2 and -5 are in chromosome 2 next to Hox-D. IGFBP-4 is in chomosome 17 next to Hox-C and IGFBP-6 in chromosome 12 next to Hox-B (Allander et al. 1993).

In the adult serum, 80-85% of the IGFs are found in a 150- kDa ternary complex formed by an IGF, IGFBP-3 (or to a smaller degree IGFBP-5), and ALS. A minority of IGFs in serum is also found in a binary complex of approximately 50 kDa together with an IGFBP (Clemmons 1993, Rajaram et al. 1997). Although most of the circulating IGFs is produced by the liver, the physiologically important autocrine and paracrine production of IGFs occurs within tissues. Due to the absence of ALS in tissues, most tissue IGFs are bound to binary complexes with IGFBPs (Wetterau et al. 1999).

IGFBPs are ubiquitously expressed, soluble extracellular proteins that bind to IGFs, and regulate their function (Jones and Clemmons 1995). IGFBPs contain conserved cysteine rich areas at the amino and carboxyl termini and a non-conserved central area (Clemmons 1993). Despite their structural and functional similarities, each IGFBP has a unique expression pattern and functional role (Collett-Solberg and Cohen 2000). IGFBP expression is regulated by several hormones, cytokines and growth factors (including IGFs) (Collett-Solberg and Cohen 2000). The activities of IGFBPs are also modified by

several distinct post-translational modifications, such as proteolysis, extracellular matrix (ECM) association, glycosylation and phosphorylation (Clemmons 1993, Clemmons 1998, Ferry et al. 1999b). IGFBPs regulate the bioavailability of IGFs by prolonging the half-life of IGFs; IGFBP bound IGF has a half-life of 12-15 h, which is significantly longer than the half-life of free IGF, which is only about 10 min (Jones and Clemmons 1995, Collett-Solberg and Cohen 2000). Consequently, e.g. in plasma, IGFBPs can act as transport proteins to sustain a certain level of inactive IGF, ready to be quickly released into tissues. For example, IGFBP-4 is cleaved by pregnancy-associated plasma protein-A (PAPP-A), which is found in high amounts in the pregnancy serum. Following this cleavage, the affinity for IGF-I is reduced. This could be a mechanism to quickly release the IGF needed in growth during pregnancy (Lawrence et al. 1999). Also in tissues, IGFs appear in a complex with an IGFBP, which can be rapidly modified e.g. by proteolysis and thus liberate the active IGF. In addition, they provide a mechanism for tissue specific localization (Jones and Clemmons 1995, Collett-Solberg and Cohen 2000). IGFBPs have also been shown to have either inhibiting or enhancing actions on the IGF activity. The binding of IGFBP to IGF can either inhibit or enhance the biological activity of IGFs. However, when the IGF is bound to IGFBP, the binding to the actual cell surface receptor is inhibited (Clemmons 1991, Clemmons 1993). The enhancing effect on IGFs may be a consequence of concentrating the IGFs in the proximity of the receptors. Thus, when subsequently released from the complex (e.g. as a result of IGFBP-5 binding to the extracellular matrix), an interaction with the receptor is more likely to occur (Jones et al. 1993). In addition, several IGF independent effects of IGFBPs, including nuclear actions and signaling through IGFBP receptors, are known (Clemmons 1993, Kelley et al. 1996, Rajaram et al. 1997, Ferry et al. 1999a, Wetterau et al. 1999, Collett-Solberg and Cohen 2000, Liu et al. 2000). For example, the effects of IGFBP-3 in apoptosis have been shown to depend on IGFBP-3 localization to nuclei and binding to retinoid X-receptor-α (RXR-α). This binding mediates the effects of IGFBP-3 in apoptosis by regulating the transcriptional activity of the RXR-α (Liu et al. 2000).

2.2.2. Transgenic models of the IGF system

Several transgenic animal studies on the IGFs show that these growth factors are important but not essential for the development and differentiation of animals. Namely, the disruption of IGF-I expression in IGF-I knock-out (KO) mice causes variable phenotypes, which might be due to the differences between strains in the capacity to increase the expression of alternative ligands, such as IGF-II, or the differences in the increase in the expression of IGF1R or the main intracellular mediators for IGF-I action (Rosenfeld and Roberts 1999). Generally, the IGF-I KO mice are significantly smaller at birth and their postnatal growth is very poor (Baker et al. 1993, Liu et al. 1993, Powell-Braxton et al. 1993). One striking feature is the increased neuronal density in the brain, which might be a result of reduced amount of neuropil and neurites (Liu et al. 1993). On the contrary, several organs of the IGF-I overexpressing (OE) mice have increased weight (Mathews et al. 1988, Behringer et al. 1990). More specifically, the increased size/weight of brain is thought to be attributable to the increased cellular survival and proliferation of neurons and oligodendrocytes, higher myelin content of the axons, and an increase in the number of dendrites followed by an increase in the size of the cells (Ye et al. 1995a, Gutierrez-Ospina et al. 1996, Ye et al. 1996). The changes in

IGF-II KO mice are less significant, the animals survive, although they suffer some deficits and a reduced birth weight (approx. 60%). It seems that IGF-II has a major role in growth *in utero* in contrast to the postnatally important role of IGF-I (DeChiara et al. 1990, Baker et al. 1993, Liu et al. 1993, Powell-Braxton et al. 1993). The disruption of the IGF1R has more severe consequences, and results in postnatal death of animals which are approx. 45% of normal weight (Baker et al. 1993). On the contrary, disruption of the IGF-II/M-6-P receptor in KO mice results in overgrowth and perinatal lethality, which might be due to impaired lysosomal enzyme trafficking combined with decreased degradation of IGF-II, and possibly also IGF-I, by IGF-II/M-6-P receptor (Seta et al. 1993, Lau et al. 1994).

Several OE or KO models of IGFBPs have been developed over the last decade. The KO studies have been somewhat disappointing with only minor effects in the phenotype (Pintar et al. 1996). It seems reasonable to speculate that other IGFBPs compensate for the functions of the missing counterpart, and prevent dramatic phenotypic changes (Schneider et al. 2000). On the contrary, overexpression of IGFBPs in transgenic mice has resulted in characterization of various expected and unexpected phenotypes (Schneider et al. 2000). To date, several transgenic mouse lines overexpressing IGFBP -1, -2, -3, and -4 have been produced, while the effects of IGFBP-5 and -6 overexpression remain to be clarified. Overall, the phenotypic changes of the existing IGFBP transgenic mice are varied depending on the mouse strain, differences on the gene construct, and expression profile of the transgene. The changes include impairment of glucose metabolism and organ specific weight and cellular structure alterations (Schneider et al. 2000). Thus, the transgenic studies have not only confirmed the importance of IGFBPs as regulators of IGFs activity, but also revealed the complexity of the IGF and IGFBP related interactions (Schneider et al. 2000).

Overall, the actions of the IGF system have been shown to be essential to the normal development of the nervous system (D´Ercole et al. 1996, Stewart and Rotwein 1996), and the expression patterns of the members of mammalian IGF system have been thoroughly characterized (Bondy 1991, Bondy and Lee 1993, Schuller et al. 1994, D´Ercole et al. 1996, Feldman et al. 1997). Interestingly, many of the transgenic models overexpressing IGFBPs have exhibited abnormal and impaired brain development (Schneider et al. 2000). For example, overexpression of IGFBP-1 causes significant reduction in the brain weight and alters the brain development and morphology similarly to IGF-I or IGF1R KO mice (Ye et al. 1995a, Ye et al. 1995b, Gutierrez-Ospina et al. 1996). Based on studies on the IGF system in the brain, it is known that brain is not a site of major IGFBP-1 expression; thus the functional role of IGFBP-1 in brain is largely unknown (Schuller et al. 1994). Since the IGFBP-1 levels in circulation of OE mice are not markedly increased, the alterations in the brain are likely to be a direct and specific result of the inhibitory effect of high expression levels of IGFBP-1 on the essentially important activity of IGF-I in the brain (Schneider et al. 2000). The main features of IGF system transgenic models are summarized in table 1.

Table 1. IGF system OE and KO mice; effects on brain development and function.

Type of alteration	Perinatal viability / effect on growth	Effect on brain
IGF-I KO	0-70% / 60 % birth weight	Cell density ↑ Oligodendrocyte no. and myelin ↓ Dentate gyrus granule cell no. ↓
IGF1R KO	0% / 45% birth weight	Cell density ↑ Oligodendrocyte no. and myelin ↓ Dentate gyrus granule cell no. ↓
IGF-I OE	normal / 130% adult weight	Weight of most regions ↑ Myelin ↑ Oligodendrocyte no. ↑ Neuron and neurite no. ↑, cell size ↑ (cerebral cortex) Cerebellar weigth ↑↑, granule (182%) and Purkinje (120%) cell no. ↑ Spinal cord weight ↑
IGFBP-1 OE	normal / 83-92 % adult weight	Abnormal brain development: brain weight ↓ dilation of ventricles disorganization and atrophy of cortex, hippocampus and corpus callosum
IGFBP-2 OE	normal / 90% adult weight	brain weight (↓)

Modified from: (Rosenfeld and Roberts 1999, Schneider et al. 2000).

Data from references: (Mathews et al. 1988, Behringer et al. 1990, Baker et al. 1993, Liu et al. 1993, Powell-Braxton et al. 1993, Schuller et al. 1994, Beck et al. 1995, Rajkumar et al. 1995, Ye et al. 1995a, Ye et al. 1995b, Gutierrez-Ospina et al. 1996, Pintar et al. 1996, Ye et al. 1996, Gay et al. 1997, Hoeflich et al. 1999, Schneider et al. 2000).

2.2.3. The IGF-insulin relationship

Although the main functions of IGFs and insulin are clearly distinguishable (i.e. IGF stimulates growth via IGF1R and insulin has effects on energy metabolism via the insulin receptor), they are still close relatives with important structural and functional similarities, which occur also in their receptors and signalling pathways (Cohick and Clemmons 1993). Moreover, they have a common ancestor, possibly an old serine protease, which has been important in the stimulation of tissue growth after food intake, with probably some “insulin-like activity” (Rinderknecht and Humbel 1978a, McRory and Sherwood 1997). Despite the ancient divergence, both insulin and IGF “remember”

their roots and display overlapping activities: IGFs have metabolic effects while insulin can stimulate growth (Froesch and Zapf 1985). Furthermore, they cross-react with each other's receptors; IGFs can act as weak insulin-like agonists and vice versa (Froesch and Zapf 1985, Lopaczynski 1999). Structural similarities between IGF-I and insulin include: 45% homology of the IGF-I A and B domain with the insulin A and B chain, the identical three dimensional structure and folding of IGF-I to pro-insulin, 50-60% overall homology in the corresponding receptors, a common mode of receptor activation by tyrosine kinase activation and many shared signalling molecules downstream of the receptors (Froesch and Zapf 1985, Rosenfeld and Roberts 1999).

Even though insulin and IGFs possess many similarities, there are also several important differences. The mature molecules are structurally different: the C peptide is cleaved away during insulin synthesis, and furthermore, insulin does not contain the D-domain, which is found in IGF-I (Froesch and Zapf 1985, Lopaczynski 1999). The corresponding receptor affinities to IGF-I and insulin differ by one to two orders of magnitude, enhancing the binding of the "original" ligand (LeRoith et al. 1995). While insulin is produced mainly by the pancreatic β-cells, IGFs are produced in most tissues with liver being the main site of production (Rosenfeld and Roberts 1999). Also the way of production is different: insulin is produced in a pulsatile fashion, while IGF-I production is more or less constant and slow (Froesch and Zapf 1985, McRory and Sherwood 1997). An important difference is that IGFBPs bind to IGFs with considerably higher affinity than to insulin (Collett-Solberg and Cohen 2000). Actually, until the discovery of the IGFBPrPs, no IGFBP was believed to bind insulin, but later, the IGFBPrPs were shown to bind both insulin and IGFs, and furthermore, it has been shown that a weak insulin binding affinity exists even in the original IGFBPs (Yamanaka et al. 1997).

2.2.4. The IGF system in the CNS

IGFs have been shown to have a plethora of growth promoting and functional effects on neuronal cells *in vivo.* IGFs are known to have effects on proliferation, differentiation, maturation of neuronal and glial precursors, and also on survival of many types of mature neurons (de Pablo and de la Rosa 1995, D´Ercole et al. 1996). Therefore, the classical definition of IGFs as mediators of growth hormone action (somatomedins), and insulin as a postnatal metabolic regulator, are far too narrow to describe their actions in the central nervous system, and other organs as well (de Pablo and de la Rosa 1995). In fact, insulin and IGFs have been shown to be active in the brain in a surprisingly varied and significant fashion. IGFs and their signaling components have been found to be an essential part of the correctly developing and functioning CNS (Folli et al. 1994, de Pablo and de la Rosa 1995, D´Ercole et al. 1996). The IGF-I expression in the rat brain begins early during the embryonic development and peaks at (E) day 14 (Bondy 1991, Rosenfeld and Roberts 1999). Subsequently, during later life, the expression of IGF-I and IGF1R decreases, but still remains in many brain areas, such as the cerebral cortex, hippocampus, olfactory bulb and striatum (Garcia-de Lacoba et al. 1999). Most of the IGF system components are synthesized in the CNS, by both neurons and glial cells, although they have cell specific expression profiles and localization. The expression pattern of IGFBPs often follows, both temporally and spatially, the IGF-I expression (Bondy and Lee 1993). In addition, both insulin and

IGFs can also cross the blood brain barrier with the assistance of transport proteins (Reinhardt and Bondy 1994). The IGFBP binding affinity in the plasma can be reduced, for example, by partial proteolysis of the IGFBP in the 150 kDa complex, which subsequently leads to an enhanced entry of IGF into the brain capillary endothelial cells. However, IGFs in the circulation are not the major source of IGFs in the CNS (D´Ercole et al. 1996).

A growing body of evidence indicates that the expression of IGFs and IGFBPs in the CNS changes in response to injury (Beilharz et al. 1993, D´Ercole et al. 1996, Saatman et al. 1997, Hammarberg et al. 1998, Hughes et al. 1999, Walter et al. 1999). However, even though the IGFs are expressed at higher levels, the adjoining expression of the IGFBPs may abate the protective effects. Therefore, it has been suggested that the displacement of IGFs from their binding proteins could be used as a treatment for stroke, liberating the endogenously produced neuroprotective IGFs (Loddick et al. 1998). However, the activities of the IGFBPs are still largely unknown and require further characterization in order to efficiently use the IGFs (either endogenous or exogenous) as neuroprotective drugs.

IGFBP-2, IGFBP-4, and IGFBP-5 are the most prominently expressed IGFBPs in the brain (Bondy and Lee 1993, D´Ercole et al. 1996). They are expressed in many areas of the brain, but each IGFBP has its own expression pattern. IGFBP-2 and -5 are expressed already in the floor plate of the developing rodent embryo at E4. The areas of postnatal expression for IGFBP-2 are in the cerebellum and pituitary. IGFBP-5 is expressed in the olfactory bulb, thalamus, and hippocampus, as well as cerebellum and pituitary (de Pablo and de la Rosa 1995). IGFBP-5 is expressed in parallel with IGF-I, especially during development (Bondy and Lee 1993, Cheng et al. 1996). Interestingly, changes in IGFBP-5 expression have been implicated in some pathological conditions in the CNS: the enhanced expression of IGFBP-5 in the cerebellum of weaver mice is thought to cause inhibition of IGF-I growth promoting activity as well as abnormal granule cell development (Lee et al. 1995). Although IGFBP-3 is the most common IGFBP in the serum, there is little evidence for the actions of IGFBP-3 in the CNS. However, IGFBP-3, and also IGFBP-5 are known to be upregulated as a consequence to hypoxic-ischemic injury (Gluckman et al. 1992, Beilharz et al. 1993, Lee et al. 1999).

In several studies on non-neuronal cells, IGFBPs have been shown to either inhibit or augment the action of IGFs. For example, overexpression of IGFBP-5 in yeast inhibits IGF-I and IGF-II stimulated DNA and glycogen synthesis (Kiefer et al. 1992). On the contrary, when IGFBP-5 is associated with the extracellular matrix, the binding affinity of IGFBP-5 to IGF-I is decreased, releasing IGF-I and thereby potentiating the effect of IGF-I on fibroblast proliferation (Jones et al. 1993). The actions of IGFBPs in the CNS remain largely unknown.

There are additional findings, which show that the insulin receptors in the periphery and CNS exhibit both structural and functional differences. When combined with the recent evidence on insulin being produced also locally in the brain and transported in the cerebrospinal fluid (CSF), this all points to a special role for insulin in the brain (Devaskar et al. 1994, van Dam et al. 2000, Zhao and Alkon 2001). In fact, both insulin and IGF have been shown to have a role in cognition, learning and memory (Saatman et

al. 1997, Markowska et al. 1998, Wickelgren 1998, Zhao et al. 1999, van Dam et al. 2000, Zhao and Alkon 2001). Interestingly, IGF signaling components have also been shown to be expressed in the CNS synapses (Abbott et al. 1999). These data implicates that IGFs have role in neuronal synaptic transmission.

Another intriguing detail is that insulin degrading enzyme (IDE) functions as the principal degrading enzyme for β-amyloid (Aβ), the major constituent of one of the neuropathological hallmarks of Alzheimer's disease (AD), amyloid plaques (Qiu et al. 1998). Moreover, glucose metabolism and energy metabolism are common factors that link the glucose dependent brain and insulin; the role of impaired glucose metabolism in AD may be of special importance. This gives insulin/IGF system a new importance in AD research (Wickelgren 1998). Taken together, these findings indicate that the IGF system has an important role in the CNS, during both normal situations and neurodegenerative diseases.

2.2.5. IGF1R mediated signaling

IGF1R belongs to the classical group of type 2 tyrosine kinase membrane receptor family (Ullrich and Schlessinger 1990). Initially, IGF-I binds to the extracellular α-chains of the disulfide-linked heterotetrameric (αββα) IGF1R (LeRoith et al. 1995). In theory, if it were present in high amounts (*in vitro* conditions), insulin could also initiate receptor activation, but IGF1R has a 10 to 100 fold higher affinity to IGF-I than insulin, which explains the specificity of the signaling (LeRoith et al. 1995). IGF-I binding induces autophosphorylation of two intracellular β-chains of the receptor on tyrosine residues located within the highly conserved tyrosine kinase domain (Ullrich et al. 1986). To transmit the message from the cell surface into the cell, several downstream signaling molecules bind to the tyrosine-phosphorylated receptor. These include members of at least five major families of protein substrates: *1)* the GRB (growth factor receptor-bound protein) family, adaptor proteins containing a Src-homology (SH) 2 and SH 3 domains; *2)* the SHC-family, adaptor proteins with SH2 domains; *3)* the IRS (insulin-receptor substrate) family, adapter proteins with a PTB (phosphotyrosine binding) domain and multiple tyrosine-residue containing docking sites for other downstream proteins; *4)* the CRK family, adaptor proteins containing SH2 and SH3 domains; *5)* and p85, the regulatory subunit of phosphatidyl-inositol 3-kinase (PI-3K), an enzyme that phosphorylates intracellular lipids (Ullrich et al. 1986, Ullrich and Schlessinger 1990, LeRoith et al. 1995). These receptor substrates trigger signaling cascades that involve at least the activation of phosphatidylinositol-3 kinase (PI-3K) and Akt, as well as the Ras-Raf-MAPK pathway (LeRoith et al. 1995). The IGFIR signaling pathways are summarized in figure 2.

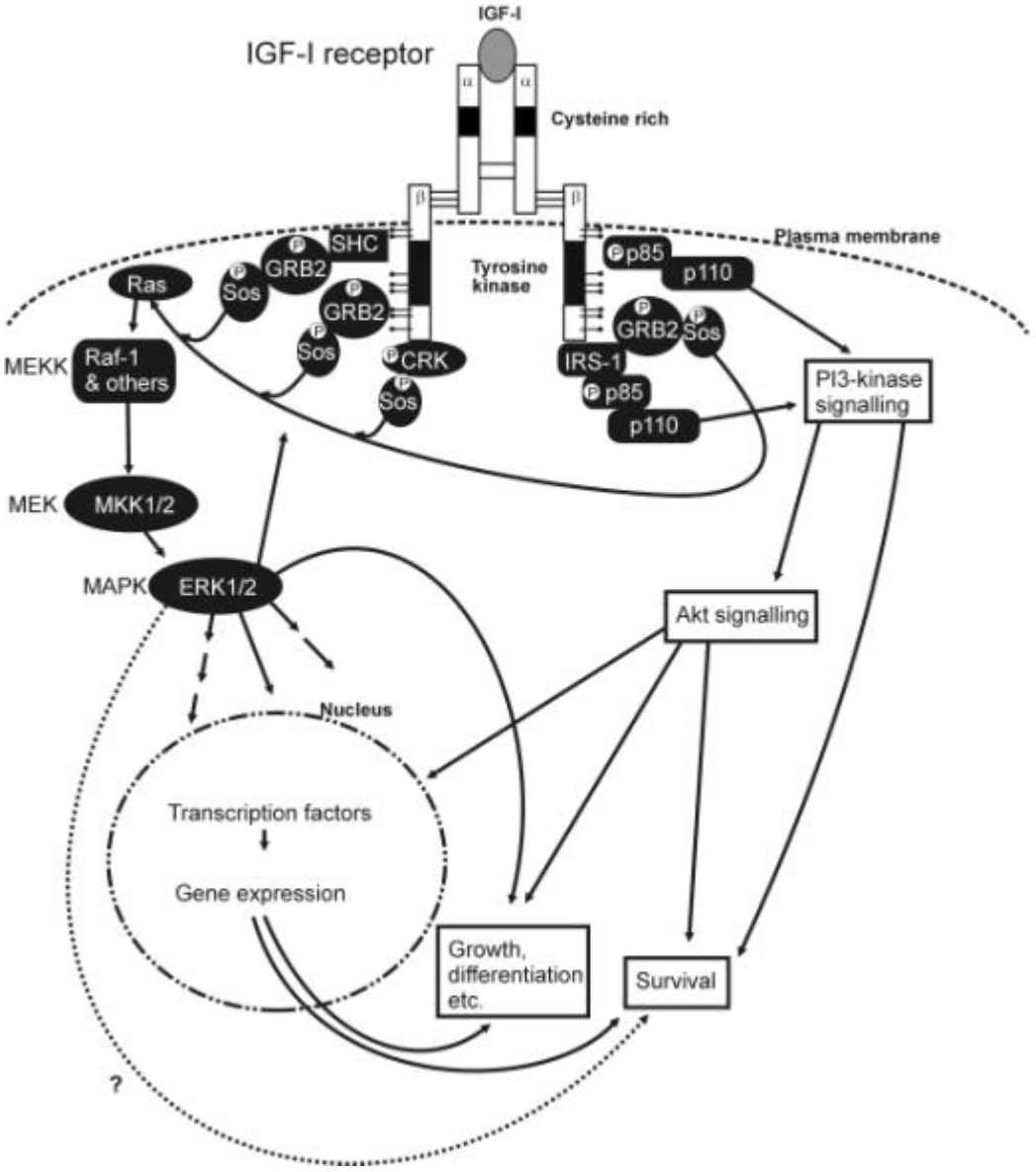

Fig. 2. The IGF-I signalling pathways (modified from LeRoith et al. 1995). The IGF binding to the IGF1R induces autophosphorylation of the receptor β-subunit at multiple tyrosine residues, and activation of the receptors intrinsic tyrosine kinase. The predominant substrate for IGF1R is the insulin-receptor substrate –1 (IRS-1), which is also the main substrate for IR. IRS-1 acts as a docking protein, which can bring together and thereby regulate the activity of many SH2 domain-containing proteins, such as the regulatory p85 subunit of PI-3K and Grb2. Activation of IGR1R also results in phosphorylation of SHC, which also complexes with Grb2 associated with the mammalian guanine nucleotide exchange factor Sos, which activates Ras. This leads to the activation of the Raf-1 - MAPK – ERK –pathway and change in the expression of early response genes. Activated PI-3K generates 3' –phosphorylated phosphoinositides (PI3,4P and PI3,4,5P), which function as downstream signaling molecules. PI-3K signaling results, for example, in the activation of Akt. The CRK-family of SH2 and SH3 domain-containing adaptor proteins are also substrates for IGF1R. When phosphorylated, they interact with Sos and thereby also activate the Ras-Raf-MAPK –pathway.

MAPK-pathway. IGF1R-bound and tyrosine-phosphorylated and thereby activated, Grb2 is known to recruit and to associate with mSos, which is a guanine nucleotide exchange protein involved in converting inactive Ras-GDP into active Ras-GTP (Egan et al. 1993, Olivier et al. 1993, Rozakis-Adcock et al. 1993). Ras is an important mediator of growth factor dependent cell survival (Downward 1998), which subsequently conveys the signal further to Raf-family of serine / threonine protein kinases. This leads to activation of kinase cascades, which ultimately promote the activation of kinases belonging to the mitogen-activated protein (MAP) kinases (Boguski and McCormick 1993, Crews and Erikson 1993). The MAP kinases (MAPKs) are serine / threonine protein kinases e.g. ERK1/2 (extracellular signal-related kinase 1/2), which are activated by MAP/ERK kinases (MEKs), which in turn is activated by MEK kinase (MEKK), such as Raf (Su and Karin 1996, Robinson and Cobb 1997, Karin 1998). The MAPKs consist of several subfamilies such as ERK, JNK/SAPK (c-Jun amino-terminal kinase / stress-activated protein kinase), and p38. They act in distinct and independent signaling pathways with a wide range of cellular responses including proliferation, differentiation and survival (Cross et al. 2000). The important pro-survival and mitogenic pathways activated by the IGF1R include signaling through a MAPK, p42/p44 extracellular signal-related kinases, ERK 1 and 2 (Boulton et al. 1991). The signaling through ERK 1 and 2 have a major role in the stimulation of cell proliferation; they have been shown to be translocated to the nucleus and induce gene expression that promotes the cell cycle entry (Greulich and Erikson 1998). Whether the signaling through the p42/p44 ERK pathway involves pro-survival mechanisms other than stimulation of cell proliferation is currently under investigation. There is evidence of direct regulation of apoptosis by ERK (downstream of b-Raf) through cytosolic caspase inhibition (Erhardt et al. 1999). In neuronal cells, the MAP-activated pp90 ribosomal S6 kinase family members, Rsks, are known to promote neuronal survival by enhancing the expression of pro-survival genes through CREB (cAMP response element-binding protein). They also promote survival by directly phosphorylating and thereby inactivating the pro-apoptotic bcl-2 family member, BAD (Bonni et al. 1999). In neuronal cells, the Ras-MAPK pathway is known to activate gene expression needed for neurite outgrowth and also to have a role in the stimulation of protein synthesis involved in memory formation (Frank and Greenberg 1994, Doherty et al. 2000, Rosenblum et al. 2000, Sweatt 2001).

The other MAPK members, p38 and JNK/SAPK represent signaling pathways homologous to the Ras-MAPK pathway, which are involved in the regulation of cellular responses to stress (Kyriakis and Avruch 1996). These pathways, in contrast, are not activated primarily by mitogens, but instead by various kinds of cellular stress and inflammatory cytokines, and result in apoptosis (Kyriakis and Avruch 1996).

PI-3K - Akt pathway. The IRS-1 is the predominant substrate of the IGF1R and IR, and initiates a very important signaling cascade involved in neuronal survival promoted by the IGFs. The IRS-1 is considered to be a “docking protein” that brings the receptor complex together with certain SH2-domain containing proteins, such as the PI-3K (Myers et al. 1992). The SH2 domain in the adapter / regulatory subunit p85 of PI-3K activates the PI-3K catalytic p110 subunit, and brings the PI-3K adjacent to the cellular membranes, where the PI-3K substrates are located (Shepherd et al. 1998). The PI-3K recruited to the inner surface of the plasma membrane generates 3’-phosphoinositides

by phosphorylating the 3-position in the inositol ring (Alessi and Downes 1998). The 3'-phosphorylated phospholipids are capable of activating a number of cellular proteins including tyrosine and serine /theonine kinases (Shepherd et al. 1998, Rameh and Cantley 1999). One crucially important protein kinase activated by PI-3K is the serine/threonine protein kinase Akt / protein kinase B (PKB) (Datta et al. 1997, Coffer et al. 1998, Datta et al. 1999). Akt was initially identified as the cellular homolog (c-Akt) of the transforming oncogene of the AKT8 retrovirus. In addition to c-Akt, at least two other differentially expressed Akt family members are known to exist: c-Akt 2 and 3 (Staal 1987, Bellacosa et al. 1991). The activation of Akt can be facilitated by direct binding of the PI-3K generated phosphoinositides to the plekstrin homology (PH) domain of Akt (Franke et al. 1997). This is suggested to lead to the translocation of Akt from the cytoplasm to the cell surface and to conformational changes in Akt that render the phosphorylation sites essential for Akt activation accessible for 3-phosphoinositide dependent kinases (PDKs), which are located near the plasma membrane (Alessi et al. 1997, Datta et al. 1997, Alessi and Downes 1998, Datta et al. 1999). In addition to PI-3K kinase related mediators; there are other factors that can also activate Akt, such as the protein kinase A (PKA) pathway, and calcium / calmodulin dependent kinase kinase (CaMKK) (Yano et al. 1998, Filippa et al. 1999).

Akt has several survival promoting effects, many of which include Akt phosphorylation on a specific Akt consensus phosphorylation site in the corresponding substrates. At least Bad, caspase-9, the Forkhead family of transcription factors, and the NF-?B regulator IKK are direct targets of Akt phosphorylation (Datta et al. 1999). Akt phosphorylation is known to prevent the interactions of Bad with its targets in the mitochondria (Wang et al. 1999). Growth factors inhibit the mitochondrial cyt c release indicative of an important role for Bad phosphorylation in survival signaling (Datta et al. 1997, Kennedy et al. 1999). Despite the fact that Akt does not act like a generalized caspase inhibitor, caspase-9 is directly phosphorylated and thereby inhibited by Akt, which enables the inhibition of apoptotic cascade to be halted also downstream of cyt c release from the mitochondria (Datta et al. 1999, Khwaja 1999).

In addition to these post-transcriptional changes, Akt can also promote cell survival through regulating gene expression. For example, Akt negatively regulates the expression of pro-apoptotic FasL through inhibition of Forkhead transcription factors (FKHRs) (Tang et al. 1999). Interestingly, the three FKHR isoforms (FKHR, FKHRL1 /AF6q21, and AFX) are the mammalian homologues of the *C. elegans* DAF-16, which is involved in the regulation of the longevity of *C. elegans* (Lin et al. 1998, Ogg and Ruvkun 1998, Paradis et al. 1999). The insulin/IGF-I receptor pathway of *C. elegans*, DAF-2 pathway, is required for normal life span, reproductive growth and metabolism. Disruption of this pathway results in arrest at the dauer larvae stage and an increased life span (Kimura et al. 1997). The major target of this pathway in *C. elegans* has been shown to be the DAF-16/FKHR transcription factor. Interestingly, the induction of dauer larvae by DAF-2 disruption can be abolished by disruption of the DAF-16 gene (Honda and Honda 1999). These studies provide evidence on the importance on the IGF-1/Akt/ FKHR pathway not only in apoptosis, but also as a probable link between growth and metabolism and aging (Thomas and Inoue 1998). This link might be of major relevance in neuronal survival during both aging and neurodegenerative diseases. In addition, the IGF / PI-3K / Akt pathway is involved in the regulation of the anti-

apoptotic transcription factors, such as NF-κB, which is a ubiquitous transcription factor that promotes the transcription of many genes involved in survival, for example, the bcl-2 family members (Zong et al. 1999).

In addition, a number of other cellular signaling pathways involved in apoptosis are regulated directly by Akt. For example, phosphorylation and inhibition of glycogen synthase kinase -3β (GSK-3β) activity contributes to cellular survival (Cross et al. 1995). Interestingly, the GSK-3β has been shown to be involved in neuronal apoptosis induced by trophic factor withdrawal, and furthermore, GSK-3β overexpression induces apoptosis in cortical neurons (Hetman et al. 2000). These results suggest that the IGF-I-induced PI-3K/Akt pathway inhibits the GSK-3β activity in normal situations, and thereby promotes survival.

One interesting additional factor regulating the IGF−PI-3K−Akt pathway is the PTEN/MMAC-1/TEP-1 tumor suppressor (PTEN, phosphatase and tensin homolog deleted on chromosome ten, MMAC, mutated in multiple advanced cancers; TEP, TGF-β-regulated and epithelial cell-enriched phosphatase) (Li and Sun 1997, Li et al. 1997a, Steck et al. 1997). PTEN functions as both a protein and a lipid phosphatase (Maehama and Dixon 1998, Vazquez and Sellers 2000). It dephosphorylates also PI-3K-generated 3'-phosphoinositides, and thereby negatively regulates Akt signaling (Gu et al. 1998, Maehama and Dixon 1998, Myers et al. 1998). PTEN is known to be mutated in many types of human cancers, especially in glioblastomas (Li et al. 1997a, Liu et al. 1997, Steck et al. 1997). In addition to the genetic data on cancers, transgenic studies on the PTEN show that it is essential for normal development: homozygotic mutants die during embryogenesis (around E8), and heterozygotic mice exhibit increased tumor incidence (Di Cristofano et al. 1998, Suzuki et al. 1998, Podsypanina et al. 1999). It is known that normally Akt activity is low in the absence of growth factor stimulation. However, in cell lines or fibroblasts obtained from PTEN-deficient mutant mice, exceptionally high basal levels of phosphorylated/activated Akt have been observed (Stambolic et al. 1998). Furthermore, the $PTEN^{-/-}$ fibroblasts are resistant to apoptotic stimuli, but when normal expression of wild-type PTEN is reconstituted, the resistance is lost and normal Akt regulation is achieved (Myers et al. 1998, Stambolic et al. 1998). Since the PI-3K-Akt-PTEN pathway has been shown to be of major importance in many cancers, and because this signaling has been shown to be highly conserved, it may be one of the key contributors of the regulation of cellular survival in response to extracellular stimuli. Evidently, "both sides of the PTEN-coin" should be evaluated: in addition to cancers (i.e. diseases with a lack of apoptosis and excess survival), the opposite situations, such as neurodegenerative diseases (excess apoptosis and lack of survival) should be studied. There is some evidence of the role of PTEN in the regulation of PI-3K mediated actions in neuronal systems, where PTEN is shown to have multiple actions and to regulate the differentiation of neuroblasts into post-mitotic neurons (Lachyankar et al. 2000).

2.3. Studying the role of trophic support in neuronal culture models

While there are obvious benefits in studying intact nervous systems *in vivo*, there are some major drawbacks, which limit the use of these methods in certain kinds of molecular studies. Therefore, various *in vitro* models have been developed to study molecular events, for example, those involved in neuronal growth, survival and apoptosis. If used in an appropriate manner and in a sensible context, these models mimic the naturally occurring phenomena of biological events, and, the metabolic conditions and biological events can still be manipulated in a meaningful and controlled way.

One of the widely used in vitro models is the rat/mouse cerebellar granule cell (CGC) culture, in which the CGCs are prepared from the neonatal (P6-8) mouse/rat cerebellum (Levi et al. 1984). Commonly, the primary cultures of neuronal cells are prepared from either embryonic (E) or early postnatal (P) mouse/rat brain (Gallo et al. 1987). The timing of the preparation is crucial for the neuronal cells to continue growth in cell culture conditions (Levi et al. 1984, Levi et al. 1989). The granule cells are the most abundant cell type in the mammalian brain (20 million cells in P7 rat brain), and in culture, the CGCs form a homogenous (98%) population of neurons that can be cultured for 2-3 weeks using the appropriate culture conditions (D´Mello et al. 1993). When cultured from the postnatal rat/mouse brain, the cerebellar granule cells continue to differentiate, and they also acquire several characteristics of mature neurons, such as dendritic processes and glutamate receptors. In addition, they release specific neurotransmitters upon depolarization (Lasher and Zagon 1972, Levi et al. 1984). The CGCs require constant trophic support, which can be established during *in vitro* using a high extracellular potassium concentration (25 mM KCl) (Gallo et al. 1987, Koike et al. 1989). When the potassium concentration is lowered to a level that is close to the physiological concentration (5 mM KCl), the cells undergo apoptosis (D´Mello et al. 1993).

The mechanisms of the high potassium support CGC survival are thought to be similar to those activated by neuronal activity during embryogenesis, and thus, the mechanism by which potassium deprivation induces apoptosis is similar to naturally occurring phenomena of trophic deprivation-induced cell death (Levick et al. 1995). Depletion of growth factors is considered to result in an increase in the amount of pro-apoptotic effectors in proportion to pro-survival effectors, and subsequently lead to apoptosis (Dudek et al. 1997). A high concentration of potassium causes also an elevation in the cytoplasmic Ca^{2+} concentration. According to the "Ca^{2+} set-point hypothesis" of neuronal survival and dependence on neurotrophic factors, an appropriate Ca^{2+} level that is related to the amount of other neurotrophic support is necessary for the survival of neurons (Franklin and Johnson 1992). The mechanisms of elevated potassium-mediated survival are considered to be mediated through calcium-initiated signaling (D´Mello et al. 1997), in which the high potassium concentration-induced depolarization is thought to induce calcium influx through a voltage-dependent calcium channel (Gallo et al. 1987). The calcium influx can affect the cellular survival through an activation of

intracellular kinases, and possibly, subsequent activation of PI-3K (Rosen et al. 1994, Franke et al. 1997). Evidence for the role of IGF-I / PI-3K in CGC apoptosis induced by low potassium comes from studies which show that neurotrophic factors, such as IGF-I, are able to prevent potassium deprivation-induced apoptosis in CGCs (Galli et al. 1995). The signaling mechanisms of these factors in the promotion of CGC survival are thought to require PI-3K activation at least in IGF-mediated survival signaling (D´Mello et al. 1993, Galli et al. 1995, Lin and Bulleit 1997). The high-potassium-induced and calcium-mediated survival pathways can also initiate the production of trophic factors, which may in turn activate survival signaling through an autocrine loop (Ghosh et al. 1994, Ono et al. 1997). However, there is some contradictory data on the role of PI-3K in high-potassium-mediated survival. Other studies show that high-potassium-induced survival is dependent on PI-3K (Shimoke et al. 1997), while evidence of PI-3K independent pathways in high-potassium-promoted survival have also been proposed (Galli et al. 1995, D´Mello et al. 1997). These discrepancies might be explained by differences in the experimental cell culture conditions used (Shimoke et al. 1997).

The pathways involved in the induction of apoptosis by a depletion of extracellular stimulation by lowering the potassium concentration and subsequent decrease in the intracellular calcium concentration are largely unknown. However, it is known that apoptosis induced by low potassium in CGCs is inhibited by RNA and protein synthesis inhibitors (D´Mello et al. 1993, Schulz et al. 1996). Thus, the neosynthesis of some “killer proteins”, which remain currently unknown, is needed for the apoptotic process. Furthermore, neurons subjected to PI 3-kinase blockade, and subsequently induced apoptosis, can be rescued by transcriptional and translational inhibitors, suggesting that IGF-1-mediated activation of PI 3-kinase leads to a suppression of "killer gene" expression (D´Mello et al. 1997). It is known that the levels of c-jun mRNA are increased 2-3 h after potassium deprivation, with a subsequent increase in c-jun protein (Miller and Johnson 1996, Watson et al. 1998). Furthermore, it is known that c-jun activates the expression of FasL in CGCs, but the downstream mechanisms of this induction in CGCs remain unknown (Le-Niculescu et al. 1999). The phosphorylation of c-jun protein is known to be essential for the low-potassium-induced apoptosis (Watson et al. 1998). However, the SAPK and p38 pathway that in other systems activate c-jun phosphorylation, are not activated in CGCs during potassium deprivation (Watson et al. 1998). This may indicate that c-jun is phosphorylated by some other still unknown kinase, and that this phosphorylation activates the production of the actual regulators and effectors of potassium deprivation-induced apoptosis (Watson et al. 1998).

3. AIMS OF THE STUDY

Many of the apoptotic stimuli not only activate various signaling pathways, but also induce gene expression. Some of the apoptosis-responsive “death genes” are known, but there still is a gap in our knowledge of specifically neuronal apoptosis related genes, since the majority of studies on apoptotic gene expression have been carried out on non-neuronal systems. To unravel some of the molecular events during neuronal apoptosis, we have employed a screening method, the differential display (DD) technique, to detect changes in gene expression during neuronal apoptosis. We employed a well-known and widely used model, the potassium deprivation-induced apoptosis in rat cerebellar granule cells.

The specific aims of this study were:

Study I**:**
- to establish a differential display method to study gene expression in neuronal apoptosis and to identify promising candidate genes for further studies

Study II:
- to characterize the PTZ-17 RNA expression during neuronal apoptosis

Study III:
- to study how insulin-like growth factor binding protein -5 and type -1 insulin-like growth factor receptor are regulated during neuronal apoptosis

Study IV:
- to identify the expression patterns of IGFBP-2,-3 and -5 in neuronal apoptosis

Study V:
-to study whether apoptotic inducers affect the neuronal PTEN expression

4. MATERIALS AND METHODS

4.1. Cell culture

4.1.1. Cerebellar granule cells

Primary cultures of cerebellar granule cells (CGCs) were prepared from isolated cerebella of 7-day-old Wistar rats; 20 rats were used at each time. The rat was decapitated and the cerebellum was detached with Dumont-style forceps and placed on a petri dish containing PBS-BSA-glucose buffer solution (Levi et al. 1989). The meninges and blood vessels were removed carefully under a dissecting microscope using Dumont-style forceps. The purified cerebellum was transferred to another petri dish containing PBS-BSA-glucose buffer containing 1.2 mM $MgSO_4$. This petri dish and the purified cerebella were kept on ice until all of the 20 cerebella were prepared.

The cerebella were cut into small pieces (~ 0.4 × 0.4 mm cubes) using a sterile surgical knife in ~3 ml of buffer on the petri dish. The pieces were then transferred into a sterile 50 ml conical tube using a 5 ml plastic pipette in a volume of 2.5 ml of buffer / cerebellum. To collect the pieces, the tube was centrifuged briefly with a Megafuge 1.0 at 150 g (900 rpm) for 10 seconds.

The supernatant was removed and the pellet was gently resuspended in PBS-BSA-glucose buffer containing 1.2 mM $MgSO_4$ and 0.25 mg/ml trypsin, and incubated in a shaking water bath at + 37 °C for 15 min. After incubation, a solution (2.5 ml / cerebellum) containing PBS-BSA-glucose buffer supplemented with 1.2 mM $MgSO_4$ and 12.8 µg/ml of DNase I and 83 µg/ml of soybean trypsin inhibitor was added to the trypsin solution to inhibit DNA precipitation and the trypsin activity. The tube was immediately centrifuged at 150 g to pellet the pieces and the supernatant was removed with a pipette.

The pieces were resuspended (2.5 ml / cerebellum) in a PBS-BSA-glucose buffer supplemented with 2.4 mM $MgSO_4$ and 80 µg/ml of DNase I and 520 µg/ml of STI. The solution was triturated gently with a 5 ml pipette ~ 25 times. To allow the supernatant to sediment the suspension was allowed to stand for 15 min. The supernatant (containing the dissociated cells) was transferred into a new 50 ml tube and triturated for a second time for 25 times. Again the supernatant was carefully collected and combined with the first supernatant. The tube was centrifuged at 150 g for 5 minutes and the supernatant was carefully removed. The remaining cell pellet was suspended in cell culture medium (Dulbecco's modified Eagle's medium, DMEM, (Sigma, St. Louis, MO) containing 4.5 mg/ml glucose) and the cell density was counted using a Bürker's hemocytometer. The average yield per cerebellum was 11-15 × 10^6 cells.

The granule cells were plated on 90 mm cell culture dishes coated with 10 µg/ml poly-D-lysine at a density of 2.3 × 10^5 cells/cm^2 and maintained at 37 °C, 10 % CO_2 for 7

days in DMEM (Sigma) containing 4.5 mg/ml glucose, supplemented with 10 % heat-inactivated (56 °C, 30 min) fetal bovine serum, FBS (10106-169, Life Technologies, Rockville, MD), 2 mM glutamine, 10 µM cytosine-β-D-arabinofuranoside (added 48 h after plating to inhibit the growth of non-neuronal cells), 100 U/ml penicillin, 100 µg/ml streptomycin, and 20 mM KCl.

4.1.2. Neuroblastoma cells

Mouse Neuro-2a neuroblastoma cells (CCL 131) were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in DMEM (Sigma) containing 1000 mg/l D-glucose and supplemented with 10% FBS (Life Technologies), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine.

4.2. Apoptosis models

4.2.1. Potassium deprivation

After 7 days *in vitro*, apoptosis was induced by switching to a fresh medium with no added KCl (D´Mello et al. 1993). As a control, fresh medium supplemented with 20 mM KCl was used after 7 days *in vitro*. To avoid the toxic effect of fresh serum (i.e. glutamate) to mature CGCs, dialyzed serum (F3260, Sigma) was used in the replaced medium (Schramm et al. 1990). Low and high KCl samples were taken at various time points during deprivation, with the first (0h) sample taken immediately after changing the medium. A control sample from untreated cells was taken in the end of the experiment.

4.2.2. Other models and drugs used

Apoptosis was induced with okadaic acid (Calbiochem San Diego, CA) in CGCs at final concentrations of 2.5 - 40 nM. Staurosporine (Sigma) was used for CGCs at a final conc. of 50 nM. Actinomycin D (Sigma) was added to the plates at a final conc. of 1 µg/ml. For mouse neuroblastoma cells, etoposide (Calbiochem) was used at a final concentration of 5 µM. Human recombinant IGF-I was used at a final concentration of 25 ng/ml (Roche Molecular Biochemicals, Indianapolis, IN) To control plates, an equal concentration of the corresponding solution without the drug was added. The experiments were started at 7DIV in CGCs.

4.2.3. Analysis of neuronal apoptosis

The level of caspase-3 activity, and the release of lactate dehydrogenase were assayed, in order to determine the time course and quantity of cell death in cerebellar granule cells. The activities of caspase-3 were assayed from the cytosolic extracts using fluorogenic substrate Ac-DEVD-AMC from BD Biosciences - PharMingen (San Diego, CA). The assays were performed according to the manufacturer's protocol. LDH leakage to the medium was assayed using a cytotoxicity kit obtained from Promega (Madison, WI).

4.3. RNA isolation and cDNA synthesis

Total RNA was isolated using TRIzol® reagent (Life Technologies). Culture dishes were washed once with 10 ml of ice-cold PBS, after which the cells were detached using a cell scraper and homogenated in 2 ml of TRIzol®. The homogenates were mixed by repeated pipetting and stored at -70 °C in 1.5 ml tubes.

RNA was isolated according to the TRIzol® protocol, dissolved in 20 µl of diethylpyrocarbonate (DEPC) treated water, and pooled when necessary. To avoid degradation of RNA by RNases, DEPC-treated reagents were used in all subsequent steps involving RNA. The RNA concentration was measured spectrophotometrically at 260 nm in TE buffer. Three µg samples were stored at −70 °C to subsequently check the quality of the RNA.

The volume of a 2–3 µg RNA sample was adjusted with formamide to 9 µl, and 1 µl of EtdBr (1 µg/µl) and 2 µl of 6× loading buffer were added. The samples were denatured at 65 °C for 5 min and loaded into a 1 % agarose gel containing 0.5 µg/ml EtdBr and run in 0.5× TBE buffer at 5 V/cm for 45 min. The RNA was visualized on a UV transilluminator (λ = 312 nm).

To remove genomic DNA contamination, 50 µg of RNA was treated with 10 U of RNase-free DNase I (Promega) in the presence of 50 mM Tris pH 7.5, 1 mM $MnCl_2$, 5 mM DTT, and 1 U/µl RNasin (Promega) at 37 °C for 30 min. TE-buffer (pH 7.5) was added up to 250 µl, and the RNA was purified by extraction with phenol-chloroform (3:1) and chloroform. The RNA was precipitated from the aqueous phase by adding sodium acetate up to 0.3 M and 2.5 V of 100 % ethanol. The RNA pellet was redissolved in 20 µl of DEPC-treated H_2O and the RNA concentration was measured with a spectrophotometer. Three µg samples of DNase-treated and untreated RNA were electrophoresed in a 1 % agarose gel to check the integrity of RNA.

Each RNA sample was reverse transcribed using three different anchored 3' primers (5'-AAGCT_{11}V, V = A, C, or G). For the reactions, 1 µg of DNase-treated RNA was diluted to 0.1 µg/µl in DEPC-treated H_2O. Then 2 µl of diluted RNA was mixed with 4 µl of 5× MMLV buffer, 2 µl of 0.1 M DTT, 2 µl of 200 µM 4dNTP mix, and 2 µl of 10 µM anchored 3' primer in a total volume of 19 µl. The mixture was incubated at 70 °C for 5 min to denature the RNA and quick chilled on ice for 1 min, microcentrifuged at 10 000 g for 10 seconds, and incubated for 2 min at 37 °C to allow primer annealing. One µl of 200 U/µl MMLV reverse transcriptase (Promega) was mixed into each tube, and the reactions were incubated at 37 °C for 60 min. Duplicate reactions were made for each RNA and 3' primer to exclude PCR artifacts in the subsequent DD-PCR step. As a genomic DNA (gDNA) control, enzyme was omitted from one reaction to check for residual gDNA contamination in DD-PCR. To inactivate the reverse transcriptase, the samples were incubated at 95 °C for 5 min, then microcentrifuged at 10 000 g for 10 seconds, and placed on ice or stored at –20 °C for later PCR amplification.

4.4. Differential display

For each cDNA prepared with a particular 3' primer, DD-PCR was carried out using 8 different arbitrary 5' primers, resulting in a total of 24 (3 × 8) different primer combinations per RNA sample (see table 2 for sequences). The 20 µl reaction mixtures included: 2 µl of cDNA, 2 µl of 2 µM 3' primer, 2 µl of 2 µM 5' primer, 2 µl of 20 µM dNTP mix, 0.5 µl of 2 U/µl DyNAzyme II thermostable DNA polymerase (Finnzymes, Espoo, Finland), 2 µl of 10× optimized reaction buffer, and 1 µCi of [α-^{33}P]dCTP (3000 Ci/mmol, Amersham Pharmacia Biotech, Piscataway, NJ or PerkinElmer Life Sciences Boston, MA). To avoid pipetting errors, a master mix was prepared containing all reagents except the 3' and 5' primers and cDNA. The master mix was divided into three parts, to which the three 3' anchored primers were added. These three premixes were aliquoted into PCR tubes, and the 5' arbitrary primer and cDNA prepared with the respective 3' primer were added.

Table 2. DD-PCR primer sequences.

Primer	Sequence
3' primers	
RT1	AAG CTT TTT TTT TTT A
RT2	AAG CTT TTT TTT TTT C
RT3	AAG CTT TTT TTT TTT G
5' primers	
DD1	AAG CTT GAT TGC C
DD2	AAG CTT CGA CTG T
DD3	AAG CTT TGG TCA G
DD4	AAG CTT CTC AAC G
DD5	AAG CTT AGT AGG C
DD6	AAG CTT CAT TCC G
DD7	AAG CCT GAT TGC C
DD8	AAG CTT TAG GCA C

As a control, the "gDNA" sample from the reverse transcription step was used as a template to check for residual genomic contamination. In addition, reactions without a template were included as a control of PCR product contamination. Temperature cycling was carried out in an Uno II thermocycler (Biometra, Gottingen, Germany) using the following conditions: initial denaturation at 94 ºC for 5 min, 40 cycles of 94 ºC for 30 s, 42 ºC for 60 s, 72 ºC for 30 s, and final extension at 72 ºC for 5 min.

After thermocycling, 3.5 µl of PCR product was mixed with 2 µl of formamide loading buffer (90 % formamide, 0.05 % xylene cyanol, 0.05 % bromophenol blue) and incubated at 80 °C for 2 min. The samples were loaded into a 6 % denaturing polyacrylamide gel (Ausubel et al. 1987) (Sequi-Gen® GT, BioRad, Hercules, CA), which had been prerun for 1 hour to reach a temperature of ~50 °C. The gel was run at 60 W for ~3 h until the xylene cyanol dye reached 10–15 cm from the bottom of the gel. After electrophoresis, the gel was dried on Whatman filter paper under vacuum at 60 °C for 3 h. The corners of the dried gel were marked with radioactive dye (prepared by mixing 1 µl of PCR reaction with 10 µl of loading buffer) for accurate alignment of the

gel with the film, after which the gel was wrapped in polyethylene film and exposed to BioMax MR film (Scientific Imaging Systems, Rochester, NY) overnight.

DD analysis was performed twice starting from the duplicate cDNA templates, and the results were compared in order to confirm that the observed differences were not due to PCR artifacts.

After the film was developed and differentially expressed bands were identified, the bands of interest were marked on the gel and excised. They were placed into sterile microcentrifuge tubes. To check that the bands had been correctly excised from the desired positions, the gel was re-exposed to X-ray film.

TE buffer (200 µl, pH 7.5) was added to the excised bands, after which the tubes were incubated at room temperature (RT) for 10 min and boiled for 20 min. The tubes were briefly microcentrifuged to remove gel and paper debris, and the supernatant was decanted into a fresh tube. Four µl of the eluted DNA was reamplified in a 40 µl PCR reaction using the same primers and PCR conditions as used in the DD-PCR step except that 20 µM dNTP instead of 2 µM and no isotope were used. A large dilution volume (200 µl) for the gel slices before the re-PCR is adopted to decrease the urea concentration to a level that does not inhibit amplification (Konecny and Redinbaugh 1997).

After amplification, 30 µl of each reaction was run in a 1.5 % agarose gel containing 0.5 µg/ml ethidium bromide (EtdBr). The reamplified cDNA bands were quickly located on a UV transilluminator (λ = 312 nm), and marked with a sterile scalpel in the gel to avoid UV damage to the DNA fragments. After switching off the UV light, the bands were cut out from the gel, and the DNA was recovered using QIAquick Gel Extraction kit (QIAGEN, Valencia, CA). The concentrations of the PCR products were determined by comparison to Low DNA Mass Ladder (Life Technologies) in a 1.5 % agarose gel.

4.5. Cloning and single-stranded conformational polymorphism -analysis

T-overhang vector was prepared from pBluescript II SK + cloning vector as described previously (Hadjeb and Berkowitz 1996). Briefly, 20 µg of pBluescript was digested with *Eco*RV, and T-overhang was added using Taq DNA polymerase (MBI-Fermentas, Hanover, MD) and dTTP. The T-tailed product was recovered by phenol-chloroform extraction and ethanol precipitation. The vector was subjected to self-ligation using T4 DNA ligase (Promega) to remove any remaining vector with no added T-overhangs. The self-ligated vector and a control sample of unligated vector were electrophoresed in a 1 % agarose gel and visualized with EtdBr. The linear T-tailed vector was purified from the gel using QIAquick Gel Extraction kit.

The purified, reamplified DD-PCR products were ligated into the pBluescript-T vector using a 1 : 1 insert to vector molar ratio. The ligation reaction consisted of 2–4 ng of insert, 50 ng of pBluescript-T, 1 µl of 10× T4 DNA ligase buffer, 3 U of T4 DNA ligase (Promega) in a total volume of 10 µl. Ligation was performed at +4 °C overnight.

Competent JM109 cells (≥ 10^8 cfu/µg, Promega) were transformed essentially according to the manufacturer's protocol. Briefly, 50 µl of JM109 cells were thawed and gently mixed with 2 µl of ligation reaction, and incubated on ice for 20 min. The cells were subjected to heat shock at 42 °C for 45 seconds and immediately placed on ice. SOC-medium (950 µl) was added and reactions were incubated at 37 °C for 1.5 h in a shaker incubator, after which 100 µl of transformation mixture was plated on X-gal-IPTG-ampicillin (XIA) plates for blue/white selection. The plates were grown overnight at 37 °C.

Ten randomly chosen white (recombinant) colonies per transformation were subjected to SSCP (single−stranded conformational polymorphism) analysis (Orita et al. 1989) in order to visualize different cDNAs originating from the same DD band. Bacterial colonies were directly analyzed using SSCP as follows: A colony was touched with a 10 µl pipette tip, inoculated on a fresh XIA plate in a 1 cm × 1 cm array by gently touching with the tip, and dipped and mixed into a PCR-reaction tube containing 1 U of DyNAzyme II thermostable DNA polymerase, 2 µl of 10× optimized reaction buffer, 200 µM of dNTP, 200 nM of T3 and T7 RNA polymerase promotor primers and 1 µCi of [α-^{33}P]dCTP (3000 Ci/mmol) in a volume of 20 µl. The primer sequences were: T3 = 5'-ATTAA CCCTC ACTAA AGGGA, T7 = 5'-TAATA CGACT CACTA TAGGG A. A "touchdown" PCR temperature profile was used: initial denaturation 94 °C for 5 min, 10 cycles of [94 °C for 30 s, 56 °C (−0.5 °C/cycle) for 30 s, 72 °C for 60 s] followed by 20 cycles of [94 °C for 30 s, 51 °C for 30 s, 72 °C for 60 s] and final extension 72 °C for 5 min.

After the colony-PCR, 2 µl of the PCR reaction was diluted 1:8 by mixing it with 5 µl of H_2O and 9 µl of loading buffer (90 % formamide, 0.03 % xylene cyanol, 0.03 % bromophenol blue, 20 mM EDTA). The samples were denatured at 85 °C for 5 min and quickly chilled on ice. Two µl of denatured sample was loaded into a 5 % non-denaturing polyacrylamide gel in a sequencing gel apparatus (Sequi-Gen® GT, BioRad) and run overnight at 650 V. After the run, the gel was dried under a vacuum at 60 °C for 1.5 h and exposed to BioMax MR (Kodak) film overnight.

4.6. Slot blot analysis

Clones originating from the same DD band (i.e. obtained from the same transformation) but producing different band patterns in SSCP analysis were selected for slot blot analysis. Inserts were amplified for slot blot analysis using colony PCR, starting from the XIA plates containing the randomly picked, arrayed clones. The PCR reaction conditions were the same as in the preceding SSCP step, except that no [α-^{33}P]dCTP was used. As controls, inserts from DD clones previously found to have constant expression in this cell culture system were amplified with the appropriate primers.

Two identical slot blots containing an array of denatured PCR products alkaline fixed on a positively charged nylon membrane were prepared as follows: 2 µl of PCR products were mixed with 400 µl of denaturation buffer containing 0.3 N NaOH, 1.5 M NaCl, and 5 mM EDTA, and incubated at RT for 10 min. Two filter papers and a Magnacharge (Osmonics, Minnetonka, MN) nylon membrane presoaked 10 min in H_2O were stacked in a Schleicher & Schuell Minifold II slot-blot apparatus (Schleicher &

Schuell, Keene, NH). 200 µl of each denatured PCR product was pipetted to the wells. After 30 min, the samples were transferred to the membrane with low vacuum, and the wells were rinsed with 300 µl of denaturation buffer. The membrane was removed from the apparatus and allowed to dry in air for 5 min, after which the membrane was rinsed 2 × 5 min in 0.1 M Tris pH 7.0 and once in H_2O. The duplicate blot was prepared identically. The membranes were not allowed to dry at any step.

Radioactive cDNA probes were prepared from apoptotic and control total RNA as follows: 4 µg of DNase-treated total RNA was reverse transcribed to cDNA in a 20 µl reaction containing 1 µl of 200 U/µl SuperScript II reverse transcriptase (Life Technologies), 4 µl of 5× reaction buffer, 2 µl of 0.1 M DTT, 1 µl of 3dNTP mix (10 mM each of dATP, dGTP and dTTP), 1 µl of 50 µM dCTP, 5 µl of [α-^{32}P]dCTP (10 µCi/µl, 3000 Ci/mmol), and 2 µl of 10 µM dT_{18} primer. The RNA was mixed with dT_{18} primer, denatured at 70 °C for 10 min and placed on ice for 1 min, after which 15 µl of premix containing all other reaction components except enzyme was added and mixed. After incubating at 42 °C for 2 min, 1 µl of enzyme was added. The reaction was incubated at 42 °C for 2 hours and terminated by denaturation at 70 °C for 15 min.

The probes were purified using ProbeQuant G-50 (Pharmacia) columns after adjusting probe volume to 50 µl with H_2O. Before and after purification, 1-µl samples were taken for measurement of label incorporation using a scintillation counter in Cerenkov counting mode. To remove the RNA strand from the cDNA-RNA heteroduplex, 5.5 µl of 10× RNA degradation solution (1.0 N NaOH, 10 mM EDTA) was added and the probes incubated at 65 °C for 30 min. The probes were neutralized by adding 55 µl of neutralization buffer (200 mM Tris pH 7.0, 100 mM HCl).

The membranes were transferred into hybridization bottles, equilibrated for 5 min in buffer containing 0.5 M (with respect to Na^+) sodium phosphate (pH 7.2) at 65 °C, and blocked in 10 ml of hybridization buffer containing 7 % SDS, 0.5 M sodium phosphate, 1 % BSA, and 1 mM EDTA at 65 °C for 2 hours.

The hybridization buffer was removed from the bottles and replaced with 10 ml of fresh, preheated hybridization buffer in which the purified probe had been mixed. The blots were hybridized overnight at 65 °C.

After hybridization, the membranes were briefly rinsed in 2× SSC / 0.1 % SDS, then washed twice for 45 min in 2× SSC / 0.1 % SDS at RT and twice for 45 min in 0.2× SSC / 0.1 % SDS at RT. The signal was detected and quantified using Storm PhosphorImager and ImageQuaNT software (Amersham Pharmacia Biotech Inc. - Molecular Dynamics Div, Piscataway , NJ).

To facilitate the selection of clones of interest, a graph was prepared showing the intensity ratio (i.e. apoptotic : control) of each signal pair from the two membranes. Intensity ratios which differed significantly from the ratios of the control signals were assumed to correspond to the differences seen in the DD gels. The respective clones were selected for sequencing and Northern analysis.

4.7. Sequencing

From the clones selected using slot-blot-analysis, 5 ml LB cultures were grown at 37 °C for 22 hours and plasmids extracted using Wizard® *Plus* SV Minipreps (Promega). The insert regions were sequenced by a commercial sequencing service (A.I.V. Institute, University of Kuopio) using the ALFexpress™ (Pharmacia) sequencing system. The clones were identified by comparing the sequences against the GenBank/EMBL nucleotide database using the BLAST 2.0 algorithm (Altschul et al. 1997).

4.8. Northern blotting

Northern blot analysis in studies I and II was performed essentially as described previously (Sambrook et al. 1989). Briefly, 10–20 µg of total RNA was separated in a 1 % agarose gel containing 1.23 M formaldehyde. The RNAs were blotted overnight onto a Magnacharge positively charged nylon membrane by downward capillary transfer. After transfer, the RNA was fixed with UV light (120 000 µJ/cm^2) (Stratalinker, Stratagene, La Jolla, CA) and stained with methylene blue to confirm equal loading and transfer efficiency (Ausubel et al. 1987). The membrane was incubated in hybridization solution (7 % SDS, 0.5 M sodium phosphate pH 7.2, 1 % BSA, 1 mM EDTA) at 65 °C for 2 hours in a roller bottle. To prepare a cDNA template for probe synthesis, 0.2 ng of plasmid from the appropriate clone was amplified with the T3/T7 primers in a volume of 50 µl. The PCR product was run in a 1.5 % agarose gel and purified using QIAquick Gel Extraction kit. A radioactive probe was prepared with the High Prime random primer labelling reagent (Roche Molecular Biochemicals, Indianapolis, IN) using 25 ng of template and 30–50 µCi of [α-^{32}P]dCTP (PerkinElmer Life Sciences, Boston, MA). The probe was filtered through a ProbeQuant G-50 column (Amersham Pharmacia Biotech), denatured at 98 °C for 5 min and added to fresh, preheated hybridization solution (0.1 ml/cm^2). The membrane was hybridized overnight (2 rpm) at 65 °C. Washes were performed as described previously (Ausubel et al. 1987), increasing the washing stringency when necessary, starting from 2× SSC / 0.1 % SDS at RT. The hybridization results were visualized and quantified using phosphorimaging.

In studies IV and V, PCR primers for IGF1R, 18S, IGFBP-2, -3 and -5 and PTEN were designed using Primer Detective 1.01 software (Clontech, Palo Alto, CA). The primers are represented in table 3. The corresponding fragments (except IGF1R and 18S) were generated by PCR and cloned into pGEM-T Easy vector (Promega), and verified by sequencing. Templates for in vitro transcription were prepared by PCR from recombinant plasmids using gene specific sense primers and modified M13 / pUC Universal and Reverse primers (depending on the orientation of the insert). IGF1R and 18S PCR-fragments were generated using composite primers and used directly for in vitro transcription after QIAquick purification (QIAGEN). For Northern blotting, equal amounts of 7/10/15 µg of total RNA were denatured and resolved in 1% agarose gel with 0.6 M formaldehyde. The RNA was transferred to Magnacharge nylon membrane (Osmonics), fixed using a UV-crosslinker (70mJ/cm^2) (Stratalinker 2400, Stratagene, La Jolla, CA). As a control of equal loading, the membranes were stained using methylene blue as decribed by Ausubel *et al* (1987).

Table 3. PCR primers used in studies IV and V (5'-3'):

GENE	SENSE	ANTISENSE	PROBE (bp)
IGFBP-2	ACGTTACGCTGTTACCCCAACC	GTGAACCCCATCATTCTCCTGC	624
IGFBP-3	CTCGCAGTAGTCAACCAAAAGC	GACCCCAAGATCAACAAACAGG	703
IGFBP-5	CCTCAACGAAAAGAGCTACGCC	AAAGTCCCCATCGACGTACTCC	484
IGF1R	TGAACTAATTAACCCTCACTAAAG GGCGTGGCGATCTCAAAAGTTATC TCCG	ACTTGATAATACGACTCACTATA GGGCATCCTTGATGCTTCCGATG ATCTCC	556
PTEN	CTCCTTTTTCTTCAGCCACAGG	GTGACTCCCTTTTTGTCTCTGG	530
18S	TGAACTATTTAGGTGACACTATAG AACCGCTAGAGGTGAAATTCTTGG	ACTTTGATAATACGACTCACTAT AGGGAGTCGGCCATCGTTTATGG TCGG	197

^{32}P-labelled riboprobes were generated using the Strip-EZ *in vitro* transcription system (Ambion, Austin, TX). The blots were hybridized overnight in modified "high-stringency" church buffer (Church and Gilbert 1984) (250 nM Na^+, 125 mM PO_4^{-2}, 7% SDS, 1% BSA, 1 mM EDTA, 50% formamide, pH 7.2, at 60-62°C. After hybridization, membranes were rinsed once with 1×SSC, 0.2% SDS, and washed with fresh 1×SSC, 0.2% SDS for 1h at 68°C. Washes were continued in 0.1×SSC, 0.2% SDS for 1h at 68°C. The signals were detected using Storm 860 PhosphorImager (Amersham Pharmacia Biotech Inc.-Molecular Dynamics Div) and quantified using ImageQuaNT program (Amersham Pharmacia Biotech Inc.-Molecular Dynamics Div). For normalization, the filters were re-probed with 18S riboprobe and relative pixel volumes were counted by comparing the corresponding pixel volume to the 18S pixel volume. Relative pixel volumes were obtained by comparing the pixel volume of each sample with the pixel volume of the corresponding 0 h sample (studies III, IV), or other corresponding control sample (study V).

4.9. Western blotting

In study III, whole-cell extracts from apoptotic and control cells were prepared by lysing CGCs on the culture dish in 800 µl of ice-cold RIPA buffer (10 mM Tris pH 7.4, 1 % Triton X-100, 0.1 % SDS, 0.1 % sodium deoxycholate, 150 mM NaCl). The suspension was transferred using a 27G needle into a microcentrifuge tube, incubated on ice for 30 min, and microcentrifuged at 10 000 g for 10 min at +4 °C. The pellet was discarded, and the protein concentration was determined using the Bio-Rad DC Protein Assay (Bio-Rad). In study V, protein samples for PTEN Western blotting were obtained from whole-cell extracts using PBS-1% Triton. The cell samples were samples were kept on ice for 15 and centrifuged at 10 000 g for 15 min at +4 °C. The supernatant was used for determination of protein concentration (Bio-Rad DC Protein Assay) and Western blotting.

Western blotting was performed according to the standard protocol (Sambrook et al. 1989). Briefly, 10–20 µg of cell lysate was resolved in a 10-12.5% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel, and electroblotted overnight onto Hybond ECL membrane (Amersham Pharmacia Biotech). The membrane was stained with Ponceau S to ensure equal loading and transfer efficiency. The blots were blocked in

phosphate-buffered saline (PBS) containing 1-3 % nonfat dried milk and 0.05 % Tween-20, and incubated with primary and secondary antibodies according to standard protocols (Sambrook et al. 1989) using the following dilutions: rabbit anti-mouse/rat IGFBP-5 (PAX1, GroPep, Adelaide, Australia), 1:1000; goat anti-mouse/rat PTEN (N-19, Santa Cruz Biotechnologies, Santa Cruz, CA), 1:100; rabbit anti-mouse/rat IGF1R (sc-713, Santa Cruz Biotechnologies), 1:300; donkey anti-rabbit–HRP (NA 9340, Amersham Pharmacia Biotech), 1:4000; donkey anti-sheep/goat-POD 1:2500 (Roche, Indianapolis, IN, 1301977). The protein bands were visualized using a chemiluminescent detection system (SuperSignal, Pierce, Rockford, IL) and Hyperfilm ECL film (Amersham Pharmacia Biotech). For quantification, the films were scanned and images were analyzed using ImageQuaNT software (Amersham Pharmacia Biotech Inc.-Molecular Dynamics Div).

5. RESULTS

5.1. Differential display (I)

DD analysis (study I) using 24 different primer combinations produced about 1500–2000 distinct bands, with lengths mostly in the 100–500 bp region. The number of bands per lane was about 50–100 (study I, Fig.1), although some of the primer combinations did not produce more than ~20 bands. The control reactions containing no PCR template produced no bands, whereas in the case of the "gDNA" controls, a few faint bands were occasionally observed, possibly due to a weak reverse transcriptase activity possessed by the PCR polymerase. After the first DD analysis, a duplicate assay starting from cDNA synthesis was performed, and results were compared. The overall reproducibility of the two parallel DD analyses was high, about 98 % of the bands appearing identically in the duplicate assays.

5.1.1. Candidate gene selection

A total of 148 differentially displayed bands were observed in the initial DD analysis. The same expression pattern was confirmed for 145 of them in the subsequent duplicate DD analysis. A total of 30 reproducible bands showing the most prominent differences between apoptotic and control cultures were selected for cloning and identification and extracted from the DD gel. Of the selected bands, 15 were upregulated and 15 downregulated during potassium deprivation (study I, Fig. 2).

5.1.2. Heterogeneity of the differential display clones

Out of the 30 selected bands extracted from the DD gels, 27 produced a sharp, clearly visible band in agarose gel. After cloning and transformation, about 40–70 % of recombinant (white) colonies were obtained.

Before the actual SSCP analysis, an initial experiment was carried out to assess the heterogeneity of the DD bands. For this purpose, four clones that were derived from a single DD band but produced differing SSCP band patterns were sequenced. Analysis of the sequences revealed that two of the clones contained the same insert but in opposite orientations. Otherwise the sequences were completely unrelated, in accordance with earlier studies where overlapping DD products in a single DD band have been detected (Callard et al. 1994, Ito et al. 1994, Li et al. 1994). Since the insert lengths of a DD clone were found to vary within only 3 bp, the different inserts appeared to be identical in agarose gel electrophoresis. In the actual SSCP analysis, the 10 colonies picked from each transformation produced an average of 5 different band patterns representing different clones (study I, Fig. 3).

5.1.3. Slot blot confirmation

In the cDNA probe synthesis reaction, the incorporation of ^{32}P label was 35–55 %. The slot blot analysis was performed on 142 clones selected using SSCP (30 DD bands ×

average of 5 clones / DD band). After a 2-day exposure onto a phosphorimager screen, more than 50 % of the inserts gave a signal with sufficient intensity for quantitation (study I, Fig. 4). . The intensity ratios of the signals (i.e. apoptotic : control) varied severalfold, the majority of them centering about the 1:1 ratio. For further analysis, 9 clones producing the most prominent signal differences were selected.

5.1.4. Identification and Northern blotting

The sequencing of nine cDNAs led to the identification of three by comparing the sequences with the GenBank database. The previously known genes were insulin-like growth factor binding protein -5 (IGFBP-5), mouse pentylenetetrazol-related cDNA 17 (PTZ-17), and mitochondrial cytochrome b (GenBank Acc. Nos L12447, D45203, and J01436; respectively. The remaining six cDNAs did not have significant homology to any sequence in GenBank and were classified as previously unknown. The main interest of this work was in known genes, thus the 6 unknown genes were excluded from further studies. Interestingly, the identified genes or functionally related genes were associated with cell death or neuropathology (Jones and Clemmons 1995, Kajiwara et al. 1995, Lee et al. 1995, D´Ercole et al. 1996, Kajiwara et al. 1997, Cai et al. 1998).

The expression differences of these clones were confirmed using Northern blot analysis. The results for IGFBP-5 and PTZ-17 were in accordance with the preceding DD assay (study I, Fig. 5). In the case of cytochrome b, however, no difference was seen in Northern analysis, which led to the exclusion of cyt b from later studies. The DD and post-DD step results of the sequenced clones are summarized in table 4.

Table 4. The follow-up of the 9 candidate genes throughout the experiments in study I. A-F are the six unknown genes from the DD analysis.

Sequence	Insert size (bp)	Expression change in DD	Expression change in slot blot	Northern blot
IGFBP-5 (L12447)	229	↓	↓	↓
PTZ-17 (D45203)	287	↑	↑	↑
cyt b (J01436)	204	↑	↑	no change
A	280	↑	↑	-
B	200	↑	↑	-
C	210	↑	↑	-
D	180	↑	↑	-
E	280	↓	↓	-
F	180	↓	↓	-

5.2. PTZ-17 is upregulated in potassium deprivation-induced apoptosis (II)

From the rat clones obtained in study I, one cDNA showed a 91 % identity with mouse PTZ-17 (pentylenetetrazol-related clone 17) cDNA (Kajiwara et al. 1995). Pentylenetetrazol (PTZ) is a potent epileptogenic agent that has been used as a tool to study the mechanism of epileptogenesis (Madeja et al. 1991). Kajiwara *et al.* used a differential hybridization technique to detect PTZ-induced alterations in neuronal gene expression and observed downregulation of the PTX-17 RNA (Kajiwara et al. 1995).

Our 287 bp fragment spans the bases 1331–1617 of the PTZ-17 sequence (GenBank Access No. D45203). The rat RNA homolog of the mouse PTZ-17 has not been cloned. However, a search in GenBank with the rat cDNA fragment for 100 % identical rat EST sequences enabled the extension of rat PTZ-17 sequence towards the 5’ end, producing 484 bp of 3’ end sequence of rat PTZ-17 RNA which was 95 % identical to mouse PTZ-17.

The PTZ-17 transcript showed a marked upregulation at 24 h (study II, Fig.1b). In Northern blot analysis, a single transcript of 2.2 ± 0.2 kb was detected, in accordance with the reported mouse PTZ-17 RNA size (Kajiwara et al. 1995). Furthermore, Northern blot analysis confirmed the result of differential display and showed that the expression of PTZ-17 RNA was upregulated at 6 h after the switch to low-potassium medium and continuously increased until 48 h. As expected, potassium-deprived,

actinomycin D-treated cerebellar granule cells did not show any increase in PTZ-17 expression (study II, Fig. 2). The level of the 2.2 kb RNA remained unchanged for at least 24 h after adding the actinomycin D, indicating a relatively high stability of the RNA.

Notably, our results show that the increase in the PTZ-17 expression level is specific for potassium deprivation. Induction of apoptosis by staurosporine or okadaic acid treatments, known to induce apoptosis in cerebellar granule cells (Candeo et al. 1992, Cagnoli et al. 1996), did not enhance the expression of PTZ-17 in this cell type, but instead reduced the expression slightly after 12 h (study II, Fig. 3). The expression of PTZ-17 RNA was also examined in cerebella of young (5 months), middle-aged (1.5 years) and old (3 years) female Wistar rats with a minor decrease observed in aged animals (study II, Fig. 4).

5.3. IGFBP-5 and IGF1R are differentially regulated during apoptosis (III)

5.3.1. Expression of IGFBP-5 is downregulated during apoptosis

Another of the cloned cDNA fragments in study I was identified as the rat homologue of the mouse 3' end of the insulin-like growth factor binding protein -5 (IGFBP-5) mRNA (GenBank Acc.# L12447). Northern blot analysis, using the IGFBP-5 cDNA fragment as a probe, confirmed the differential expression of the IGFBP-5 transcript during potassium deprivation-induced apoptosis (study I). We continued to study the expression pattern of IGFBP-5 more closely (study III) using Northern and Western blotting. In our experiments, a single IGFBP-5 transcript at 6 kb was detected, as has been observed in all adult rat tissues examined (Cohick and Clemmons 1993). The IGFBP-5 mRNA was markedly downregulated as early as 6 h after switching to low KCl medium and it remained at a low level for up to 48 h (study III, Fig. 1). This is in line with the original finding from study I.

To further characterize the expression in different apoptosis models, the IGFBP-5 expression was examined during okadaic acid and staurosporine -induced apoptosis in cerebellar granule cells. The same pattern of downregulation was observed also in these cultures, appearing as early as 4 hours after administration of the drugs (study III, Fig. 2a). To determine whether this downregulation of IGFBP-5 was an early event, we analyzed the time course of major cell death and caspase-3 activation in okadaic acid and staurosporine treated CGCs (study III, Fig. 2b). The major cell death occured at 24 h, which is shown by the amount of LDH released in the medium. Although, some caspase-3 activation could be seen at 8 h, major activation of caspase-3 occurs at 24 h. These results indicate that a rapid downregulation of IGFBP-5 mRNA is one of the early responses of cerebellar granule cells to apoptotic stimuli.

Apoptosis induced in cerebellar granule cells using potassium deprivation can be suppressed using IGF-I or the transcriptional inhibitor actinomycin D (D´Mello et al. 1993). We examined the effect of these factors on IGFBP-5 mRNA expression during potassium deprivation in CGCs. In KCl-deprived CGCs treated with actinomycin D (1 µg/ml) IGFBP-5 mRNA level was restored when compared to cells without actinomycin D with a statistically significant difference at 24 h (study III, Fig. 3). This

result demonstrates that the rapid and prominent downregulation of IGFBP-5 mRNA during apoptosis is dependent on new RNA synthesis, indicating that the decrease in the downregulation of IGFBP-5 is due to the inhibition of apoptosis by actinomycin D (D'Mello et al. 1993).

Addition of fresh serum has been shown to be toxic to mature CGCs at 7 DIV, possibly due to the presence of glutamate (Schramm et al. 1990, D'Mello et al. 1993). Consequently, in order to induce apoptosis via potassium deprivation, either dialyzed serum or the withdrawal of both KCl and serum at 7 DIV (days *in vitro*) is used. However, dialyzed serum may be deficient in small-M_W protein factors, and withdrawal of serum causes oxidative stress in CGCs (Atabay et al. 1996), in addition to apoptosis. We analyzed the effects of KCl concentration, serum and dialyzed serum to test whether IGFBP-5 expression is altered by the medium change *per se* (study III, Fig. 4). In addition, we studied the effect of IGF-I which both inhibits CGC apoptosis and upregulates IGFBP-5 mRNA expression *in vivo* (Ye and D'Ercole 1998). Withdrawal of both KCl and serum from CGCs caused a prominent downregulation of IGFBP-5 mRNA (study III, Fig. 4). Of the effectors tested, the KCl concentration was found to have the largest effect on IGFBP-5 mRNA level. In low KCl, neither dialyzed serum nor IGF-I restored the expression of IGFBP-5 mRNA. However, in high KCl, IGF-I markedly increased the IGFBP-5 mRNA level. Interestingly, in cells cultured in high KCl with dialyzed serum for 6 h after changing the medium at 7 DIV, the level of IGFBP-5 mRNA was significantly lower as compared to untreated control cells. This might be due to the removal of some extracellular factors from the medium, which had been secreted by the cells prior to the medium change at 7 DIV.

To study the expression at the protein level, Western blot using polyclonal IGFBP-5 antiserum was performed. A prominent band at ~33 kDa and a much weaker band at ~35 kDa were detected, as observed previously (Ye and D'Ercole 1998, Gregory et al. 1999). In addition, a weak band was seen at ~20 kDa, possibly corresponding to a proteolytic fragment of IGFBP-5 (Clemmons 1998). The expression of IGFBP-5 protein was found to decrease markedly during potassium deprivation-induced apoptosis at 6–24 h (study III, Fig. 5a). In contrast to the mRNA level, IGF-I caused a major increase in IGFBP-5 protein in KCl-deprived samples, restoring IGFBP-5 expression close to the level before potassium deprivation (study III, Fig. 5b). These findings may indicate that the increase in IGFBP-5 protein level by IGF-I could be independent from *de novo* synthesis and involve posttranscriptional mechanisms as observed also previously (Camacho-Hubner et al. 1992).

5.3.2. IGF1R expression is regulated by potassium deprivation and IGF-I

In a preceding cDNA array study (data not shown), a pair of Atlas mouse cDNA expression arrays (Clontech, Palo Alto, CA) were hybridized to cDNA probes from apoptotic and control samples to reveal potassium deprivation-induced mRNA expression changes in cerebellar granule cells. Based on the preliminary results of this array study, we analyzed the IGF1R mRNA in our system.

In Northern blot analysis, an 11 kb transcript was detected in CGCs, corresponding to IGF1R mRNA (Ullrich et al. 1986). The IGF1R mRNA showed a statistically

significant, but transient upregulation in CGCs at 6 h after switching to low KCl (study III, Fig. 6). At later time points, the expression of IGF1R mRNA decreased to the basal level. Interestingly, however, the level of IGF1R mRNA remained higher in potassium-deprived cells than in control cells maintained in high KCl. IGF-I treatment significantly decreased the expression of IGF1R mRNA in both potassium-deprived CGCs and in cells maintained in high KCl (study III, Fig. 7). This is in accordance with observations in a muscle cell line and a neuroblastoma cell line (LeRoith et al. 1995, Hernandez-Sanchez et al. 1997). The transient upregulation of IGF1R mRNA during early events of apoptosis may indicate the presence of an adaptive response that is exhibited in an attempt to enhance survival IGF signaling during cell stress (D´Ercole et al. 1996).

To study changes at the protein level, Western blot analysis from total GCC extracts was performed. A polyclonal antibody against IGF1R β-subunit recognized a major band at ~95 kDa and a much weaker band at ~100 kDa, corresponding to the sizes of the heterogenous IGF1R β-subunit isoforms (Garofalo and Rosen 1989, Moss and Livingston 1993, LeRoith et al. 1995). The protein level of IGF1R β did not show any transient upregulation, in contrast to the mRNA level, but instead decreased markedly in KCl-deprived cells at 24 h (study III, Fig. 8). This suggests that due to the short commitment time (6-12 hours in low KCl) of the apoptotic program (Nardi et al. 1997), the upregulation in the mRNA expression of IGF1R may not be reflected at the protein level.

As observed at the mRNA level, IGF-I treatment of CGCs caused a decrease in the IGF1R β protein level during potassium deprivation (study III, Fig. 8). In addition, in cells maintained in high KCl, IGF-I treatment decreased the IGF1R β level. This is in agreement with previous studies on IGF-I regulation of IGF1R expression (LeRoith et al. 1995, Hernandez-Sanchez et al. 1997).

5.4. IGFBP-2, -3 and -5 are differentially expressed during neuronal apoptosis (IV)

We studied the expression of IGFBPs -2, -3 and -5 in neuronal apoptosis *in vitro* (study IV). Originally, the differential expression of the IGFBP-5 was found in the differential display analysis of apoptosis related genes in cerebellar granule cells (study I). Consequently, this original finding was also followed by the study of other IGF system members.

Two models of neuronal apoptosis in rat cerebellar granule cells were used: potassium deprivation and okadaic acid induced apoptosis (Candeo et al. 1992, D´Mello et al. 1993, Cagnoli et al. 1996). Riboprobing with IGFBP-2, -3 and -5 specific probes showed mRNAs of approx. 1.5, 2.5 and 6 kb, respectively. The sizes of the mRNAs corresponded to previously defined mRNAs from rodent tissue (Shimasaki et al. 1991, Schuller et al. 1994, Hwa et al. 1999). We described the changes in IGFBP-5 mRNA during neuronal apoptosis in study III. In this study (study IV), IGFBP-5 riboprobing showed a reproducible expression pattern when compared to study III, with a marked downregulation during apoptosis.

Interestingly, the IGFBP-2 mRNA level showed a marked increase during potassium deprivation-induced apoptosis in cerebellar granule cells, with a peak at 24 h (study IV, Fig. 1). The IGFBP-2 mRNA level started to increase around 9 h, and thereby continued to increase to 24 h, which was the last time-point studied. In contrast, IGFBP-3 mRNA level decreased markedly as early as 3 hours (study IV, Fig. 1). The expression pattern of IGFBP-3 was similar to the one observed in IGFBP-5, but the initial drop in the mRNA was more rapid in IGFBP-3 mRNA. These results showed that the IGFBPs are regulated in a distinct fashion and support the idea that each of them has a special functional role.

To study the regulation of IGFBP mRNAs in a different model of apoptosis, okadaic acid was used to induce apoptosis (study IV, Fig. 2). Again, IGFBP-5 mRNA levels were identical to those previously observed, with a rapid and marked decrease. IGFBP-2 mRNA, however, did not increase as in potassium deprivation-induced apoptosis, but instead, showed a decrease. In addition, IGFBP-3 mRNA showed a differing pattern in okadaic acid treated cells: it showed an increase with a peak at 3 h.

IGF-I is known to regulate the expression of IGFBPs (Camacho-Hubner et al. 1991, Ye and D'Ercole 1998, Duan et al. 1999), and thus we tested whether it has any effect in our *in vitro* system. Neither IGFBP-2 nor IGFBP-3 mRNA levels changed in response to IGF-I addition. As expected, IGFBP-5 mRNA level became slightly increased when IGF-I was added to the cells (study IV, Fig. 3).

We used the RNA synthesis inhibitor, actinomycin D, to test the effects on changes in mRNA levels of IGFBP-2 and -3. We observed that the downregulation of IGFBP-5 mRNA during CGC apoptosis could be suppressed by actinomycin D. Interestingly, the IGFBP-2 mRNA, which was markedly increased during potassium deprivation (at 24 h), was reduced to the control level when actinomycin D was added (study IV, Fig. 4). These point to an interconnection between apoptotic signaling and regulation of IGFBP expression. However, actinomycin did not prevent the marked decrease of IGFBP-3 mRNA during apoptosis (study IV, Fig. 4). On the contrary, when added to the control samples, actinomycin D almost completely abolished the IGFBP-3 mRNA. This may indicate a shorter mRNA turnover for IGFBP-3 than for IGFBP-2 and IGFBP-5. A decrease in IGFBP-2 mRNA was also observed in control samples in which actinomycin D was added.

5.5. PTEN is downregulated in trophic factor withdrawal induced apoptosis (V)

Since the expression of the IGF system members was shown to be changed during neuronal apoptosis (studies I, II, III and IV), we wanted to test whether the expression of IGF signaling -related genes, such as PTEN, is altered during neuronal apoptosis. PTEN tumor suppressor is an important regulator of the IGF system related survival signaling (Stambolic et al. 1998, Cantley and Neel 1999, Vazquez and Sellers 2000).

PTEN mRNA expression showed a major 5.5 kb transcript and a lower abundance transcript of 2.5 kb, both in cerebellar granule neurons and Neuro-2a cells (study V). Similar transcripts have been earlier described in several glioma cell lines (Furnari et al. 1997). The nature of these two transcripts is still unknown.

We induced apoptosis also in Neuro-2a cells either by serum withdrawal or by exposing cells to okadaic acid or etoposide. Serum withdrawal induced a prominent decrease in the level of PTEN mRNA expression in Neuro-2a cells. The decrease appeared both in the density of 5.5 kb and 2.5 kb bands and continued up to 24 h after switching to serum deprivation (study V, Fig. 1). In contrast to serum deficiency, okadaic acid and etoposide, classical inducers of apoptosis, seemed to only slightly decrease the expression of 5.5 kb transcripts and even to enhance the level of 2.5 kb transcript.

The changes in total level of PTEN protein after serum withdrawal and after treatment with okadaic acid or etoposide were characterized (study V, Fig. 2). Western blot assays showed a major 56 kD band both in Neuro-2a and cerebellar granule cells. The same size of PTEN protein has been observed in several other cells and tissues (Li et al. 1998, Stambolic et al. 1998). Verifying the PTEN mRNA results, in Neuro-2a cells the protein level of PTEN decreased during apoptosis induced by serum deprivation but no changes were observed during apoptosis induced by okadaic acid or etoposide. The immunoblot analysis showed that the protein level of PTEN is already decreased 12 h after serum withdrawal (study V, Fig. 2). Earlier studies have shown that serum deprivation from Neuro-2a cells induces typical apoptotic characteristics, caspase-3 activation and poly(ADP-ribose) polymerase cleavage, but not earlier than 24 h after the withdrawal (Solovyan et al. 1998). It seems that the decrease in PTEN expression precedes apoptotic changes after serum withdrawal in Neuro-2a cells.

Interestingly, okadaic acid treatment of cerebellar granule neurons induced a prominent increase in PTEN mRNA expression after 6 h treatment. Both, 5.5 kb and 2.5 kb bands were affected (study V, Fig. 3). This early response in PTEN mRNA expression of the 5.5 kb transcript disappeared already at 12 h, in contrast to the 2.5 kb transcripts, which lasted for up to 24 h or longer. Western blot analysis verified this later downregulation of PTEN expression by showing that okadaic acid treatment for 24 h induced a decrease in PTEN protein level (study V, Fig. 2b). The decrease was more prominent after 36 h. A lower concentration of okadaic acid did not affect the level of PTEN protein.

6. DISCUSSION

6.1. The CGCs and DD as tools to analyze gene expression during apoptosis

The differential display (DD) analysis of gene expression allows the systematic comparison of mRNA expressed in various biological processes or as a response to experimental treatments (Liang and Pardee 1992, Liang et al. 1993, Liang et al. 1994). However, it is not the only method of choice today. Analysis of gene expression using different kinds of DNA microarrays has become extremely popular. Using these high-throughput methods, the simultaneous analysis of expression profiles of thousands of genes is possible. However, at the time of starting this thesis, microarrays with the requirement for facilities for equipment and data analysis were out of our reach. Moreover, when considered as a method for finding a few novel and interesting or previously unsuspected genes to be further characterized, the DD approach is adequate. Together with the potassium deprivation-induced apoptosis in rat cerebellar granule cells (CGCs), it establishes a model that is well applicable to screening studies on neuronal apoptosis.

The CGCs form a culture of mature post-mitotic neurons, in which apoptosis can be induced by potassium deprivation. This mimics the mechanisms of cell death *in vivo,* which occurs in response to lack of trophic stimulation (D´Mello et al. 1993). The prerequisite for obtaining reproducible results in this system is well fulfilled because the cell population is very homogenous (Levi et al. 1989). This reduces the possibility of artefacts caused by differences in the cell population. In addition, since the total amount of granule cells in the cerebellum is high, the amount of RNA and protein obtained from CGCs is sufficient to enable one to perform further analysis, such as Northern and Western blots. However, previous neurobiological DD studies have concentrated on development (Joseph et al. 1994), and aging (Salehi et al. 1996), and have not utilized this system.

Although the potassium deprivation-induced apoptosis possesses the essential prerequisites that make it a good model for screening studies, it does suffer from some drawbacks. One of the major difficulties is created by the fact that the induction of apoptosis occurs by changing the cell culture medium, and thus there are several unknown factors that are lost because of the medium change *per se*. The use of undialyzed, fresh serum is not possible, due to the presence of a small M_W substance in serum, probably glutamate, which is toxic to mature CGCs in culture (Schramm et al. 1990). Some effects can be avoided by using dialyzed serum, but the loss of factors that are under the dialysis membrane M_w cut-off limit, e.g. 1000 or 10 000, cannot be avoided.

Our DD analysis produced ~150 differentially displayed bands, from which 30 bands with the most prominent difference in the intensity, were selected for further analysis. The DD results were highly reproducible (>98%), when the analysis was repeated starting from the cDNA synthesis (study I, Table 2). However, because of the suspected heterogeneity of DD products obtained from a single band, an SSCP analysis was

performed in order to reduce the amount of false positive results in the subsequent post-DD analysis. SSCP showed that although the bands appear as single bands in the DD gel, the band still consisted of several cDNAs which had the same length but different sequences. This is in accordance with earlier reports where overlapping DD products in a single DD band have been detected (Callard et al. 1994, Ito et al. 1994, Li et al. 1994). Thus, the ability of SSCP to detect sequence variations (in addition to differences in length) was efficient in distinguishing between inserts of similar length (study I, Fig. 3).

In the following slot blot analysis of the DD results, the amount of detected changes was reduced, possibly due to the relatively low detection sensitivity of this method. In addition, only those bands showing the most prominent differences in the slot blot were chosen for further analysis. The sequenced clones which appeared as previously known were chosen for subsequent studies. In addition some functionally related genes were studied.

6.2. Upregulation of PTZ-17 expression

In study I we observed the upregulation of pentylenetetrazol-related RNA, PTZ-17, during apoptosis induced by potassium deprivation. The expression pattern was characterized further in study II, which surprisingly demonstrated the expression to be upregulated specifically in potassium deprivation-induced apoptosis and not in other apoptotic models used.

Kajiwara *et al* (Kajiwara et al. 1995) cloned and characterized a cDNA designated PTZ-17, the RNA expression of which showed a major downregulation after PTZ treatment. Interestingly, the injection of PTZ-17 RNA into *Xenopus* oocyte provoked a large calcium inward current following the extracellular application of PTZ. The nucleotide sequence of PTZ-17 (1618 bp) is 99 % identical to the 3' end of the 3.1 cDNA cloned earlier by Studler *et al.* (Studler et al. 1993). The 3.1 RNA is abundant in mouse cerebellum, hippocampus and heart (Studler et al. 1993).

PTZ-17 RNA shows an interesting expression pattern: depolarization of neurons induces downregulation (Kajiwara et al. 1995), whereas repolarization by potassium deprivation upregulates the PTZ-17 expression (study II, Fig. 1b). How might PTZ-17 be related to apoptosis? The PTZ-17 RNA and cytoplasmic binding proteins are involved in the regulation of calcium entry into neurons in the case of PTZ-induced epileptic bursts. Recently, Kajiwara *et al.* reported that a particular sequence within the 3'-untranslated region (3'UTR) of PTZ-17 RNA binds to 60 and 47 kDa intracellular proteins (Kajiwara et al. 1997). The injection of this 3'UTR sequence into *Xenopus* oocyte induced a calcium current similar to that caused by the injection of whole-length PTZ-17 RNA. Moreover, they showed that the mouse strains with different susceptibilities to epileptic convulsions, DBA/2 and BALB/c, have sequence differences in the 3'UTR of PTZ-17 (Kajiwara et al. 1997). The injection of PTZ-17 RNA derived from DBA/2 mice, which have a high susceptibility to epileptic convulsions, caused a large calcium current in *Xenopus* oocytes after PTZ treatment, but the RNA from BALB/c mice with a low susceptibility to convulsions showed no PTZ response in oocyte calcium currents.

It may be that PTZ-17 is involved in apoptosis via regulation of calcium homeostasis. It is known (Franklin and Johnson 1992) that the prolonged depolarization of cultured neurons, for example, by a high potassium concentration in medium, maintains an elevated intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration and promotes neuronal survival, while low potassium concentration reduces the intracellular Ca^{2+} concentration and triggers an apoptotic cell death (Galli et al. 1995). Franklin and Johnson (Franklin and Johnson 1992) have presented a "Ca^{2+} set-point hypothesis" to postulate the presence of steady-state levels of calcium that affect survival. According to this hypothesis, the epileptogenic insult induced by PTZ might represent an excitotoxic level of $[Ca^{2+}]_i$ and may induce necrosis, whereas potassium deprivation decreases $[Ca^{2+}]_i$ which triggers an apoptotic cell death. PTZ-17 expression is clearly related to processes that respond to changes in the calcium and potassium concentrations. We showed that PTZ-17 RNA remained at the control level when actinomycin D was present, which may be a result of inhibition of apoptosis (study II, Fig. 2). PTZ-17 is upregulated late in the process (24 h- 48 h) (study II, Fig. 1b), which may indicate that it is involved in the processes during the execution phase of apoptosis. A physiological role for PTZ-17 not only in development but also in adults is supported by the fact that the expression is sustained in adult rats (Studler et al. 1993). Interestingly, Studler *et al.* (Studler et al. 1993) failed to detect any 3.1/PTZ-17 protein in tissues that express the mRNA at a high level although the antibodies could recognize the protein produced by *in vitro* translation of synthetic 3.1 RNA. The role of PTZ-17 and its interacting RNA-binding proteins in this complex regulation of neuronal excitability and calcium homeostasis should be examined in future studies.

6.3. Expression of IGFBPs and IGF1R is altered during neuronal apoptosis

In study I IGFBP-5 mRNA was observed to be markedly downregulated during potassium deprivation-induced apoptosis. The expression profiles of IGFBP-5 and IGF1R were further characterized in study III. In addition, the studies were extended to cover also other IGFBPs: the expression patterns of IGFBP-2, -3, -5 were profiled during neuronal apoptosis (study IV).

A prominent downregulation of IGFBP-5 mRNA and protein during potassium deprivation was observed (study III, Fig. 1). In subsequent studies also IGFBP-2 and -3 were shown to be differentially expressed during potassium deprivation-induced apoptosis in cerebellar granule cells (study IV, Fig. 1). IGFBP-2 mRNA was markedly increased, while IGFBP-3 mRNA was significantly and rapidly decreased. These results show that the expression of each IGFBPs is altered in a specific way.

IGF-I increased the level of IGFBP-5 protein (study III, Fig. 5b), in accordance with previous findings in a non-neuronal system (Clemmons 1998). Also in IGF-I overexpressing mice, IGFBP-5 expression is increased (Ye and D'Ercole 1998). Conceivably, if IGF-I is available in sufficient amounts, the upregulation of IGFBPs serves to sequester excess IGF-I molecules. However, during potassium deprivation, the observed increase in IGFBP-5 expression by IGF-I was more prominent at the protein than at the mRNA level. This is in agreement with the additional findings in study IV, which show that IGF-I has no major impact on IGFBP-2 and -3 mRNA levels in CGCs. These findings are consistent with observations in fibroblasts, in which the increase in

IGFBP-5 protein level by IGF-I appears to be mostly attributable to post-transcriptional mechanisms (Camacho-Hubner et al. 1992).

We observed that the downregulation of IGFBP-5 mRNA during CGC apoptosis could be suppressed by actinomycin D (study III, Fig. 3). Furthermore, in the presence of actinomycin D, IGFBP-2 mRNA stayed at control level (study IV, Fig. 4). Also, when actinomycin D was present, the downregulation of IGFBP-3 mRNA observed during potassium deprivation was slightly inhibited. There is increasing evidence for a role of IGF signaling pathways in apoptosis (Werner and Le Roith 1997). These results may also point to an interconnection between apoptotic signaling and regulation of IGFBP expression. However, when added to control samples, actinomycin D decreased the expression of all of the IGFBP mRNAs studied. In contrast to IGFBP-2 and IGFBP-5 mRNAs, which remained at a detectable level, IGFBP-3 mRNA was almost completely abolished when actinomycin was added to control samples for 24 h (study IV, Fig. 4). This may indicate a shorter mRNA turnover for IGFBP-3 than for IGFBP-2 and IGFBP-5.

In addition to potassium deprivation, apoptosis was induced in CGCs using protein kinase and phosphatase inhibitors, staurosporine and okadaic acid (studies II, III, IV and V). In sharp contrast to the upregulation of IGFBP-2 during potassium deprivation, IGFBP-2 mRNA was decreased during apoptosis induced by okadaic acid. Interestingly, even though IGFBP-3 mRNA was significantly and rapidly decreased during potassium deprivation, it was increased during okadaic acid-induced apoptosis (study IV, Fig. 2). IGFBP-5 mRNAs were decreased in all apoptosis models used (studies III and IV). The exact mechanisms of how okadaic acid or potassium deprivation induce apoptosis are not known. Evidently there is a difference between the apoptotic processes triggered, which is demonstrated by the individual regulation of the IGFBPs.

The main effects of IGFs on cell proliferation, survival and differentiation are mediated through type 1 insulin-like growth factor receptor (IGF1R) (LeRoith et al. 1995). We observed that the mRNA expression of IGF1R was transiently upregulated after switching to low KCl in CGCs (study III, Fig. 7). The transient upregulation of IGF1R mRNA in the early phase of apoptosis may support the adaptive response that is exhibited in an attempt to enhance survival IGF signaling during cell stress (D´Ercole et al. 1996). However, at the protein level, a downregulation of IGF1R protein was observed (study III, Fig. 8). This suggests that due to the short commitment time (6-12 hours in low KCl) of the apoptotic program (Nardi et al. 1997), the upregulation in the mRNA expression of IGF1R may not be reflected at the protein level. We also observed a negative effect of IGF-I on IGF1R expression both in KCl-deprived and high KCl cultures, in agreement with previous studies on IGF-I regulation of IGF1R expression (LeRoith et al. 1995, Hernandez-Sanchez et al. 1997).

6.4. Expression of PTEN is modulated by trophic factor signaling

We observed that the IGF system members undergo specific regulation during potassium deprivation-induced apoptosis (studies I, III, and IV). Surprisingly, we observed that potassium deprivation alone did not affect PTEN mRNA expression.

However, combining serum withdrawal with potassium deprivation-induced a clear decrease in the density of 5.5 kb PTEN band (study V, Fig. 3b). Also in Neuro-2a cells, serum deprivation in decreased PTEN expression (study V, Fig. 1). Interestingly, okadaic acid treatment of cerebellar granule neurons increased PTEN mRNA expression at 6h. This early response in PTEN mRNA expression of the 5.5 kb transcript disappeared already at 12 h, in contrast to the 2.5 kb transcripts, which lasted for up to 24 h or longer (study V, Fig. 3). Western blot analysis verified this later downregulation of PTEN expression by showing that okadaic acid treatment for 24 h induced a decrease in PTEN protein level (study V, Fig. 2).

Etoposide treatment did not have a major effect on PTEN expression although we have observed that etoposide treatment does induce caspase-3 activation and DNA disintegration in cerebellar granule neurons (Solovyan et al. 1998).

These results indicate that apoptosis may involve changing the expression of several members at distinct levels of the IGF-PI3K signaling pathway. More specifically, we have shown that this regulation occurs at the level of ligand binding through the regulation of IGFBP expression (studies III and IV). It can also occur downstream of the receptor, through the maintenance of PI-3K phosphorylation effects by PTEN downregulation (study V). This regulation may be specifically involved in growth factor deprivation-induced apoptosis, and when taken together, indicate that potassium/serum deprivation and okadaic acid-induced apoptosis involve at least partly distinct processes. The results (I-V) are summarized in figure 3.

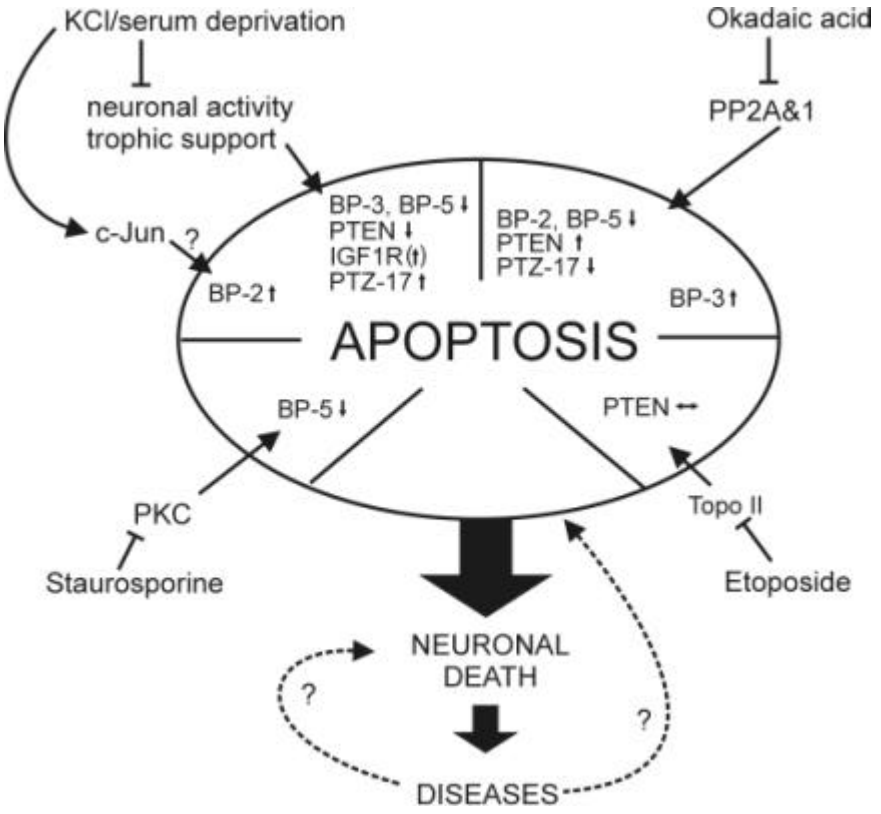

Figure 3. A schematic representation of the results showing observed changes in gene expression during different apoptotic conditions studied.

6.5. The distinct regulation of the candidate genes

We characterized the expression of the candidate genes (studies II and III), which were identified with the differential display (study I) along with two other related genes (studies IV and V) in different apoptosis models and made the following observations: *1)* IGFBP-2, -3 and -5 are regulated distinctively, with each of them having a characteristic expression pattern; *2)* the expression pattern of IGFBPs varied according to the apoptosis inducers used; *3)* the candidate gene expression repeatedly differed in okadaic acid vs. potassium deprivation-induced apoptosis indicating different mechanisms of apoptosis for these models.

According to our results, a deficiency of trophic signaling in neurons induces a response that clearly involves an ordered regulation of the IGF-system expression. It is possible that the consequences of a rapid downregulation of IGFBP-5 (study III) may involve an attempt to promote IGF-I activity. This idea is supported by the fact that IGF1R mRNA is also rapidly upregulated (study III). Since the exact role of IGFBP-5 in the CNS is not known, and because IGFBP-5 has been shown to be able also to promote IGF-I signaling (Jones et al. 1993, Perks et al. 2000), the consequences of this downregulation remain currently unknown. On the contrary, since the activities of IGFBP-3 involve mainly inhibition of IGF-I signaling and promotion of apoptosis (Oh et al. 1995, Butt et al. 1999, Butt et al. 2000, Cohen et al. 2000, Hollowood et al. 2000), it is possible that the expression is downregulated in order to maximize the availability IGFs and survival.

Interestingly, we observed that the withdrawal of serum mitogenic factors decreased the expression of PTEN phosphatase, both at the mRNA and protein level in Neuro-2a cells as well as in cerebellar granule cells together with potassium deficiency (study V). It seems that neuronal cells try to compensate for the deficiency of extracellular mitogen - inducing the activation of receptor tyrosine kinases by downregulating the expression of antagonistic PTEN phosphatase to maintain PI-3K/Akt -mediated signaling. The decline in PTEN expression clearly precedes the first indications of apoptosis we have described in both neuronal models (Solovyan et al. 1998). However, PTEN expression was only slightly affected by okadaic acid or etoposide treatments in Neuro-2a cells although they evoke a prominent apoptosis in Neuro-2a cells (Solovyan et al. 1998). It is possible that the changes in PTEN expression level and apoptosis are not related to each other in general but the expression of PTEN phosphatase might regulate certain apoptotic signals affecting phosphoinositide 3-kinase function.

The IGF-PI-3K-Akt signaling is not an isolated pathway, however, and thus the interaction between different neurotrophic factors in a multifactorial manner is apparent, although the precise mechanisms are still unclear. Whether depolarization induces the endogenous production of autocrine trophic factors, which include an autocrine loop between different neurotrophic factors, such as IGF and BDNF, remains to be clarified. It is known that in the case of BDNF knockout mice, the survival of hippocampal neurons of these animals can be enhanced in culture using both IGF-I and BDNF, showing that there exists some complementary actions between these factors (Lindholm et al. 1996). It has been also debated whether there is a common and united pathway among the signals promoting neuronal survival. Hence, a good candidate for such a

"converge point" is the PI-3-kinase, which plays a significant role in the enhanced survival induced by several neurotrophic factors, such as IGF-I and BDNF (D´Mello et al. 1997, Shimoke et al. 1997). On the other hand, there exists a large body of data about membrane depolarization promoted neuronal survival (Galli et al. 1995, D´Mello et al. 1997, Miller et al. 1997, Shimoke et al. 1997). The involvement of PI-3-kinase in neuronal survival induced by membrane depolarization is somewhat controversial. However, the elevation of intracellular calcium plays most likely a key role in the depolarization-induced survival (Koike et al. 1989, Franklin and Johnson 1992).

The majority of the genes studied during potassium deprivation in CGC are downregulated (Miller and Johnson 1996). This downregulation may be a result of a general downregulation of the cell functions, or it can also be part of adaptive responses to a decline in trophic factor support. However, a few genes that show increased expression are known: e.g. an AP-1 transcription factor c-Jun (Watson et al. 1998); glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (Ishitani and Chuang 1996, Ishitani et al. 1997); PTZ-17 RNA (study II); SFN1-related-kinase (Yoshida et al. 2000), and IGF1R (study III).

The transcriptional commitment point, i.e. the time-point when 50 % of CGCs can be rescued from potassium deprivation-induced apoptosis by actinomycin D, is at 3 h (Watson et al. 1998). This suggests that the genes that are needed to trigger apoptotic process are transcribed during this time (Watson et al. 1998). The time-point when the CGCs are no longer rescued by re-addition of high potassium concentration occurs much later: around 6 to 12 hours (Chang and Wang 1997, Nardi et al. 1997, Watson et al. 1998). This clearly demonstrates that trophic support can silence the already produced harmful apoptotic mediators and prevent the execution of apoptosis.

c-Jun forms together with c-Fos the ubiquitous AP-1 transcription factor that is responsible for the regulation of various genes in response to exogenous and endogenous stimuli (Lee et al. 1987, Ullman et al. 1990). c-Jun mRNA and protein levels are rapidly increased during potassium deprivation: mRNA peaks already at 2h and protein at 4 h (Watson et al. 1998). In addition, the phosphorylation of c-Jun has been shown to be essential for potassium deprivation-induced apoptosis to proceed (Watson et al. 1998). Although c-Jun activation has been shown to be crucial for apoptosis in many types of neuronal cells, only a few c-Jun target genes that are important for neuronal death have been characterized (Herdegen et al. 1998, Watson et al. 1998, Levkovitz and Baraban 2001). FasL is one of the potential death-inducing targets of c-Jun (Le-Niculescu et al. 1999). Interestingly, the IGFBP-2 promoter region has also been shown to include a putative AP-1 (c-Fos/c-Jun) binding site (Brown and Rechler 1990). We observed the IGFBP-2 mRNA to be markedly increased with a peak of expression as late as 24 h after switching the CGCs to low potassium medium. The continuous increase in the mRNA level started around 9 h (study IV, Fig. 1). Furthermore, c-Jun has been shown to regulate also the expression of other IGFBPs in response to different cellular stresses. For example, IGFBP-1 expression is significantly increased by interleukin-6 (IL-6) in hepatocytes as an adaptive response to hepatic injury (Leu et al. 2001). The change in IGFBP-1 expression is known to be dependent on AP-1 transcription factor (Leu et al. 2001). Taken together, since the IGFBP-2 upregulation occurs around 5 hours after the peak in c-Jun protein expression and

because the IGFBP-2 gene contains a putative AP-1 binding site, it seems highly possible that IGFBP-2 is one of the c-Jun target genes and therefore upregulated as a response to potassium deprivation.

6.6. Differences in the apoptotic models used

Apoptosis can be induced in cerebellar granule cells (among several other cell types) by a protein phosphatase inhibitor okadaic acid, which is a selective inhibitor of protein phosphatases 1 and 2A (PP2A and 1) (Candeo et al. 1992). The exact mechanisms of how okadaic acid or potassium deprivation induces apoptosis are not known. However, it is assumed that the okadaic acid-induced prolonged phosphorylation of proteins may be the trigger for apoptosis (Candeo et al. 1992). The calcium homeostasis may not be a key player in okadaic acid induced apoptosis, which is in contrast to the involvement of calcium in the apoptotic process induced by potassium deprivation (Koike et al. 1989, Galli et al. 1995).

The expression patterns of the candidate genes change almost totally when another form of apoptosis-induction is used. In fact, IGFBP-5 is the only candidate gene from study I that was clearly downregulated with all the apoptosis models used (studies III and IV). Thus, the expression of IGFBP-5 is clearly downregulated by the apoptotic process *per se*. The upregulation of IGFBP-2 mRNA was not found during okadaic acid-induced apoptosis in CGCs (study IV). In contrast, the IGFBP-2 mRNA was downregulated. Furthermore, an opposite form of regulation was observed for IGFBP-3 mRNA in both of these apoptotic models: it was markedly downregulated in potassium deprivation-induced apoptosis and rapidly upregulated in okadaic acid-induced apoptosis.

Our results, which show an opposite regulation of IGFBP-2 and IGFBP-3 in response to apoptotic inducers in CGCs, support the concept that okadaic acid and potassium deprivation can act on “opposite mechanisms” to trigger apoptosis in CGCs. Indeed, we observed differences in the expression of apoptosis related genes between okadaic acid and potassium/serum deprivation-induced apoptosis models in CGCs. PTZ-17 RNA was significantly upregulated in potassium deprivation-induced apoptosis, whereas okadaic acid caused PTZ-17 to decrease (study II). In addition, PTEN expression was shown to decrease in response to growth factor withdrawal, whereas okadaic acid-induced apoptosis evoked PTEN upregulation (study V).

As far as we know, the effect of okadaic acid, an inhibitor of PP2A and 1, on the activity of PTEN phosphatase has not been studied. However, okadaic acid is a strong tumor promoter and mimics some effects of insulin (Cohen et al. 1990, Fujiki et al. 1992). Although the molecular mechanism of okadaic acid-induced apoptosis is not known, there is evidence that okadaic acid may induce a mitotic attempt in postmitotic neurons (Nuydens et al. 1998). PTEN phosphatase, as a tumor suppressor protein, might provide a rescue route to postmitotic neurons to inhibit the cell cycle entry mediated by PI-3K/Akt pathway (Cantley and Neel 1999).

Interestingly, protein phosphatase 2A was recently shown to dephosphorylate BAD in interleukin-3 dependent FL5.12 lymphoid cells (Chiang et al. 2001). When the dephosphorylation of BAD was prevented by okadaic acid, a significant increase in cell

survival was observed during survival factor withdrawal in the FL5.12 cells (Chiang et al. 2001). Deprivation of growth factors is known to induce BAD dephosphorylation and apoptosis (Datta et al. 1997). More specifically, in CGCs, potassium deprivation is known to induce BAD dephosphorylation, which can be inhibited by IGF-I (Gleichmann et al. 2000). Thus, it is tempting to speculate that okadaic acid could compensate for the decrease in growth factor dependent phosphorylation, and even be protective during survival factor withdrawal by preventing BAD dephosphorylation by PP2A. On the contrary, if added when normal survival signaling is present, okadaic acid could induce apoptosis by disturbing the phosphorylation status by protracting the phosphorylation of some key regulators of cellular survival. The upregulation of IGFBP-3 during okadaic acid-induced apoptosis may be a result of this aberrant phosphorylation and may be a significant player in the apoptotic process, since IGFBP-3 has been shown to be able to induce growth inhibition and apoptosis (Oh et al. 1995, Shen and Glazer 1998). However, the exact mechanisms remain unknown. It is possible that IGFBP-3 acts through inhibition of IGF1R signaling by binding to IGF-I (Nickerson et al. 1997). On the other hand, several lines of evidence point to IGF-independent effects for IGFBP-3 (Butt et al. 1999). IGFBP-3 can localize to nuclei and bind to retinoid X-receptor-α (RXR-α). This binding mediates the effects of IGFBP-3 in apoptosis by regulating the transcriptional activity of the RXR-α (Liu et al. 2000). Also the tumor suppressor p53 is known to induce IGFBP-3 expression (Buckbinder et al. 1995).

7. SUMMARY

The cerebellar granule cell cultures in combination with differential display analysis were used in order to identify gene expression changes and neuronal apoptosis related candidate genes. The potassium deprivation-induced apoptosis in CGCs is evidently a suitable model for screening studies and analysis of gene expression. Surprisingly, many genes belonging to the IGF system were found to be differentially expressed. The IGFs are recognized as potential drugs for neurodegenerative diseases. However, little is known about the actions of IGFs and IGFBPs in the nervous system. Thus, our data on the regulation of the IGF system expression during neuronal apoptosis provides basic knowledge to help unravel the role of the IGF system in neuronal pathology. We have shown that IGFBP-2, IGFBP-3 and IGFBP-5 are differentially regulated in neuronal apoptosis, with very distinct patterns depending on the apoptotic inducers used. Also the expression of the IGF1R and PTEN phosphatase changes during apoptosis, indicating the presence of an adaptive response to the decline in trophic factor stimulation. However, the changes in gene expression vary depending on the apoptosis model used, which reveals the existence of several apoptotic mechanisms in neuronal cells. The presence of differing apoptotic mechanisms in neuronal cells is further demonstrated by the expression pattern of one of our candidate genes, PTZ-17, which is specifically involved in potassium deprivation-induced apoptosis in cerebellar granule cells. The consequences of the observed gene regulation and their significance in the apoptotic process remain to be clarified.

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