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PAULIINA KORHONEN

Gene Regulation in Neuronal Degeneration – Role of mSin3 and YY1 factors

Doctoral dissertation

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Department of Neurology, University of Kuopio
Department of Neurology, Kuopio University Hospital

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Author's address: Department of Neurology
University of Kuopio
P.O.Box 1627
FIN-70211 KUOPIO
FINLAND
Tel. +358 17 163 446
Fax +358 17 162 048

Supervisors: Docent Antero Salminen
Department of Neurology
University of Kuopio

Professor Hilkka Soininen
Department of Neurology
University of Kuopio

Pre-examiners: Docent Tapio Visakorpi
Institute of Medical Technology
University of Tampere

Docent Jukka Westermarck
Turku Centre for Biotechnology
University of Turku

Opponent: Professor Lea Sistonen
Department of Biology
Åbo Academi University

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ABSTRACT

In neurodegenerative disorders, neural cells can die through two distinctive cell death processes, apoptosis and excitotoxicity. Both of these cell death processes involve changes in mRNA and protein synthesis rates and thus alterations in the level of transcription and translation. Age-related neuronal degeneration involves changes at the transcriptional and protein synthesis level. Transcription factor YY1 and adaptor molecule mSin3 are two proteins which are involved in transcriptional repression. YY1 and mSin3 are units of separate complexes where their repressive function is attributable to several other participants, mostly to histone deacetylases (HDACs). HDACs remove the acetyl groups from the core histone proteins of nucleosomes, and this leads to a conformational change in the nucleosome and further to transcriptional repression. Thus, through HDACs, the YY1 and mSin3 complexes regulate gene expression activity. Our aim was to investigate the changes in the expression of transcripton factor YY1 and adaptor molecules mSin3A/B at the mRNA and protein levels as well as to measure changes in the DNA binding activity of YY1 in neuronal cells and tissues during apoptosis, excitotoxicity and aging. Mouse Neuro-2a neuroblastoma cells, as well as rat primary hippocampal and cerebellar granule cells were used in the apoptosis and excitotoxicity studies. Tissue samples were taken from young and old rats. Several apoptotic inducers increased the mSin3A protein levels, but mSin3B levels remained unchanged. Trichostatin A (TSA), a specific inhibitor of HDACs, induced the strongest increase in the mSin3A protein expression. All apoptotic inducers significantly increased the caspase-3 activities in Neuro-2a cells, whereas etoposide did not induce any caspase-3 activity in primary cerebellar granule cells. Caspase-3 activity is considered to be a reliable marker of apoptosis. A brief exposure of primary neuronal cells to glutamate induced a dramatic change in the size of the binding complex of YY1 to its DNA binding site. The binding of the normal YY1 complex present in control samples disappeared while a new, smaller complex appeared. However, both complexes shifted to the same level with anti-YY1 antibody in EMSA-supershift assays. Interestingly, also okadaic acid -induced apoptosis caused similar changes in the DNA binding complexes of YY1 as did glutamate-induced excitotoxicity. These results indicate that apoptosis and excitotoxic shock in neuronal cells can change the protein levels of mSin3A and YY1. Also the binding complexes of YY1 were disrupted in excitotoxicity and okadaic acid induced apoptosis, highlighting the role of changes in YY1 transcriptional regulation. The changes seen in the DNA binding complexes of YY1 may be due to the glutamate induced Ca^{2+} influx, which further activates several signalling cascades in the cell, or okadaic acid induced changes in the phosphorylation status of the cellular proteins. Aging *in vivo* did not have any effect on either the expression of mRNA or protein of mSin3A/B and YY1 or on the complex formation of YY1 in rat brain samples. Thus, aging *in vivo* does not seem to have a role in the regulation of mSin3 and YY1, although protein synthesis slows down during aging. As a conclusion, apoptosis and excitotoxicity, but not aging, affected the transcriptional repressor factors mSin3A and YY1 indicating that the roles of those protein change during neuronal degeneration.

National Library of Medicine Classification: WL 359, WL 102.5
Medical Subject Headings: transcription, genetic; apoptosis; aging; neurons; neuroblastoma/cytology; gene expression; blotting, Northern; blotting, Western; electrophoretic mobility assay; transcription factors; excitatory amino acids; gene expression regulation; down-regulation/genetics; DNA-binding proteins/genetics

To Jarmo, Akuliina and Aukusti

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Kuopio, May 2002
Pauliina Korhonen

ABBREVIATIONS

aa	amino acid
AD	Alzheimer's disease
AIF	apoptosis inducing factor
AMC	7-amino-4-methyl coumarin
AML	acute myelogenic leukemia
ALL	acute lymphoblastic leukemia
Apaf-1	apoptotic protease-activating factor-1
APL	acute promyelocytic leukemia
APP	amyloid precursor protein
Aβ	β-amyloid
Ara-C	cytosine-β-D-arabinofuranoside
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
Caspases	cysteinyl aspartate-specific proteinases
CBP	CREB-binding protein
CDK	cyclin dependent kinase
cDNA	complementary DNA
CNS	central nervous system
CREB	cAMP response element binding protein
cyt c	cytochrome c
dATP	deoxyadenosine triphosphate
DIV	day *in vitro*
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
Dnmt	maintenance methylase
dNTP	deoxynucleotide triphosphate
DTT	dithiotreitol
ECL	enhanced chemiluminescence
EDTA	ethyldiamine tetraacetic acid
EED	embryonic ectoderm development
EMSA	electophoretic mobility shift assay
FADD	Fas-associated death-domain
Fas L	Fas ligand
FBS	fetal bovine serum
G	guanine
HAT	histone acetyltransferase
HD	Huntington's disease
HDAC	histone deacetylase
HID	HDAC interacting domain
HRP	horseradish peroxidase
kD	kilodalton
KO	knock-out
LF	long form
LZ	leucine zipper
MBD	methyl-CpG-binding domain
MeCP	methyl-CpG-binding protein
mRNA	messenger RNA
MTA	metastasis associated protein
M_W	molecular weight
NAD^+	nicotinamide adenine dinucleotide
N-Cor	nuclear hormone receptor corepressor
NF-κB	nuclear factor kappa B
NFT	neurofibrillary tangle
NMDA	N-methyl-D-aspartate
NMP-1	nuclear matrix binding protein 1
NP	neuritic plaque
NRS	neural restrictive silencer
NRSF	neural restrictive silencer factor
nt	nucleotide
NuRD	nucleosome remodelling and histone deacetylation
PAH	paired amphipathic helix
PARP	poly(ADP-ribose)polymerase

PCAF	p300-CBP associated factor
PBS	phosphate buffered saline
PcG	polycomb group
PCR	polymerase chain reaction
PD	Parkinson's disease
PHF	paired helical filament
PKA	protein kinase A
PKC	protein kinase C
PP	protein phosphatase
RAR	retinoic acid receptor
Rb	retinoblastoma
RbAp	retinoblastoma associated protein
REST	RE1-silencing transcription factor
RNA	ribonucleic acid
RE	repressor element
RT-PCR	reverse transcription PCR
RXR	retinoid X-receptor
SAP	Sin associated protein
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SF	short form
SID	Sin interacting domain
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
SPSS	Statistical Package for Social Sciences
STI	soybean trypsin inhibitor
SV-40	Simian virus 40
TAF	TATA binding protein associated factors
TBP	TATA binding protein
TF	transcription factor
TopoII	topoisomerase II
TR	thyroid hormone reseptor
TRADD	TNF-receptor associated death domain
TSA	trichostatin A
UV	ultraviolet
YY1	Yin Yang 1

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** and on two unpublished experiments in Appendix.

I Korhonen P, Huotari V, Soininen H and Salminen A. Glutamate-induced changes in the DNA-binding complexes of transcription factor YY1 in cultured hippocampal and cerebellar granule cells. Brain Res Mol Brain Res 1997;52:330-333.

II Korhonen P, Kyrylenko S, Suuronen T and Salminen A. Changes in expression and DNA binding of transcription factor YY1 in neuronal degeneration. (manuscript)

III Salminen A, Tapiola T, Korhonen P and Suuronen T. Neuronal apoptosis induced by histone deacetylase inhibitors. Brain Res Mol Brain Res 1998;61:203-206.

IV Korhonen P, Tapiola T, Suuronen T and Salminen A. Expression of transcriptional repressor protein mSin3A but not mSin3B is induced during neuronal apoptosis. Biochem Biophys Res Commun 1998;252:274-277.

V Kyrylenko S, Korhonen P, Kyrylenko O, Roschier M and Salminen A. Expression of transcriptional repressor proteins mSin3A and 3B during aging and replicative senescence. Biochem Biophys Res Commun 2000;275:455-459.

CONTENTS

1. INTRODUCTION

Degeneration of neuronal cells during aging when it remains at a low level is a normal physiological process whereas the degeneration rate in neuronal diseases is acclerated and more severe. The primary cause of neurodegeneration during aging and diseases is a phenomenon still poorly understood, although it represents the target of a multitude of investigations. The expected human lifespan is now longer than ever, thus also the problems of aged populations are continuously growing. Treatments to prevent age related neurodegeneration would have economic benefit as well a major impact on the quality of life of the patient and his/her relatives. A great deal is already known about the pathology of neuronal diseases, but the molecular mechanisms underlying many of these diseases remains unknown. Thus, more research is needed to find the cause and to improve the treatments for these problems.

Changes in the level of gene transcription and protein synthesis take place due to several circumstances in life, since some cells continue to devide all throughout life as other cells arekilled and removed from the body. The transcriptional activity rate is very high during the period of embryonic development whereas during aging there is extensive variation in the transcription rate between tissues, cell types and genes. The two opposite processes, cell division and cell death, are both extremely important aspects of life. Without functioning cell death machinery, the organism would suffer from serious pathological problems, since all defective cells need to be removed from the body. Nature has resolved this problem by establishing a suicide mechanism for cells called apoptosis. It is tightly controlled process and is activated intrinsically after a deleterious signal. It is a sophisticated way to get rid of unwanted cells, since apoptotic cell death does not damage any of the surrounding cells. Apoptosis has major significance during development and aging, and both excessive and reduced amounts of apoptosis are involved in the pathogenesis of a variety of diseases such as cancer and AIDS and some neuronal degenerative disorders, including Alzheimer's disease and Parkinson's disease.

Also other types of cell death are important. These include necrosis induced by neuronal cell excitotoxicity. Necrosis is a pathological or accidental mode of cell death, which affects a number of contiguous cells and is involved in inflammatory responses. The increased glutamate release in the brain is involved in several serious neurological diseases, including stroke and epilepsy. Glutamate is the most common and abundant excitatory transmitter in the brain, being involved in transporting the activating signal from the presynaptic to the postsynaptic neuron. However, in some circumstances, excess glutamate can be toxic to the neuronal cells, leading to a process called excitotoxic cell death. The excitotoxicity causes massive activation in the signalling cascades within the cell and interferes with the protein synthesis control, as does apoptosis, even though the signalling routes and proteins involved may differ in these two cell death types. .

The life and death of cells are both controlled and a balance exists in the healthy organism. If either process, excess cell division or enhanced cell death gains an advantage, the balance is disrupted and a pathological situation occurs. The controlling system is complicated and many details remain unknown. Thus, more knowledge is needed if we are to understand the mechanisms involved in the inappropriate control processes in these diseases.

2. REVIEW OF THE LITERATURE

2.1 Mechanism of cell death

Cell death mechanism is determined by signalling cascades activated by an intrinsic or extrinsic primary insult. These mechanisms include apoptosis (also known as programmed cell death), necrosis, aponecrosis and excitotoxicity. Apoptosis is a silent and controlled type of cell death, the opposite of necrosis. Apoptosis requires energy to occur, whereas necrosis can be accomplished without ATP. Aponecrosis is a combination of these two processes, and the energy level of the cell finely decides, whether the cell dies through apoptosis or necrosis. Excitotoxic cell death is attributable to excess calcium influx into the neuronal cell and it also has features of both apoptosis and necrosis, although the primary cell death mechanism in excitotoxicity is necrosis.

2.1.1 Apoptotic cell death

Apoptosis is a type of cell death that Kerr, Wyllie and Currie described and named in 1972 (Fig. 1). The word apoptosis comes from Greek language meaning “dropping or falling off” and it represents a situation that is complementary to mitosis in the regulation of eukaryotic cell populations (Kerr et al. 1972). Apoptosis is initially defined by its morphological characteristics such as cell shrinkage, membrane “blebbing”, chromatin condensation and nuclear fragmentation (Kerr et al. 1972). The nucleus condenses completely and is segregated into several fragments, although the other organelles are generally left intact (Hacker 2000). The entire condensed cell is reorganized into “apoptotic bodies” which are small membrane-bound vesicles varying in size and composition (Kerr et al. 1972). The “apoptotic bodies” can contain whole organelles, parts of condensed nuclei or cytosolic elements, which are phagocytosed by neighbouring cells. Apoptosis is a silent type of cell death, since it does not induce a local inflammatory response, and has no effect to the surrounding cells. In this respect it differs from the necrotic type of cell death (Fig. 1). The dying apoptotic cells simply disappear without leaving any traces.

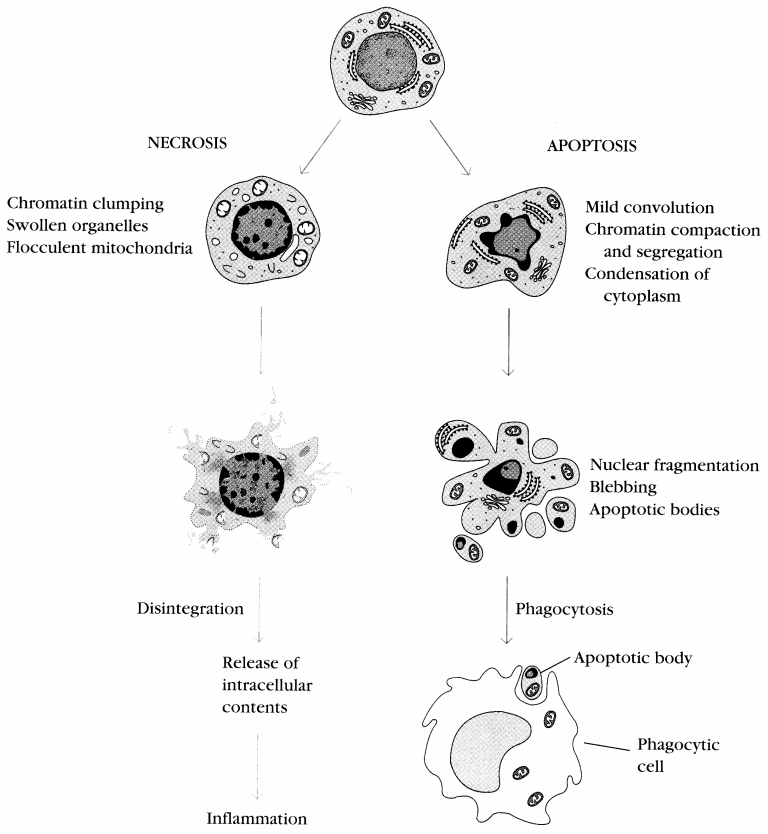


Fig. 1 The two major type of cell death mechanism, necrosis and apoptosis, and their morphological changes. The picture is modified from Kuby Immunology by Goldsby at al. (2000) pp.31.

Apoptosis is an active, energy requiring process that is genetically controlled and involves protein synthesis (Wyllie 1995). However, reports where apoptosis was shown to occur without protein synthesis has recently been published (Ui-Tei et al. 2000, Jeffrey et al. 2002). The whole apoptotic process can be divided into three functionally separate phases, the induction, execution and degradation phases (Yue et al. 1999). In the induction phase, signals reach the cell and activate the apoptotic machinery. These apoptotic signals are transmitted via cell surface death receptors and/or they can affect mitochondria leading to the activation of the apoptotic signal cascade (Ashkenazi and Dixit 1998, Green and Reed 1998). The execution phase includes processes that occur inside the cell after it has received the apoptotic stimuli. The degradation phase consists of processes leading to a “point of no return” after which the cell no longer has the possibility to recover and/or escape from cell death (Yue et al. 1999).

Several signals originating either from the cell itself or from its extracellular environment are known to induce the apoptotic type of cell death. These signals include cell-lineage information, ionising radiation, viral infection, extracellular growth factors, cell-cell interactions, hormones *etc.* (Steller 1995). Central components of the apoptotic death machinery include caspases (cysteinyl aspartate-specific proteases), Bcl-2 protein family members and Apaf-1 (apoptotic protease-activating factor 1).

2.1.1.1 Caspases

Caspases are a family of cysteine proteases that cleave their substrate proteins on a specific recognition site flanked by aspartic acid residues (Thornberry and Lazebnik 1998). After the aspartic acid residue at least four followed amino acids need also to be recognized for a successful cleavage (Thornberry et al. 1997). The variability among these amino acids has shown to be significant between the caspases (Thornberry et al. 1997). Caspases are synthesized initially as inactive pro-enzymes (zymogens) that are converted to active proteases upon different death signals. When activated, a single-chain zymogen is cleaved into two distinct subunits forming a heterodimer and further two heterodimers associate into an active heterotetramer protease with two catalytic sites (Thornberry and Lazebnik 1998). Caspases are divided into two categories, called initiators and effectors (Thornberry and Lazebnik 1998). Initiators are those enzymes that are activated in the early phase of apoptosis and further have the capacity to cleave and activate downstream caspase zymogens. These downstream effectors are involved in the cleavage processes of cellular proteins.

A total of over ten caspases have been cloned (Budihardjo et al. 1999). Because all caspases are cleaved at specific aspartic acid residues, some caspases may activate other caspases, establishing a hierarchy of these proteases (Cohen 1997). It is known that the initiators (caspase-8 and -9) activate the effector caspases, such as caspase-3 and -7. These (caspase-3 and -7) further activate the downstream caspases, like caspase-6. Proteins cleaved by the effector caspases include poly(ADP-ribose)polymerase (PARP), DNA-dependent protein kinase (DNA-PK), Bcl-2, lamin B, fodrin and histone H1 (Cohen 1997). Many of the proteins cleaved by caspases are

DNA repairing enzymes, anti-apoptotic proteins and important cytoskeletal proteins. By breaking down these proteins, the caspases are responsible for most of the dramatic morphological events occurring in apoptosis.

There are two well characterized cascades known to regulate apoptosis due to caspase activation: one acts via cell surface death receptors and the other is activated by changes in mitochondrial integrity (Budihardjo et al. 1999). Although the apoptotic signals do not always affect the function of mitochondria immediately, at some stage in the apoptotic process mitochondria will become involved (Green and Reed 1998). Death receptors are cell surface receptors, that belong to the tumor necrosis factor (TNF) receptor gene superfamily and transmit apoptosis signals initiated by specific "death ligands" (Ashkenazi and Dixit 1998). They all contain a homologous sequence, the so called "death domain" in their cytoplasmic region. Binding of extracellular ligands to the receptors, such as CD95L/Fas ligand to CD95/Fas/Apo1 receptor and TNF to TNF receptor 1 (TNFR1), induces trimerization of these receptors such that their "death domains" bind together. These further recruit some additional adaptor proteins, like FADD (Fas-associated death domain) or TRAD (TNFR-associated death domain) to the clustered receptor death domains (Ashkenazi and Dixit 1998). These adaptor molecules then bind and activate pro-caspase-8 from zymogen into active protease, which in turn goes on to cleave downstream caspase zymogens, such as pro-caspase-3 (Stennicke et al. 1998). These receptors have the capability to activate caspases very quickly, within seconds of ligand binding, evoking the cell's apoptotic death within hours (Ashkenazi and Dixit 1998).

During apoptosis, cytochrome c is released from the mitochondria, which in turn associates with Apaf–1 and pro-caspase-9, forming a complex called the "apoptosome" (Green and Reed 1998). This complex with adenosine triphosphate (ATP) or with deoxyadenosine triphosphate (dATP) further activates the caspase-9 and leads to the activation of the other downstream effector caspases (Green and Reed 1998).

A lot of discussion is going on with respect to different possibilities to treat diseases that involve an apoptotic type of cell death, but some basic questions still need to be

resolved. In the case of neurodegenerative diseases, it is still unclear whether apoptosis is the cell's reaction to a permanent neuronal injury caused by the disease or vice versa (Nijhawan et al. 2000). If the disease itself is directly involved in activating the apoptosis, there may be possibilities to prevent the cell death by using anti-apoptotic drugs, such as caspase inhibitors (Nijhawan et al. 2000).

2.1.1.2 Apoptosis during development and degeneration

Apoptosis plays very important role during normal development and tissue homeostasis but its inappropriate regulation is associated with several diseases, including neurodegenerative diseases (like Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS)), ischaemic stroke, AIDS, cancer and autoimmune disorders (Thompson 1995). In AD, HD, ALS and PD, the neurodegeneration and cell death is associated with intracellular or extracellular protein aggregation (Kakizuka 1998). These aggregates are thought to have an association with caspase activation, at least in the cases of AD, HD and ALS, and thus to apoptosis (Nijhawan et al. 2000).

In the developing brain, the huge original cell population is eliminated as a result of apoptosis. According to some estimates this may involve at least half of the original cell population (Burek and Oppenheim 1999, Nijhawan et al. 2000). To avoid apoptosis during development, a neuronal cell needs connections to a postsynaptic target and also neurotrophic factors derived from the target cells, glial cells (Lindsay 1979, Gomes et al. 2001) and presynaptic cells (Linden 1994). Also steroid hormones have been shown to provide trophic support for neurons (Chew and Gallo 1998).

The two different events, cell division and apoptotic cell death share some common processes, like chromatin condensation and volume loss. Thus it was thought that the cell machinery could be involved in both of those processes (Evan and Littlewood 1998). Depending on the level of appropriate stress signals received by the postmitotic cells, also their cell cycle machinery and apoptotic processes may become activated, leading to cell death (Liu and Greene 2001). This is mainly due to the cell cycle regulatory protein Rb and its related protein p130 (Liu and Greene 2001). Their

function in the mature neuronal cell is to repress the cell cycle machinery and keep the cell in the quiescent stage of the cycle, *i.e.* in the G0/G1 state. Phosphorylation of Rb and p130 reduces their ability to work as repressors, and the activation of cell cycle process occurs. This evokes the neuronal cells to activate the apoptotic mechanisms leading ultimately to cell death. In addition, there are studies indicating that in AD brains the characteristic neurofibrillary tangles and neuritic plaques may be a consequence of inappropriate cell cycle events (Nagy et al. 1997b, Nagy et al. 1998). It has been suggested that neuronal cells that suffer from cell cycle disturbances are committed to one of two fates; they either die via apoptosis or they produce Alzheimer-type pathology (Nagy et al. 1998).

2.1.2 Necrosis

Apoptosis is a silent type of cell death, since it does not harm the surrounding cells. The dying cell just disappears without leaving any trace. Necrosis is the opposite process to apoptosis, because it often involves a local inflammation and recruits several types of inflammatory cells to the site (Columbano 1995). Necrotic cells swell and burst, and release their intracellular contents into the extracellular space. Necrosis is a result of injury and does not involve genetic control nor protein synthesis in the way achieved during apoptosis. (See Fig. 1 in 2.1.1)

2.1.3 Excitotoxic cell death

Glutamate is the most abundant excitatory neurotransmitter in the brain. It acts through activation of glutamate receptors. These include ionotropic glutamate receptors (NMDA, AMPA, Kainate) and metabotropic glutamate receptors (Quisqualate-B). Initially the term excitotoxicity described a situation, where cell death was induced in neurons with a very high concentration of exogenous glutamate or its agonists. Subsequently the term was broadened to mean also endogenous glutamate-mediated neurotoxicity, which is reported to occur in neurological disorders including epilepsy (Bradford 1995) and ischemia (Nishizawa 2001). Excitotoxicity is triggered by the excessive release of glutamate from presynaptic and glial cells into the extracellular space. This neurotransmitter then over-activates glutamate receptors, especially N-

methyl-D-aspartate (NMDA) receptors, leading to excessive Ca^{2+} (and Na^{+}) influx into the cell.

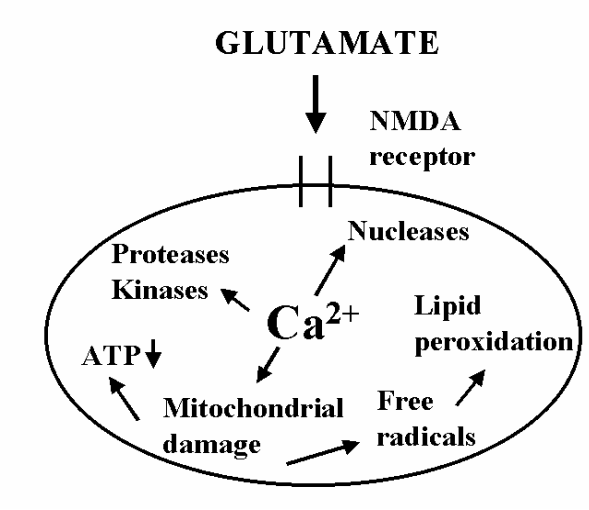


Fig. 2 Schematic diagram of the effects triggered by glutamate induced Ca^{2+} influx into the neuronal cell (modified from Adam Doble, 1999). Elevated levels of Ca^{2+} ions can activate several proteases and nucleases that damage the cytoskeleton and chromatin of the cell. Lipases are also activated, which further destroy the plasma membrane and other cellular organelles. Also the mitochondria are affected, leading to release of free radicals. Impaired function of mitochondria lowers the ATP levels of the cell and promotes cell death.

Glutamate induced excitotoxicity has been suggested to cause either necrosis or apoptosis. There has been some controversy about the cell death type which glutamate triggers. Originally, it was thought that excitotoxicity typically leads to necrosis (Zipfel et al. 2000). Nowadays it is also thought that apoptosis may have a role in excitotoxicity, at least under certain conditions, such as when the exposure to glutamate is mild (Zipfel et al. 2000). Also another kind of neuronal death is triggered in glutamate induced cell death, since the caspases are not always activated, although the nuclear morphology may seem apoptotic-like (Bezvenyuk et al. 2000). There is also a growing consensus about the role of both processes, apoptosis and necrosis occurring within a single cell; a phenomenon called aponecrosis (Formigli et al. 2000). Aponecrosis shares the molecular, morphological and dynamic features of both apoptosis and necrosis (Formigli et al. 2000). It is suggested that in early phase of the cell death process, the cell may share some common events with apoptotic and necrotic processes and later, depending on the intensity of the insult and on the energy stores (ATP) present in the cell, the cell finally dies in an apoptotic, aponecrotic or necrotic manner (Nicotera et al. 1999, Formigli et al. 2000). Thus, the two execution processes,

apoptosis and necrosis, may share similar mechanisms in their death program and are considered to represent only the extremes in the cell death processes (Formigli et al. 2000). Furthermore, there are also studies indicating, that glutamate-induced excitotoxicity is not the cause of neuronal cell death or neurodegenerative diseases (Obrenovitch et al. 2000). These studies claim that glutamate is removed the extracellular space so rapidly that it cannot cause any toxic insult to the neuronal cells (Obrenovitch et al. 2000). Thus, no unequivocal consensus on the role of excitotoxicity exists, although in general excitotoxicity is thought to play a significant role in neuronal death processes.

2.2 Neuronal aging

One aspect of aging is a decrease in the rate of protein synthesis and turnover and this can lead to inefficient removal of damaged and inactive macromolecules from the cell (Rattan 1996). Aging in the nervous system is characterized as being an irreversible process with increasing deviations from an ideal functional state present immediately after the maturation of neuronal cells (Cotman and Peterson 1989). Mature neurons are postmitotic cells thus they are incapable of undergoing cell division. Aging can also described as an event with functional losses, even though not all functions are affected in aged individuals (Cotman and Peterson 1989). Also heterogeneity within the aged population is one characteristic feature, meaning that some elderly persons suffer only mild functional losses, where others exhibit marked deficiencies. Heterogeneity exists also in different brain regions, and even among different cell groups in the brain (Cotman and Peterson 1989). Age related changes are thought to be a sum of genetic, lifestyle and environmental factors (Cotman and Peterson 1989). The markers involved in neuronal aging include neuron shrinkage, loss of neurons and synapses, accumulation of lipofuscin (lipoprotein pigment) in cell, and also formation of some neuritic plaques (NP) and neurofibrillary tangles (NFT) (Dani 1997, Esiri et al. 1997).

In addition to mature postmitotic cells, also neuronal precursor cells (stem cells) which posses a capability to divide still exist in the aged brain (Eriksson et al. 1998, Goldman 1998, Steindler and Pincus 2002). Thus replacement of degenerated or dead neurons is possible to some extent, although the deprivation of appropriate growth factors and

migratory support to the stem cells is suggested to be the limiting factor to efficient neurogenesis in adult brain tissue (Goldman 1998).

Neuritic plaques and neurofibrillary tangles are characteristic signs of protein precipitation in neuronal aging and Alzheimer disease, although the distribution and density is different in AD than in the normal aged brain (Morrison and Hof 1997). Extracellular NP contains amyloid fibrils comprise an insoluble form of the 4 kD peptide, ß-amyloid (Aß) surrounded by degenerating nerve terminals and often with the presence of astrocytes and microglial cells (Esiri et al. 1997). Also paired helical filaments (PHF) are involved in NP in degenerating nerve terminals (Esiri et al. 1997). NFT contains as well deposits of structural proteins, mainly abnormal PHFs in the nerve cell body. This type of neurodegeneration leads to a decrease in the number of functioning synapses in brain, and further to severe intellectual decline called dementia. The exact relationship between these pathological hallmarks is not known though it has been shown that NPs precede NFTs in neocortical areas (Esiri et al. 1997). However, dementia is thought to correlate better with the density of NFT rather than the density of ß-amyloid plaques (Berg et al. 1998).

Oxidative stress and DNA damage are known to play a role in aging (Martin et al. 1996, Sohal and Weindruch 1996). In addition, oxidative stress and DNA damage have also been shown to induce apoptotic cell death (Buttke and Sandstrom 1994, Canman et al. 1994). However, dysregulation of apoptosis is suggested to play a role in the aging process (Higami and Shimokawa 2000). It is thought that over a long period of time, postmitotic cells will be exposed to a certain amount of oxidative stress and this can drive certain cells into apoptosis (Higami and Shimokawa 2000).

It has been shown that low calorie intake can increase the lifespan of experimental animals (Mattson 2000b). It is also known that dietary restriction increases the resistance of neurons to damage and death in several brain disease models, including AD, PD and stroke (Bruce-Keller et al. 1999). Reduction in calorie intake has been reported to increase neuronal cell activity and induce the expression of some cellular stress proteins and neurotrophic factors that further protect the cell against aging and

injury (Mattson 2000b). Moreover, dietary restriction is claimed to increase the numbers of newly-generated neurons in the adult brain, which in turn may increase brain plasticity and self-repair (Lee et al. 2000). According to this theory, it has been proposed that dietary restriction is one way to prevent age-related disorders, including AD (Mattson 2000a). In addition, aerobic physical activity is also claimed to improve some neurocognitive function in aged individuals (Kramer et al. 1999). Moreover, in a study where mice were allowed to voluntarily exercise on a running wheel, their cell proliferation rate and neurogenesis in dentate gyrus increased, and the animals showed improved learning and memory (van Praag et al. 1999). Environmental enrichment, such as larger housing and more social interactions, gave also similar results (van Praag et al. 1999). Thus, the possibility to stimulate already existing neural stem cells to proliferate and differentiate into mature functional neurons in aged or damaged brain tissue is today one of the most exciting research topics. Mattson and co-workers have proposed that exercise with reduced calorie intake and mental activity may be the key elements in preventing age-associated problems, such as neurodegenerative disorders (Mattson 2000b).

2.3 Transcriptional regulation

The genetic information for proteins is coded into the sequences of DNA. In order to produce proteins, the cell needs first to translate the DNA code into a sequence of messenger RNA molecules (mRNA). This process is called transcription. Transcription is followed by translation, the synthesis of proteins according to the mRNA sequence. Regulation at the level of transcription allows the organism to have several types of differentiated cells, which despite all having the same set of inherited genes, can have separate functions. It is very important for the cell to regulate what genes are transcripted or repressed at a given time. Thus there are major differences in the gene expression pattern between the cell types. Various specific proteins control the transcription process, giving the cell the possibility to adapt to situations where there are changes in the intracellular or extracellular signalling cascades.

2.3.1 General transcriptional machinery

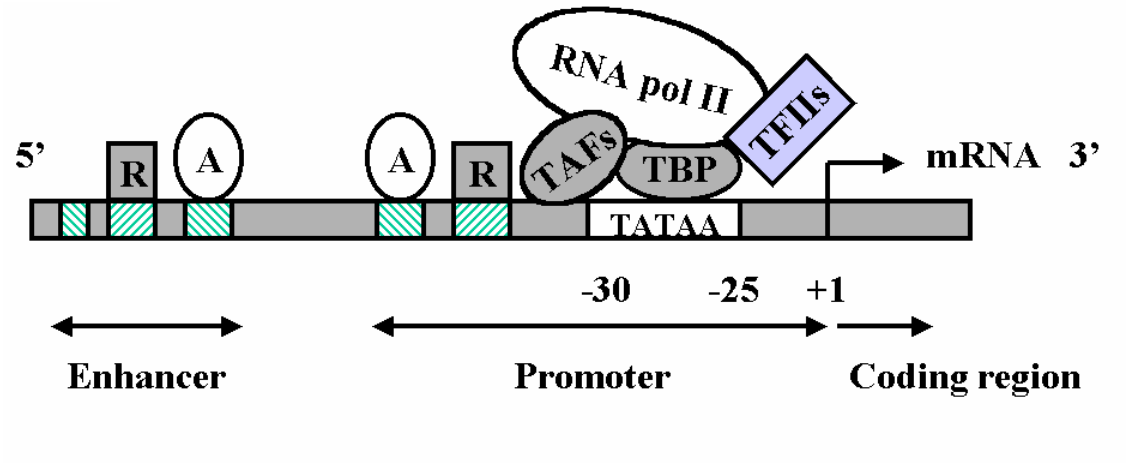
Transcriptional activators or repressors bind to their specific DNA sequences, which are usually located upstream of the core promoters (Goodrich et al. 1996). Their function is to increase or repress the rate of transcription by interacting with the basal transcription apparatus. Transcription factors can bind just upstream of the core promoter region or they can bind to a distant enhancer region, but their role as transcriptional regulators is similar (Cooper 2000). DNA can bend and form loops, thus even those transcription factors located somewhat distant from the promoter can still make physical contacts with the basal transcription machinery. Enhancer elements are usually sites for tissue specific or gene specific transcription factors (Lewin 2000). Another group of proteins, called co-activators/co-repressors or adaptors are also involved with regulation of transcription. These cofactors favour transcription, probably by promoting interactions between transcription factors, the general transcriptional machinery and core histone proteins (Burke and Baniahmad 2000, Haus-Seuffert and Meisterernst 2000, Tetel 2000).

Transcription contains five separate stages; 1) template recognition, 2) initiation, 3) promoter escape, 4) elongation and 5) termination. In the template recognition stage, the preinitiation complex, composed from RNA polymerase and five general transcription factors, binds to the promoter sequence of the double stranded DNA. Melting of the DNA strands inside of the RNA polymerase complex follows this process, enabling pairing of the template strand of DNA and ribonucleotides. During initiation, the RNA polymerase remains in the promoter and the first nucleotide bonds of RNA are synthesized and released. Following initiation, the RNA polymerase moves away from the promoter, a process called promoter escape (Conaway et al. 2000). Elongation is a stage when the polymerase enzyme moves along the DNA as the RNA chain grows. It is regulated by specific elongation factors as well as by phosphorylation of the C-terminal end of the biggest subunit of RNA polymerase II (Conaway et al. 2000). DNA strands are unwound and again rewound as the enzyme moves along the chain. The RNA-DNA hybrid is formed in the unwound region, which is the site where nucleotides are added to the 3’ end of the RNA chain. In the termination stage, the

polymerase complex recognizes a sequence, which stops the transcription leading to release of the RNA polymerase and the formed RNA. (Lewin 2000)

Eukaryotic genes are transcribed by three distinct RNA polymerases, namely RNA polymerase I, II and III (Paule and White 2000). RNA polymerase II is the enzyme responsible for all messenger RNA (mRNA) synthesis, and it requires several classes of proteins that function in a highly organized manner (Hampsey 1998). It is highly conserved between yeasts and humans (Feaver et al. 1997). The other two RNA polymerises, I and III, transcribe nuclear genes coding the ribosomal RNAs and transfer RNAs (Paule and White 2000). The general transcription factors for polymerase II are TFIIB, TFIID, TFIIE, TFIIF and TFIIH, which are required for initiation and elongation of mRNA (Zawel and Reinberg 1993, Zawel et al. 1995). The TFIID complex is responsible for promoter recognition (Burley and Roeder 1996). The core TATA box is recognized by the components of TFIID complex, TATA-binding protein (TBP), and TBP-associated factors (TAFs) (Burley and Roeder 1996). TBP binds then the TFIIB, which further recruits the RNA polymerse II – TFIIF complex to the promoter (Hampsey 1998). Initiation of transcription still needs the binding of two other factors, namely TFIIE and TFIIH (Hampsey 1998). TFIIH contains ATP-dependent helicases that unwind the double helix DNA around the initiation site and trigger the transcription process (Svejstrup et al. 1996).

A.



B.

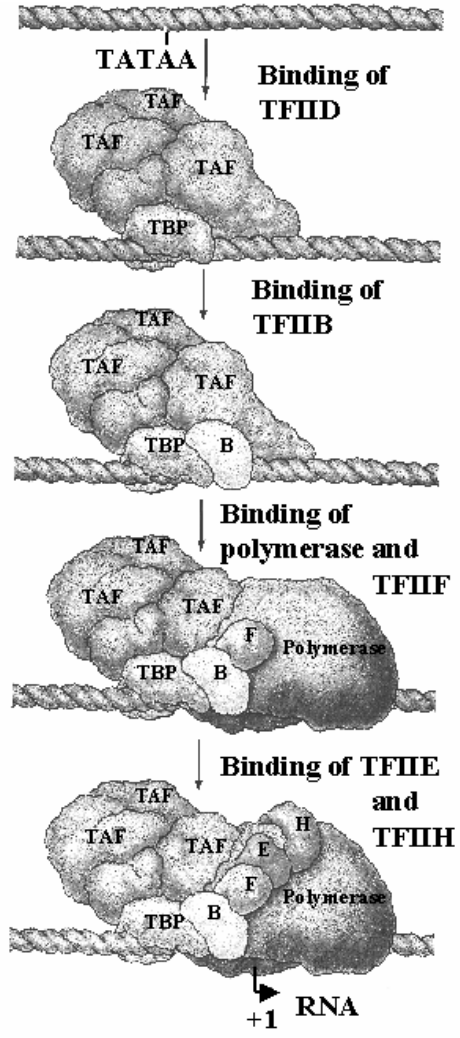


Fig. 3

A. A schematic diagram of the structure of a gene constructed from the promoter, enhancer and coding regions. The TATA sequence is located usually 25 to 35 base pairs upstream from the initiator (+1) sequence and binds TATA- binding protein (TBP). Bound TBP is the core of the transcription preinitiation complex, shown in picture A. Promoter and enhancer regions contain also sequences for transcription factors. The enhancer region is located upstream from the promoter. Transcription factors can either activate or repress the transcription. The coding sequence of the gene starts from the initiator sequence and stops at the termination site. A, activating transcription factor; R, repressing transcription factor; TAFs, the TBP associated factors; TFIIs, the general transcription factors.

B. The formation of RNA polymerase II complex according to Geoffrey M. Cooper (The Cell, A Molecular Approach (2000) pp.237).

2.3.2 Transcriptional repression

Regulation at the level of transcription, in activation or repression, has paramount importance in life. Even though each individual has the same set of genes inherited in its somatic cells, the structure and function of these cells still differ from each other. This type of regulation mechanism, where the function of the gene is selectively activated or inactivated in the cell is called epigenetics (Wolffe and Matzke 1999). Transcriptional repression of those genes that are not required in specific cell types is the basic foundation of normal development. Abnormalities in these regulatory systems are involved in human diseases, such as in cancer and in developmental defects (Nakao 2001). Mechanisms that are involved in the epigenetic control are DNA methylation and modification of chromatin (Nakao 2001).

2.3.2.1 DNA methylation

Methylation of DNA is associated with condensed nuclease-resistant heterochromatin, transcriptional repression and silencing of genes (Keshet et al. 1986, Bird 1992). In animal genomes, the cytosine residues in the sequence 5'-CpG-3' (cytosine–guanine) (and occasionally 5'-CpNpG-3') are often methylated (Nan et al. 1998). This methylation is a reversible covalent modification and it plays an important role in several physiological processes. Approximately 60% of genes have promoters containing dense regions of CpGs, called CpG islands (Antequera and Bird 1993). For some unknown regulation mechanism, these CpG islands are usually unmethylated. The best-known examples of repression by methylation are the expression of tumor suppressor genes in cancer (Baylin et al. 2001), X chromosome inactivation (Huber et al. 1999), and parental imprinting (Bartolomei and Tilghman 1997). Exceptions also exist, but they are often related to the repression of tumor suppressor genes and involved in the development of cancer by influencing many of the abnormalities in cancer including loss of cell cycle control, altered function of transcription factors and receptors, loss of apoptotic signals, loss of DNA repair enzymes and loss of cell-cell recognition (Baylin et al. 2001). Also the CpG islands in X-linked housekeeping genes in one of the female X chromosomes are methylated (Razin and Kafri 1994, Huber et al. 1999). This is because there is equal X-linked gene dosage between the sexes, so one of the chromosomes needs to be inactivated in females. Weak promoters can be

silenced already by a low density of methylated CpGs, whereas a higher density of methylation is required to repress a strong promoter, highlighting the importance of the density of methylation (Boyes and Bird 1992). Methyltransferases are enzymes that are responsible for the maintenance of methylation (maintenance methylase 1, Dnmt1) as well as *de novo* methylation during embryogenesis (Dnmt3a, Dnmt3b) (Newell-Price et al. 2000). Methylated DNA, histone deacetylation and gene silencing are linked together through methyl-CpG-binding proteins (MeCPs)(Jones et al. 1998, Nan et al. 1998).

Mutations in the MeCP2 are related into neurodevelopmental disorder, Rett syndrome, which is the major cause of mental retardation and autistic behaviour in girls (Amir et al. 1999). This disease, initially recognized by Andreas Rett in 1966, has been shown to affect 1 of 10,000 to 1 of 15,000 females (Wan et al. 1999, Amir et al. 2000). The *MeCP2* gene coding for MeCP2 protein is located in the X chromosome and the syndrome almost exclusively occurs in females (Amir et al. 1999). Because of this, it was hypothesized that the mutation of this gene is lethal to male embryos. Studies with MeCP2-null animals have been consistent with this theory, since the MeCP2-null embryos have been lethal (Chen et al. 2001, Guy et al. 2001). Since this gene is located in the X chromosome, it also undergoes X chromosome inactivation. Most interestingly, deletion of *MeCP2* gene affects almost only the nervous system, very few abnormalities are seen elsewhere (Chen et al. 2001, Guy et al. 2001). The reasons that make neuronal cells so more vulnerable to MeCP2 mutation than other cell types are now being studied (Carter and Segal 2001).

2.3.2.2 Histone acetylation and deacetylation

The activity of transcription is not only due to the regulation of DNA, since also the structure of chromatin plays a role in transcriptional regulation. Nucleosomal proteins, histones, can be modified in several ways, which further affects to the activation of gene expression.

2.3.2.2.1 Nucleosome structure

The nucleosome is the basic structural element of chromatin located in the cell nucleus. The nucleosome is built from core histone proteins, with DNA wrapped around them. The core histone proteins H2A, H2B, H3 and H4 are organized into dimers and linked end to end (H2A/H2B)-(H4/H3)-(H3/H4)-(H2B/H2A) to form an octamer (Arents et al. 1991). Almost two turns of DNA (~146 bp) are wrapped around this core histone octamer (Grunstein 1997). All core histone proteins have a similar structure consisting of a basic N-terminal unstructured domain, a globular domain formed by a histone fold, and a C-terminal unstructured tail (Davie and Chadee 1998). In addition to this, H2A has also C-terminal tail domain (Wolffe and Hayes 1999). These tail domains contain ~ 25% of the core histone mass and were originally defined by their sensitivity to proteases (Bohm and Crane-Robinson 1984). In contrast to the structural core histone proteins, histone H1 is associated into linker DNA (0-80 base bairs), which connects the nucleosomes together, resulting in the formation of "beads-on-a-string" chromatin structure (Davie and Chadee 1998). At physiological ionic strength, chromatin is folded into 30 nm chromatin fibers and this folding is stabilized by the H1 histone protein (Shen et al. 1995, Davie 1997). Even though linker histones can help the nucleosomes to form higher order chromatin structures, they are not essential for chromatin and chromosome assembly in the same way as the core histones (Dasso et al. 1994). The core histones can be modified by several post-translational modifications, such as acetylation, phosphorylation, methylation, ubiquitination, glycosylation, and ADP-ribosylation, affecting their charge and function (Grunstein 1997, Davie 1998). Most of these modifications occur on the basic N-terminal tail domain.

2.3.2.2.2 Histone acetylation

It has been known for several years that there is a functional link between transcriptional activity and acetylation of histones. This led to the idea that gene expression might be regulated by covalent modifications of histone proteins (Pogo et al. 1966). However, the final evidence connecting the chromatin function and acetylation together was discovered later when the coactivator complexes required for transcriptional activation were found to act as histone acetyltransferases (HAT) as well as the discovery of corepressors containing histone deacetylases (HDAC) which

repressed the transcription (Brownell et al. 1996, Taunton et al. 1996). The HAT family of proteins are classified into two distinct groups, HAT-A and HAT-B, where HAT-A regulates the acetylation in the nucleus and HAT-B acetylates newly synthesized core histone proteins in cytoplasm (Kleff et al. 1995). The acetylation of core histone proteins is highly evolutionary conserved (Couppez et al. 1987, Clarke et al. 1993). Acetylation occurs at specific lysine residues in the N termini of the core histone proteins. Acetylation neutralizes the positive charge of the tails and weakens the electrostatic interaction between the histone and the DNA backbone (Hong et al. 1993, Ayer 1999). There are several theories about how this acetylation mediates the activation of transcription. First it may cause 'loosening' of the nucleosome structure so that the transcription activators and the basal transcription machinery have better access to their specific binding sites in the gene promoter and enhancer sites (Grunstein 1997, Ayer 1999). Also it has been suggested that the hyperacetylated histone tails may bind directly into some transcriptional activators as well as chromatin-remodelling complexes (Lee et al. 1993, Vettese-Dadey et al. 1996, Georgel et al. 1997). It has been shown that many such proteins that have initially described having functions in transcription regulation have intrinsic histone acetylase or deacetylase activity. Some acetyltransferases are actually tightly associated with the RNA polymerase II transcription machinery itself, whereas other acetyltransferases and deacetylases are linked with transcriptional regulatory factors (Struhl 1998). The related proteins CREB-binding protein (CBP) and p300, often termed p300/CBP, are both histone acetyltranferases, and they bind to transcription factors involved in cell-cycle control, differentiation, DNA repair and apoptosis (Kornberg 1999). Thus, due to their transcriptional stimulation activity, they are called co-activators.

2.3.2.2.3 Histone deacetylation

Histone deacetylases are enzymes that remove the acetyl groups from the lysine residues of core histone proteins. Deacetylation influences the nucleosome structure in the opposite way to acetylation, *i. e.* reducing the ability of activating factors to bind to their specific binding sites. A mammalian histone deacetylase was originally purified and identified by Stuart Schreiber and co-workers using a novel method where HDAC1 was isolated on the basis of its high affinity for the histone deacetylase inhibitor,

trapoxin (Taunton et al. 1996). This HDAC1 was found to have 60% homology with yeast global transcriptional repressor Rpd3 (Taunton et al. 1996).

Human histone deacetylases have been divided into three classes; class I for HDACs that have high homology with yeast Rpd3, class II for Hda1 family of HDACs and class III for Sir2 family of HDACs (Gray and Ekstrom 2001). Histone deacetylases 1, 2, 3 and 8 belong to class I, and it has been shown in several studies that these HDACs associate with corepressor complexes to direct gene-specific transcriptional repression (Gray and Ekstrom 2001). It has also been demonstrated that all these class I HDACs are sensitive to histone deacetylase specific inhibitors, like trichostatin A (TSA), trapoxin and sodium butyrate (Gray and Ekstrom 2001, Nakayama and Takami 2001). While HDAC1 and 2 usually are found in the same complexes, HDAC3 appears to be uncomplexed with either of the two other HDACs, 1 or 2 (Hassig et al. 1998). Class II of the HDAC family comprises HDAC 4, 5, 6, 7, 9 and 10 (Fischer et al. 2001, Nakayama and Takami 2001, Zhou et al. 2001). These HDACs show tissue specificity, unlike HDACs 1, 2 and 3, which are expressed in various tissues (Cress and Seto 2000). Class III HDACs belongs to the Sir2 family of proteins. *Sir2* gene homologs have been found in many organisms, seven *Sir2* homologs have been isolated in human tissues. Each member of the HDAC family may exhibit a different, individual substrate specificity and function *in vivo* (Nakayama and Takami 2001).

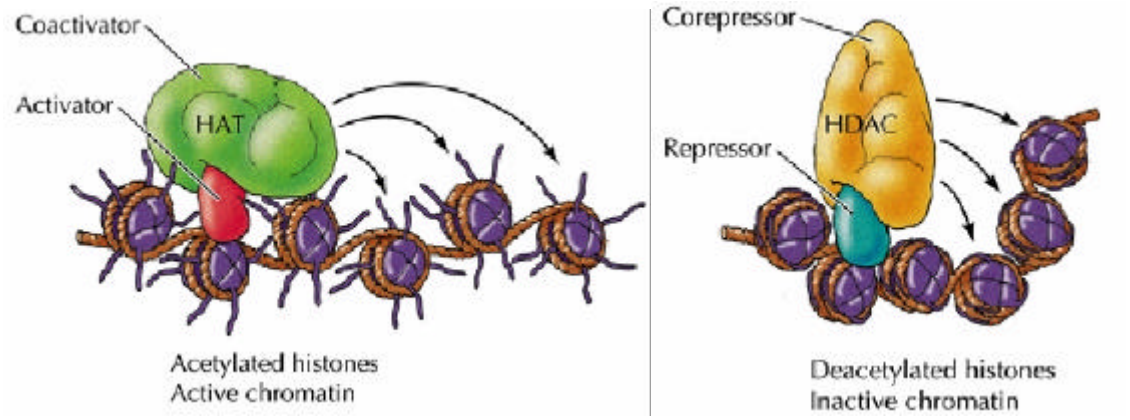


Fig. 4 Transcriptional activators and repressors form complexes with cofactors containing histone acetyltransferace (HAT) or histone deacetylase (HDAC) activity, thus modulating the conformation of nucleosomes. The diagram is from Cooper (2000) pp. 254.

2.4 Histone deacetylase complexes

There are several proteins that form complexes with histone deacetylases (HDACs). These complexes participate in transcriptional regulation by deacetylating the nucleosomal core histone proteins and changing the chromatin from the relaxed and active acetylated state to a condensed state. Only the best-characterized HDAC complexes are discussed below and the main interest has been focused towards complexes formed by mSin3 and YY1.

2.4.1 mSin3 adaptor proteins

Adaptor protein mSin3 is a cofactor, which binds other regulatory proteins including transcription factors and histone deacetylases. It forms complexes of variable composition depending on the situation and need. Since mSin3 is not a transcription factor, it requires docking proteins to gain access to DNA. Through these binding partners, mSin3 can repress the transcription of several genes. The adapter protein, mSin3, is a widely expressed protein with no tissue specificity, even though the mSin3 complexes between tissues and even cells within a tissue may differ. Two forms of

mSin3 proteins have been found in mammals, mSin3A and mSin3B (Schreiber-Agus and DePinho 1998).

2.4.1.1 Structure of mSin3 proteins

The *SIN3* gene encodes for a protein with four putative paired amphipathic helix (PAH) domains, which are thought to be involved in mediating protein-protein interactions (Wang and Stillman 1990). Initially *SIN3* gene was identified as a negative regulator of the yeast *HO* gene, which encodes for an endonuclease that initiates mating-type switching in yeasts (Nasmyth et al. 1987, Sternberg et al. 1987). Two mammalian Sin3 proteins have been identified, mSin3A and mSin3B. mSin3A is a protein consisting of 1219 amino acids (Schreiber-Agus and DePinho 1998). mSin3B is found as two different spliced forms in the cell, namely mSin3B long form ($mSin3B_{LF}$) and mSin3B short form ($mSin3B_{SF}$), consisting 954 aa and 294 aa, respectively (Schreiber-Agus and DePinho 1998). Both mSin3A and $mSin3B_{LF}$ have all four PAH regions in their structure, while $mSin3B_{SF}$ has only PAH1 and PAH2 (Alland et al. 1997). Sin3 protein can mediate transcriptional repression by interacting with sequence-specific DNA-binding proteins (Wang and Stillman 1993) as well as unliganded nuclear hormone receptors through corepressors N-Cor or SMRT (Heinzel et al. 1997, Nagy et al. 1997a). Sin3 has been shown to associate with transcription factor Mad and its related protein Mxi1, which both further associate with Max, forming a transcriptional repressor heterodimer, Mad/Max or Mxi1/Max (Ayer et al. 1995, Schreiber-Agus et al. 1995). This association occurs through PAH2 region of mSin3 that associates with the amino terminus of the transcription factors Mad/Mxi1, also termed the 'repression domain' or the SID (Sin interacting domain) (Ayer et al. 1995, Schreiber-Agus et al. 1995). A corepressor, N-Cor, interacts with two different places in mSin3 proteins, with the PAH 1 domain and with a region including PAH3 and the linker between PAHs 3 and 4 (Alland et al. 1997, Heinzel et al. 1997). SMRT associates only with the PAH1 domain of mSin3A but not with mSin3B (Nagy et al. 1997a). Conserved region between PAH3 and PAH4 domains has been shown to bind HDAC2 in mSin3A and is named as HID (HDAC interacting domain) (Laherty et al. 1997).

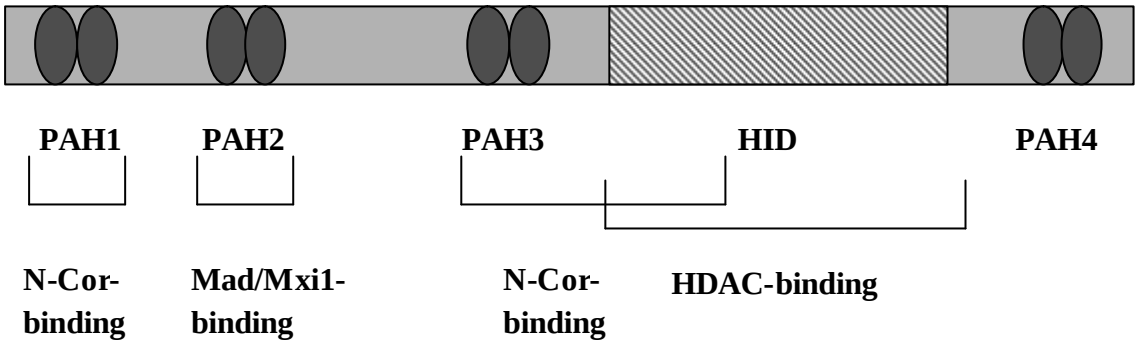


Fig. 5 A schematic diagram of mSin3A and mSin3BLF structure according to Schreiber-Agus and DePinho, 1998. Paired helical filaments (PAH) and HDAC interaction domain (HID) are shown with their typical binding partners.

2.4.1.2 mSin3 complex

The mammalian Sin3 complex consists of several subunits, including the core complex and some additional binding proteins that differ from time to time according to their use as repressors of transcription of different genes. The core is built from the mSin3, HDAC1, HDAC2, RbAp46, RbAp48, SAP18 and SAP30 proteins.

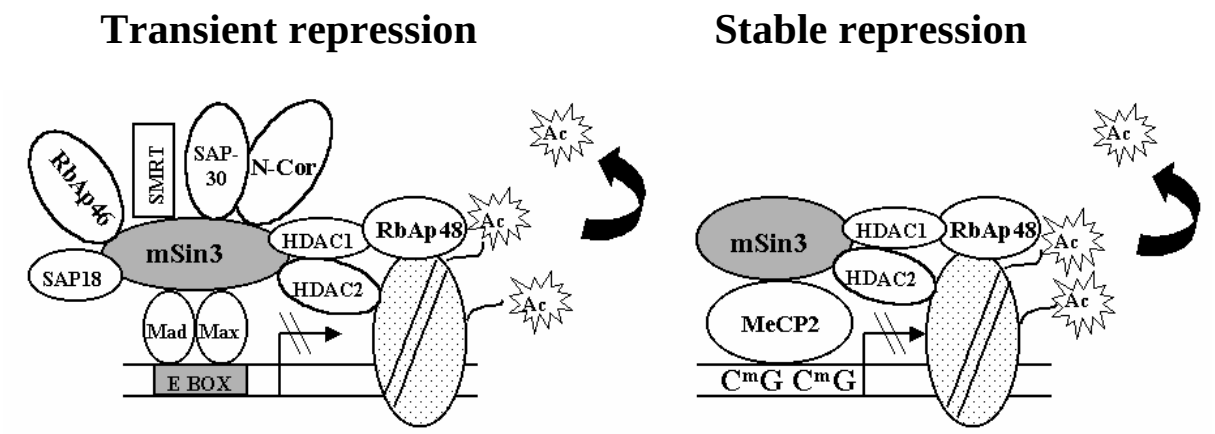


Fig. 6 A schematic diagram of mSin3 complex and role in transcriptional repression according to Razin, 1998.

RbAp-proteins have been initially found as retinoblastoma associated proteins (Tyler et al. 1996). RbAp48 has been shown to interact with histone H4 and it has been proposed that RbAp48 may have a function as a molecular bridge between histone metabolic enzymes and core histones (Parthun et al. 1996, Verreault et al. 1996).

Two novel proteins, SAP18 and SAP30, have been identified as mSin3-associated polypeptides by Zhang et al. (1997). They demonstrated a direct link between SAP18 and mSin3 and noted that SAP18 enhances the ability of mSin3 to mediate repression of transcription. SAP30 is known to bind mSin3 and to mediate transcriptional repression via histone deacetylases (Laherty et al. 1998). SAP30 associates also with N-Cor and is required for N-Cor mediated repression (Laherty et al. 1998).

Intracellular nuclear hormone receptors are structurally related transcription factors which, in conjunction with their interacting ligands (retinoid, steroid and thyroid hormones), regulate a set of genes involved in cell proliferation, differentiation, morphogenesis, and homeostasis by stimulating or repressing target gene expression (Chambon 1994, Mangelsdorf et al. 1995). Nuclear receptor corepressor (N-Cor) (Alland et al. 1997, Heinzel et al. 1997) and the related factor SMRT (silencing mediator of retinoid and thyroid receptors) (Chen and Evans 1995) associate with several unliganded nuclear hormone receptors and mediate transcriptional repression. Their repression effect is thought to be due to the complex that N-Cor and SMRT establish with mSin3 and HDACs, thus they recruit histone deacylase activity to DNA (Alland et al. 1997, Nagy et al. 1997a). The addition of a ligand results in conformational changes in specific receptor domains, with the displacement of corepressors and recruitment of coactivators, such as Creb-binding protein (CBP)/p300 with intrinsic HAT activity (Misiti et al. 1998).

One set of transcription factors which form a complex with Sin3 is the Mad(Mxi) family of Max binding proteins, containing four members (Mad1, Mxi1, Mad3 and Mad4) and related repressor Mnt (or Rox) (Ayer et al. 1995, Schreiber-Agus et al. 1995, Hurlin et al. 1997, Schreiber-Agus and DePinho 1998). All these proteins contain N-terminally located region known as the Sin interaction domain (SID), which is both

necessary and sufficient for Sin3 association and for transcriptional repression. The interaction region in Sin3 part of this complex is PAH2. Mad(Mxi) family proteins form heterodimers with the highly stable and ubiquitously expressed protein Max through their respective basic helix-loop-helix/leucine zipper (bHLH/LZ) structure (Ayer et al. 1993, Hurlin et al. 1996). This heterodimer can antagonize the transcriptional activation and proliferation-promoting functions of the Myc/Max heterodimer, by binding to the same E-box related DNA sequence (Ayer et al. 1993, Zervos et al. 1993). Thus, Myc and Mad compete for dimerization to Max and also for the same binding site in DNA, suggesting that during the normal situation, a balance in the cell is regulated between the complexes of Myc/Max and Mad/Max, where Myc/Max is a transcriptional activator and Mad/Max causes powerful repression of gene expression.

	Protein	Function	Reference
Complex proteins	HDAC 1, 2	Histone deacetylation	(Yang et al. 1997)
	HDAC 3	Histone deacetylation	(Dangond et al. 1998)
	SAP18/SAP30	Transcriptional co-repressor	(Zhang et al. 1997)
	RbAp46/RbAp48	Transcriptional co-repressor	(Ng and Bird 2000)
	Ski	Transcriptional co-repressor	(Nomura et al. 1999)
	RBP1	Rb binding protein	(Lai et al. 2001)
Trans-cription factors	N-Cor	Nuclear receptor co-repressor	(Heinzel et al. 1997)
	SMRT	Nuclear receptor co-repressor	(Nagy et al. 1997a)
	Alien	Nuclear receptor co-repressor	(Dressel et al. 1999)
	Mad/Max	Oncoproteins	(Schreiber-Agus and DePinho 1998)
	REST	Neural specific gene regulation	(Naruse et al. 1999)
	P53	Tumor suppressor	(Murphy et al. 1999)
	Rb	Tumor suppressor	(Lai et al. 2001)
	MeCP2	Methyl-cytosine binding	(Nan et al. 1998)
	Ikaros, Aiolos	B and T cell differentiation	(Koipally et al. 1999)
	ETO	Oncoprotein	(Wang et al. 1998)
	PLZF	Hematopoiesis	(Wong and Privalsky 1998)

Table I A list of the most common mSin3A/B associated proteins

2.4.1.3 Biological function and related diseases

The biological function of the mSin3 protein complex has been shown to be important in development and in some diseases of humans (Hu and Lazar 2000, Carter and Segal 2001). There are important phases during embryonic development where the effective repression of a certain set of genes is one of those necessary regulatory events needed for the perfect result, a healthy individual (Razin 1998, Van den Veyver and Zoghbi 2000).

The partners in the mSin3 complex vary in nature, thus those multiple co-repressors known to associate with mSin3 may or may not bind concomitantly (Hassig et al. 1997). Also the fact that there are two splice forms of mSin3B, a long and a short form, makes these naturally occurring complexes even more heterogeneous (Alland et al. 1997). It has been suggested that the complex created by the $mSin3B_{SF}$ is involved in an attenuated/reversible type of regulation, since it contains only the PAH1 and PAH2 domains, thus lacking the HDAC interaction domain (Schreiber-Agus and DePinho 1998). According to this theory, them$Sin3B_{SF}$ complex is not capable of evoking core histone deacetylation (Schreiber-Agus and DePinho 1998).

The fact that mSin3 has the potential to form a complex es either with Mad/Mxi protein family members or with N-Cor/SMRT, allow the cell to use distinct and separate repression pathways that may even be functionally integrated (Schreiber-Agus and DePinho 1998). These two types of mSin3 complexes can compensate for each other when necessary or potentiate the repressed situation in the cell, by utilizing alternative pathways.

Chromosomal translocations may cause human acute leukemias (Look 1997). Rearrangement of the regulatory and coding regions of genes may produce fusion proteins that can cause either enhanced activation or repression of target genes important in the cellular regulatory cascades (Look 1997). Some acute leukemias are attributable to failures in these processes. Translocation results in fusion proteins that have increased binding to corepressors and this leads to enhanced repression of target genes (Burke and Baniahmad 2000). This is the case in acute myelogenic leukemia (AML), B cell acute lymphoblastic leukemia (ALL) and acute promyelocytic leukemia (APL) where translocation results in fusion proteins that recruit silencing transcription factors and further bind mSin3-HDAC complex associated with N-Cor and/or SMRT (Look 1997, Burke and Baniahmad 2000).

Hypoxia is a common feature in malignant tumorswhich can influence tumor progression in hypoxic conditions by enhancing angiogenesis. HDAC1 has been demonstrated to induce angiogenesis *in vivo* and *in vitro* by a mechanism involving

downregulation in the expression of tumor suppressor genes, *p53* and *von Hippel-Lindau* (Kim et al. 2001). In addition, two recent studies has shown that histone deacetylase inhibitors, TSA and FK228 inhibit tumor angiogenesis *in vivo and in vitro* (Williams 2001, Kwon et al. 2002).

When stable repression is prevented during embryogenesis, as in the X-chromosome located *MeCP2* gene mutation, the consequence can be lethal or it can cause serious developmental disorders (Van den Veyver and Zoghbi 2000). This is the case in Rett-syndrome, which is the leading cause of mental retardation in girls (Armstrong 2001). *MeCP2* encodes the methyl-CpG-binding protein 2, a ubiquitous DNA-binding protein, which binds to the mSin3-HDAC complex and silences transcription (Nan et al. 1998). In studies with *MeCP2*-null mice, their nervous system is severely affected whereas very few abnormalities are seen elsewhere in the body (Chen et al. 2001).

A relationship between N-Cor, mSin3, histone deacetylases and disease has also been observed in Huntington's disease (HD) (Boutell et al. 1999). In HD brain cortex and caudate, the cellular localisation of N-Cor and mSin3A proteins were shown to be exclusively cytoplasmic, whereas in control brain, they are localized both in nucleus and cytoplasm. This phenomenon indicates that the relocalization of these transcriptional repressor proteins in HD brain may alter transcription and be involved in the pathology of the disease. HD belongs to the neurodegenerative disorder group, where the same type of mutation in different genes leads to the production of proteins with expanded polyglutamine repeats (Fischbeck 2001). These polyglutamine expansions are toxic to brain tissue (Fischbeck 2001). It has also been shown in a Drosophila model that in polyglutamine expansion neurodegenerative diseases, the repeat sequences can bind proteinswhich have acetyltransferase activity, *i.e.* CBP and p300/PCAF (Steffan et al. 2001). This reduces the acetylation state of the histones and leads to the transcriptional dysregulation (Steffan et al. 2001). HDAC inhibitors prevented this reduced histone acetylation state and were able to arrest the ongoing neuronal degeneration (Steffan et al. 2001). These studies have supported the possible use of HDAC inhibitors as therapy to treat HD patients as well as individuals with other

polyglutamine-repeat diseases to prevent or to slow down the ongoing neurodegeneration (Steffan et al. 2001).

Histone deacetylation through the mSin3 complex plays an essential role in normal development as well as maintaining the balance in cells between transcriptional activation versus repression of gene expression. Defects in this type of regulation lead to several different human diseases as discussed above. Since knowledge concerning the mechanisms of those diseases already exist, it is likely in the future that therapeutic treatments will be devised to prevent the symptoms caused by these regulatory defects.

2.4.2 Transcription factor YY1

Transcription factor Yin Yang 1 (YY1) is a multifunctional zinc finger protein, which can either activate or repress transcription of several genes depending on its binding partners (Shi et al. 1991). YY1 protein is highly conserved among different species and it is ubiquitously expressed (Shi et al. 1997). The number of promoters containing potential YY1 binding sites as well as the promoters that can be regulated by YY1 is overwhelming (Thomas and Seto 1999). YY1 recognition sites have also been identified as the initiator element of some promoters (Seto et al. 1991). YY1 interacts with either histone acetylase or histone deacetylase depending on its role in regulating the transcription process (Thomas and Seto 1999).

2.4.2.1 Structure of transcription factor YY1

YY1 protein was previously known by many names (UCRBP, NF-E1, d, CF1) and the name Yin Yang 1 reflects its dual transcriptional activities (Shi et al. 1997). For example, YY1 is thought to be involved in the activation of a number of genes, such as myelin proteolipid protein (PLP) (Berndt et al. 2001), neuron specific Fe65 (Zambrano et al. 1997), c-Myc (Riggs et al. 1993), p53 (Furlong et al. 1996), HIV-1 long terminal repeat (Coull et al. 2000), histone genes (Eliassen et al. 1998, Last et al. 1999) and some ribosomal protein genes (Hariharan et al. 1991).

The human *YY1* gene is located in chromosome 14 and the protein is expressed ubiquitously in mammalian cells (Yao et al. 1998). Human YY1 protein contains 414

amino acids and its predicted molecular weight is about 44 kDa (Shi et al. 1997). However, in SDS page gel YY1 migrates as a 65-68 kDa protein, which may be due to the protein's modifications (Shi et al. 1997). YY1 is a zinc finger protein belonging to the GLI-Kruppel-like zinc finger family (Ruppert et al. 1988, Shi et al. 1991). Zinc fingers are known as DNA binding domains, although nowadays they are also known to participate in protein-protein interactions (Mackay and Crossley 1998). YY1 contains four zinc fingers in its C-terminus, which are responsible for its sequence specific DNA-binding activity as well as its repression capacity (Hariharan et al. 1991, Lee et al. 1994). Further identification of these zinc fingers by Galvin and Shi has revealed that fingers 2 and 3 are required for DNA binding (with 2 being absolutely required) and fingers 1 and 2 for repression (Galvin and Shi 1997). There are also studies indicating the presence of another repression domain in the middle part (between amino acids 170-220) of YY1 protein, distinct from the zinc fingers, containing a long glycine-alanine stretch (Lewis et al. 1995, Yang et al. 1996). These two separate repression domains are both regulated by acetylation/deacetylation, but the exact mechanism of how the domains function together is not known (Yao et al. 2001). The N-terminal part of the protein involves the activation domain containing a stretch of 11 consecutive acidic amino acids followed by 11 histidines (Flanagan et al. 1992, Lee et al. 1995b).

The most preferred recognition sequence for YY1 in DNA is CGCCATNTT. This includes a consensus core region of 5'-CAT-3' and in many cases there is considerable heterogeneity in thebase pairs on either side of this core CAT-region (Hyde-DeRuyscher et al. 1995). This offers YY1 sites the ability to overlap with a wide range of other DNA binding sites (Hyde-DeRuyscher et al. 1995). The regulatory capacity of YY1 is mostly dependent on its binding partners. It is known to bind many proteins, such as E1A, c-Myc, PARP, Rb, Sp-1, TBP, TFIIB, HDAC1-3, CBP and p300 that all have a role in YY1's regulatory activity (see table II). There are two domains in YY1 where protein/protein interactions can take place (Thomas and Seto 1999). The first is the central domain (around residues 150-200) and the second is located in the C-terminus and contains all four zinc fingers and some spacer region. As listed above, YY1 is known to bind many cofactors, both coactivators and corepressors that are

important in regulating many alternating promoters. Coactivators known to be bound to YY1 are CBP and p300, both containing intrinsic histone acetyltransferase activity (Lee et al. 1995a, Austen et al. 1997b).

	Protein	Function	Reference
Associated enzymes	HDAC 1, 2	Histone deacetylation	(Yang et al. 1996)
	HDAC 3	Histone deacetylation	(Yang et al. 1997)
	CBP	Acetylation	(Zhou et al. 1995)
	p300	Acetylation	(Lee et al. 1995a)
	PARP	Poly(ADP-ribosyl)ation	(Oei et al. 1997b)
Trans-cription factors	c-Myc	Oncoprotein	(Shrivastava et al. 1996)
	TBP	Transcriptional activation	(Austen et al. 1997b)
	TFIIB	Transcriptional activation	(Usheva and Shenk 1994)
	TFIID	Transcriptional activation	(Chiang and Roeder 1995)
	Sp-1	Transcriptional activation	(Seto et al. 1993)
	Rb	Tumor suppressor	(Petkova et al. 2001)
	ATF/CREB	Transcriptional regulation	(Zhou et al. 1995)
	E1A	Transcriptional co-regulator	(Lewis et al. 1995)

Table II A list of the most common YY1 associated proteins

2.4.2.2 Posttranslational modifications of YY1

The details of the posttranslational modification of YY1 protein have remained unclarified, although some evidence for phosphorylation of the protein has been reported previously (Becker et al. 1994, Austen et al. 1997b). There are several consensus Serine/Threonine phosphorylation sites in YY1 protein (Becker et al. 1994). It has been reported that the binding activity of YY1 was abolished by phosphatase treatment in the murine leukemia virus long terminal repeat, suggesting that phosphorylation plays an important role in YY1 binding activity to DNA (Becker et al. 1994). Also in neonatal rat cardiac myocytes IL-1ß has been shown to enhance YY1 DNA binding activity by inducing its phosphorylation (Patten et al. 2000).

Acetylation and deacetylation have been thought to regulate the activity of YY1 but until recently there has been no direct proof for this hypothesis. Recently, Seto and coworkers reported that YY1 protein is acetylated by p300 and PCAF and deacetylated by HDACs (Yao et al. 2001). There are two acetylation regions in the protein, the central region containing amino acids 170-200 and C-terminus containing the zinc fingers, and the acetylation of the central region is required for the full transcriptional repressor activity of YY1 (Yao et al. 2001).

Poly(ADP-ribosyl)ation of certain transcription factors by poly(ADP-ribose)polymerase 1 (PARP-1) prevents them binding to their specific DNA binding sites (Oei et al. 1998). PARP is the key enzyme involved in the regulation of several nuclear processes including DNA repair and transcription (Oei et al. 1997a). YY1 has been shown to be the target for PARP mediated ribosylation (Oei and Shi 2001a). However, when bound to DNA, the transcription factors are inaccessible to poly(ADP-ribosyl)ation by PARP-1 (Oei et al. 1998). PARP-1 is proposed to work as a regulator or a molecular switch between transcription and DNA repair to evade expression of damaged genes (Oei et al. 1998). However, there are also results indicating that YY1 regulates the PARP-1 activity and thus stimulates the synthesis of poly(ADP-ribose) which further contributes to DNA repair (Oei and Shi 2001b).

2.4.2.3 Mechanisms of YY1 mediated transcriptional regulation

There are several models of YY1 mediated transcriptional activation and repression (Shi et al. 1997, Thomas and Seto 1999). First, in the question of activation, YY1 may stimulate transcription by interacting with general transcriptional factors directly. This is the case when YY1 binds to the initiator element and recruits RNA polymerase to the initiation complex (Seto et al. 1991). Further support for this model comes from the fact that E1A can convert YY1 from being a repressor to an activator by mediating the protein-protein interaction between YY1 and p300 (Lee et al. 1995a). Secondly, YY1 may bind to other regulatory proteins, which inhibit YY1's repressor properties and/or unmask its activation domain. It has been suggested that the amino acids in C-terminus of YY1 protein contain a region that regulates the activation domain of the YY1 by masking it, since deletion of this part of the molecule converts YY1 into a constitutive

activator (Lee et al. 1995b, Bushmeyer and Atchison 1998). Thirdly, as already mentioned, YY1 binds to several cofactors that can modify or interact with other proteins and factors involved in transcriptional activation. By binding to CBP/p300, which are well known proteins which both possess intrinsic histone acetyltransferase activity, YY1 may participate in the regulation of the chromatin acetylation state, thus possibly allowing other transcription factors and activators to interact with DNA and further promote gene transcription (Lee et al. 1995a, Austen et al. 1997b).

As a transcriptional repressor, YY1 has also been shown to use various mechanisms (Shi et al. 1997, Thomas and Seto 1999). First, YY1's binding sites overlap with many activating transcription factors binding sites in DNA, suggesting that YY1 may displace the activator from the DNA and repress the transcription (Shrivastava and Calame 1994, Guo et al. 1997). Also YY1-induced bending of DNA has been claimed to cause repression, when it prevents interaction between upstream activators and the basal transcription machinery (Natesan and Gilman 1993, Galvagni et al. 1998). Secondly, YY1 regulates transcription as an active repressor by physically interacting with other activators or general factors bound to the promoter and interfering with their function (Galvin and Shi 1997). Thirdly, YY1 associates with corepressor proteins, like HDAC1, 2 and 3, and this modifies either YY1, the activator proteins or chromatin by deacetylation (Yang et al. 1996, Yang et al. 1997, Yao et al. 2001).

2.4.2.4 Biological functions and related diseases

YY1 has been shown to play a crucial role in early mouse development, since mice lacking YY1 exhibit early embryonic lethality at the time around implantation (Donohoe et al. 1999). Also a subset of YY1 heterozygote mice suffer growth retardation and neurobiological defects, suggesting that both alleles of YY1 are needed for normal embryonic growth and development (Donohoe et al. 1999). Shi and his coworkers postulated that YY1 may work as a key molecule in regulating genes whose products are vital for differentiation and rapid proliferation during early embryogenesis (Donohoe et al. 1999). Furthermore, the differences in the levels of YY1 expressed in certain organs as well as the phenotype variants in heterozygote animals have raised the

question of whether YY1 may also be required for late stage embryogenesis (Donohoe et al. 1999).

A cellular memory system that ensures the maintenance of the differentiation status of cells in developing organs during embryogenesis and which is inherited through multiple cell divisions is very important for normal development (Mahmoudi and Verrijzer 2001). Genes of the polycomb group (PcG) maintain repression of several developmental regulators, like homeotic genes (Mahmoudi and Verrijzer 2001). Even though the PcG proteins are known to be involved in the transcriptional repression, the details of their mechanism of action remain to be clarified. Most of the PcG proteins are not transcription factors by themselves, thus they do need a link between them and DNA (Satijn et al. 2001). Some years ago it was shown that the *Drosophila* PcG protein Pho is a DNA binding protein and it has extensive homology to YY1 (Brown et al. 1998). Recently, YY1 and human PcG protein EED (embryonic ectoderm development) have been revealed to interact specifically together, suggesting a direct link between the DNA and the PcG protein complex (Satijn et al. 2001). Taken in conjunction, with the results showing that a subset of YY1 heterozygote mice suffer neurobiological defects, it has been suggested that YY1 and the PcG protein EED play a crucial role in neuronal tissue development during early embryogenesis (Satijn et al. 2001).

YY1 has also been found to be a nuclear matrix binding protein, initially identified as NMP-1 (Guo et al. 1995). Thus YY1 is not only present in the soluble nuclear extract but also in the insoluble nuclear matrix compartment. The nuclear matrix has a role in the organization and function of nuclear DNA and it is associated with several nuclear processes, like transcription, replication and histone acetylation/deacetylation (Davie 1997). The C-terminus of YY1 has been shown to be necessary for high-affinity interactions with the nuclear matrix, whereas its N-terminal part has a low-affinity association with this compartment (McNeil et al. 1998). This suggests that YY1 interfaces with different components of the nuclear matrix to facilitate its various different transcriptional functions (McNeil et al. 1998). Also some YY1 interacting

proteins, such as c-Myc and E1A, are known to be associated with the nuclear matrix (Eisenman et al. 1985, Carvalho et al. 1995).

YY1 has been shown to interact with the proto-oncoprotein c-Myc (Shrivastava et al. 1996) and also to regulate *c-myc* gene transcription by binding to its promoter (Riggs et al. 1993). The transcription factor, c-Myc, is an essential protein known to regulate cell growth and proliferation and to inhibit differentiation (Grandori et al. 2000). Over-expression of c-Myc is involved in tumorigenesis although in some cases it also promotes apoptosis (Grandori et al. 2000). An interaction with YY1 has been shown to inhibit the capability of c-Myc to bind to Max and form a heterodimer (Shrivastava et al. 1993). This heterodimer Myc/Max is claimed to be necessary for all c-Myc induced transcriptional functions (Grandori et al. 2000). Another report suggests that YY1 regulates c-Myc through indirect mechanisms, probably involving those where YY1 regulates upstream modulators of c-Myc (Austen et al. 1998). It has been shown that YY1 represses c-Myc transactivation and transformation functions *in vitro* cell culture experiments (Austen et al. 1997a). The interaction between YY1 and c-Myc has also been observed to inhibit YY1's function both as an activator or a repressor of transcription (Shrivastava et al. 1993). This modulating property of c-Myc to YY1 functions may interfere in YY1's ability to make contact with basal transcription proteins TBP and TFIIB (Shrivastava et al. 1996). However, a decreased binding between YY1 and c-Myc has been observed in human herpes virus 6 infected $CD4^+$ T lymphocytes (Hasegawa et al. 2001). This leads to the enhanced binding of YY1 to the CXC chemokine receptor gene and causes repression of this gene transcription (Hasegawa et al. 2001). Thus, both YY1 and c-Myc have been shown to regulate each other's functions and activity.

Retinoblastoma tumor-suppressor protein (Rb) is known to interact physically with YY1 (Petkova et al. 2001). Rb prevents YY1 from interacting with its DNA binding sites by affecting YY1's C-terminal four zinc fingers (Petkova et al. 2001). Usheva and co-workers also described that the YY1-Rb complex was mainly found in growth arrested but not in serum stimulated S-phase cell cultures, indicating that YY1-Rb complex might have role in cell cycle regulation (Petkova et al. 2001).

Expression of human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) has shown to be repressed by the function of two transcription factors, YY1 and LSF (Coull et al. 2000). LSF has the capacity to bind the HIV LTR where it also recruits YY1. Repression of the HIV LTR transcription (and because of that, also viral production) is further mediated by the HDAC1 associated with YY1 (Coull et al. 2000). This repression is blocked by TSA (Coull et al. 2000). LSF alone cannot repress the transcription of HIV LTR and, on the other hand, YY1 cannot alone bind to this promoter region (Romerio et al. 1997). It has been suggested that LSF may work as a docking protein for YY1, allowing it to recognize a site on the LTR (Romerio et al. 1997). The complex, LSF-YY1-HDAC, is proposed to maintain the deacetylated nucleosomal structure of HIV LTR corresponding to the repressed state in gene expression (Coull et al. 2000). Thus, LSF and YY1 may control the transcriptional and virological latency of HIV and this may have considerable implications for the treatment of AIDS (Coull et al. 2000).

The connection between YY1 and Alzheimer's disease comes from the finding that YY1 is known to function as a transcription activator of the Fe65 gene promoter (Zambrano et al. 1997). Fe65 is a neuron-specific adaptor protein that binds to the cytoplasmic domain of amyloid precursor protein (APP) (Fiore et al. 1995, Bressler et al. 1996). In AD, APP is proteolytically cleaved into small extracellular peptides including ß-amyloid peptide and an intracellular tail fragment, which is released into the cytoplasm (Selkoe 1998, Haass and De Strooper 1999, Vickers et al. 2000). This released tail domain has been demonstrated to associate with Fe65 protein and further with histone acetyltransferase Tip 60 (Cao and Sudhof 2001). This complex is suggested tobe active in regulating gene expression (Cao and Sudhof 2001). APP is a transmembrane protein, and association of its cytosolic part with Fe65 is also suggested to have a role in regulating cell movement (Sabo et al. 2001). This function recruits several other cellular proteins concerned with membrane motility including Mena, a-actin and some integrins (Sabo et al. 2001). Furthermore, Fe65 has been found to co-localize with tau proteins in the intracellular tangles found in the brain of AD patients (Delatour et al. 2001).

Serotonin acts as neurotransmitter in brain and it is synthesized from amino acid tryptophan. Tryptophan 2,3-dioxygenase (TDO2) is a rate-limiting enzyme in this synthesis process and thus controls the serotonin level in the brain. Many behavioural disorders (including depression, anxiety, aggression dysregulation and alcoholism) have been reported to be linked with abnormalities in serotonin and/or tryptophan levels as well as serotonin metabolism in the body (Apter et al. 1990, Comings 1990, Virkkunen and Linnoila 1990). Four different polymorphisms of the human *TDO2* gene have been identified (Comings et al. 1996). Two distinct single base mutations in intron 6 of *TOD2* gene have been shown to associate with a variety of psychiatric disorders (Comings et al. 1996). Both of these mutations have been shown to damage the YY1 binding site in the *TOD2* gene and disturb its gene expression (Vasiliev et al. 1999).

2.4.3 NuRD/Mi-2

The NuRD (nucleosome remodelling and histone deacetylation) complex (also known as Mi-2) shares many proteins with the Sin3 complex, since HDAC1, HDAC2, RbAp46 and RbAp48 are also part of NuRD corepressor complex. The major difference between these two complexes is that the NuRD complex contains also ATP-dependent nucleosome-remodelling activity due to its distinctive Mi-2 protein (Tong et al. 1998, Zhang et al. 1998). The mammalian NuRD complex contains also MTA2 and MBD3 subunits (Zhang et al. 1999). MTA2 is related to MTA1 (metastasis-associated protein 1), which was originally identified as being overexpressed in metastatic carcinomas (Toh et al. 1994). MTA2 interacts with MBD3 (methyl-CpG-binding-domain protein 3) and modulates the enzymatic activity of the histone deacetylase core complex (Zhang et al. 1999). MBD3 belongs to the same family with MeCP2, but it does not bind directly to methylated DNA. However, MBD3 is closely related to MBD2, which is a protein that binds to methylated DNA and has been claimed to have DNA demethylase activity (Bhattacharya et al. 1999). MBD2 is able to interact directly with components of the NuRD complex, which may connect the NuRD complex to DNA methylation (Zhang et al. 1999).

2.4.4 Retinoblastoma protein

There are also other proteins associating with histone deacetylases and repressing transcription of a specific set of genes. One of those proteins is the retinoblastoma tumor-suppressor protein (Rb). Rb silences a subset of genes that are active in the S phase of cell cycle and which are regulated by E2F family of transcription factors (Weinberg 1995). Rb does not bind DNA directly but associates with the E2F and represses the transcription via that connection. The 110 kD Rb protein is an important regulator of cell cycle progression and differentiation, and the Rb gene is mutated in many human cancers (Weinberg 1995). It has been shown by several groups that Rb protein physically interacts and cooperates with HDAC1 thus forming a trimeric complex that modulates the nucleosome structure surrounding the promoter region by deacetylating the core histones (Brehm et al. 1998, Luo et al. 1998, Magnaghi-Jaulin et al. 1998). The small pocket region of Rb constitutes the repressor motif of Rb, where HDAC1 is known to bind. This region was originally identified as the binding site for viral oncoproteins, and the importance of this domain comes from the fact that all Rb-mutations identified in cancer patients map to the pocket domain (Weinberg 1995, Brehm and Kouzarides 1999). In addition, mutations in the Rb pocket domain have been shown to disturb the interaction between Rb and HDAC1 (Brehm et al. 1998, Luo et al. 1998, Magnaghi-Jaulin et al. 1998). Recently, HDAC3 was also shown to bind Rb (Lai et al. 1999) and form a bridge between Rb and RbAp48 (Nicolas et al. 2001). In addition, Rb protein binds also to Ski, which associates with the Sin3 complex (Tokitou et al. 1999). When the Rb protein is phosphorylated by cyclin dependent kinase (CDK), the HDACs dissociates from the repression pocket region thereby relieving active repression by E2F (Harbour et al. 1999). Also treatment with TSA inhibits the transcriptional repression mediated by Rb complex (Luo et al. 1998, Magnaghi-Jaulin et al. 1998). Interestingly, Rb and YY1 have also been reported to form a complex in vitro that prevents the ability of YY1 to bind DNA and also inhibits its transcription initiator function (Petkova et al. 2001). Furthermore, Rb is claimed to recruit the mSin3 complex through retinoblastoma binding protein 1 (RBP1) (Lai et al. 2001).

2.4.5 Neural restrictive silencer factor

There are several neuron-specific genes that are under the control of negative transcriptional regulation, since their promoters contain a transcriptional regulatory element known as the neural restrictive silencer (NRS), also named as repressor element 1 (RE1) (Schoenherr et al. 1996). Its binding protein, NRSF/REST or its mRNA, are not present in most neuronal cells but are found in the majority of nonneuronal cell types (Kraner et al. 1992, Mori et al. 1992, Schoenherr and Anderson 1995). Thus, NRS binds to NRSF/REST. The NRSF/REST protein is a transcription factor, which represses the transcription of neural genes in nonneural tissues. It has been shown that NRSF recruits the mSin3 and HDAC complex to repress transcription (Naruse et al. 1999, Roopra et al. 2000). When this repression was inhibited using TSA, several neuronal-specific genes became detectable (Naruse et al. 1999). In addition, a human repressor protein called CoREST is also suggested to have a role in NRSF/REST mediated transcriptional repression together with mSin3A (Andres et al. 1999, Grimes et al. 2000). However, there is a study suggesting that CoREST can form a complex with HDACs 1 and 2 without any association with NRSF/REST or with mSin3 or NuRD (You et al. 2001).

2.5 Therapeutic aspects

Histone deacetylase inhibitors are being intensively investigated as new drugs to treat acute leukemias, solid tumors, aberrant hormonal signalling and polyglutamine-repeat diseases (Kramer et al. 2001, Marks et al. 2001, Steffan et al. 2001). Already several molecules are in phase I or phase II clinical trials, mostly to find new therapeutic strategies to treat different cancers *e.g.* leukemia (Warrell et al. 1998, Kramer et al. 2001) and neuroblastoma (Coffey et al. 2001, Cinatl et al. 2002). The power of these histone deacetylase inhibitors is based on their possibility to release the inhibited transcription process from its repressed state, such as reactivation of the inhibited transcription of tumor supressors. These compounds have been shown to activate either the apoptotic process or the differentiation of the cancer cells and thus repress their further proliferation (Marks et al. 2000). Also in disease models of expanded polyglutamine repeats, the histone deacetylase inhibitors have been shown to arrest neurodegeneration (Steffan et al. 2001). Polyglutamine pathogenesis is thought to result

from the reduced acetylation of histone proteins leading to transcriptional dysfunction, since expanded polyglutamine repeats binds acetyltransferase domains of CBP and p300 and trap them into aggregates (Steffan et al. 2001). The presence of expanded polyglutamine (CAG) repeats has been noted in several neurological diseases, such as HD and Kennedy's disease (Fischbeck 2001). By inhibiting the function of HDACs, the transcriptional activity in cells may be upregulated as well as causing a reduction in the toxic effect of the excess amount polyglutamine repeats (McCampbell et al. 2001, Steffan et al. 2001). According to this theory, inhibition of HDACs may also reduce other kinds of atrophic diseases by enhancing transcription. Thus, by understanding better the repression protein complexes formed by YY1 and mSin3 and their role in the transcriptional regulation, also information may be found on the possibility of treating serious diseases with HDAC inhibitors.

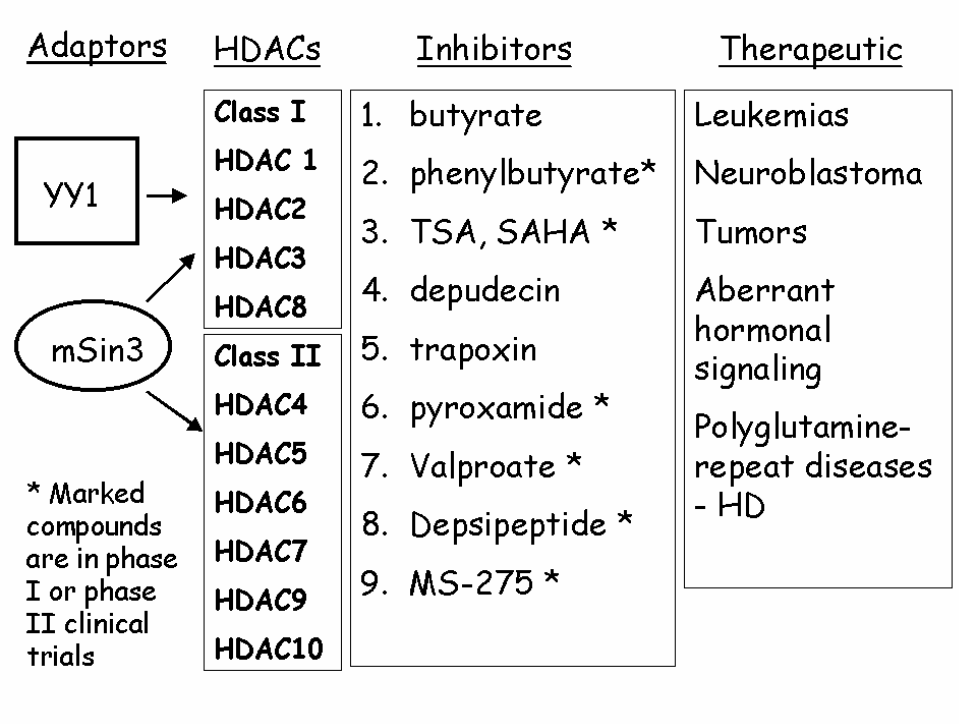


Table III A list of some of the common histone deacetylase inhibitors and their therapeutic targets

3. AIMS OF THE STUDY

Glutamate induced excitotoxicity and apoptosis are two types of cell death involved in many known neuronal degenerative diseases, including Alzheimer's disease, epilepsy and stroke. Also neuronal loss and a decrease in the protein synthesis rate occur during normal aging. Apoptosis is a tightly controlled and organized process and involves transcriptional regulation. Also in excitotoxicity, several transcriptional regulators are activated, even though the primary cell death type in excitotoxicity is necrosis. The basic mechanisms under these cell death processes have been widely studied but their regulation cascades are still not fully understood. Diseases related to aging are increasing throughout the world, since the expected lifespan of humans is now longer than ever. The pressure to find potent therapeutic treatments for diseased people is growing, because neurodegenerative diseases often cause dementia and this require institutional care. For these reasons we wanted to investigate whether the transcription regulatory factors, YY1 and mSin3, which are implicated in transcriptional repression, are regulated during excitotoxicity, apoptosis and aging in neurons.

The specific aims of the study were:

Study 1: To investigate the influence of glutamate induced excitotoxicity ontranscription factor YY1 in cultured primary hippocampal and cerebellar granule cells.

Study 2: To analyse whether neuronal apoptosis and aging *in vivo* affects the expression and the DNA binding complexes of transcription factor YY1.

Study 3: To investigate, whether the histone deacetylase inhibitors, trichostatin A and n-butyrate, induce neuronal apoptosis in neuroblastoma cells and cultured primary neuronal cells.

Study 4. To study the effects of neuronal apoptosis on the expression of mSin3 proteins.

Study 5. To study whether aging *in vivo* and replicative senescence can affect the expression levels of mSin3A and mSin3B proteins.

4. MATERIALS AND METHODS

4.1 Reagents

L-glutamic acid, trichostatin A, sodium butyrate, dialyzed fetal bovine serum (1000 molecular weight cut-off), Hoechst 33258, 2-mercaptoethanol, papain, igepal, AMC, benzamide, MK-801, poly-DL-ornithine and poly-D-lysine were purchased from Sigma. Dulbecco's modified Eagle's medium (DMEM) with 1 g/l and 4.5 g/l glucose, fetal calf serum, neurobasal, B27-supplement, trypsin-EDTA, penicillin/streptavidin, glycine, etoposide, staurosporine and TRIzol reagent were from Gibco BRL. AC-DEVD-AMC fluorogenic substrate was from Pharmingen. Hybond ECL-nitrocellulose membrane, Hyperfilm ECL, ^{35}S-methionine, ?^{32}P-ATP, ProbeQuant G-50 Micro Colums, Amplify and A-sepharose were purchased from Amersham Pharmacia Biotech. Okadaic acid, calcimycin (A23187) and Ara-C were from Calbiochem and ECL-kit from Pierce. DC Protein Assay Kit, TEMED and 30% Acrylamide/Bis solution were from Bio-Rad. Ammonium persulfate, PolyATract and pGEM-T easy vector were from Promega as well as T4 polynucleotide kinase. DNase I, insulin and Complete ^{TM} proteinase inhibitors were from Roche and Strip-EZ kit from Ambion. Consensus and mutated double-stranded oligonucleotides for YY1, AP-1, CREB, NF-?B and SP-1 were purchased from Santa Cruz Biotechnology. Autoradiography film, X-OMAT Blue XP-1, was from Kodak.

Rabbit polyclonal anti-mSin3A antibody (AK-11, 200 µg/ml), rabbit polyclonal anti-mSin3B antibody (A-20, 200 µg/ml), rabbit polyclonal anti-YY1 antibodies (C-20, 100 µg/ml) and (H-414, 200 µg/ml) and rabbit polyclonal anti-NF-κB p52 (K-27) were from Santa Cruz Biotechnology, Inc. Polyclonal anti-poly-(ADP-ribose)-polymerase antibody (PARP) from rabbit serum was from Boeringer Mannheim. Horse radish peroxidase-conjugated donkey anti-rabbit immunoglobulin F(ab)^2 fragment was from Amersham.

4.2 Animals

Wistar Hannover male rats were obtained from the National Laboratory Animal Center, Kuopio, Finland. They consisted of two main age-categories: young rats, 4-6, and old rats, 26-30 months old. They were killed using CO_2. Samples from liver, cerebellum, hippocampus and frontal cortex were removed, frozen in liquid nitrogen and stored at -70°C.

4.3 Cells and cell culture

Cell lines used in these studies were Neuro-2a (mouse neuroblastoma cell line), WI-38 (human lung fibroblast cell line) and SV-40 virus transformed WI-38 subline. All lines were obtained from American Type Culture Collection (ATCC). Cells were cultured in DMEM medium supplemented with 10% FCS, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and glutamine. Fibroblasts were replicatively senesced by culturing from the passage 15 to 32. Early passage cells (p20) represent young, actively proliferating cells whereas late passage fibroblasts (p32) were replicatively senescent with very low proliferating capacity. Mitotic quiescence was induced in SV-40-transformed WI-38 fibroblasts by culturing the cells without FCS for 3 or 5 days.

Primary cerebellar granule cells were isolated from the cerebelli of 7-day-old Wistar rats and cultured essentially as described by Schousboe and coworkers (Schousboe et al. 1989). The rat pups were decapitated and the heads were washed with 70% ethanol. The skulls were opened and the cerebelli were removed to a petri dish containing BSA (3 mg/ml)-glucose (10 mM)-PBS solution. The cerebelli were purified from peduncles, meninges and blood vessels under a dissecting microscope. Then the cerebelli were cut into small pieces, transferred to a conical tube and centrifuged briefly to the bottom of the tube. The supernatant was removed gently. The pellet was resuspended into BSA-glucose-PBS-solution containing 0.25 mg/ml trypsin (2.5 ml/cerebellum) and incubated 15 min in shaking water bath at +37°C. Immediately after the incubation, BSA-glucose-PBS-solution containing 12.8 µg/ml DNase and 83 µg/ml soybean trypsin inhibitor (STI) was added and the cells were centrifuged briefly as before and the supernatant was removed gently. The pellet was resuspended again to BSA-glucose-

PBS solution containing STI 520 µg/ml (2.5 ml/cerebellum) and the cells were triturated gently with a pipette for 25 times. The suspension was left at room temperature (RT) for 15 min to allow sedimentation of clumps. Then the supernatant containing dissociated cells was transferred to a conical tube and the trituration of the pellet was repeated. The supernatants were collected together and centrifuged at 150 g for 10 min. The supernatant was removed and the cells were resuspended to the culture medium and counted using Bürker's hemocytometer. The culture medium was DMEM (4.5 mg glucose/ml) supplemented with 10% FCS (inactivated), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ML streptomycin, 20 mM KCl, and 5 µg/ml insulin. After 2 days in culture, 1-β-D-cytosine-arabinofuranoside (Ara-C) was added to avoid proliferation of glial cells (final concentration was 10 µM). The average yield of granule cells was 12-15x10^6 cells per cerebellum. Cells were cultured on poly-D-lysine-coated culture plates. The desired density in these studies was 2-2.5x10^5 cells/cm^2. The cells were maintained in a cell culture incubator at +37°C in 10% CO_2.

Primary hippocampal neurons were isolated from 17-day-old Wistar rat embryos and cultured as described by Brewer and coworkers (Brewer et al. 1993). The 17-day-pregnant rat was anesthetized with CO_2 and then decapitated. The abdomen was wiped with 70% ethanol, the skin and the abdominal wall were opened and the uterus was removed to sterile PBS. The fetuses were removed from the uterus, decapitated and the heads were placed to a petri dish containing BSA (1 mg/ml)-glucose (10 mM)-PBS solution. The brains were removed and the hippocampi were dissected and cleaned from the meninges under the dissecting microscope. Then the hippocampi were transferred to a conical tube containing BSA-glucose-PBS solution supplemented with papain (0.5mg/ml), DNase (10 µg/ml) and $MgSO_4$ (2.4 mM) and incubated at +37°C for 10-15 minutes. After the incubation the papain-solution was removed and a new BSA-glucose-PBS solution containing DNase (40 µg/ml) was added. The hippocampi were triturated gently for 10 times through a pipette, the solution was let to stand for few minutes and the homogeneous supernatant was collected to a new tube. This was repeated once and the supernatants were collected and centrifuged for 5-10 minutes at 120 g. The supernatant was removed, the hippocampal cells were suspended in culture medium and the cells were counted. The cells were plated to poly-DL-ornithine-coated

culture plates and the cultures were maintained in a cell culture incubator with 5% CO_2. The culture medium was Neurobasal supplemented with 0.5 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1x B27-supplement. For the initial attachment of the cells, 5% inactivated FCS in Neurobasal was used. This medium was replaced the next day with the culture medium.

4.4 Apoptotic and excitotoxic models

Apoptosis was induced in cells using different compounds known to cause apoptosis to various cell types. Exponentially growing Neuro-2a cells were induced to apoptosis by okadaic acid (final concentration used was 30-40 nM), etoposide (5-10 µM), staurosporine (40 nM), trichostatin A (TSA) (1-3 µM), sodium butyrate (1-10 mM) and serum withdrawal. Exposure times were 0 h, 3 h, 6 h, 12 h, 24 h, 36 h or 48 h. Cerebellar granule cells (after 7 days of differentiation in culture) were treated with okadaic acid (5-25 nM) for 12 and 24 h, and with etoposide (10 µM) and TSA (3 µM) for 12, 24 and 48 h. Okadaic acid is a specific inhibitor of protein phosphatase 2A, etoposide is an inhibitor of topoisomerase II, staurosporine is an inhibitor of protein kinase C and TSA as well as sodium butyrate are inhibitors of histone deacetylases.

Primary neurons were treated with L-glutamate, which causes an excitotoxic shock in the cells. Cells were exposed to glutamate after 9-10 days of the differentiation period in culture. The glutamate treatment was done mainly as described earlier (Pizzi et al. 1993). In brief, the primary cerebellar granule cells and hippocampal cells were treated with L-glutamate (5-100 µM) for 15-30 min in Locke's salt solution at + 22°C (RT). The glutamate shock was terminated by washing the cells twice with Locke's salt solution containing 1mM $MgSO_4$. After this treatment, the incubation was continued in the conditioned (old) medium for 1-24 h after which the cells were scraped from the plates and the soluble nuclear and cytoplasmic proteins were isolated and separated for EMSA and Western blot studies. Primary cerebellar granule cells were also preincubated with benzamide (5 mM) or with MK-801 (10 µM) in Locke's salt solution 1 h before glutamate treatment in +37 °C. Benzamide is an inhibitor of Poly(ADP-ribose)polymerase and MK-801 is an antagonist of NMDA glutamate receptor. The

effect of ionophore (A23187) on the cultured cerebellar granule cells was also analyzed by treating the cells with 5-25 µM of calcimycin in Locke's salt solution for 1 h in +37 °C. After the treatment, the samples were treated as described above.

4.5 Laboratory techniques

4.5.1 Protein isolation

Isolation of cytoplasmic and nuclear proteins was achieved according to Dignam's method with slight modifications described in detail earlier (Helenius et al. 1996). Frozen tissues were weight and homogenized with tissue grinder (Potter-Elvehjem) in ice cold hypotonic buffer (1.5 mM $MgCl_2$, 10 mM KCl, 1.0 mM dithiotreitol (DTT), 10 mM Hepes, pH 7.9, protease inhibitors from Complete). To release the cytoplasmic proteins, homogenates were incubated on ice for 15 min and then centrifuged for 10 min at 4000 g, +4°C (Bio Fuge, Heraeus). Cytoplasmic proteins were collected from the supernatants. Pellets were washed again with a large volume of hypotonic buffer after which the pellets were suspended in ice cold low-salt buffer (25 % v/v glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1.0 mM DTT, 20 mM KCl, 20 mM Hepes pH 7.9, protease inhibitors from Complete). Nuclear proteins were released by adding a high-salt buffer (25 % v/v glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1.0 mM DTT, 1.2 M KCl, 20 mM Hepes pH 7.9, protease inhibitors from Complete) slowly to achieve a final KCl concentration of 0.4 M. The lower concentration does not release nuclear proteins but higher concentrations release also genomic DNA. Samples were incubated on ice for 30 min with frequently, gently mixing. Soluble nuclear proteins were then separated from insoluble nuclear structures by centrifugation for 30 min 13 000 g, +4°C (Bio Fuge, Heraeus). Supernatants were aliquot and stored at -70°C.

Proteins were isolated from cultured cells according to the same protocol as described above. Before isolating the proteins, the cells were washed twice with ice cold PBS and scraped from the cell culture plates in 1 ml of ice cold PBS containing protease inhibitors. The cells were then centrifuged quickly, PBS was removed and the cell pellets were stored at -70°C before protein isolation. The total protein concentration in

samples was measured with the Protein Assay Reagent kit of Bio Rad and the absorbance was analysed with Microplate reader (Bio Rad Model 550).

4.5.2 Western blotting

Soluble protein samples (10 µg protein/sample) were denatured by boiling in Laemmli-PAGE sample buffer (10% glycerol, 1% SDS, 0.25 M Tris-HCL pH 6.8, 5% mercaptoethanol, bromophenol blue) for 5 min. Proteins were loaded to SDS-PAGE gel (6% gel for Sin3A/B assays, 10% for YY1 and PARP studies). Molecular weight markers (low/high) were purchased from Pharmacia Biotech. Proteins were separated in SDS-PAGE-gel electrophoresis and transferred electrophoretically to Hybond ECL-nitrocellulose membrane as described (Towbin et al. 1992) (Trans-Blot Electrophoretic Transfer Cell, Bio Rad).

Membranes were blocked prior to immunostaining with 3% BSA-PBS-0.05% Tween 20 (Sin3A/B studies), 1% fatfree milk-PBS-0.05% Tween 20 (YY1) and 1% BSA-0.5% fatfree milk-0.05% Tween 20 (PARP) for 1 h at room temperature. This was followed by primary antibody incubation in blocking buffer for 2 h at room temperature. After washing three times for 5 min with PBS containing 0.05% Tween-20, the membranes were incubated with secondary antibody for 1 h at room temperature. Membranes were again washed as described above and an additional wash with only PBS was also carried out. Finally, the membranes were incubated in Chemiluminescence Reagent from Pierce and exposed against ECL-Hyperfilm.

4.5.3 Northern blotting

Total RNA was isolated from cells and tissue samples using Trizol reagent according to the manufacturers protocol. The poly(A)-mRNA was further purified with PolyA-Tract Kit. PCR primers were designed with Primer Detective 1.01 software (Clontech). Gene specific fragments for riboprobes were generated by PCR, cloned into pGEM-T Easy vector and verified by sequencing. The probes used in this study were mSin3A for rat and human samples (gene bank number U22349, nucleotides 773-1567), mSin3B for human (ABO14600, nt 614-1420) and rat (L38622, nt 312-663) samples, YY1 for rat (M73963, nt 818-1363), cyclophilin (X52803, nt 32-543) and 18S (X00686, nt 952-

1096) for all samples. Templates for in vitro transcription were prepared by PCR on recombinant plasmids as the template, with gene-specific sense primers and modified M13/pUC Universal (5'-ggttttcccagtcacgacg) or reverse (5'-cacacaggaaacagagctatgac) primers depending on the orientation of the insert. ^{32}P-labelled riboprobes were generated with Strip-EZ kit and used without further purification for hybridising the blots in high-stringency Church buffer (Church and Gilbert 1984) with 250 mM Na^+ and 50% formamide. Membranes were reprobed with "housekeeping" probes (18S rRNA for total RNA and cyclophilin for poly(A) mRNA) to normalize for quantities of RNA loaded. Signals were visualized and quantified with Storm 860 PhosphorImager and ImageQuaNT 4.2a software (Molecular Dynamics).

4.5.4 EMSA (Electrophoretic mobility shift assay)

Binding activities of transcription factors YY1, AP-1, CREB, NF-κB and Sp-1 in nuclear extracts were analysed by the EMSA technique. Consensus and mutated double-stranded binding site oligonucleotides were end-labelled using γ^{32}P-ATP and T4 polynucleotide kinase according to the protocol of Promega. Labelled oligonucleotides were purified using either ProbeQuant G-50 Micro Columns as described in the manufacturer's protocol (Amersham Pharmacia Biotech), or gel purification (4% PAGE). In the gel purification method, the labelled oligonucleotides were first eluted from the gel into distilled water in eppendorf tube at +4 °C overnight and then stored at –20 °C.

In protein-DNA binding reactions, 3-10 µg of nuclear proteins were incubated with radiolabelled probe (20 000 cpm) in a medium containing 4% glycerol, 1.0 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 1.0 mM DTT, and 10 mM Tris-HCl, pH 7.5. The salt concentration was adjusted to the same level in all samples by adding low salt/high salt (2:1) buffer. Unspecific binding was blocked by using poly(dI/dC). In NF-κB binding assays, 1.0 % (v/v) igepal was included. Samples were incubated at room temperature for 15 min. DNA loading buffer without SDS was added and the samples were loaded to a native 4% polyacrylamide gel to separate bound and unbound probes in electrophoresis. The gel was dried on a vacuum gel dryer (Savant GP110-200) for 1 h

at 80°C and an autoradiography film was exposed for 1-2 d at -70°C or the membrane was analysed using Storm PhosphoImager (Molecular Dynamics).

4.5.5 Supershift assay

The binding complex of transcription factor YY1 was identified using a specific antibody against it in a supershift assay. First, a binding reaction was done according the protocol described above. After 10 min incubation, 3 µl of a specific antibody, (anti-YY1 (C-20)X, 200 µg/0.1 ml) or antibody against proteins,anti- c-Fos (sc-52X) or anti-NF-κB p52 (K-27X) was added to the reaction mixture (total of 20 µl) and the incubation was continued for 30 min on ice. This was followed by the separation procedure using a native gel electrophoresis and visualisation of results as described above.

4.5.6 Immunoprecipitation

Subconfluent Neuro-2a cells were labelled with ^{35}S-methionine (50 µCi/ml) for 12 h in DMEM (without methionine) with 10% dialyzed FBS, with or without apoptotic inducers. After washing twice with DMEM, the cells were lysed with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate). Cell extracts were centrifuged at 12 000 *g* for 1 min and the supernatants were removed to clean tubes. Immunoprecipitation was performed by incubating an equal amount of radioactivity (10^7 cpm) per sample with rabbit polyclonal antibody against mSin3A (AK-11) for 2 h on ice. Then the antibody-protein complexes were collected with Protein A-Sepharose during overnight incubation with a constant rotation at +4°C. Immunoprecipitates were then washed five times and dissolved to Laemmli-PAGE sample buffer, boiled and electrophoresis was performed using 6% SDS-PAGE gel (described in 4.5.2). After electrophoresis, the gel was fixed for 30 min with isopropanol:H_2O:acetic acid (25:65:10), incubated in Amplify liquid for 30 min, dried under a vacuum and visualized by PhosphorImager (Storm, Molecular Dynamics).

4.5.7 Caspase-3 assay

Cytoplasmic extracts were prepared by lysing the cells with hypotonic buffer as described in 4.8. Lysed cells were centrifuged for 13 000 g, 30 min, +4°C (Bio Fuge, Heraeus) and the supernatant was removed to a clean tube. Protein concentration was measured with the Protein Assay Reagent kit (BioRad). Caspase-3 activity was analysed by its capability to cleave Ac-DEVD-AMC fluorogenic substrate. This synthetic substrate contains the specific cleavage site of caspase-3, the same amino acid sequence that is in the PARP cleavage site. When caspase-3 is activated, it cleaves the substrate releasing a fluorescent AMC compound. The enzyme assay was carried out according to the manufacturer's protocol (Pharmingen) in black microplates (Nunc). The reaction mixture contained 200 µl protease assay buffer (20 mM Hepes, pH 7.5, 10 % glycerol, 2 mM DTT), 30-50 µg protein in 50 µl (adjusted with assay buffer) and 30 µl of substrate (final conc. 20 µM). The plate was incubated in +37°C and the fluorescence was measured after 1 h and 2 h with Victor, 1420 Multilabel Counter (Wallac Oy) (excitation wavelength 380 nm, emission 460 nm, 0.1 s). Values were expressed as nmol AMC/(s x g protein). AMC (7-amino-4-methyl coumarin) is a fluorescence standard used in the caspase-3 assay.

Neuro-2a cells were fixed with 4% paraformaldehyde and stained with nuclear dye Hoechst 33258 (0.5 µg/ml, Sigma) to detect nuclear condensation and fragmentation to apoptotic bodies as described earlier in detail (Gerschenson and Rotello 1992).

4.6 Statistics

The autoradiographs for Northern blot, EMSA and immunoprecipitation were analysed by Storm 860 PhosphoImager (Molecular Dynamics) and pixel volumes of specific bands were calculated with ImageQuaNT 4.2 software (Molecular Dynamics). The autoradiographs for western blots were analysed by scanning the films with ScanJet IIC (Hewlett Packard) and analysing the mean densities of binding bands with ImageQuaNT software. The statistical analysis was performed using either Mann-Whitney *U* test or nonpaired Student's *t* -test.

5. RESULTS

5.1 YY1

5.1.1 YY1 and excitotoxic cell death (I, II)

L-glutamate exposure to the cultured primary cells was brief, 50 µM for 15 min for hippocampal cells and 5-100 µM for 15 or 30 min for cerebellar granule cells in RT and the samples were collected between 1 and 24 hours after reincubation in the old culture medium. There was approximately 20% cerebellar granular cell death after 2 hours of the glutamate exposure (100 µM for 30 min), which increased up to 70% death after 24 hours of treatment (study I). This cell death was not thought to be apoptotic, since the enzymatic activity of caspase-3 did not increase either in primary hippocampal or in cerebellar granule cells after the glutamate insult (data not shown).

Glutamate induced considerable changes in the DNA-binding complexes of YY1 protein (Fig. 1, study I, and Fig. 1A, study II). The DNA binding studies were done using the EMSA technique, where the soluble nuclear samples from control and glutamate treated cells were allowed to react either with consensus double strand oligonucleotides containing the YY1 binding site or with mutated sequences. The changes seen in these binding complexes between the control and glutamate treated samples were prominent both in cultured hippocampal and cerebellar granule cells (Fig. 1A and 1B, study I). A very intense binding was found at the smallest YY1 complex, while the binding activity of two larger complexes remained constant or decreased in the glutamate exposed cells, compared to control samples. The intensity of the switch between the binding complexes was dependent on the concentration of glutamate exposure, since already 5 µM glutamate for 15 min at RT was able to induce an increase in the smallest complex, but the binding activity of the largest complex still remained more constant than in the cells treated with 50 µM glutamate (Fig. 1B, study II). In control cells, the greatest binding activity was found to occur in the largest complex (Fig. 1A and 1B, study I and Fig. 1A, study II). Excess of unlabelled or mutated oligonucleotide did not affect the binding pattern of YY1 (Fig. 2, study I and Fig. 1A, study II). Interestingly, in supershift assays with antibody against YY1 protein

(an assay to further verify that the complex contains YY1 protein), all three complexes both in control and glutamate treated samples shifted to the same level (Fig. 2, study I and Fig. 1C, study II). However, the bands did not shift when the antibody against some other proteins were used (Fig. 2, study I and Fig. 1C, study II). The changes in the DNA binding complexes of YY1 lasted for 6 h after glutamate exposure to cerebellar granule cells, even though the 6 h bands seen in the gel had started to decrease compared to the 1, 2 or 3 h bands (Fig. 1A, study II). The effect of glutamate to the DNA binding complexes of YY1 was able to inhibit by pretreating the cells with benzamide (5 mM) or MK-801 (10 μM) for 1 h before the glutamate shock (Fig. 2, study II). Incubation of the primary cerebellar granule cells with calcimycin (A23187) (concentrations 5, 10 and 25 μM) in Locke's salt solution for 1 h did not cause any changes in the DNA binding complexes of YY1 protein (Fig 2, study II).

Western blot studies verified, that the YY1 protein expression in the cerebellar granule cells had decreased considerably already 1 h after the excitotoxic insult, although the protein was still present even 9 h after the glutamate treatment (Fig. 1D, study II). The location of YY1 protein remained constant in excitotoxicity, since all of the specific protein was found from the nuclear fraction (Fig. 1D, study II).

5.1.2 YY1 and apoptosis (II, III)

The effect of apoptosis on YY1 protein expression was analysed from mouse Neuro-2a neuroblastoma cells and cultured primary cerebellar granule cells. Apoptosis was induced with TSA, n-butyrate, okadaic acid, etoposide, and additionally in Neuro-2a cells by serum withdrawal.

In cultured primary cerebellar granule cells, okadaic acid was the only apoptotic inducer that caused changes in the DNA binding complexes of YY1, thus TSA (3 μM) and etoposide (10 μM) had no effect on the complex formation (Fig. 3A, study II). In this cell model, okadaic acid was a very potent inducer, since already at a 5nM concentration it increased the formation of the smallest binding complex during the first 12 h incubation (Fig. 3A, study II). Incubation for 24 h with 5 nM okadaic acid decreased also the binding activity of the largest complex (Fig. 3A). Incubation with

the 25 nM okadaic acid concentration showed considerably decreased binding of the largest complex, while the binding activity of the smallest complex increased. Even 48 h incubation of the primary cerebellar granule cells with TSA or with etoposide did not cause changes in the binding complexes of YY1 protein (data not shown). The protein expression of YY1 decreased in okadaic acid treated primary cerebellar granule cells after the first 12 h incubation and was almost undetectable after a further 12 h treatment (Fig. 3B, study II). However, the protein expression of YY1 was increased in cultured cerebellar granule cells after TSA and etoposide treatment already after 12 h exposure and the increase continued for the next 12 h (Fig. 3B, study II). The indicator for neuronal apoptosis, the caspase-3 activity increased significantly in okadaic acid treated cells already after 12 h exposure, whereas TSA and etoposide treatments had no significant effect on the caspase-3 activity levels during the 24 h incubation (Fig. 3C, study II). However, after 48 h incubation also the TSA treated cerebellar granule cells had significantly increased caspase-3 activities, while etoposide still had no effect (data not shown). Even though the caspase-3 activities were increased in TSA treated cells after 48 h incubation, no changes were seen in the DNA binding complexes of YY1 (data not shown).

Apoptosis in Neuro-2a cells induced by any of the agents used did not cause changes in the DNA binding complexes of YY1 factor (Fig. 4B, study II). The only effect seen was the decrease in the binding activity of the largest complex in okadaic acid treated cells after 36 h incubation (Fig. 4B). The same treatment also decreased the protein expression of YY1 (Fig. 4A, study II). The first 12 h incubation with TSA first decreased the protein expression of YY1 but after another 12 to 24 h exposure the protein levels returned to the control levels (Fig. 5A, study II). This same effect was seen in the DNA binding activities of YY1, but this affected only the largest binding complex (Fig. 5B, study II). Etoposide and serum withdrawal did not have any effect on the protein expression of YY1 (Fig. 4A, study II). However, northern analysis showed that YY1 mRNA level was significantly upregulated in etoposide treated cells, whereas other apoptotic inducers decreased the YY1 mRNA levels (Fig. 4D, study II). Caspase-3 activity increased almost five fold during the 36 h in TSA treated Neuro-2a cells compared to the control samples (Fig. 5D). Also the ability of the all inducers

used in study II to activate the caspase-3 enzyme in Neuro-2a cells and thus induce apoptosis in these cells were analysed in study IV (Fig. 4).

5.1.3 YY1 and neuronal aging (II)

Brain and liver tissue samples from young and old rats were collected to analyse the effect of normal aging on YY1 mRNA and protein expression. No statistically significant differences in protein levels either in hippocampus or cerebellum were found between the two age groups indicating that aging does not have any role on the YY1 protein expression (Fig. 6B, study II). The DNA binding activity of YY1 remained also constant and no changes were found in the binding complexes of YY1 between the young and old rat brain samples studied (Fig 6C, study II). In concordance with the western assays, the expression levels of YY1 mRNA in brain and liver samples from the rats studied did not show any age-related changes (Fig. 6A, study II).

5.2 mSin3

5.2.1 Regulation of mSin3 in apoptosis (III, IV)

First the localization of the proteins, mSin3A and B were studied from Neuro-2a cells using western blot assay. Both forms were located to the soluble nuclear fraction and neither mSin3A nor mSin3B were detected from the cytoplasmic fraction (Fig. 1, study IV). The protein of mSin3A appeared as “doublet” bands (approximately 150 kD in size) due to the alternative splicing isoforms, whereas mSin3B showed only a single band (approximately 130 kD) (Fig. 1, study IV).

Treatments of Neuro-2a cells with okadaic acid, etoposide, TSA and serum deprivation all induced an apoptotic cell death process in these cells, that being detected by measuring the caspase-3 activity levels from the cytosolic fraction of the treated cells (Fig, 4, study IV). In addition, the effect of TSA and n-butyrate as an apoptotic inducers in Neuro-2a and cultured cerebellar granule cells were studied in more detail (study III). Both of the compounds were able to induce caspase-3 activity in Neuro-2a and primary cerebellar granule cells, although the extent of the increase was lower in the sodium butyrate treated cells (Fig. 1, study III). The effect of TSA on caspase-3

activities was shown to be dose-dependent and time-dependent in both cell models studied (Fig.1, study III). Cycloheximide protected Neuro-2a cells against TSA induced apoptosis, since the caspase-3 activities were significantly lower in cells treated with cycloheximide (0.2 µM/ml, final conc.) and TSA (3 µM/ml) together than in cells treated with only TSA (Fig. 2, study III). This verifies that protein synthesis is needed in the TSA induced apoptosis. The activation of caspase-3 was further analysed in Western assays by studying the apoptotic cleavage of poly(ADP-ribose)polymerase in Neuro-2a cells after 48 h incubation with TSA (3 µM) and n-butyrate (10 mM), since PARP is one of the specific targets of activated caspase-3 enzyme (Fig. 2, study III). In cells treated with either of these compounds the 116 kD full-length PARP protein was cleaved into 85 kD fragment (Fig. 2, study III). However, the apoptotic cleavage capacity was greater in TSA than in n-butyrate treated Neuro-2a cells (Fig. 2, study III). Also the ability of TSA and n-butyrate to induce apoptotic morphology into the Neuro-2a cells was analysed by staining the cells with Hoechst 33258. Approximately 6% of the Neuro-2a cells treated with TSA (2 µM) for 48 h revealed apoptotic bodies caused by nuclear fragmentation (study III). In n-butyrate (10 mM) treated cells, the level was approximately 2%.

Apoptosis in Neuro-2a cells showed a prominent upregulation in mSin3A protein levels while the mSin3B levels remained unchanged. This was studied in western blot assays using isolated soluble nuclear proteins from Neuro-2a cells (Fig. 2, study IV). TSA induced the earliest upregulation of mSin3A, which is interesting since TSA is a specific inhibitor of histone deacetylases and induces hyperacetylation of histones. This increase took place already after 12 h exposure time while the other apoptotic inducers affected the mSin3A levels after 24 or 36 h of exposure. Also in cultured primary cerebellar granule cells treatment with TSA (3 µM) for 12 and 24 h increased the mSin3A expression most rapidly compared to the other compounds (Appendix, Fig. 1). In addition, etoposide (10 µM) induced mSin3A expression in primary cerebellar granule cells, while okadaic acid (25 nM) decreased the protein level. Expression of mSin3B remained constant in the apoptotic cerebellar granule cells during the first 12 h (Appendix, Fig.1). Only okadaic acid decreased the mSin3B protein levels after 24 h

exposure time, while in TSA and etoposide treated cells, the protein expression remained unchanged.

To determine whether the increase in mSin3A protein levels was due to the increased protein synthesis or for example retarded protein degradation, the synthesis rate of mSin3A was studied in Neuro-2a cells using metabolic labelling with ^{35}S-methionine and immunoprecipitation techniques. After 12 h exposure with different apoptotic inducers, TSA, etoposide and okadaic acid induced an increase in mSin3A protein synthesis (Fig. 3, study IV). Again the TSA induced increased in the mSin3A expression at this time point was shown to be the most potent, which is in agreement with the western blot studies. However, okadaic acid and etoposide induced almost as high expression of mSin3A protein as TSA (Fig. 3, study IV).

The enzymatic activity of caspase-3 increased significantly in all studied Neuro-2a apoptosis models after 36 and 48 h exposure with apoptotic inducers (Fig. 4, study IV). Interestingly, the protein expression of mSin3A was upregulated earlier than the increase in caspase-3 activity occurred.

5.2.2 Regulation of mSin3 by excitotoxicity (appendix)

Glutamate treatment of cultured cerebellar granule cells (25 µM for 15 min RT) decreased the expression of mSin3A proteins already after 1 h, and after 3 and 6 h incubation almost no mSin3A protein was detectable (Fig 2, appendix). Specifically, mSin3B seemed to be a more stable protein than mSin3A, since 1 h incubation after the glutamate shock did not affect the mSin3B protein levels (Fig. 2, appendix). The levels of mSin3B were decreased after 3 h of glutamate exposure but a further 3 h did not change mSin3B levels. Interestingly, mSin3A protein appeared as a “triplet” band instead of the usual “doublet” one (Fig. 2, appendix). This is similar to the mSin3A protein expressed in the human WI-38 fibroblasts (Fig. 1B, study V). The reason for the additional band in cerebellar granule cells may be due to posttranslational modification or proteolytic fragmentation.

5.2.3 mSin3, aging and replicative senescence (V)

Aging *in vivo* was analysed from the rat tissue samples taken from young and old animals. Rats from 4 to 6 months of age represented young animals and from 26 to 30 month of age represented old animals. Frontal cortex and liver samples were used for mRNA studies and samples from hippocampus, cerebellum and liver for protein studies. Transcripts of 6.0 and 3.2 kb mSin3A mRNAs were found in rat frontal cortex samples whereas only the 6.0 kb transcript was present in liver samples (Fig. 2, study V). Also mSin3B showed two mRNA transcripts in frontal cortex, 4.8 and 1.1 kb in size, but only the shorter one (1.1 kb) in liver (Fig. 2, study V). Aging had no influence on either the mSin3A or mSin3B mRNA expression levels in either frontal cortex or liver. This was also noted in the protein level, when mSin3A and B protein expression was studied in western blot assays analysing samples from rat hippocampus, cerebellum and liver (Fig. 3, study V).

One widely used model for aging is replicative senescence, which is based on the Hayflict limit of division capacity of diploid fibroplasts in culture (Hayflick and Moorhead 1961). Cultured human WI-38 fibroblasts were utilized as the model of replicative senescence in our studies, since passage 20 (p20) cells represented early, potentially replicative cells and p32 cells represented late passage cells with very low proliferation capacity. Also mitotic quiescence was studied in a model where SV-40 transformed WI-38 fibroblasts were cultured without foetal calf serum for 3 and 5 days.

Two transcripts of mSin3A mRNA were found in northern blots of human WI-38 fibroblasts, 6.0 and 2.5 kb in size (Fig. 1A, study V). The SV-40 transformed WI-38 cells showed a significantly higher expression of the longer mSin3A mRNA transcript than either the early passage or the late passage cells, and this expression was increased significantly in quiescent cells (Fig. 1A, study V). The expression of the shorter mSin3A transcript in these samples was not changed. Furthermore the expression levels of both mSin3A transcripts remained unchanged between the early and late passage WI-38 cells (Fig. 1A, study V). Only one mSin3B mRNA transcript was found from human WI-38 fibroblast of size 5.0 kb (Fig. 1A, study V). This transcript remained

unchanged in early and late passage fibroblasts as well as in quiescent SV-40 transformed cells.

AS in Neuro-2a cells, the mSin3A protein was also seen in human WI-38 fibroblasts as a double band approximately 150 kD in size (Fig. 1B, study V). A faint additional band was also seen below this “doublet”, possibly due to proteolytic breakdown of the mSin3A protein. Although there were no changes in mRNA levels between the early and late passage WI-38 fibroblasts, the cells in replicative senescence showed a significant decline in protein expression level of mSin3A (Fig. 1B, study V). The quiescence of SV-40 transformed WI-38 fibroblasts did not change their mSin3A protein expression.

6. DISCUSSION

This study was undertaken to analyse the effect of two different types of cell death, apoptosis and excitotoxicity, on the transcription factor YY1 and adapter molecule mSin3. The impact of normal aging on these proteins was also evaluated. Even though the existence of both of these cell death types are known to occur in several neuronal diseases, the specific mechanisms of their regulation and role are still largely unknown.

6.1 YY1 and mSin3 in apoptosis

In Neuro-2a cells, all apoptotic inducers tested induced an increase in mSin3A expression while the mSin3B levels remained constant. The earliest increase in mSin3A protein expression and caspase-3 activity was induced with TSA treatment. This is interesting, since mSin3 is known to bind histone deacetylases (HDACs) and TSA is a specific inhibitor of HDACs. The synthesis rate of the mSin3A protein was upregulated already 12 h after the TSA exposure, which was an earlier event than the increase in caspase-3 activity. This suggests that the transcriptional repressor mSin3A complex might have a regulatory role in the early phase of apoptotic process. TSA affected the mSin3 levels in the same way also in cultured cerebellar granule cells, since it increased the mSin3A protein expression. Also in this model, the mSin3B levels remained constant. When only mSin3A levels were affected in Neuro-2a cells induced to undergo apoptosis, it seems that mSin3 proteins are regulated differentially, at least in stressful situations leading to apoptotic cell death. The inhibition of histone deacetylases induces hyperacetylation of core histone proteins in nucleosomes and activates the transcriptional activity of several genes (Yoshida et al. 1995, Van Lint et al. 1996). Interestingly, this inhibition also induces an increase in mSin3A protein expression and synthesis rate, suggesting a feedback regulation mechanism. When these HDAC-enzymes are inhibited, the cell possibly tries to compensate for this situation at least by two ways. First by making more of these proteins that can recruit the HDACs, such as mSin3A and YY1, and secondly the cells synthesize more of the HDAC-enzymes themselves. Our group has data where class III HDACs (Sir2 family of proteins) have been studied after the TSA exposure to neuronal cells, and we determined that the mRNAs of SIRT2, 4 and 7 are increased in Neuro-2a cells treated

with TSA while the mRNAs from SIRT1, 3, 5 and 6 are either unaffected or decreased (S. Kyrylenko et al, unpublished data). Also the same effect has been found in human Hep3 cell line cells, where TSA induced HDAC1 mRNA level expression (Gray et al. 2000). The inhibitors for HDACs have been studied as therapeutic compounds to treat different kinds of cancers as well as polyglutamine-repreat diseases (Kramer et al. 2001, Marks et al. 2001, Steffan et al. 2001). This is due to their ability to re-initiate the repressed transcription and drive the diseased cells to apoptosis or activate their differentiation (Marks et al. 2000). In our studies, TSA induced apoptosis in the Neuro-2a cells as well as in the cultured primary cerebellar cells and increased the protein synthesis rate of mSin3A and YY1. However, the increase in the caspase-3 activities (an indicator of apoptosis) was a more prominent and earlier event in the Neuro-2a cells than in primary cerebellar granule cells. In addition, in Neuro-2a cells TSA increased the DNA binding activity of some early response transcription factors, e.g. AP-1, CREB and NF-κB, whereas in cultured cerebellar granule cells only the DNA binding activity of NF-κB was increased. This reflects the possible differences in the regulatory processes between the different cell types.

Okadaic acid was the only apoptotic inducer that decreased the YY1 protein expression in cultured cerebellar granule cells and induced changes in the DNA binding complexes of YY1. Also the expression of mSin3A and B was decreased in cultured cerebellar granule cells after okadaic acid treatment. In addition, the caspase-3 activities were extremely high in okadaic acid treated cells compared to the levels achieved with the other apoptotic inducers used (approximately 10 times higher than in TSA treated granule cells), suggesting that the okadaic acid treated cells were already in late apoptosis. Okadaic acid is an inhibitor of serine/threonine protein phosphatase 2A (Walker and Watson 1992, Favre et al. 1997). It has been shown that the cause of neuronal cell death in cerebellar granule cells after treatment with okadaic acid is caused by increased phosphorylation (Candeo et al. 1992). However, YY1 is shown to be a stable phosphorylated protein (Austen et al. 1997b). In a recent study by Galasinski et al. (2002), the okadaic acid treatment of cells was shown to induce hyperphosphorylation of HDAC1 and 2 and disrupt the protein complex formation between HDAC1, YY1 and mSin3A. It was also shown, that YY1 and mSin3A

proteins were phosphorylated after okadaic acid treatment (Galasinski et al. 2002). In our experiments, the changes in the DNA binding complexes of YY1 induced by okadaic acid occur between the largest and the smallest complex from the three complexes seen. The binding activity of the largest complex decreased while it increased in the smallest complex. This could be interpreted that the increased phosphorylation in cells caused dissociation of the binding partners of the YY1 protein and the smallest complex seen in EMSA experiments would represent the YY1 protein alone bound to its specific oligonucleotide. Alternatively, the phosphorylation could cause changes in YY1's binding partners and lead to the formation of a new smaller complex with a possible novel role in transcriptional regulation. Since YY1 can act as a transcriptional activator or repressor, the dissociation of the HDAC proteins from the YY1 complex could change the nature of this "new complex".

It is more complicated to understand how the other apoptotic inducers used in our studies affect the mSin3A and YY1 levels and binding properties of YY1. Etoposide is an inhibitor of topoisomerase II. Topo II is a nuclear enzyme that catalyses the reversible breakage and rejoining of the double helix DNA between its relaxed and supercoiled states (Felix 2001). Etoposide stabilizes the complex between DNA and DNA topoisomerse II, resulting a decrease in the re-ligation rate of the DNA strands, interference in the cleavage-ligation ratio and finally as a net effect it causes an increase in the cleavage of the DNA (Felix 2001). It could be speculated that one possible connection between transcriptional regulation and etoposide induced apoptosis is the enzyme poly(ADP-ribosyl)polymerase 1, since PARP enzymatic activity increases greatly in response to DNA damage. It has been shown that activated PARP induces poly(ADP-ribosyl)ation of YY1 and in this way abolishes its binding affinity to DNA (Oei et al. 1998, Oei and Shi 2001a). Etoposide increased the expression of both mSin3A and YY1 proteins in Neuro-2a cells, as well mSin3A expression in cultured cerebellar granule cells. In Neuro-2a cells, etoposide induced apoptotic processes, since the caspase-3 activities increased significantly after the36 h exposure. However, in cerebellar granule cells etoposide did not increase the caspase-3 activities, even after 48 h exposure. The mechanism whereby etoposide regulates the levels of YY1 and

mSin3A as transcriptional repressors remains to be elucidated. They may have a role as general transcriptional repressors during the time of DNA repair.

During serum deprivation, the cells suffer from starvation of growth factors. Deficiency of the growth factors affects the cells more slowly but evokes the apoptotic processes to be turned on as well. As long as there is energy left, the apoptosis may continue (Pieper et al. 1999). Otherwise the process turns down anecrotic route. The exact cascade, how this process increases the mSin3A protein level, is not known. It could be suggested that the increase in mSin3A protein expression in serum deficiency, is due to the increased need for general transcriptional repressors in cell. In order to save energy and avoid necrosis, the cell tries to turn off all unnecessary protein synthesis and requires mSin3A and HDACs for that purposes.

In summary, okadaic acid induced apoptosis in cultured cerebellar granule cells altered the DNA binding complexes of transcription factor YY1. This is speculated to be due to enhanced phosphorylation of HDACs and YY1 causing dissociation of the formed complex in cell. This may lead to the changed activity of YY1, since its role as a transcriptional factor is dependent on the complex formed (Shi et al. 1997). Neuronal apoptosis induced an increase in the expression of transcriptional repressor mSin3A but not mSin3B protein, suggesting that they are differently regulated in stress situations. The increase in the expression of transcriptional repressors during apoptosis may reflect the general need for a cell to shut off all unnecessary transcription processes to save energy and to prevent transcription of possibly damaged chromatin. However, differences in the expression of YY1 and mSin3A/B proteins and also in the formation of DNA binding complexes of YY1 between these two studied cell models occurred, suggesting differences in the regulation processes among different cell types.

6.2 YY1 and mSin3 in excitotoxicity

Glutamate is the major excitatory neurotransmitter in the brain. However, in certain conditions, where excessive amounts of glutamate are released into the extracellular space, it can be toxic to neurons evoking cell death and this process in involved in stroke, head trauma and epilepsy (Bradford 1995, Doble 1999). In the normal situation

the synaptically released glutamate is removed rapidly by surrounding cells but after a neuronal tissue insult their glutamate uptake may be depressed (Pieper et al. 1999). The combination of lowered uptake and increased release of this excitatory amino acid leads finally to neuronal cell death. However, there are also conflicting opinions about glutamate's role in excitotoxicity (Obrenovitch et al. 2000). It has been calimed that in studied models of stroke and head trauma, the concentration of glutamate in the extracellular space would be far too low to be able to induce neuronal death (Obrenovitch et al. 2000). Also the removal of glutamate from the extracellular space is suggested to occur so rabidly that it would not provoke excitotoxicity (Obrenovitch et al. 2000). The glutamate concentrations applied in our primary cell culture models are those widely used (5-100 µM) (Dessi et al. 1993, Cosi et al. 1994). In rat spinal cord injury studies, glutamate concentrations as high as 1 mM were measured at two millimeters from the center of impact (McAdoo et al. 1999).

Glutamate induces Ca^{2+} influx into the cell mainly through NMDA receptors (Coyle and Puttfarcken 1993, Zipfel et al. 2000). This accumulation of Ca^{2+} ions into intraneuronal space will further activate proteases, such as calpains, leading to generation of oxygen radical formation, and stimulate NO synthetase activation (Pieper et al. 1999). Elevated levels of NO damage DNA and disrupt energy homeostasis in cell. Pumping the excess of Ca^{2+} out of the cell and/or into endoplasmic reticulum consumes energy and decreases the ATP levels of the cell (Pieper et al. 1999). Accumulation of Ca^{2+} into mitochondria disturbs their metabolism as well, and causes more oxidative stress and damage and also impairs the production of ATP (Ward et al. 2000).

The cause of the changes seen in the DNA binding complexes of YY1 protein after glutamate insult has been studied extensively. The greatest differences seen in EMSA assays occurred between the largest and the smallest complex. In control primary neuronal cell samples, the greatest binding affinity was found from the first complex, whereas glutamate induced a switch from the first to the third complex. Interestingly, with an antibody against YY1 protein, both of those formed complexes shifted to the

same level in the gel. Thus, in the supershift assay no, differences between the control and glutamate treated samples were seen.

We propose that the smallest complex seen in EMSA studies after glutamate insult is the single YY1 protein itself without any partner proteins. Or alternatively the smallest complex may represent YY1 with a new set of binding partners. It seems that the glutamate induced Ca^{2+} influx influences the signalling cascades which disturbs the complex formation. The YY1 protein may be modified, and hence prevent the binding of other proteins and repress the complex formation. It is known, that increased levels of Ca^{2+} in the cell can activate calcium-dependent kinases, like protein kinase C (Favaron et al. 1990). This may lead to the phosphorylation of YY1 and its binding partners causing the complex to dissociate (Galasinski et al. 2002). It is unlikely that the smallest band seen is a fragment of YY1, since the antibody against the whole YY1 polypeptide (H-414) shifted the control and glutamate treated samples to the same level. If the YY1 protein had broken down, the formed fragments would have produced several bands in the EMSA gel instead of the single one seen. However, calcimycin (A23187) did not induce changes in the DNA binding complexes of YY1, although it is known to cause enhanced Ca^{2+} influx into the cell. This suggests that the Ca^{2+} is not the only trigger in excitotoxicity causing the changes in YY1 binding complexes, but some other regulatory signals are also involved.

Interestingly, both benzamide (PARP inhibitor) and MK-801 (NMDA receptor antagonist) inhibited the changes seen in the DNA binding complexes of YY1. The inhibition of the changes in the binding complexes of YY1 by MK-801 verified that the signalling cascades modifying YY1 factor goes through NMDA receptors. As in apoptosis also in excitotoxicity, the poly(ADP-ribosyl)polymerase is activated and this ribosylates nuclear proteins, including YY1 (Oei et al. 1998). The overactivation of PARP decreases the energy levels of cells leading to cell death. It has been shown that the PARP inhibitors repressed the glutamate-induced excitotoxicity in cultured cerebellar granule cells (Cosi et al. 1994). Thus, glutamate induced excitotoxicity is thought to activate PARP, which may further ribosylate YY1 protein as well as its binding partners leading to the changes in the DNA binding complexes of YY1.

However, ribosylation of transcription factors has been shown to decrease their binding affinity to the DNA binding sites (Oei and Shi 2001a). This may involve factorswhich are free in the cytosol, but those already bound to DNA could be regulated differently.

Also the effect of the glutamate insult to the expression of mSin3 proteins was analysed (appendix). Since the mSin3A levels decreased significantly earlier than the mSin3B levels after the glutamate insult, it seems that the mSin3B protein represents a more stable and constitutively expressed form of the mSin3 proteins. This is also seen in apoptosis, since the expression rate of mSin3A was increased, but the mSin3B levels remained constant. It could be suggested that since the expression of transcriptional repressor protein mSin3A declines in excitotoxicity, also the genes regulated by the mSin3A recruited complex are no longer subject to repression during excitotoxic cell death.

The hypothesis about the type of cell death provoked by glutamate to neuronal cells is controversial, since both necrosis and apoptosis have been suggested (Dessi et al. 1993, Bezvenyuk et al. 2000, Zipfel et al. 2000). We have not analyzed the precise type of cell death occurring in these cultured primary neurons. However, the time points at which we drew our excitotoxic samples are too early to detect caspase-3 activation. In our experimental model for excitotoxicity, the enzymatic activity of caspase-3 did not increase. In this model, approximately 20% of the primary cerebellar granule cells died during the first two hours due to glutamate treatment, whereas approximately 70% of the cells died in 24 hours. Our recent results show that glutamate treatment induces cell death independently of caspase-3 activation and without any oligonucleosomal DNA fragmentation but still shows apoptotic like nuclear morphology (Bezvenyuk et al. 2000). According to these studies it has been suggested that glutamate induced cell death is an unusual type of cell death somewhere between apoptosis and necrosis without fulfilling the criteria of either classical apoptosis or necrosis (Bezvenyuk et al. 2000). However, results where mild excitotoxic treatment has been shown to induce caspase-3 activation in cerebrocortical neurons also have been presented (Tenneti and Lipton 2000). In addition, the hypothesis of how the energy levels available in the cell would finally decide the cell death type, must be considered (Formigli et al. 2000). If

the cell has enough ATP, it would go into apoptosis, whereas cell with insufficient energy stores would die in necrotic way. Finally the cell could exhibit features from both, apoptosis and necrosis (aponecrosis), but the final death process would depend on the intracellular ATP level (Formigli et al. 2000). Furthermore, also the heterogeneity of different cell populations and the state of maturation has been observed to cause variations in the intensity of the glutamate induced insult (Zipfel et al. 2000).

In summary, glutamate induced changes in the DNA binding complexes of YY1 transcription factor in cultured primary cerebellar granule cells. Glutamate caused a switch from the largest binding complex to the smallest, suggesting that there are changes in the binding partners of the DNA binding complexes of YY1. The change from the largest DNA binding complex of YY1 to the smallest one was dependent on the level of toxicity. The changes were similar in okadaic acid treated cerebellar granule cells. These changes were inhibited by PARP inhibitor, benzamide and by NMDA receptor antagonist, MK-801. Calcimycin (A23187) did not cause any changes in the DNA binding complexes of YY1, suggesting that Ca^{2+} is not the only or even a sufficient trigger for the changes seen. We propose that the changes in the binding complexes are induced by either phosphorylation of ribosylation of the YY1 protein and its complex proteins. The expression of mSin3A decreased dramatically in glutamate treated cultured cerebellar granule cells, while the expression of mSin3B protein remained more constant. This suggests differences in the regulation of mSin3 proteins during excitotoxicity, a phenomenon also seen in neuronal apoptosis between these proteins.

6.3 YY1 and mSin3 during aging

Aging is known to decrease the rate of transcription, protein synthesis, modification and turnover (Rattan 1996, Ryazanov and Nefsky 2002). It remains to be determined how the transcription might be regulated due to aging. We wanted to investigate whether aging had any effect on YY1 and/or mSin3 factors, since both of the proteins are known to function as transcriptional repressors.

The changes in YY1 and mSin3 mRNA and protein expression during aging in vivo was studied using brain and liver samples collected from young and old rats. Additionally also human WI-38 fibroblasts were studied in mSin3 assays to investigate the difference between *in vitro* senescence and *in vivo* aging in tissue. Aging did not seem to have any specific effect on YY1 and mSin3 mRNA and protein expression levels *in vivo,* studied in brain and liver tissues. Also aging did not influence onthe DNA binding activity or complex formation of YY1. It has been shown previously that the DNA binding activity of YY1 protein increases during aging in liver samples of mice (Adrian et al. 1996). In that study, it was shown, that human transferrin gene containing the YY1 binding site bindsthe YY1 transcription factor in an age-related manner and the changes were seen in the middle and in the smallest complex from the three complex present (Adrian et al. 1996). In contrast to those results, we could not find any YY1 protein in our western blot studies from rat liver samples, but this may be due to the antibody used (data not shown).

In vitro model, replicative senescence in human lung WI-38 fibroblasts showed a decline in mSin3A protein levels, even though the mRNA levels remained unchanged. The differences between *in vivo* and *in vitro* models in aging have been reported also from transcription factor NF-?B studies (Helenius et al. 1996). The differences in the results between the two aging models studied may be due to the dissimilar regulation systems operating in proliferating cells and postmitotic neuronal cells. The replicative senescence is based on the Hayflick limit of division capacity of the cultured diploid fibroblasts (Hayflick and Moorhead 1961).

In conclusion, the transcriptional repressor proteins YY1, mSin3A and B are not regulated during aging *in vivo.*

As a summary, the roles of transcription factor YY1 and adaptor protein mSin3 are depicted below.

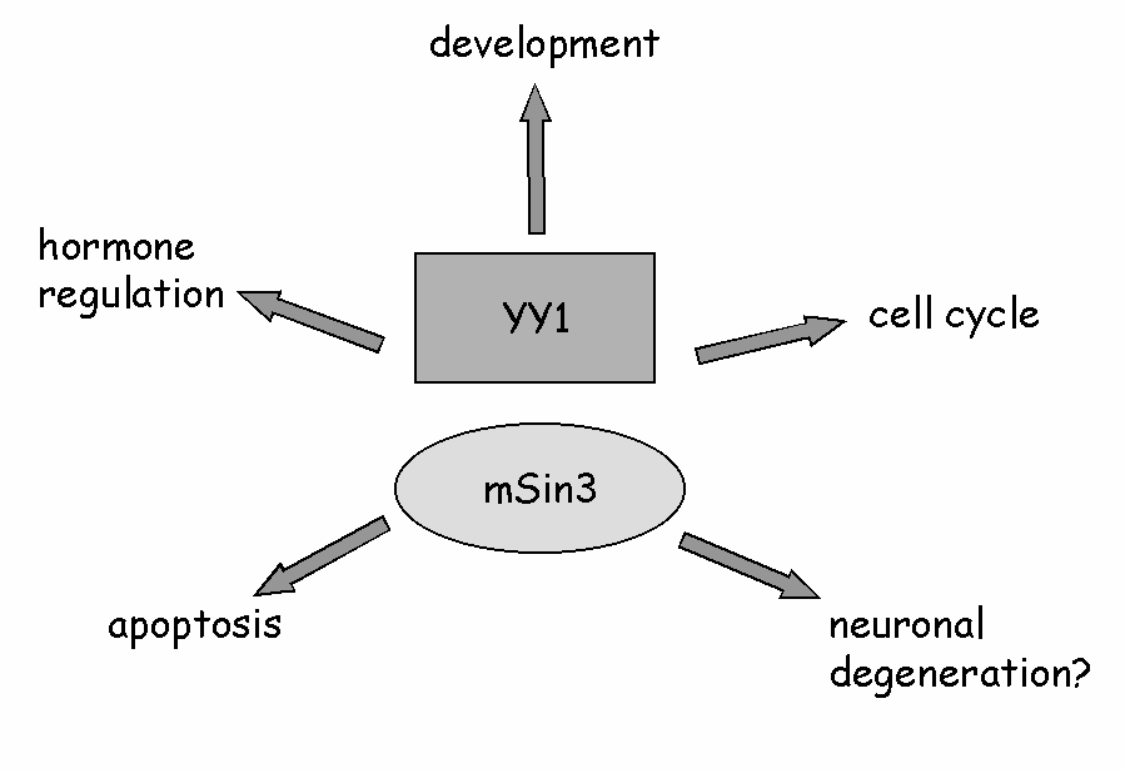


Fig. 7 Cellular regulatory events involving the transcription factor YY1 and the adaptor protein mSin3.

7. CONCLUSIONS

The aim of the study was to investigate the effect of neuronal apoptosis, excitotoxicity and aging on the expression of transcriptional repressor factors, YY1 and mSin3.

The following conclusions can be drawn:

1. Glutamate induced excitotoxicity in cultured primary cerebellar and hippocampal cells caused changes in the DNA binding complexes of transcription factor YY1. The DNA binding complexes of YY1 protein in the control and the glutamate treated primary neurons shifted to the same level in EMSA supershift assays demonstrating that YY1 protein was present in both complex forms.

2. Okadaic acid induced apoptosis in primary cultured cerebellar granule cells showed similar changes in the DNA binding complexes of YY1 to that seen in the same cells during glutamate induced excitotoxicity. Also the expression of the YY1 protein decreased considerably after both treatments.

3. Apoptosis in Neuro-2a cells induced an increase in the expression of transcriptional repressor protein mSin3A, whereas mSin3B levels remained unchanged.

4. The expression pattern of the YY1 and mSin3A/B proteins as well as the DNA binding complexes of YY1 varied between the cell models studied, pointing to the involvement of cell specific regulation.

5. YY1 and mSin3 protein levels are not changed during aging *in vivo.*

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APPENDIX

Experiment 1

In this experiment, the effect of apoptosis in primary cerebellar granule cells on the expression levels of mSin3A and mSin3B protein was analysed (see 4.4). Primary cells after 7DIV were treated with apoptotic inducers TSA (3 µM final concentration), okadaic acid (25 nM) and etoposide (10 µM) for 12 and 24 h. The cells were lysed with RIPA-buffer and the protein expression was detected by western blot method as described in 4.5.2 using antibodies from Santa Cruz Biotechnology, AK-11 (200 µg/ml) against mSin3A and A-20 (200 µg/ml) against mSin3B.

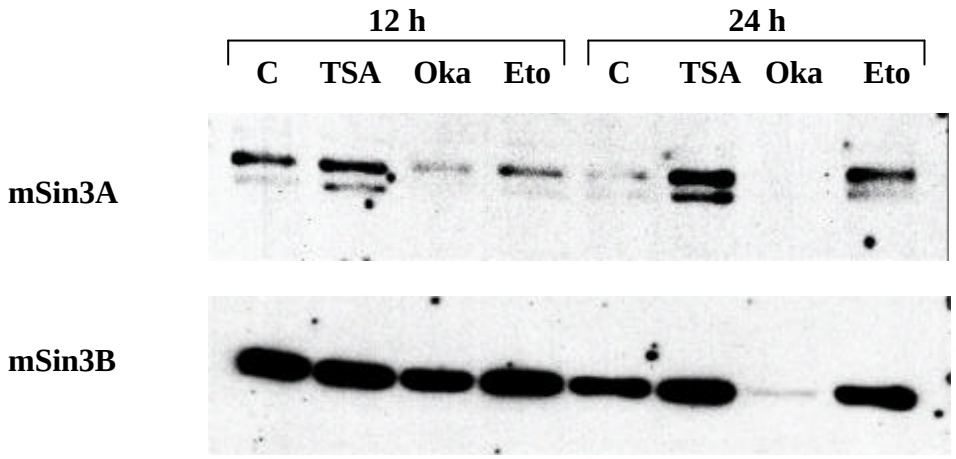

Fig. 1 TSA and etoposide increased the expression of mSin3A protein in cultured primary cerebellar granule cells, whereas okadaic acid decreased it. The expression of mSin3B remained constant after all apoptotic treatments but not okadaic acid, since okadaic acid for 24 h reduced the mSin3B levels to zero. C, control; TSA, Trichostatin A; Oka, okadaic acid; Eto, etoposide.

Experiment 2

The influence of glutamate induced excitotoxicity in primary cerebellar granule cells on the expression of mSin3A and mSin3B proteins was studied. Primary cells after 10 DIV were treated with 25 µM glutamate in Locke's solution for 15 min at RT followed by washings with Locke's solution containing Mg^{2+}. The conditioned medium was returned to the cells and the samples were taken after 1, 3 and 6h. The cells were lysed with RIPA-buffer and the western blot assay was performed as described in 4.5.2 using the same antibodies as in experiment 1.

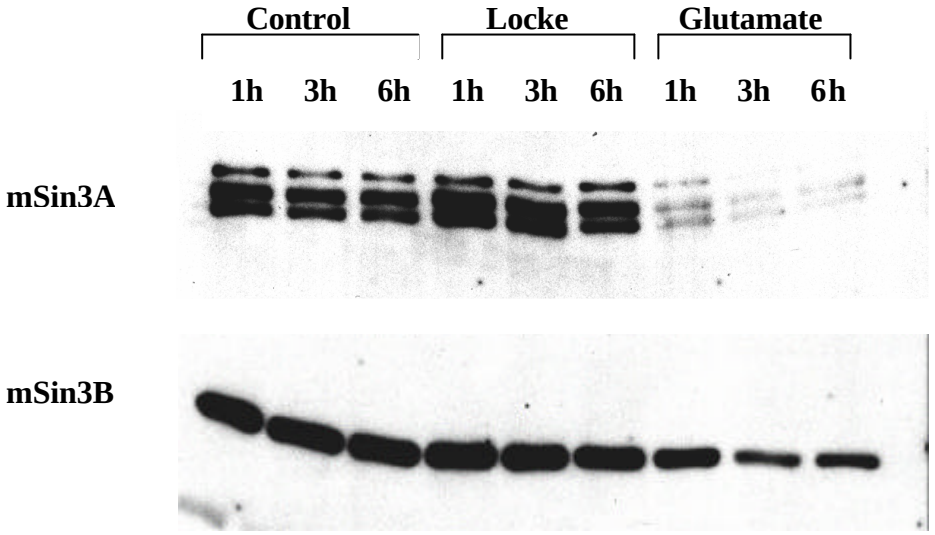

Fig. 2 L-glutamate induced insult to the primary cerebellar granule cells decreased the levels of mSin3A protein already after 1h, whereas the protein expression of mSin3B was more constant and stable at that time point. The mSin3B protein showed overall a more stable expression, since after 6 h there was still a significant amount of mSin3B protein expressed, while mSin3A had almost disappeared. Interestingly, mSin3A appeared as a "triplet" band as in WI-38 fibroblasts (study V). Locke's salt solution did not have any effect on the protein expression.