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Organelle specific mechanisms of neuronal cell death

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After all the loving and the losing,
the heroes and the pioneers,
the only thing that’s left to do is live,
and get another round in at the bar.
-Frank Turner

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LIST OF ORIGINAL PUBLICATIONS

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

I Sokka, A.L.*, Putkonen, N.*, Mudo, G., Pryazhnikov, E., Reijonen, S., Khiroug, L., Belluardo, N., Lindholm, D. & Korhonen, L. 2007, "Endoplasmic reticulum stress inhibition protects against excitotoxic neuronal injury in the rat brain", *The Journal of Neuroscience,* vol. 27, no. 4, pp. 901-908.

II Putkonen, N., Kukkonen, J.P., Mudo, G., Putula, J., Belluardo, N., Lindholm, D. & Korhonen, L. 2011, "Involvement of cyclin-dependent kinase-5 in the kainic acid-mediated degeneration of glutamatergic synapses in the rat hippocampus", *The European Journal of Neuroscience,* vol. 34, no. 8, pp. 1212-1221.

III Reijonen, S., Putkonen, N., Norremolle, A., Lindholm, D. & Korhonen, L. 2008, "Inhibition of endoplasmic reticulum stress counteracts neuronal cell death and protein aggregation caused by N-terminal mutant huntingtin proteins", *Experimental Cell Research,* vol. 314, no. 5, pp. 950-960.

* Equal contribution

SUMMARY

Neuronal cell death caused by excitotoxicity accompanies neurodegenerative disorders, such as Alzheimer's disease (AD) and Huntington's disease (HD), epilepsy and ischaemia. Glutamate is the major excitotoxin in the CNS and causes activation of glutamate receptors. Ionotropic glutamate receptors can directly cause calcium influx that further enables activation of cell death pathways. Kainic acid (KA) is a specific agonist for ionotropic non-NMDA glutamate receptors, namely KA and AMPA receptors. KA induces epiletic activity in rodents and causes hippocampal sclerosis, similar to human temporal epilepsy. HD, a neurodegenerative disease characterized by accumulation of mutant huntingtin protein, and causing cell death in the striatum of affected individuals, has also been shown to involve excitotoxic cell death. Intracellular organelles have been implicated in stress sensing and contribute to cell death signaling. Mitochondria have been closely linked to apoptotic pathways and recent research has also implicated other organelles, such as the endoplasmic reticulum (ER), lysosomes and Golgi apparatus in cell death.

In this thesis, the involvement of ER stress was shown to accompany hippocampal cell death caused by KA *in vivo* and *in vitro* as well as in a cell model of HD. KA induced activation of ER stress sensors that aim to restore homeostasis via activation of the unfolded protein response (UPR). In prolonged stressful conditions, the UPR activates apoptotic pathways. Treatment with an ER stress inhibitor, Salubrinal (Sal), significantly attenuated cell death in hippocampal neurons *in vivo* and *in vitro*. ER stress was also activated in a cell model of HD and treatment with Sal reduced cell death and mutant hungtingtin aggregation. These data indicated for the first time the involvement of the ER in cell death pathways caused by excitotoxicity, and that inhibition of ER stress could be a potential treatment against neuronal cell death in HD and other disorders involving excitotoxicity.

In search of other cell death mediators we focused on Cdk5 that has been implicated deregulated in excitotoxicity. Involved in multiple signaling pathways, Cdk5, has been implicated, for instance, in regulation of synaptic proteins, ER stress and cell death. In this thesis, a KA receptor important for mediating cell death in the hippocampus, GluR6, was shown to be regulated by Cdk5. Inhibition of Cdk5 reduced GluR6 downregulation by KA as well as cell death caused by KA *in vitro*. These data indicated Cdk5 involvement in KA excitotoxicity and could also present a potential drug target in neurological disorders. Moreover, this was the first time that Cdk5 was shown to contribute to KA receptor regulation.

ABBREVIATIONS

AD	Alzheimer's disease
ADP	Adenosine diphosphate
ALS	Amyotrophic lateral sclerosis (Lou Gehrig's disease)
AMP	Adenosine monophosphate
AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid
ATF	Activating transcription factor
ATP	Adenosine triphosphate
Aβ	Amyloid beta peptide
Bcl-2	B-cell lymphoma 2
BH	Bcl-2 homology
BiP	Binding immunoglobulin protein (BiP); 78 kDa glucose-regulated protein (GRP-78); heat shock 70 kDa protein 5 (HSPA5)
BIR	Baculovirus IAP repeat –domain
CARD	Caspase recruitment domain
Cdk5	Cyclin-dependent kinase 5
CED	Cell death abnormal
CNS	Central nervous system
DED	Dead effector domain
ER	Endoplasmic reticulum
ERAD	ER associated degradation
ERSE	ER stress element
GluK	Glutamate receptor, kainate subtype
GluR	Glutamate receptor
Grik	Glutamate receptor, ionotropic, kainate (gene)
GRP	Glucose regulated protein
GTP	Guanine triphosphate
HD	Huntington's disease
iGluR	Ionotropic glutamate receptor
IAP	Inhibitor of apoptosis –protein
KA	Kainic acid, kainate
KAR	Kainate receptor
KO	Knockout
mGluR	Metabotropic glutamate receptor
mRNA	Messenger RNA
NMDA	*N*-methyl-*D*-aspartic acid
PD	Parkinson's disease
PDZ	Postsynaptic density-95/Discs large/Zona occludens-1
PSD	Postsynaptic density
Sal	Salubrinal
TLE	Temporal lobe epilepsy
TNF	Tumor necrosis factor
TRAF2	TNF receptor associated factor 2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
WB	Western blotting

Review of the Literature

1.1 Cell death

1.1.1 Classification of cell death

Cell death has classically been divided to apoptosis and necrosis. While apoptosis is a controlled, active form of cell death, necrosis is characterized by uncontrolled, passive breakdown of cellular contents to the surroundings, often leading to spread of inflammation in nearby cells. In addition, morphological classification of cell death by Schweichel and Merker adds autophagy to the list of cell death mechanisms (Schweichel, Merker 1973, Kroemer et al. 2009). Nowadays, several other types of cell death or subtypes of apoptosis are proposed; however, the characteristics can be overlapping making the distinction challenging.

1.1.2 Necrosis

Necrosis (from Greek νεκρός, "dead") is considered as uncontrolled death of cells in unbearable conditions. Necrosis is often caused by external factors that can occur after sudden trauma, hyperthermia, infection, or toxin. Morphologically, necrotic cell death involves clumping of chromatin, swelling of mitochondria, disruption of cellular membranes and disintegration of organelles. For a long time necrosis was thought to be a passive process. Recently, certain cell types have been shown to undergo so called "programmed necrosis" or "necroptosis" that has been shown to involve specific molecular machinery (Bizik et al. 2004).

Necroptosis was described as a form of ordered cellular explosion (Vandenabeele et al. 2010). As early as in 1988 it was noted that stimulation of certain cell types with tumor necrosis factor (TNF) could result both in apoptotic-like cell death without DNA fragmentation or necrosis-like cell death (Laster, Wood & Gooding 1988). The dying cells exhibited either classical apoptotic or balloon like morphology without nuclear condensation, characteristics of necrotic and apoptotic cell death, respectively (Laster, Wood & Gooding 1988). Nowadays, many proteins are known to contribute to necroptosis, including TNF receptors, receptor interacting proteins 1 and 3 (RIP1, -3) and caspase inhibitors, among others (Vandenabeele et al. 2010). Caspase inhibitors expressed in some cell lines and primary cells, including cIAP, as well as pharmacological caspase inhibitors can block the apoptotic pathways and thus favor necroptotic cell death pathway (Fiers et al. 1995, Vercammen et al. 1998).

1.1.3 Apoptosis

Apoptosis is a genetically programmed form of cell death required for embryonic and postnatal development as well as for tissue homeostasis, aging and removal of unwanted cells. Especially, during development an excess number of cells are produced that later are removed by apoptosis. In addition, a large number of diseases are associated with disturbances in apoptotic cell death pathways, including deregulation of the pathways in cancers and autoimmune diseases, as well as excessive cell death seen e.g. in

neurodegenerative diseases, AIDS (Acquired Immune Deficiency Syndrome), and ischaemic injuries (Agostini, Tucci & Melino 2011).

Apoptosis is characterized by cell shrinking, membrane blebbing, nuclear chromatin condensation and fragmentation while integrity of the membranes and organelles are sustained (Kroemer et al. 2009, Kerr, Wyllie & Currie 1972, Williams, Little & Shipley 1974). More detailed biochemical criteria nowadays define apoptosis including caspase activation, energy consumption (ATP), and exposure of phosphatidylserine on the cell outer membrane. Definite criteria of apoptotic characteristics, however, remain to be fully described, since they are overlapping with other forms of cell death (Kroemer et al. 2009, Kroemer et al. 2005, Galluzzi et al. 2011).

1.1.4 Non-apoptotic cell death

Cell death is a growing area of research and since the first descriptions of programmed cell death (PCD) non-apoptotic types of cell demise have been proposed (Kroemer et al. 2009, Kroemer et al. 2005, Galluzzi et al. 2011). Certain tentative definitions of atypical cell death include mitotic catastrophe, anoikis, paraptosis, pyroptosis, pyronecrosis, and entosis. *Anoikis*, for instance, is cell death of adherent cells caused by loss of cell-to-matrix interaction. However, anoikis is mostly executed by intrinsic apoptosis machinery (Frisch, Francis 1994). Another similar form of cell death is *entosis* that is characterized as a non-apoptotic cell death process. Entosis was found to occur in epithelial cells detached from the matrix, subsequently eaten by neighboring cells (Overholtzer et al. 2007). The peculiar finding was that the cells were alive and seemed normal by the time of engulfment, also the cell death was driven by lysosomal degradation machinery rather than apoptosis or autophagy (Overholtzer et al. 2007).

Autophagic cell death

Autophagy, also called self-eating, is a catabolic pathway of cytoplasmic constituents and organelles by delivery to and fusion with the lysosome (Mizushima 2007). The lysosome contains hydrolases that require acidic environment (pH 4,8) and the organelle maintains this acidic environment by pumping protons from the cytosol via proton pumps and chloride ion channels. These hydrolases are able to proteolyse the constituents delivered to the lysosome and recycle basic materials such as amino acids and lipids. Autophagy is mediated by a specialized organelle, the autophagosome, that sequesters portions of cytosol in response to certain stimuli. Originally autophagy was found to be an adaptive response to nutrient deprivation but now it is also linked to clearance of damaged organelles and aggregated proteins and serves to protect cells from different stressors (Banerjee, Beal & Thomas 2010). Existence of autophagic vacuoles in dying cells has introduced the term “autophagic cell death” or more conveniently, “cell death with autophagy” (Mizushima 2005). Dysregulation of autophagy has emerged in numerous diseases, especially in neurodegenerative diseases where observation of increased amounts of autophagosomes have provoked discussion about their roles in neuronal cell death (Banerjee, Beal & Thomas 2010). However, in neurons it has been thought that defective rather than excessive autophagy results in cell death.

Autophagy can be divided into three types: macro-, micro- and chaperone-mediated autophagy (Mizushima 2007). Macroautophagy, which is usually referred to as bare autophagy, involves de novo formation of autophagosomes that sequester proteins targeted to lysosomes. Microautophagy is basically direct pinocytosis of cytosol by the lysosome and functions continuously in resting state (Banerjee, Beal & Thomas 2010). Chaperone-mediated autophagy (CMA) is a selective degradation pathway of proteins containing a pentapeptide motif (KFERQ) that is recognized by a cytosolic chaperone, heat-shock cognate 70 (HSC70), targeting the substrates to lysosomes. CMA can be induced for prolonged times, for instance, during nutritional deprivation (Kon, Cuervo 2010).

The machinery of autophagy includes over 30 genes named autophagy-related genes (ATG) (Klionsky 2007). Classical pathway to autophagy involves mTOR (mammalian target of rapamycin), a kinase that negatively regulates autophagy. mTOR-mediated inhibition can be removed by e.g. starvation resulting in autophagosome formation. Furthermore, a protein involved in autophagy, Beclin-1 (Atg-6), is bound to Bcl-2 under normal conditions but released in response to starvation (Pattingre et al. 2005) emphasizing the roles of one cell death pathway beyond its borders. Another example of interconnections between cell death pathways comes from the finding that Atg5 and Beclin-1 deficient cells are unable to express phosphatidylserine (PS) on their surface and secrete less lysophosphatidylcholine (LPC), a signal for phagocytic clearance, during apoptosis (Qu et al. 2007). Thus, autophagy might also be needed for proper function of phagocytosis since PS exposure is a prerequisite for apoptotic cell engulfment by phagocytes (Marguet et al. 1999). Rapamycin has been shown to ameliorate amyloid and tau pathology in a mouse model of AD (Spilman et al. 2010). Moreover, autophagy has been linked to HD, where sequestration of mTOR as well as Beclin-1 to mutant huntingtin aggregates might both induce but also inhibit autophagy, respectively, resulting in inefficient clearance of aggregates (Ravikumar et al. 2004, Shibata et al. 2006, Hyrskyluoto et al. 2012).

PARP-1 –dependent cell death
Poly(ADP-ribose) polymerase 1 (PARP-1) is a nuclear DNA repair enzyme that modulates the activities of histones and topoisomerases by incorporating polymers of ADP-ribose in response to DNA damage. PARP-1 –dependent cell death divides opinions, some considering it as a form of necrosis (Duprez et al. 2009), others as a separate cell death mode, also termed *parthanatos* (Andrabi, Dawson & Dawson 2008). Regardless, early activation of PARP-1 occurs during excitotoxicity, ischaemia, traumatic brain injury and several neurodegenerative disorders. The molecular determinants include accumulation of poly (ADP-ribosyl)ated proteins and translocation of AIF (Apoptosis inducing factor) to the nucleus (Yu et al. 2002). PARP-1 is also a substrate for caspases (Lazebnik et al. 1994).

1.2 Mediators of apoptosis

1.2.1 Apoptosis in the nematode *Caenorhabditis elegans*

Pioneering work in the characterization of apoptotic proteins was conducted in the nematode *Caenorhabditis elegans* (*C. elegans*) during the 1990s. *C. elegans* has provided many advantages in cell death research mostly due to its small size and short generation time (3d).

The development of *C. elegans* hermaphrodite has been fully characterized: 1090 somatic nuclei are generated out of which 131 undergo programmed cell death to yield an adult worm with 959 somatic cells (Metzstein, Stanfield & Horvitz 1998). Furthermore, 116 of all dying cells are derived from the lineage forming the nervous system, highlighting the importance of cell death in formation of functional neuronal networks.

Genetic studies on *C. elegans* have defined over 20 genes involved in programmed cell death (Lettre, Hengartner 2006). Apoptosis in *C. elegans* can be described by a linear model as shown in Figure 1. Genes involved in all these steps have been characterized and divided into four groups according to which phase they take part in: decision, execution, engulfment, and degradation. The decision-making is assumed to be controlled by several mechanisms. However, two neurosecretory motor (NSM) neurons that normally die during development are rescued by mutations in *ces-1* (*ce*ll death *s*pecification abnormal) or *ces-2* genes. Both *ces-1* and *ces-2* are transcription factors and *ces-1* is thought to inhibit NSM sister cell death while *ces-2* inhibits the pro-survival function of *ces-1* thereby causing cell death (Lettre, Hengartner 2006) (Fig 1).

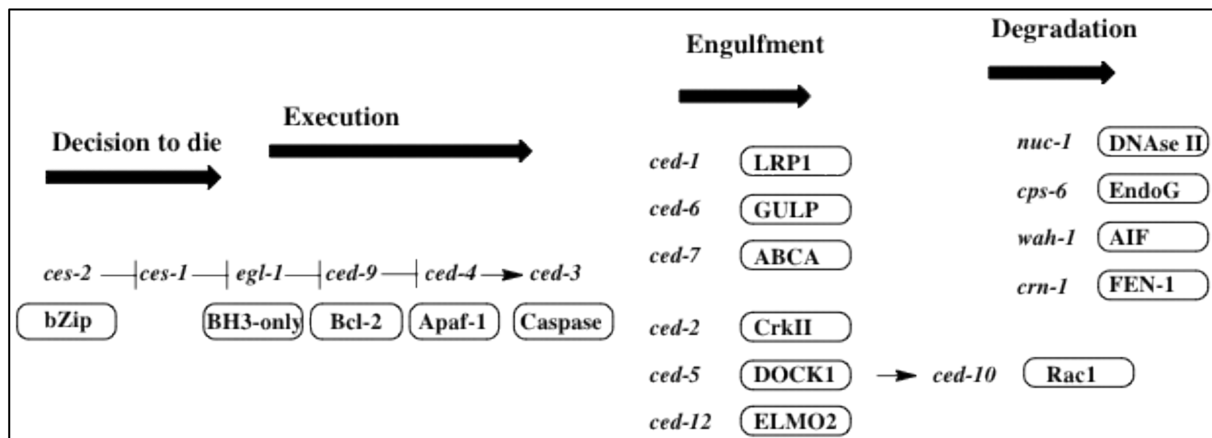


Figure 1 Cell death in *C. elegans*. Mammalian homologs are circled. Abbreviations: ABCA: ATP-Binding Cassette, subfamily A; AIF: Apoptosis Inducing Factor; Apaf-1: Apoptotic Protease-Activating Factor-1; Bcl-2: B-Cell Lymphoma 2; BH3: Bcl-2 Homology-3; bZIP: basic region leucine-ZIPper; *ced*: CEll Death abnormal; *ces*: CEll death Specification abnormal; *cps-6*: CED-3 Protease Suppressor 6; *crn-1*: Cell death Realated Nuclease 1; DOCK1: Dedicator Of CytoKinesis 1; *egl-1*: EGg-Laying abnormal; ELMO-2: Engulfment and celL Motility gene-2; FEN-1: Flap structure-specific EndoNuclease-1; GULP: phosphotyrosine-binding (PTB) domain-containing enGULfment adaptor Protein 1; LRP1: Low density lipoprotein receptor-Related Protein 1; *nuc-1*: NUClease abnormal; Rac1: RAs-related C3 botulinum toxin substrate 1; *wah-1*: Worm AIF Homolog.

Three apoptotic genes were found to be essential for the developmental PCD to occur, therefore called the "killer genes". Loss-of-function in the *egl-1* (*e*gg-*l*aying abnormal), *ced-3* (*ce*ll *d*eath abnormal) or *ced-4* genes were found to resist the PCD of all 131 cells that normally are removed during wild-type development (Conradt, Horvitz 1998, Ellis, Horvitz 1986). Egl-1 shares similarity to mammalian BH3-only proteins, Bax and Bid. The other two "killer genes", *ced-3* and *ced-4*, also have mammalian counterparts; caspase and Apaf-1, respectively. Loss-of-function mutations in these genes result in similar phenotype as seen in *egl-1* mutant. Another gene involved in the execution phase of apoptosis is *ced-9* that on the contrary to the "killer genes" is anti-apoptotic. Mutations in c*ed-9* gene lead to ectopic cell death and embryonic lethality (Hengartner, Ellis & Horvitz 1992). As a consequence,

overexpression of *ced-9* or its mammalian homolog *bcl-2* protects against cell death (Hengartner, Horvitz 1994). Normally, CED-9 resides in the outer mitochondrial surface and binds CED-4 in an inactive conformation. When destined to apoptosis, EGL-1 is produced that binds to CED-9 thereby releasing CED-4 dimer. CED-4 then forms a tetramer that recruit proCED-3 to an apoptosome. The apoptosome activates CED-3 by proteolysis and subsequently CED-3 continues to execute apoptosis by cleaving important cellular proteins (Lettre, Hengartner 2006) (Fig 1). The engulfment by neighboring cell is mediated by several *ced* –genes as shown in figure 1. These genes are involved i.e. in cytoskeletal rearrangements. Finally the degradation of the engulfed cell involves activation of several nucleases that degrade the DNA from the cell corpse (Fig 1).

Mechanisms of apoptosis are evolutionarily conserved with much more complex variations in higher order animals as compared to *C. elegans*. Nonetheless, apoptosis in *C. elegans* is considered as a simplified model of mammalian apoptosis (Fig. 1).

1.2.2 Caspase family proteins

Apoptotic signaling often leads to activation of cysteine specific proteases called caspases (cysteinyl aspartate-specific proteinases) (Thornberry 1997, Cohen 1997). Caspase activation is an important step in the cell's commitment to apoptosis and can be regarded as a point of no return. Caspases are responsible for most of the visible characteristics of apoptosis, shown by deletion studies, and using inhibitors that block caspase activation and subsequent apoptosis (Earnshaw, Martins & Kaufmann 1999). Caspases are translated as inactive zymogens (pro-caspases) and they reside dormant in the cytosol waiting for a signal of activation. The apoptotic caspases are divided into upstream initiator caspases (caspase-2, -8, -9, -10, and -12) that upon apoptotic stimulus are activated via dimerization. Dimerization is brought about by interaction of apoptotic regulator proteins with the initiator caspase prodomains containing either caspase recruitment domain (CARD) or dead effector domain (DED). This interaction enables clustering of procaspases and subsequent caspase activation cascade (Creagh, Conroy & Martin 2003). Caspase-2 was initially considered as an initiator caspase since it is activated by dimerization, however, recent studies have indicated caspase-2 also in other functions apart from apoptosis, including cell cycle arrest and in tumor suppression (Bouchier-Hayes, Green 2012). Thus caspase-2 has been designated as an "orphan" caspase (Bouchier-Hayes, Green 2012).

Table 1 Caspase family proteins.

Group		Members	Prodomain	Special
Group I	Pro-inflammatory caspases	Caspase-1, -4, -5, -12 (in humans), -13, and -14	Long	Involved in cytokine maturation, inflammatory response
Group II	Apoptotic initiator caspases	Caspase-(2), -8, -9, -10, and -12 (rodents)	Long	Contain DED (8 and 10), or CARD (2, 9 and 12) domain
Group III	Apoptotic executor caspases	Caspase-3, -6, and -7	Short	Contain NED domain

Abbreviations: CARD: Caspase Recruitment Domain; DED: Dead Effector Domain; NED: Non-Enzymatic Domain.

As the name implies, caspases are specific for substrate proteins that contain an aspartate (Asp/D) residue and use a conserved cysteine residue in their active site for catalyzing the peptide bond cleavage (Pop, Salvesen 2009). Caspases posses fairly conserved substrate pockets or amino acid recognition sites with slight variations (Chowdhury, Tharakan & Bhat 2008). Caspase substrates usually contain a tetrapeptide that usually ends with the critical aspartate residue; however, variations in the tetrapeptide sequence are numerous for different caspases (Pop, Salvesen 2009). The means by which caspases exert the destruction occurs by either inactivation of the target protein, or by activation via cleavage of a regulatory domain (Hengartner 2000).

Once initiator caspases are activated they cleave downstream effector caspases or "executors" (caspase-3, -6, and -7) in a cascade –like manner (Slee et al. 1999). These executors eventually perform the destructive work by cleaving the key enzymes and structural proteins resulting in the formation of apoptotic bodies. It is estimated that caspases have in the order of several hundred substrates, especially, cytoskeletal and nuclear proteins, as well as important signaling proteins (Chowdhury, Tharakan & Bhat 2008). Important caspase substrates include the nuclease responsible for the nucleosomal laddering, used to detect apoptosis (Wyllie 1980). Nowadays known as CAD, caspase-activated DNase, is translated with an inhibitory subunit (ICAD) that is released by activated caspase-3 (See fig 3). As a result, active CAD cuts DNA to fragments of approximately 180 base pair (Liu et al. 1997, Enari et al. 1998). Other downstream effects of caspase activation include cleavage of nuclear lamins, resulting in nuclear shrinking and budding (Rao, Perez & White 1996), cleavage of cytoskeletal proteins, such as spectrin, gelsolin and PARP (Wang et al. 1998, Kothakota et al. 1997, Lazebnik et al. 1994) and constitutive activation of p21 activated kinase 2 (PAK2) by cleavage of the negative regulatory subunit (Rudel, Bokoch 1997).

Non-cell death related functions

In addition to apoptosis, some caspases have roles beyond cell death. These include both proteolytic and non-proteolytic processes, involving their catalytic- and prodomains, respectively. A subset of caspases is involved in inflammation, specifically, in the maturation of lymphocytes. The first caspase to be described was interleukin-1 (IL-1) beta converting enzyme (ICE; caspase-1) that, as the name implies, converts the precursor of the pro-inflammatory cytokine IL-1 beta to its mature form (Thornberry et al. 1992). Together with caspase-1, caspase-4 and -5 belong to the group I pro-inflammatory caspases involved in cytokine maturation. Moreover, certain bacteria have been shown to use caspase-1 to kill their host cells, and this caspase-1 –dependent cell death has been termed *pyroptosis* (Labbe, Saleh 2008). Other non-cell death related functions of caspases include regulation of cell survival, proliferation, and differentiation (Lamkanfi et al. 2007).

In addition, it has been proposed that only caspase-14 might function as a true non-apoptotic caspase (Pop, Salvesen 2009). Caspase-14 is strictly expressed in suprabasal layers of epidermis and involved in keratinocyte differentiation and cornification, and is essential in protection against UVB photodamage (Denecker et al. 2007).

The activation of caspase-2 occurs via a signaling platform consisting of PIDD (p53-induced protein with a death domain) and RAIDD (Receptor interacting protein (RIP)-associated ICH1/CAD-3 homologous protein with a death domain), the PIDDosome (Tinel, Tschopp 2004). Importantly, PIDD has been implicated in NFκB (Nuclear factor κB) activation in a signaling complex consisting of RIPK1 (Receptor interacting protein kinase 1) and NEMO (NFκB essential modulator)/IKKγ (Inhibitor of κB (IκB) kinase γ) (Janssens et al. 2005). Activation of PIDD occurs in response to DNA damage and which signaling module is assembled is determined dose-dependently and thus by the extent of damage. PIDD can be considered therefore as a molecular switch between cell death (caspase-2 activation) and survival (NFkB activation) (Bouchier-Hayes, Green 2012).

Table 2 Phenotypic effects of caspase knock-out in mice (Earnshaw, Martins & Kaufmann 1999, Degterev, Boyce & Yuan 2003).

Group	Member	Developmental effect in knock-out mouse	Other defects in knock-out mice or other remarks
Group I Pro-inflammatory caspases	Caspase-1	Normal	Defective production of IL 1 α, 1β, 18 and IFNγ. Resistant to septic shock
	Caspase-4, -5	-	-
	Caspase-11	Normal	Defective production of IL 1 α, 1β, 18 and IFNγ. Defective lymphocyte apoptosis during sepsis and ischemic brain injury
Group II Apoptotic initiator caspases	Caspase-2	Normal	Reduced numbers of facial motor neurons at birth. Resistance to granzyme B + perforin induced B-lymphocyte cell death. Defective in developmental and doxorubin-induced apoptosis of oocytes. Accelerated aging (Zhang et al. 2007)
	Caspase-8	Embryonic lethal	Impaired development of cardiac muscles, abdominal hemorrhage. Defects in death—receptor activated apoptosis
	Caspase-9	Embryonic/ neonatal lethal	Abnormal brain development, neuronal hyperplasia
	Caspase-10	-	Deletion in humans cause systemic juvenile idiopathic arthritis (Tadaki et al. 2011)
	Caspase-12	Normal	Resistance to ER stress-induced cell death and Aβ toxicity. Resistance to sepsis (Saleh et al. 2006)
Group III Apoptotic executor caspases	Caspase-3	Embryonic lethal	Defective brain development, neuronal hyperplasia. Defect in DNA fragmentation during apoptosis
	Caspase-6		-
	Caspase-7	Normal	Appear normal, substituted by other caspases.

Abbreviations: Aβ: Amyloid beta; IFNγ: Interferon gamma; IL: Interleukin.

Lessons from caspase knock-out mice – relevance for brain development
While deletion of *ced-3* gene in *C.elegans* resulted in almost total inability to developmental apoptosis, deficiency in mammalian caspases exert often tissue-specific defects in apoptosis due to wider selection of caspases. For instance, caspase-8 deficiency in mouse is embryonic lethal, impairs cardiac muscle development and blocks signaling through the death receptors TNFR1, Cd95/Fas/Apo1 and Dr3 (Varfolomeev et al. 1998). Knockout of either caspase-1 or -11 in mice produce viable animals that show resistance to ischaemic brain injury and lipopolysaccharide (LPS)-induced endotoxic shock (Li et al. 1995, Kang et al. 2000, Schielke et al. 1998). Caspase-3 and caspase-9 deletions have been shown to affect neuronal development, inducing neuronal hyperplasia and perinatal lethality (Kuida et al. 1996, Hakem et al. 1998).

Caspase-3
Caspase-3 is a major executor caspase that acts downstream of caspase cascade and has important substrates, such as, ICAD, PARP, sterol regulatory element-binding proteins (SREBPs), lamins, β-catenin and other caspases (Chowdhury, Tharakan & Bhat 2008). The activation of caspase-3 occurs by two sequential cleavages of the full initial 32kDa proform to yield a heterodimer of 20kDa and 12kDa subunits (Nicholson et al. 1995). Procaspase-3 can be cleaved, for instance, by caspase-8 that is activated by APO-1 (Fas/CD95)-receptor pathway involving the DISC signaling complex formation (classically termed the extrinsic pathway) (Kischkel et al. 1995). In addition, apoptotic signaling from mitochondria, formation of the apoptosome and subsequent activation of caspase-9 results in procaspase-3 cleavage (classically termed the intrinsic pathway) (Li et al. 1997).

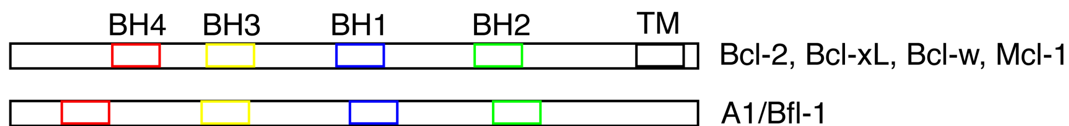
Caspase-12
The murine caspase-12 is an ER resident caspase with most homology (48%) to human caspase-4. Human caspase-12 is a pseudogene with the exception of some populations of African heritage (Saleh et al. 2004, Kachapati et al. 2006). The human functional variant of caspase-12 has been linked to pro-inflammatory caspases and increased risk of sepsis (Saleh et al. 2004). For comparison, caspase-12 defective mice show resistance to septic shock (Saleh et al. 2006). The murine caspase-12, has been shown to promote apoptosis in ER stressed cells and caspase-12 deficient mice are resistant to ER stress-induced apoptosis (Nakagawa et al. 2000). In addition, caspase-12 was required for Aβ -mediated cell death in mouse cortical neurons but is not involved in other apoptotic pathways (Nakagawa et al. 2000). Previously, caspase-12 activation has been closely linked to cell death involving ER stress, such as, transient ischaemic injury and oxygen/glucose deprivation (Osada et al. 2010, Badiola et al. 2011).

The ER-mediated cell death pathway can be activated by means of accumulation of misfolded or unfolded proteins or by perturbation of calcium homeostasis (Kaufman 1999, Ferri, Kroemer 2001). Caspase-12 locates to the cytoplasmic side of the ER where stress – induced translocation of cytosolic caspase-7 results in proximity-induced caspase-12 cleavage and activation (Nakagawa et al. 2000, Rao et al. 2001). Caspase-12 subsequently activates procaspase-9 that in turn activates downstream effector caspases (Rao et al. 2002a).

1.2.3 The Bcl-2 family proteins

As the caspase family proteins can be viewed as a group of true cell demolitioners, the Bcl-2 (B-cell lymphoma 2) family proteins are a group of cell death regulators, including both anti- and pro-apoptotic members. This family consists of approximately 20 members, divided into three interacting groups according to which Bcl-2 Homology (BH) domains they exhibit (Fig 2).

Anti-apoptotic



Pro-apoptotic

BH3-only

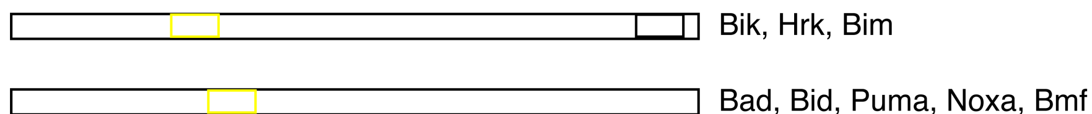


Figure 2 The Bcl-2 family proteins. The three subfamilies of Bcl-2 family proteins, domains present in each subfamily and their members. Abbreviations: A1/Bfl-1: Bcl-2-related protein A1; Bad: Bcl-2 Antagonist of cell Death; Bak: Bcl-2 antagonistic killer; Bax: Bcl-2-Associated X protein; Bcl-2: B cell lymphoma-2; Bcl-X_L: Bcl-extra long; Bid: BH3 interacting domain death agonist; Bik: Bcl-2 interacting killer; Bim: Bcl-2 interacting mediator of cell death; Bmf: Bcl-2 modifying factor; Bok: Bcl-2 related ovarian killer; Hrk: hara-kiri; Mcl-1: myeloid cell leukemia 1; Noxa: named for "injury"; Puma: p53 promoter upregulated modulator of apoptosis; TM: transmembrane domain.

Anti-apoptotic Bcl-2 family proteins

The founding member, proto-oncogene Bcl-2, as well as Bcl-xL, Bcl-w/Bcl2L2, A1/Bfl-1, and Mcl-1 (Fig 2) promote cell survival by preventing mitochondrial permeabilization and cytochrome c release (Fig 3). These anti-apoptotic members of Bcl-2 family contain all of the four BH domains 1-4 with conserved homology among all members. Bcl-2 is an integral membrane protein residing at the mitochondria, the ER or the nucleus (Hockenbery et al. 1990, Zhu et al. 1996). Expression of Bcl-2 can block apoptotic features such as membrane blebbing and nuclear condensation. Furthermore, mice lacking the *bcl-2* gene show excessive cell death and Bcl-2 is required for kidney, lymphocyte and melanocyte stem cell survival (Veis et al. 1993, Ranger, Malynn & Korsmeyer 2001) (Table 3). In addition, deletion of Bcl-X_L in mice results in massive cell death of developing neurons (Motoyama et al. 1995) (See table 3).

Pro-apoptotic Bcl-2 family proteins

The pro-apoptotic Bcl-2 proteins are divided to the multidomain (BH 1-3) pro-apoptotic or effector proteins, e.g. Bak and Bax, and to BH3-only proteins, e.g. Bim, Bid, Bad, and Puma (Fig 2). Many pro-apoptotic members are cytosolic or in contact with the cytoskeleton in healthy cells while death signals induce their conformational changes and targeting to membranes, especially to the mitochondrial outer membrane (Korsmeyer et al. 2000) (Fig 3).

The multidomain pro-apoptotic Bak and Bax are crucial for the mitochondrial outer membrane permeabilization (MOMP) (Wei et al. 2001) (Fig 3). Bak and Bax are normally guarded by interaction with Bcl-2, ensuring neutralization and inhibition of Bak/Bax oligomerization (Youle, Strasser 2008). Apoptotic signals remove this interaction and allow pore formation through Bax/Bak oligomerization to release the mitochondrial cell death machinery. Bax and Bak are related to each other having overlapping functions. Knockout mice of either gene produce viable animals, although Bax deficient mice are sterile (Lindsten et al. 2000, Knudson et al. 1995) (Table 3). However, combined deletion of Bax and Bak, $bax^{-/-}/bak^{-/-}$, was shown to be mostly lethal and causing wide developmental disturbances underlining their importance in cell death during development (Lindsten et al. 2000) (Table 3).

BH3-only proteins

The BH3-only proteins function mostly as the sensors of apoptotic signals and pass it on to other Bcl-2 members, for instance, by antagonizing their anti-apoptotic relatives (See fig 3). The BH3-only protein subfamily consists of over 12 members found from mice and humans, and given their highly specialized tasks, this subfamily provides a higher level of fine-tuning for apoptotic pathways.

For instance, the BH3-only protein, Bid, can be cleaved by caspase-8 in response to APO-1 (Fas/CD95)-receptor pathway resulting in formation of truncated Bid (tBid) that translocates to mitochondria (Li et al. 1998). At mitochondria, tBid inserts to the outer membrane where it induces Bak and/or Bax oligomerization and pore formation (Wei et al. 2001, Wei et al. 2000) resulting in MOMP (See fig 3).

Table 3 Major phenotypes in certain Bcl-2 family knock-out mice (Ranger, Malynn & Korsmeyer 2001).

Bcl-2 subfamily	**Member**	**Developmental effect in KO mouse**	**Other defects**
Anti-apoptotic	Bcl-2	Death within a few months of birth	Renal failure, apoptosis in thymus, spleen, melanocytes, postnatal neuronal death
	Bcl-X_L	Embryonic lethal (E13)	Extensive neuronal death, apoptosis of hematopoietic cells of liver
	Mcl-1	Embryonic lethal (E3,5-4)	
	A1	Viable	Accelerated neutrophil apoptosis
	Bcl-w	Viable	Male infertility
Pro-apoptotic	Bax	Viable	Modest lymphoid and neuronal hyperplasia, male infertility, increased oocyte lifespan
	Bak	Viable	Fertile
	Bax/Bak	90% perinatal lethal	Persistence of interdigital webs, accumulatin of CNS neurons, splenomegaly, resistance to various cell death stimuli
BH3-only	Bid	Viable	Resistance to anti-Fas-induced hepatocellular apoptosis
	Bim	Partially embryonic lethal before E9,5	Perturbation of thymic development, lymphadeno-pathy, splenomegaly. Premature death due to vasculitis, cardiac infarction.

Abbreviations: A1: Bcl-2-related protein A1; Bak: Bcl-2 antagonistic killer; Bax: Bcl-2-Associated X protein; Bcl-2: B cell lymphoma-2; Bcl-X_L: Bcl-extra long; Bid: BH3 interacting domain death agonist; Bim: Bcl-2 interacting mediator of cell death; CNS: central nervous system; KO: knock-out; Mcl-1: myeloid cell leukemia 1.

1.2.4 Inhibitor of apoptosis –proteins

The inhibitor of apoptosis (IAP) -proteins are a family of anti-apoptotic proteins. The first IAP genes were found from baculovirus where they are believed to prevent apoptotic response in the host insect cells (Harvey et al. 1997). The IAP family comprises of eight members: Neuronal apoptosis inhibitory protein (NAIP/BIRC1), cellular IAP1/human IAP2 (c-IAP1/HIAP1/MIHB/BIRC2), cellular IAP2/human IAP1 (c-IAP2/HIAP1/MIHC/API2/BIRC3), X-linked inhibitor of apoptosis (XIAP/MIHA/hILP/BIRC4/ILP-1), Survivin (TIAP/BIRC5), BIR-containing ubiquitin conjugating enzyme (BRUCE/Apollon/BIRC6), Livin (KIAP/ML-IAP/BIRC7) and testis-specific IAP (Ts-IAP/hILP2/BIRC8/ILP-2).

All IAPs are structurally characterized by one or more ~70-residue Baculovirus IAP Repeat (BIR) domains that are zinc-containing globular structures with a conserved cysteine and histidine core sequence (Miller 1999). The BIR domains are essential for the anti-apoptotic functions of IAPs since they enable association with certain caspases and can inhibit their actions (Ni, Li & Zou 2005) (Fig 3). As it turns out, all IAPs have been shown to inhibit the caspase cascade, however, physical interactions and direct caspase inhibition have been only shown for some (Salvesen, Duckett 2002). Taken their anti-apoptotic functions, it is not surprising that IAPs have been found upregulated in certain cancers and are studied as targets for cancer therapy (Hunter, LaCasse & Korneluk 2007). Furthermore, IAPs are a good example of how the ubiquitin-proteasomal system is linked to apoptotic signaling. Five of the IAPs contain a carboxy-terminal RING (Really Interesting New Gene) domain that targets both the IAPs themselves (autoregulation) as well as pro-apoptotic proteins, such as caspase-3 and -7, for proteasomal degradation, thus enhancing their anti-apoptotic properties (Ni, Li & Zou 2005).

The RING domain is characteristic for ubiquitin E3 ligases, a family of proteins that add ubiquitin to target proteins in a series of enzymatic reactions, collectively termed the ‘ubiquitination’ (Ciechanover 1998). Other enzymes involved in ubiquitination include E1 ubiquitin activating enzyme and E2 ubiquitin conjugating enzyme. The addition of ubiquitin protein to target protein at a lysine recidue signals most often for degradation of the target protein in the proteasome but can also be a sign for endocytosis, activation or other signaling mechanism (Ciechanover 1998). The ability to form isopeptide bonds between other ubiquitin polypeptides makes ubiquitin a versatile signal molecule. Seven lysine residues in the ubiquitin polypeptide enable multible different types of linkages. Of these a polyubiquitin chain formed via Lys48 has been shown to be the signal for proteasomal degradation. Others, like Lys63 linked polyubiquitin chains are involved in regulatory functions and Lys11 linked polyubiquitin chains have been shown to control cell division (Strieter, Korasick 2012).

XIAP is ubiquitously expressed and might be a “housekeeping” IAP functioning in prevention of apoptosis induced by various extracellular stimuli such as UV light, chemotoxic drugs, and activation of the tumor necrosis factor (TNF) and Fas receptors (Duckett et al. 1996, Duckett

et al. 1998, Hunter, LaCasse & Korneluk 2007). XIAP mediates its protective effect downstream of mitochondrial cytochrome c release in contrast to Bcl-x_L that protects mitochondrial integrity (Duckett et al. 1998). XIAP contains three BIR domains, the third of which (BIR3) inhibits caspase-9 activity by binding monomeric forms of mature caspase-9 (Shiozaki et al. 2003). On the contrary, the linker region between BIR1 and BIR2 of XIAP inhibits cleaved caspase-3 and -7 (Riedl et al. 2001, Scott et al. 2005, Chai et al. 2001). Smac/DIABLO binds the same region as caspase-9 and can antagonize the inhibitory effect of XIAP on caspase-9 (Srinivasula et al. 2001). Ablation of XIAP in mice is viable and perhaps partially compensated by c-IAP-1 and c-IAP-2 (Harlin et al. 2001).

Expression of XIAP has been detected during rat brain development and in adult rat brain (Korhonen, Belluardo & Lindholm 2001). Caspase-3 activation by kainic acid results in XIAP degradation in the rat hippocampus. In addition, it has been shown that caspase-3 induces cleavage of XIAP BIR3 motif that releases caspase-9 inhibition and allows additional caspase activation (Zou et al. 2003). XIAP has been indicated protective for neuronal cells in several ways. Overexpression of XIAP reduced caspase-12 cleavage and calpain activation in a mouse model of ALS, carrying human mutant superoxide dismutase 1 (SOD1) (Wootz et al. 2006). Furthermore, overexpression of XIAP induces brain neurotrophic factor (BDNF) (Kairisalo et al. 2009) and mitochondrial antioxidant SOD2 (Kairisalo et al. 2007) via nuclear factor-κB (NFκB) dependent mechanisms. Interestingly, resveratrol, an antioxidant polyphenol found in grapes and red wine was shown to increase XIAP levels and mitochondrial antioxidants in rat PC6.3-cells (Kairisalo et al. 2011) and provides an intriguing therapeutic agent in multiple disorders.

c-IAP-1 and c-IAP-2, the two closest IAP family proteins to XIAP, have also been shown to bind to caspases, however, their capacity to inhibit caspases is poor as compared to XIAP (Eckelman, Salvesen 2006). Despite their poor caspase inhibition ability, c-IAPs may still contribute to apoptosis pathways via their ubiquitin ligase activity. Indeed, it was shown that c-IAP-1 and c-IAP-2 bind and ubiquitinate Smac/DIABLO (Hu, Yang 2003). In addition, c-IAP-1 ubiquitinates TRAF2 in response to TNF receptor II activation (Li, Yang & Ashwell 2002, Samuel et al. 2006). The rat inhibitor of apoptosis protein-2 (RIAP-2), homologue of human c-IAP-1, is expressed mainly by neurons in the adult rat brain and KA was shown to regulate RIAP-2 expression in vulnerable areas of the hippocampus (Belluardo et al. 2002). KA induced similar expression patterns for XIAP and RIAP-2 with initial increase in the levels in certain areas, including the CA3 area of the hippocampus, followed by a decrease in longer time-points (Korhonen, Belluardo & Lindholm 2001, Belluardo et al. 2002).

The first mammalian IAP to be found was NAIP (Roy et al. 1995). Deletion of NAIP in patients with severe form of the neurodegenerative disease spinal muscular atrophy (SMA) was initially thought to be the gene responsible for the disease, however, later identified as a modifier of the severity of the disease (Roy et al. 1995, Scharf et al. 1998). NAIP has been shown to inhibit neuronal cell death *in vitro* and *in vivo* (Simons et al. 1999, Xu et al. 1997b). In addition, NAIP expression was shown to be elevated in response to ischaemia in surviving neurons (Xu et al. 1997a) and might have broad neuroprotective effects in disorders involving neuronal damage. The protective effect of NAIP has been linked to interaction with

hippocalcin, a neuronally expressed calcium-binding protein that increased the neuroprotective effect of NAIP BIR-domains against calcium-induced motor neuron death (Mercer et al. 2000). The fact that XIAP is considered the only true caspase inhibitor and many of the other IAPs likely use their ubiquitin ligase activity to inhibit apoptosis has raised the question how NAIP functions since it does not contain the RING domain. Recently, it was shown that NAIP binds and inhibits pro-caspase-9 via its BIR3 domain and has been evolved resistant to inhibition by Smac-type proteins (Davoodi et al. 2010).

The giant 530kDa IAP, Bruce, contains a ubiquitin conjugating domain (UBC), characteristic for E2 ubiquitin conjugating enzymes, in its C-terminus, in addition to the BIR domain (Hauser et al. 1998). Apollon, the human homolog of Bruce, was first identified in human brain cancer cells, gliomas, showing resistance to chemotherapy (Chen et al. 1999). Interestingly, Bruce deletion is embryonic lethal in mouse and it was identified as indispensable for Smac ubiquitination and degradation (Hao et al. 2004). KA treatment in the rat decreased Bruce levels in the hippocampus in coordination with caspase-3 activation and cell death that was further increased by an antisense oligonucleotide for Bruce (Sokka et al. 2005). Taken together, Bruce, as well as c-IAP-1/RIAP-2 and XIAP are downregulated in cells undergoing KA-induced apoptosis underlining their importance in cellular survival.

In addition to Smac and Omi, another IAP inhibitor, XAF1 (XIAP-Associated Factor 1) has been characterized that inhibits most IAPs, including XIAP, c-IAP-1, c-IAP-2, Livin, TsIAP, and NAIP. XAF1 was also required for XIAP-mediated downregulation of Survivin via the ubiquitin-proteasome system (Arora et al. 2007). Downregulation of XAF1 has been linked to cancer with a coinciding IAP upregulation. Overexpression of XAF1 can sensitize cells for Apo2L/TRAIL-induced apoptosis and result in cancer cell death showing promise as a therapeutic target (Hunter, LaCasse & Korneluk 2007).

1.2.5 Other cell death mediators

Calpain

Among other cell death mediators is the calcium-dependent neutral proteinase calpain that was identified in 1968 (Huston, Krebs 1968). The human genome encodes at least 13 homologs of the large subunit of calpain superfamily (Dear, Boehm 2001). Only the major isoforms, m-calpain and μ-calpain have been found to be expressed in neuronal tissues (Wu, Lynch 2006). Calpains participate in numerous signaling pathways regulated by calcium. Calpain activation has been closely linked to neurotoxic insults often involving rises in intracellular calcium, occurring, for instance, during ischaemia, AD, PD, and HD (Bevers, Neumar 2008, Frederick et al. 2008, Yamashima 2000, Saito et al. 1993a, Bizat et al. 2003, Crocker et al. 2003).

Calpains are activated in response to cytosolic calcium elevation. Threshold for half-maximal activation of m- and μ-calpains are 400-800μM and 3-50μM, respectively (Goll et al. 2003). Generally, calpains are composed of a distinct 80kDa catalytic subunit and a common 30kDa regulatory subunit, both of which can bind calcium. A natural inhibitor of calpain, calpastatin, binds the substrate binding site in calcium-dependent manner (Goll et al. 2003).

Neuronal calpain substrates include synaptic and post-synaptic density proteins, cytoskeletal proteins, and signaling proteins. In addition, calpain substrates important for cell death include AIF (Polster et al. 2005), Cdk5 cofactors, p35 and p39 (Kusakawa et al. 2000, Patzke, Tsai 2002), and spectrin (Harris, Morrow 1988, Czogalla, Sikorski 2005). Importantly, calpain can be activated by glutamate receptor stimulation and process also numerous glutamate receptors, including NMDA- and AMPA-receptor subunits, PSD95 and MAP2 (microtubule-associated protein-2) that affect the levels, localization and stability of the proteins and synapses (Wu, Lynch 2006). Spectrin is an important substrate of calpain cleavage (Harris, Morrow 1988) and often used as a marker of calpain activation. Together with caspase-3, calpain cleaves at specific sites in spectrin polypeptide, and the breakdown product profiles can be used to distinguish the activation of these proteases.

1.3 Organelle-specific initiation of cell death

The initial discovery of apoptosis described it morphologically as controlled cell shrinking with sustained organelle and membrane integrity (Kerr, Wyllie & Currie 1972). Later on it was, however, recognized that intracellular organelles participate in cell death and are capable of sensing stressful conditions. Classic apoptotic signaling was divided to extrinsic or death receptor pathway and intrinsic or mitochondrial pathway characterized by MOMP and release of mitochondrial cell death mediators (See fig 3).

The evolution of eukaryotes includes the formation of compartmentalized, highly specialized membrane-bound structures, organelles. This compartmentalization has provided evolvability through microenvironments that are maintained by controlled ion and small molecule flux across their membranes. Importantly, coupled to this is the vesicle transfer between organelles, which allows distribution of molecules to secretion or sites where they are needed in the cell. Organelle-specific responses that aim to adaptation and restoration of homeostasis or, after surpassing a threshold, lead to activation of cell death pathways are now acknowledged and under intense investigations. At the level of the whole cell, it should be noted that perturbations in any organelle could ultimately lead to activation of the common apoptotic pathway (Ferri, Kroemer 2001).

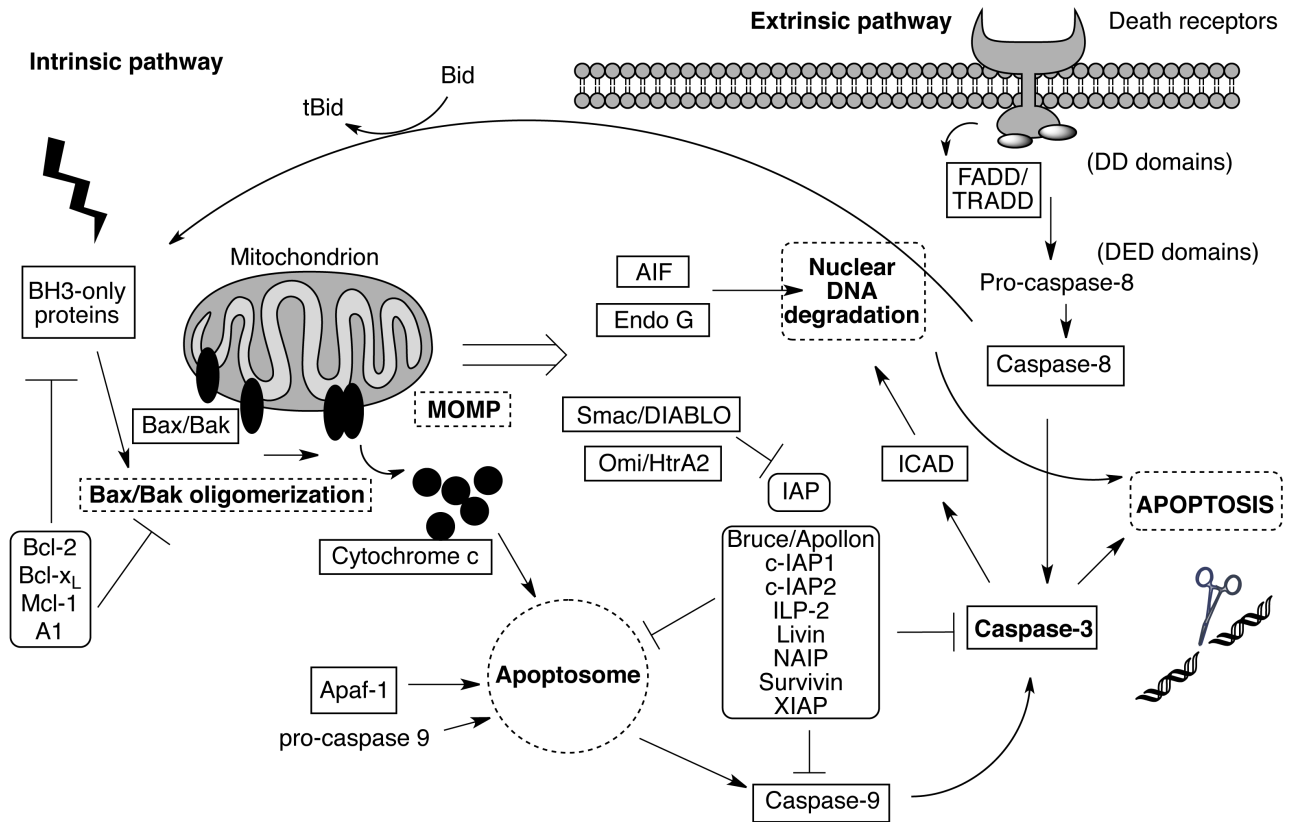


Figure 3 Intrinsic and extrinsic cell death pathways. The intrinsic or mitochondrial apoptotic pathway can be induced by DNA damage, free radicals or other stressors leading to activation of BH3-only proteins, release of anti-apoptotic Bcl2 members from pro-apoptotic Bcl-2 members and Bax/Bak oligomerization. Bax/Bak oligomerization results in mitochondrial outer membrane permeabilization (MOMP) and release of mitochondrial cell death mediators. Cytochrome c forms part of the apoptosome with Apaf-1 and pro-caspase-9 that is a platform for activation of caspase-9. Active caspase-9 can further activate the executioner caspase-3 that further cleaves major cellular substrates and executes apoptosis. AIF and Endo G, release from mitochondria results in their translocation to the nucleus where they take part in nuclear DNA degradation. Smac/DIABLO and Omi/HtrA are both inhibitors of IAP proteins and their release enables release of cell death molecules that are normally kept in guard by IAPs. Extrinsic or death receptor pathway can be activated by extracellular stimuli by binding of ligands to their cognate death receptor. Ligand binding induces receptor interaction with adapter proteins FADD or TRADD and formation of a caspase-activating platform for initiator caspases, such as caspase-8. Active caspase-8 can directly further activate the executioner caspases, such as caspase-3 but also activate Bid that in turn enhances the mitochondrial cell death pathway. Caspase-3 cleaves ICAD releasing CAD from the inhibitory subunit that allows its translocation to the nucleus for DNA degradation. Abbreviations: A1: Bcl-2-related protein A1; AIF: apoptosis inducing factor; Apaf-1: apoptotic protease-activating factor-1; Bak: Bcl-2 antagonistic killer; Bax: Bcl-2-Associated X protein; Bcl-2: B cell lymphoma-2; Bcl-X_L: Bcl-extra long; Bid: BH3 interacting domain death agonist; Bruce/Apollon: BIR-containing ubiquitin-conjugating enzyme; c-IAP1/2: cellular IAP1/2; IAP: inhibitor of apoptosis –protein; DD: death domain; DED: death effector domain; Endo G: endonuclease G; FADD: Fas-associated death domain; ICAD: caspase-activated DNAse with inhibitory subunit; ILP-2: testis-specific IAP; Mcl-1: myeloid cell leukemia-1; NAIP: neuronal apoptosis inhibitory protein; Omi/HtrA2: high temperature requirement A2; Smac/DIABLO: second mitochondria-derived activator of caspases; TRADD: TNFR-1-associated death domain; XIAP: X-linked IAP.

1.3.1 Extrinsic / death receptor pathway

Some cells express cell surface receptors that upon binding their cognate ligands can form a platform for caspase activation. These, so called ‘death receptors’ belong to tumor necrosis factor receptor (TNFR) family and include CD95/Fas/Apo-1 (Cluster of differentiation 95/Fas/apoptosis antigen 1) receptor that binds FasL, type 1 tumor necrosis factor α (TNFα) receptor that binds TNFα, death receptor 3 (DR3) that binds Apo3 ligand, and DR4 and DR5 that bind Apo2/TRAIL (Degli-Esposti 1999, Pan et al. 1997, Friesen et al. 1996). Receptor activation follows interaction with specific adapter proteins, containing death domains (DD),

such as, Fas-associated death domain (FADD) and TNFR-1-associated death domain (TRADD) that initiate apoptosis by activating caspase-8 or caspase-10 via their death effector domain (DED) (Chinnaiyan et al. 1995, Boldin et al. 1995, Hsu, Xiong & Goeddel 1995) (See fig 3).

For instance, binding of FasL to CD95/Fas/Apo-1 induces association with the adapter molecule FADD that has the ability to form oligomers when bound to receptor (Chinnaiyan et al. 1995, Boldin et al. 1995, Tourneur, Chiocchia 2010). Death effector domain (DED) motifs located in the N-terminal part of FADD activate pro-caspase-8 through homotypic interaction and this allows efficient activation of caspase-8 (Tourneur, Chiocchia 2010). Active caspase-8 then directly cleaves caspase-3 and Bid, a pro-apoptotic protein that can translocate to mitochondria to release cytochrome *c*, leading to further enhancement of caspase activation (Luo et al. 1998, Budihardjo et al. 1999) (Fig 3).

1.3.2 Mitochondrial cell death pathway

Mitochondrial or intrinsic pathway can be triggered by various stressors, including DNA damage, free radicals or damage by toxins, leading to apoptotic signaling with central role played by mitochondria. Mitochondrial outer membrane permeabilization (MOMP) occurring after apoptotic stimulus promotes the release of several pro-apoptotic proteins to the cytosol. These include cytochrome c, Smac/DIABLO, AIF, EndoG and Omi/HtrA2. MOMP is thus the major driving force in cell death pathways initiated by mitochondria. In addition to Bak and Bax, mediating MOMP, it was previously shown that upon genotoxic DNA damage, release of histones promotes mitochondrial damage that occurs via a strong interaction between histones and mitochondria, and that this results in the permeabilization of the mitochondrial membranes (Cascone et al. 2012) (Fig 3).

Cytochrome c

Cytochrome c is an essential protein for cell life and death. It resides in mitochondria and involves in cellular respiration as part of the electron transport chain as well as scavenges reactive oxygen species (ROS) (Landes, Martinou 2011). In addition to supporting cell's life and wellbeing it was recognized to take crucially part in apoptosis (Liu et al. 1996). Induction of MOMP allows cytochrome c to flow to the cytosol where it activates an adaptor protein, Apaf-1 (Fig 3).

Apaf-1

Apaf-1 (Apoptotic Protease-Activating Factor-1) is a multidomain protein containing caspase-recruitment domain (CARD), a nucleotide-binding and oligomerization domain (NOD) and a regulatory Y-domain composed of WD40 (named after their terminal tryptophan (W) and aspartic acid (D) dipeptide) repeats (WDRs) (Zou et al. 1997). In healthy cells, Apaf-1 exists in an inactive form as monomer while binding of cytochrome c to the WDRs after MOMP induces conformational changes in Apaf-1, including ATP hydrolysis of the NOD domain and subsequent nucleotide exchange (Kim et al. 2005). Through several steps of activation, Apaf-1 molecules are able to oligomerize into a heptameric apoptosome complex (Riedl, Salvesen 2007) (Fig 3). Oligomerization is coupled to procaspase-9 recruitment via the CARD domains in both procaspase-9 and Apaf1 (Qin et al. 1999). Thus,

the apoptosome serves as a platform and greatly raises the local concentration of procaspase-9 further allowing subsequent caspase cascade and caspase-3 activation (Bratton, Salvesen 2010) (Fig 3).

Omi/HtrA2

HtrA2 (High temperature requirement A2), also known as Omi, is a serine protease located normally in the mitochondria (Suzuki et al. 2001). After translation, HtrA2 is delivered to mitochondria by mitochondrial targeting sequence (MTS) and inserted to the inner mitochondrial membrane. Maturation requires proteolysis and release of HtrA2 to the inner mitochondrial space (Vande Walle, Lamkanfi & Vandenabeele 2008). Apoptotic stimulus allows HtrA2 to relocate to the cytosol where it can bind and neutralize inhibitor of apoptosis (IAP)-proteins (Fig 3), such as the X-linked inhibitor of apoptosis protein (XIAP), and directly promote apoptosis with its proteolytic activity (van Loo et al. 2002). Deletion of HtrA2 gene in mice has been linked to parkinsonian, neurodegenerative phenotype and premature death (Jones et al. 2003). Consequently, mutations in the HtrA2 gene were associated to Parkinson's disease (PD), altered mitochondrial morphology and susceptibility to stress-induced cell death (Strauss et al. 2005). In addition, Cdk5 was recently found to phosphorylate HtrA2 at S400 during p38 activation, increasing its protease activity and possibly neuroprotection by maintaining mitochondrial membrane potential (Fitzgerald et al. 2011).

AIF

AIF (Apoptosis-inducing factor) was the first mitochondrial protein linked to cell death independent of caspases (Susin et al. 1996, Susin et al. 1999). Normally AIF resides in the mitochondrial intermembrane space (IMS) but can be proteolysed and released in response to MOMP. During cell death, AIF traverses from the mitochondria to the nucleus to condense and fragment DNA (Fig 3). Furthermore, AIF has functions as part of the respiratory chain and metabolic redox reactions (Joza et al. 2009). AIF is released after most proapoptotic signals inducing MOMP; however, certain signals can promote AIF release specifically, such as glutamate excitotoxicity and ischemic insults (Zhu et al. 2003, Wang et al. 2004). In addition, excessive DNA damage can activate the PARP-1 –dependent cell death pathway independent of caspases but dependent on AIF (Yu et al. 2002). The release of AIF without MOMP was proposed to involve calpain-mediated proteolysis (Polster et al. 2005). Interestingly, PARP-1-mediated cell death can be induced by excitotoxins, including NMDA and KA that are also linked to calcium-mediated calpain activation (Wang et al. 2004, Polster et al. 2005, Cheung et al. 2005). A mouse model with reduced AIF expression, the "Harlequin" (*Hq*), showed less neuronal damage in the hippocampus after KA treatment suggesting that AIF is an important mediator of excitotoxic cell death (Cheung et al. 2005).

Smac/DIABLO

Smac (Second Mitochondria-derived Activator of Caspases) is a natural inhibitor of IAP-family proteins (Du et al. 2000). Mitochondrially located in healthy cells, Smac is cleaved and released into the cytosol in response to apoptotic stimulus (Shiozaki, Shi 2004) (Fig 3).

EndoG

Endonuclease G released from mitochondria upon apoptosis is translocated to the nucleus where it cleaves chromatin DNA into nucleosomal fragments (Li, Luo & Wang 2001) (Fig 3). EndoG functions independently of caspases.

1.3.3 Lysosomal initiation of cell death

The lysosomes are involved in hydrolysis of intracellular macromolecules and filled with different types of hydrolytic enzymes such as proteases, lipases, nucleases, glycosidases, and phosphatases, among others, that are enzymatically active at low pH. Lysosomal membrane permebilization (LMP) might occur when uncharged lysosomotropic amines are taken by the lysosomes and accumulate in excess due to the their ability to become protonated in the acidic environment. These protonated forms then act as detergents to destabilize the lysosomal membranes (Boya, Kroemer 2008). In addition, ROS, Bax, as well as various other agents have been indicated in LMP (Terman et al. 2006). LMP usually results in cell death as it involves the release of proteases, cathepsins B, D and L that are active also in neutral pH and able to activate caspases and other apoptotic effectors (Boya, Kroemer 2008). Balance between complete and partial lysosomal breakdown determines the cell death pathway. Complete disruption usually induces necrosis while partial breakdown of a subset of lysosomes may cause apoptotic cell death. For instance, in mouse hepatocytes it was shown that TNF-α induces Bid-mediated LMP and subsequent activation of caspase-2 that further facilitated mitochondrial permeabilization (Guicciardi et al. 2005). Apart from LMP, deficiency in lysosomal function has been linked to neuronal cell death in certain disorders. For instance, deficiency in cathepsin B and L cause neurodegeneration in mice that is similar to human neuronal ceroid lipofuscinoses (Felbor et al. 2002, Siintola et al. 2006, Jalanko, Braulke 2009).

1.3.4 Golgi apparatus and cell death

The Golgi apparatus is an ancient organelle with a central function in the secretory pathway. In mammalian cells the Golgi is most often composed of a stack of flattened cisternal membranes arranged in a polarized fashion (*cis*-to-*trans*). On the contrary, plants, invertebrates and the fungi exhibit isolated Golgi stacks and, for instance, in the yeast *Saccharomyces cerevisiae* the Golgi cisternae are separate compartments distributed thorough the cell (Latijnhouwers, Hawes & Carvalho 2005, Preuss et al. 1992). Mammalian Golgi is dependent on and tightly associated with the microtubule network around the centrosome (Rios, Bornens 2003). The Golgi apparatus is structurally composed of a number of golgins and Golgi reassembly stacking proteins (GRASPs) that are mostly transmembrane proteins (Rios, Bornens 2003). The Golgi has been implicated in multiple functions, including transport and sorting, protein and lipid biosynthesis, microtubule nucleation centre formation, and calcium storage (Wilson et al. 2011). Neuronal Golgi is especially important for axoplasmic transport of proteins and macromolecules in ortho- and retrograde as well as transsynaptic routes and, therefore, dysfunction of Golgi could be detrimental for neuronal survival. At present, the role played by the Golgi in neurological disorders, however, remains controversial.

The Golgi undergoes reversible structural disassembly/reassembly under different conditions, especially during mitosis and upon massive flow of cargo proteins or membranes. Also many

drugs can cause fragmentation of the Golgi with different morphologies. For instance, brefeldin A inhibits ER to Golgi transport and induces breakdown and redistribution of Golgi membranes with the ER (Klausner, Donaldson & Lippincott-Schwartz 1992). During apoptosis, the Golgi has been shown to undergo irreversible fragmentation as an early sign (Mukherjee et al. 2007). Importantly, several apoptotic factors are found at Golgi membranes, including caspase-2 (Mancini et al. 2000). Caspase-2 was found to have a unique role in the initiation of apoptosis at the Golgi where it cleaves Golgin-160 at a specific site not cleaved by other caspases (Mancini et al. 2000). Furthermore, preventing this cleavage delayed apoptotic Golgi fragmentation in Staurosporin-treated cells indicating that caspase-2 cleavage of golgin-160 is critical in fragmentation of the Golgi (Mancini et al. 2000). Following caspase-2 cleavage of golgin-160, caspase-3 cleaves golgin-160 at other sites and also other Golgi proteins, including GRASP65 and the vesicle tethering protein p115 (Chiu et al. 2002, Lane et al. 2002). Cleaved Golgi proteins often translocate to the nucleus to either block (golgin-160) or promote apoptosis (p115) (Chiu et al. 2002, Hicks, Machamer 2005).

Induction of apoptosis by death receptor ligation has been shown to trigger a clustering of mitochondria, lysosomes and endosomes at the Golgi/microtubule organizing center (MTOC) site (Degli Esposti et al. 2009, Ouasti et al. 2007). It has been proposed that the Golgi might serve as a converging site during apoptosis and promote apoptosis via organellar cross talk (Aslan, Thomas 2009).

Recently, Golgi fragmentation was shown to occur during neuronal injury caused by NMDA, reactive oxygen and nitrogen species as well as ER stress (Nakagomi et al. 2008). The study also pointed out that inhibition of Golgi fragmentation was at least partially preventive or delayed cell death implicating that the Golgi has an important role in apoptotic signaling (Nakagomi et al. 2008). Golgi fragmentation has been observed in several neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), AD, corticobasal degeneration and Creutzfeldt-Jacob disease, among others (Gonatas, Stieber & Gonatas 2006). The relevance of Golgi fragmentation in dissecting cell death (the cause or the consequence) in these diseases, however, remains to be verified.

1.4 ER stress and cell death

The endoplasmic reticulum has a number of important functions: it is the first compartment in the secretory pathway, the site of secreted and membrane protein synthesis, an important intracellular calcium store (5mM versus cytosolic 0,1μM) (Sammels et al. 2010) and the site of lipid and sterol biosynthesis (Chang et al. 2006). Thus, perturbations of ER homeostasis in any of the functions mentioned can trigger stress reactions and signaling pathways called the unfolded protein response (UPR).

In general, the folding of a typical secreted protein is driven by the hydrophobic effect to minimize the amount of hydrophobic amino acids on the surface of the protein, which also ensures the native conformation with the lowest free energy. Unfolded conformations are characterized by hydrophobic amino acids on the protein surface which leads to abnormal interactions and aggregation with other unfolded proteins. To ensure correct folding of proteins, ER and cytosolic chaperones catalyse rate-limiting folding reactions. The ER

harbors several chaperones from which at least three general folding systems can be distinguished: the HSP70 molecular chaperones BiP/GRP78/Kar2p (Gething 1999, Kleizen, Braakman 2004), and Lhs1p/GRP170/ORP150 (Saris et al. 1997), the HSP90 chaperone GRP94/HSP94 (Argon, Simen 1999), and the lectin chaperones calnexin and calreticulin (Williams 2006). In addition, the ER contains many other essential foldases, enzymes catalyzing steps that increase their folding rate. For instance, ER harbors the peptidyl prolyl isomerase (PPI) that catalyzes the *cis-trans* isomerization of peptidyl proline bonds, and the protein disulfide isomerase (PDI) catalyzing the formation of disulfide bonds. Furthermore, ER is the site of N-linked glycosylation system, which is part of the protein folding and maturation process in the ER.

Translation of proteins destined for secretion and membranes is guided by a signal sequence that leads the ribosomes to the endoplasmic reticulum. A signal recognition particle (SRP) in the ribosome enables the binding of the ribosome to the rough endoplasmic reticulum (rER) (Lutcke 1995) and insertion of the growing peptide to the lumen of the ER via the Sec61p translocation channel. GRP78 (glucose-regulated protein 78kDa) also known as BiP (Immunoglobulin binding protein) is a major regulator of ER homeostasis and involved in binding of newly synthesized proteins and translocation of misfolded proteins out of the ER to the proteasomes, a process called ER associated degradation (ERAD). BiP binds the unfolded peptides with its peptide-binding domain to promote proper folding and to prevent aggregation (Kleizen, Braakman 2004). BiP has a low affinity to short hydrophobic peptides allowing a wide substrate spectrum (Flynn et al. 1991). Characteristic of HSP70 class chaperones is a conserved N-terminal ATPase domain and a C-terminal substrate-binding domain. Binding and release of substrates from BiP is catalyzed by ATP-ADP cycling. ATP hydrolysis releases the peptides from BiP in a reaction stimulated by DnaJ-like co-chaperones, such as MTJ1 (murine transmembrane protein) (Chevalier et al. 2000). GrpE co-chaperone Sil1p/BAP (BiP-associated protein), a nucleotide exchange factor, catalyzes the ADP-ATP exchange reaction (Chung, Shen & Hendershot 2002). ATP depletion inhibits protein folding and it has been suggested that protein folding capacity is limited by ATP import during active secretion and ER stress (Schroder 2008). Upregulation of BiP is a marker of accumulation of unfolded proteins in the ER. Functionally, BiP is also regulated at the level of oligomerization. In oligomeric state BiP is phosphorylated and ADP-ribosylated while the monomeric and unmodified form of BiP alone is able to associate with unfolded proteins (Freiden, Gaut & Hendershot 1992). The oligomeric pool has been suggested to present a BiP storage pool and during unfolded protein response BiP is released from the oligomeric pool to monomeric form (Freiden, Gaut & Hendershot 1992, Laitusis, Brostrom & Brostrom 1999).

Other chaperones that bind partially folded peptides include, for instance, the GRP94 chaperone that acts as a holdase, presenting unfolded substrate peptides to foldases, such as PDIs and PPIs. Unfavorable folding conditions, such as increased ATP consumption by HSP70 foldases induce the buffering activity of holdases (Winter, Jakob 2004). A common post-translational modification of ER-translated proteins is glycosylation that begins in the ER and continues in the Golgi apparatus. The lectin chaperones assist in the correct glycosylation reactions and retain the unfolded N-linked glycoproteins in the ER. The folding

protein is de- and reglycosylated a few cycles in reactions catalyzed by α-glucosidase and uridine diphosphate (UDP)-glucose:glycoprotein glycosyl transferase (UGGT), respectively. The lectins calnexin and calreticulin retain monoglycosylated proteins in the ER during the glycosylation cycle. UGGT acts preferentially on unfolded conformations to start the reglycosylation reaction and improperly folded proteins are finally transferred via calnexin to the Mn11p/Htm1p/EDEM (ER degradation-enhancing α-mannosidase-like protein) that induces retrotranslocation to the cytosol for proteasomal degradation (Molinari et al. 2003).

1.4.1 Endoplasmic reticulum-associated degradation (ERAD)

Only proteins that have been correctly folded are packaged to COPII (coatamer protein II) vesicles for ER exit. Failure to adopt a stable conformation is sensed by the chaperones in a process termed quality control resulting in dislocation of unfolded proteins from the ER through the retrotranslocon channel, composed of at least Sec61. Recently a ubiquitin-like domain (UBL) containing transmembrane protein homoCys -responsive ER resident protein (HERP) was implicated as a receptor for BiP substrates (Schulze et al. 2005). HERP is known to bind derlin, another protein implicated in retrotranslocation machinery (Okuda-Shimizu, Hendershot 2007). Taken together, it is assumed that these factors link the unfolded proteins to cytosolic ubiquitin-proteasome system (Vembar, Brodsky 2008). In the cytosol unfolded proteins are ubiquitinated by cytosolic ubiquitin ligases for targeting to proteasomal degradation (Kopito 1997).

1.4.2 ER stress

Stress in general is a response of perturbation of the normal homeostasis or state in a system. In the ER, stress can be seen as loss of normal homeostasis due to accumulation of unfolded proteins, disturbance in calcium levels or lipid metabolism. If the cell is in threat it triggers a conserved tripartite transcriptional program called the unfolded protein response (UPR) (see fig 4) aiming to restore ER homeostasis (Schroder, Kaufman 2005).

1.4.3 Unfolded protein response

The UPR consists of activation of three transmembrane proteins at the ER membrane: 1) IRE1 (inositol-requiring 1)/ERN1 (ER to nucleus signaling 1), 2) PERK [double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase] / PEK [pancreatic eukaryotic initiation factor 2α (eiF2α) kinase] and 3) ATF6 (activating transcription factor 6) (Fig 4). All these stress sensors are associated with BiP by their ER luminal domains in normal conditions (Bertolotti et al. 2000). Increase in the amount of unfolded proteins strips the ER membrane-bound BiP resulting in release of stress sensor proteins.

PERK pathway

During resting conditions, PERK is found as a monomer at the ER membrane bound by BiP via the ATPase binding region. Induction of the UPR results in the release of BiP allowing dimerization of PERK and subsequent *trans*-autophosphorylation (Sood et al. 2000). The activation mechanisms as well as the domain involved are very similar in PERK and IRE1. The most acknowledged function of activated PERK is transient phosphorylation of the α-subunit of the eukaryotic translational initiation factor 2 α (eiF2α) that inhibits translation of most mRNAs (Sood et al. 2000) (Fig 4). Phosphorylation of eiF2α inhibits the GDP-GTP

exchange by the guanine nucleotide exchange factor (GEF) eIF2β that is necessary for the activity of eiF2α. This results in sequestration of $tRNA_{met}$ needed for translation initiation (Bertolotti et al. 2000). Not all translation is, however, blocked. Certain mRNAs with specific upstream open reading frames (uORFs) in the 5' leader are translated, among them the transcription factor ATF4 (Harding et al. 2000, Scheuner et al. 2001). ATF4 induces both pro-survival (early) and pro-apoptotic (late) gene expression, including the pro-apoptotic C/EBP (CCAAT/enhancer binding protein) homologous protein (CHOP) (Ma et al. 2002).

IRE1α pathway
Ire1α is the most conserved ER stress sensor. Like the other sensors, Ire1α is a transmembrane protein with an ER luminal domain and cytosolic kinase and RNase domains (Tirasophon, Welihinda & Kaufman 1998, Wang et al. 1998). During UPR, dissociation of BiP or direct binding of unfolded proteins induces the ER luminal domains of Ire1α to undergo homo-oligomerization (Hetz, Glimcher 2009). This results in conformational changes that enable trans-autophosphorylation of the cytosolic kinase domains and subsequent RNase activation. The endoribonuclease activity of Ire1α is responsible for excision of a 26-nucleotide fragment from *XBP1* (X-box binding protein 1) mRNA (Fig 4). This modification yields a spliced form of *XBP1* mRNA (*XBP1s*) that is translated into an active transcription factor (Yoshida et al. 2001, Calfon et al. 2002). Translated XBP1 binds to promoters containing the *ERSE* (ER stress element) consensus sequences and promote transcription of BiP/Grp78 as well as other chaperones (Yoshida et al. 2001) (Fig 4). In addition, XBP1 binds another cis acting element, *UPRE*, found specifically in genes involved in ERAD (Yamamoto et al. 2004).

Alternatively, Ire1α can be phosphorylated and serve as platform for activation of TRAF2 (Tumor necrosis factor (TNF)-receptor associated factor 2), ASK1 (Apoptosis signal-regulating kinase 1) and the IκB (Inhibitor of kappa B) kinase IKK. These events lead to activation of stress-activated JNK, ASK1, ERK (Extracellular signal-regulated kinase) and p38 kinases (Hetz, Glimcher 2009). Subsequently, caspase-12 is released from ER membranes linking ER stress to JNK pathway and apoptosis (Yoneda et al. 2001).

ATF6 pathway
In basal conditions, ATF6 is localized to the ER and bound to the chaperone BiP (Shen et al. 2002) (Fig 4). The interaction masks two Golgi localization signals that are exposed during accumulation of unfolded proteins and following BiP sequestration. The Golgi resident site-1 and site-2 proteases (S1P and S2P) cleave ATF6 (p90ATF6), which generates a cytoplasmic basic leucine zipper (bZIP) transcription factor N-ATF6 (p50ATF6) (Shen et al. 2002) (Fig 4). N-ATF6 translocation to the nucleus induces transcription of genes containing the ERSE-I, -II, or CRE consensus sequences. For instance, BiP, XBP1 and CHOP are target genes upregulated by ATF6 under stressed conditions (Haze et al. 1999, Yoshida et al. 2001).

Additionally, ATF6 is bound by calreticulin, an ER calcium- and glycoprotein -binding chaperone (Hong et al. 2004b). In normal conditions, ATF6 is glycosylated and another mechanism to induce UPR is to sense underglycosylated forms of ATF6. Underglycosylation occurs, for instance, during calcium depletion from the ER (Hong et al. 2004b). Furthermore,

chemical inhibition of N-glycosylation by tunicamycin (Tu) results in ATF6 activation (Haze et al. 1999). Another ER stress inducer, thapsigargin (Tg), causing ER calcium depletion via inhibition of the SERCA pumps (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase) also induces cleavage and underglycosylation of ATF6 (Hong et al. 2004b, Haze et al. 1999). In addition, Tg and Tu were shown to induce proteasomal degradation of ATF6 independently from S1P/S2P cleavage (Hong et al. 2004a). Consequently, inhibition of proteasomal activity was sufficient to prevent the decrease in the levels of ATF6 in response to ER stress but also stabilize ATF6 levels under normal conditions (Hong et al. 2004a). Rapid turnover of ATF6 by UPS-dependent mechanism is thought to have evolved to keep ATF6 in guard from accidental triggering of the UPR.

1.4.4 Pharmacological inducers of ER stress

ER quality control is tightly linked in with the UPS. Thus it is not surprising that ER stress increases cytosolic UPS substrates (Menendez-Benito et al. 2005). Vice versa, proteasomal inhibition by lactacystin upregulated ER stress-associated genes (Choy et al. 2011). Thus, proteasomal dysfunction occurring during normal aging and in neurodegenerative diseases (Grune et al. 2004) might contribute to the level and vulnerability of the ER to cope with different stressors.

Experimental ER stress can also be induced by pharmacological agents affecting different functions of the ER. Tunicamycin, for instance, disturbs N-linked glycosylation of proteins by inhibiting i.e. GlcNAc phosphotransferase (GPT) in the first step of glycoprotein synthesis (Breckenridge et al. 2003). Thapsigargin specifically inhibits the ER Ca^{2+} -ATPase and induces rapid release of stored Ca^{2+} with the disruption of calcium homeostasis (Thastrup et al. 1990). Furthermore, dithiotreitol (DTT) inhibits disulfide bond formation by its reducing properties and thus inhibits normal protein maturation in the ER (Breckenridge et al. 2003). Moreover, Brefeldin A (BFA) is an inhibitor of certain Arf1 GTP-exchange factors (GEFs; GBF1, BIG1 and BIG2) and inhibits the anterograde ER to Golgi transport of proteins without affecting retrograde transport resulting in accumulation of proteins in the ER (Breckenridge et al. 2003, Zhao, Lasell & Melancon 2002).

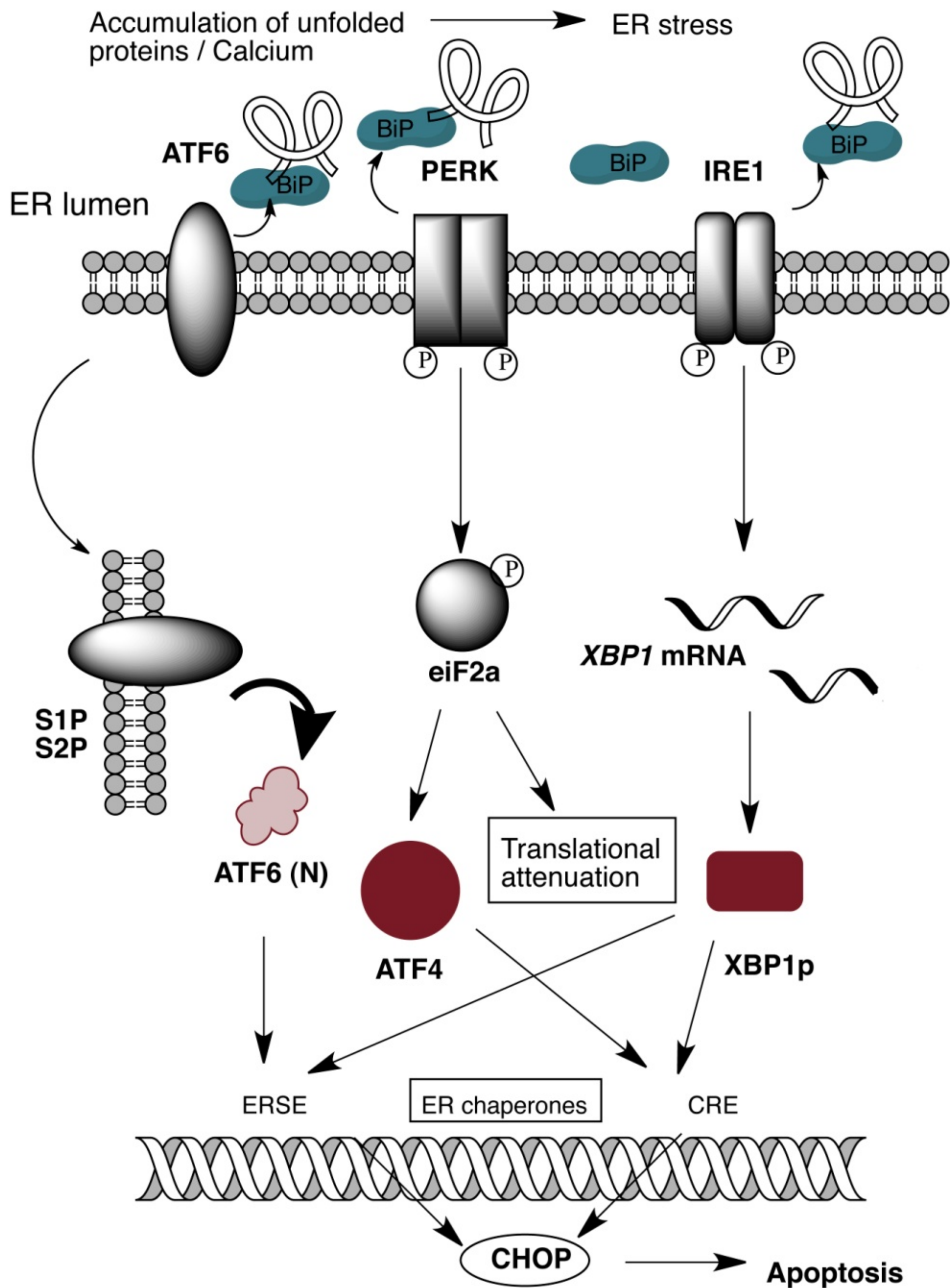


Figure 4 ER stress pathways. See text for details. Abbreviations: ATF4/6: activating transcription factor 4/6; BiP: immunoglobulin binding protein; CHOP: C/EBP homologous protein CRE: cAMP response element; eIF2α; eukaryotic translational initiation factor 2α; ERSE: ER stress element; IRE1: inositol requiring 1; PERK: PKR-like endoplasmic reticulum kinase; S1P/S2P: site 1/2 protease; XBP1: X-box binding protein 1.

1.4.5 Cell death induced by ER stress

In case adaptive responses to ER stress fail, the UPR switches into apoptosis promoting mode. PERK and IRE1 signaling pathways have evolved mechanisms to trigger apoptotic pathways during irremediable ER stress. Apoptosis is triggered by at least three pathways, including activation of CHOP, JNK pathway, and caspase-12, that all promote activation of caspase-3 (Oyadomari, Mori 2004). ATF6 and ATF4, downstream of PERK, and induce transcription of the CHOP/GADD153 transcription factor (Yoshida et al. 2001, Ma et al. 2002). CHOP can downregulate expression of the anti-apoptotic Bcl-2 and on the other hand upregulate

expression of ERO1α inducing oxidative reactions in the ER (McCullough et al. 2001, Marciniak et al. 2004). CHOP is ubiquitously expressed at low levels in the cytosol of non-stressed cells. Induction of stress in the ER leads to CHOP expression and accumulation to the nucleus (Ron, Habener 1992). CHOP depleted mice exhibit reduced levels of ER stress-induced apoptosis (Oyadomari et al. 2002). Vice versa, overexpression of CHOP leads to cell cycle arrest and/or apoptosis (Maytin et al. 2001, Barone et al. 1994).

Apoptosis induced by ER stress has been proposed to consist of a combination of intrinsic and extrinsic apoptotic pathways with essential roles played by the Bcl-2 family proteins (Schroder, Kaufman 2005). Bcl-2, keeping the proapoptotic Bcl-2 family proteins at guard, localizes to the ER membranes in addition to the mitochondrial and nuclear membranes (Lithgow et al. 1994). Overexpression of Bcl-2 and Bcl-xL protect cells from thapsigargin-induced cell death by inhibiting caspase-3 activation and the JNK pathway (Srivastava et al. 1999). On the other hand, ER stress induces the activation of several BH3-only proteins; Bid, Bim, Noxa and Puma that further target Bax and Bak and signaling to the mitochondrial cell death machinery (Li, Lee & Lee 2006, Upton et al. 2008, Puthalakath et al. 2007). Furthermore, in addition to mitochondrial membrane localization, Bax and Bak have been shown to localize to the ER in response to ER stress (Zong et al. 2003). At ER membranes Bax and Bak oligomerize to form pores and induce progressive calcium depletion and caspase-12 cleavage leading to cell death. These findings are also supported by studies with *bax*$^{-/-}$/*bak*$^{-/-}$ cells that are resistant to ER stress induced apoptosis (Zong et al. 2003, Zong et al. 2001). In addition, IRE1 associates with the TNF receptor associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1) to form a complex that activates the c-Jun N-terminal kinase (JNK) (Urano et al. 2000). JNK activates the proapoptotic Bim and inhibits the anti-apoptotic Bcl-2.

Caspase-12 was shown to localize to the ER and be involved in ER stress-induced cell death (Nakagawa et al. 2000). Deletion of caspase-12 from mice results in inhibition of ER stress mediated cell death, although the mice are capable of other types of cell death pathways. Moreover, it was shown that inhibition of caspase-9 blocked this cell death pathway while caspase-8 was not involved (Rao et al. 2002b). Apart from Caspase-12, also caspase-2 was linked to ER stress-induced apoptosis in multiple myeloma cells treated with the 26S proteasome inhibitor, bortezomib (Gu et al. 2008). Caspase-2 is also involved in Bid cleavage contributing to apoptotic signaling downstream of ER stress, an event counteracted by inhibition of caspase-2 (Upton et al. 2008).

1.4.6 ER stress in neurological disorders

The very focal roles of the ER in cellular functions sets it vulnerable for a number of environmental and genetic factors. ER stress has been detected in numerous human diseases from diabetes to neurological disorders, such as AD, ALS, and generally in diseases with accumulation of mutant proteins (Schroder, Kaufman 2005, Lindholm, Wootz & Korhonen 2006). Disruption of proteostasis occurs, for instance, in ALS and HD, where mutant proteins superoxide dismutase-1 (SOD1) and huntingtin, respectively, aggregate and result in proteasomal dysfunction and accumulation of proteins in the ER (Nishitoh et al. 2008, Nishitoh et al. 2002). Mutations in the presenilins were also linked to disruption of calcium

homeostasis and oxidative stress in AD with implications in ER dysfunction (Guo et al. 1997, Mattson et al. 2000). Moreover, it was recently shown that presenilins actually form Ca^{2+} leak channels at ER membranes that when mutated, as in familial AD, disrupt Ca^{2+} signaling (Tu et al. 2006). In addition, as previously described, Aβ -mediated cell death involves caspase-12 and ER -specific apoptosis (Nakagawa et al. 2000).

1.5 Excitotoxicity

1.5.1 Glutamate and other excitatory amino acids

The amino acid glutamic acid, or glutamate as it exists in physiological pH, is the major excitatory neurotransmitter in the mammalian central nervous system (CNS). It is essential for development, differentiation and synaptic plasticity of neurons. Toxicity of glutamate was recognized in 1957 by Lucas and Newhouse (Lucas, Newhouse 1957) with their observation that subcutaneous injection of glutamate to mouse retina resulted in neuronal degeneration. Later Olney coined the term "glutamate excitotoxicity" in studies characterizing brain lesions and neuronal necrosis after subcutaneous injections of glutamate in infant and adult mice as well as in an infant rhesus monkey (Olney 1969, Olney, Sharpe 1969). Association of glutamate excitotoxicity to acute brain damage, such as hypoxia and ischaemia, was noticed when Rothman used a post-synaptic blocker of excitatory transmitters, y-D-glutamylglycine (DGG), to inhibit the effects induced by anoxic atmosphere and exogenous glutamate (Rothman 1984). Since, it has been noted that glutamate excitotoxicity occurs in acute ischaemic injury and as secondary injury after traumatic brain injury as well as in epilepsy. Moreover, chronic over-excitation of glutamate receptors occurs in neurodegenerative diseases, such as ALS, AD, PD and HD.

Although glutamate is by far the most important and potent excitotoxin in the CNS, also other natural and exogenous molecules can mediate excitotoxicity. The natural excitatory amino acids include aspartic acid/aspartate, quinolinic acid (QA), sulphur-containing excitatory amino acids, such as homocysteic acid and the dipeptide *N*-Acetylaspartylglutamic acid (NAAG) (Doble 1999). Exogenous excitatory molecules include *N*-methyl-D-aspartic acid (NMDA) and kainate (KA).

1.5.2 Glutamate receptors

Glutamate gates two main receptor types in the CNS: the ionotropic and metabotropic glutamate receptors. These receptors belong to the ligand-gated ion channel protein family sharing the common property of rapid activation by an agonist followed by desensitization, uncoupling of agonist from receptor, a process that inhibits unregulated ion flow and cell death in the continued presence of neurotransmitters (Jones, Westbrook 1996). Both receptor types are further divided to three families.

1.5.2.1 Ionotropic glutamate receptors

Ionotropic glutamate receptors (iGluRs) are named according to which specific agonist they bind. These are the *N*-Methyl-D-Aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and kainic acid (KA). Classically, AMPA and KA receptors have been categorized together as non-NMDA receptors. Ionotropic glutamate receptors

function as ligand-gated ion channels that upon binding of glutamate open and allow ion flux through the channel, more precisely, influx of sodium and/or calcium and efflux of potassium. The structure of iGluRs in general is a tetramer in which each monomer possesses its own ligand-binding site and a hydrophobic domain (M2) that contributes to the channel lumen formation (Madden 2002). Moreover, three additional transmembrane segments, M1, M3 and M4, contribute to the channel formation and are arranged so that the N-terminal part of the polypeptide lies extracellularly and C-terminus intracellularly. Specific residues distributed in the agonist-binding site, especially in the S2 loop contribute to the receptor sensitivity and agonist specificity (Stern-Bach et al. 1994).

Excitotoxicity is most potently triggered via NMDA receptor overactivation, however, other glutamate receptors can induce excitotoxicity either directly (KARs, AMPARs) or indirectly (AMPARs, mGluRs) by activating calcium channels or NMDA receptors via sodium influx - mediated depolarization of the cell membrane (Carriedo, Yin & Weiss 1996).

1.5.2.2 NMDA receptors

The most studied of ionotropic glutamate receptor subtypes are the NMDA receptors (NMDARs). The NMDARs form highly calcium permeable glutamate-gated ion channels from a combination of three different subunits GluN1-3 (formerly NR1-3). The GluN1 subunit has eight splice variants (GluN1-1a-4a; GluN1-1b-4b), while GluN2 subunits include four (GluN2A-D) and GluN3 two isoforms (GluN3A-B) (Lau, Tymianski 2010). Classically, gating of NMDARs require both binding of various ligands as well as cellular depolarization. In basal, unstimulated conditions the channel currents are inhibited by a magnesium ion in the pore that can be removed by activation of other ionotropic glutamate channels (AMPA; KA). The amino acid glycine is also an essential cofactor for NMDAR opening; very small concentrations (10nM) potentiate NMDAR activation (Johnson, Ascher 1987). In addition, changes in physiological pH can affect NMDAR functions, for instance, after an injury or ischaemia. NMDA receptor signaling can be coupled to another calcium-permeable channel, the acid-sensing ion channel (ASIC) enhancing calcium signaling and acidotoxicity in ischaemia (Gao et al. 2005).

1.5.2.3 AMPA receptors

AMPA receptors include four subunits GluA1-4 that make up heteromeric channels. Their calcium permeability varies between subtypes; for instance, GluR1 and GluR3 are permeable while GluR2 containing channels are impermeable to calcium (Hollmann, Hartley & Heinemann 1991). AMPARs mediate most of the low frequency glutamatergic neurotransmission. In addition, AMPARs are thought to regulate NMDAR excitation by removing the magnesium block via postsynaptic depolarization.

1.5.2.4 Kainate receptors

KA was originally isolated from the seaweed Kainin-sou (or Makuri) in Japan. Before the characterization of KARs, KA was known as an agonist for non-NMDA receptors. However, cloning of KARs enabled the differentiation of KARs from AMPARs and their functional separation (Hollmann, Heinemann 1994, Bettler, Mulle 1995). KA has high affinity to KARs; 5-15nM for KA1 and KA2 and 70-100nM for GluR5-7. AMPARs can also be activated by

KA, but exhibit much lower affinity for KA (1000 fold lower as for KARs) (Hollmann, Heinemann 1994).

The KA receptor family comprises of five subunits, that have been renamed as GluK1-5 (encoded by the genes *Grik1-5*); GluR5 (GluK1/*Grik1*), GluR6 (GluK2/*Grik2*), GluR7 (GluK3/*Grik3*), KA1 (GluK4/*Grik4*) and KA2 (GluK5/*Grik5*) (Contractor, Mulle & Swanson 2011). KARs form functional receptors in homo- or heteromeric combinations. GluR5-7 are capable of forming both homo- and heteromeric channels (Lerma 2006). The structure of KARs is homologous to other iGluRs. The extracellular N-terminal domains of KARs including the S1 and S2 (the loop between M3 and M4) regions form the glutamate binding site while the C-terminal intracellular domain is responsible for receptor targeting and signal transduction (Fig 5). The transmembrane domains; M1, M3 and M4, with the re-entrant membrane loop M2 line the channel pore (Fig 5).

RNA editing of GluR5 and GluR6 at the Q/R site in the M2 domain by RNA deaminase gives rise to further structural variety. Substitution of glutamine (Q) by arginine (R) decreases the calcium permeability of the receptor and ultimately its conduction properties (Swanson et al. 1996). The physiological relevance of the Q/R site has remained unclear, but there seems to be developmental control that might contribute, for instance, to remission from childhood epilepsies (Vincent, Mulle 2009) (Fig 5). GluR6 has additionally two editing sites in the M1 domain that affect the ion selectivity of the receptor (Kohler et al. 1993).

Furthermore, GluR5-7 subunits undergo alternative splicing that affects their cytoplasmic C-terminal domains. Splice variants include GluR5a-c, GluR6a-b, and GluR7a-b (Barbon, Barlati 2000, Barbon, Vallini & Barlati 2001, Jaskolski et al. 2005). All splice variants vary in their amino acid sequences of the C-terminal domains, which in turn affects their interactions with stabilizing and trafficking proteins. Thus, the splice variants may show very different properties in trafficking to the cell surface or subcellularly, and even activity of the KARs. The two splice variants of GluR6, GluR6a and GluR6b, differ by two distinct sequences of their C-terminal domains (Barbon, Vallini & Barlati 2001). GluR6a has been shown to be highly expressed in the plasma membrane of neurons and also affect the surface targeting of other KARs thus being critical in targeting of heteromeric KARs (Jaskolski et al. 2004). GluR6a carries a high surface expression determinant of four charged amino acids that is indispensable in membrane targeting. In addition, GluR6a has a PDZ (postsynaptic density-95/discs large/zona occludens-1) interaction motif that allows interaction with e.g. PSD95, GRIP and PICK1. PDZ proteins can regulate trafficking of receptor proteins from the ER to the plasma membrane, however, for KARs they seem to function only as to stabilize their postsynaptic localization (Garcia et al. 1998, Hirbec et al. 2003).

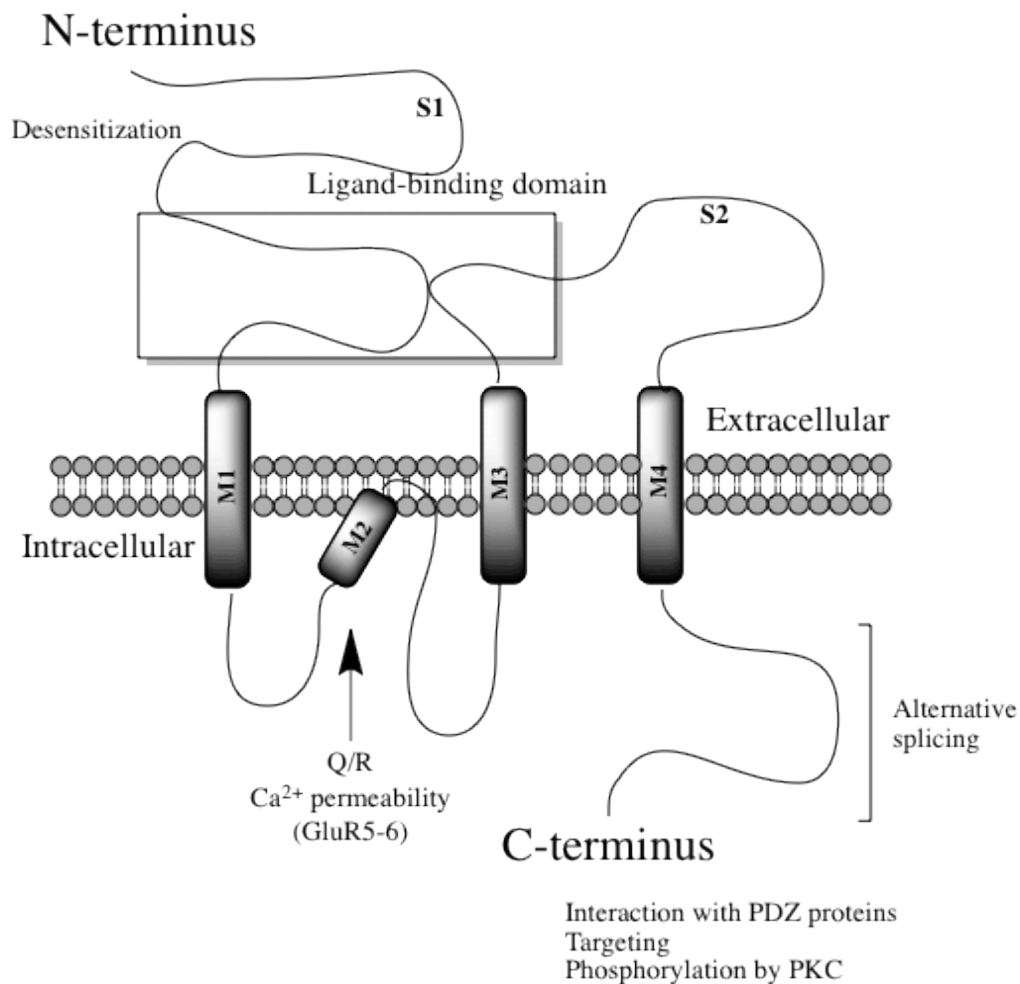


Figure 5 Schematic structure of KARs. Extracellular, intracellular and transmembrane domains found in KARs, with sites of known interactions and amino acid substitutions important for the receptor function. Abbreviations: M1-4: transmembrane domains 1-4; Q/R: glutamine/arginine substitution site; PDZ: postsynaptic density-95/discs large/zona occludens-1; PKC: protein kinase C; S1-2: extracellular domains 1-2.

Kinetic profiles of postsynaptic KARs vary from extremely low to rapid responses (Castillo, Malenka & Nicoll 1997, DeVries, Schwartz 1999). In the hippocampus, KARs are involved in direct excitatory post-synaptic currents (EPSCs) between synapses of mossy fibers and CA3 pyramidal cells (Castillo, Malenka & Nicoll 1997, Vignes, Collingridge 1997). KA-mediated EPSCs are lower in amplitude as compared to AMPA-mediated EPSCs and show slower rise and decay times. The slow kinetics result in summation of repetitive stimuli and may also arise from non-saturation of postsynaptic KARs (Jaskolski, Coussen & Mulle 2005). CA3 pyramidal cell dendrites show KAR (GluR6, KA2, and KA1) expression and EPSCs specifically in the stratum lucidum region where they contact with mossy fiber axons (Castillo, Malenka & Nicoll 1997). GluR6$^{-/-}$ mice are devoid of the KAR component of the mossy fiber EPSCs as well as the presynaptic actions (Mulle et al. 1998, Contractor, Swanson & Heinemann 2001). Other neurons, such as the CA1 pyramidal neurons and striatal medium spiny neurons exhibit unconventional synaptic transmission (Bureau et al. 1999, Chergui et al. 2000). Studies indicate that these KARs localize extrasynaptically and can also be activated by glutamate release from astrocytes (Liu et al. 2004). The CA1-localized KARs possibly increase neuronal excitability through a metabotropic function (Melyan, Wheal & Lancaster

2002). Presynaptic KARs have been detected for instance in the mossy fiber-CA3 synapses and function as autoreceptors sensing glutamate release. This function increases the likelihood of future release of glutamate thus modifying the strength of the synaptic stimulation and short-term synaptic plasticity (Contractor, Swanson & Heinemann 2001, Lauri et al. 2001).

Activation of KARs is modulated by ions and and small molecules. Different from other iGluRs, KARs are dependent on both Na^+ and Cl^- in the extracellular solution (Paternain et al. 2003, Plested, Mayer 2007). Interestingly, Cl^- ions play an essential role by binding between subunits and stabilizing the dimer structure while in the absence of Cl^- ions the receptors are desensitized and glutamate-induced activation diminished (Plested, Mayer 2007).

Desensitization mechanisms are not fully clear for iGluRs but most is known of AMPARs and includes rearrangements in dimer interfaces of the receptors as well as transmembrane AMPAR regulator proteins (Hansen, Yuan & Traynelis 2007). KARs were shown to have similar mechanism of desensitization that seems to be conserved among AMPARs and KARs. Desensitization requires the domain 1 surfaces of a dimer and large conformational rearrangements (Weston et al. 2006). KAR desensitization can be relieved by concanavalin A treatment that is achieved possibly via interaction with surface sugar chains of KARs (Fletcher, Lodge 1996).

In addition to the repertoire of different KARs and their variants, combinations of different subunits to functional channels as well as other proteins can affect the properties of the KARs. Previously an auxiliary protein, Neto1, was shown to interact and affect GluR6 expression in the hippocampal PSDs (Straub et al. 2011, Tang et al. 2011).

KARs are widely distributed throughout the nervous system (Wisden, Seeburg 1993) (See table 4). Their function in both pre- and postsynaptic neurons allows diverse roles in glutamatergic signaling; facilitating glutamate release, influencing other glutamate receptors and GABAergic signaling. In addition, variations in subunit composition and KAR expression levels may influence their impact on the glutamatergic signaling. Expression of different KARs occurs in complex patterns, indicating that there are numerous functionally diverse receptor complexes in the CNS. For instance, KARs have been implicated in mood regulation, for GluR5 being encriched in the amygdala (Li et al. 2001b) as well as GluR6 and KA1-2 prominently expressed in the dentate gyrus and hippocampal CA3 area (Wisden, Seeburg 1993). (Table 4) In addition, presynaptic KARs can regulate GABA release in the hippocampus and amygdala (Rodriguez-Moreno, Herreras & Lerma 1997). To further complicate their involvement in neuronal systems, they seem to produce metabotropic actions via interaction with G-proteins (Melyan, Wheal & Lancaster 2002, Melyan, Lancaster & Wheal 2004). Thus, KARs seem to be involved in a vast array of modulatory functions in the hippocampus and the whole nervous system.

Table 4 Expression of high -affinity KAR mRNAs in the rat brain. The areas of highest expression are highlighted.

KAR subtype	**Expression** (Wisden, Seeburg 1993)
GluK1/ GluR5	Neocortex, **pyriform cortex**, **septum**, **anterior ventral thalamus**, **hypothalamus**, **pontine and rhabdoid nuclei of the hindbrain**, cerebellar purkinje cells
GluK2/ GluR6	Neocortex, **pyriform cortex**, **hippocampus**, caudate putamen, dorsal raphe nucleus, **cerebellar granule cells**
GluK3/ GluR7	**Neocortex, cingulate cortex**, dentate gyrus, caudate putamen, septum, **reticular** and anterior ventral nuclei of the thalamus, hypothalamus, dorsal raphe and pontine nuclei of the hindbrain, cerebellar stellate/basket cells
GluK4/ KA1	Neocortex, **hippocampus**, globus pallidus, medial septum, medial preoptic hypothalamus, hindbrain, corpus callosum, cerebellar purkinje cells
GluK5/ KA2	**Neocortex**, **hippocampus**, **caudate putamen**, **septum**, medial habenula, anterior ventral thalamus, hypothalamus, **hindbrain**, **cerebellar granule cells**, **pineal gland**

KAR signaling can be divided into two characteristic forms; the canonical pathway involves ion flux, and the noncanonical pathway links KAR activation to G protein activation and GABA release in GABAergic interneurons (Melyan, Lancaster & Wheal 2004, Rodriguez-Moreno, Lerma 1998, Rozas, Paternain & Lerma 2003). In addition, KARs can be divided to having both pre- and postsynaptic functions. In other words, the presynaptic functions include modulation of release of neurotransmitters while postsynaptically KARs mediate synaptic currents as ion channels like the NMDA and AMPA receptors.

KARs have been implicated in synaptic plasticity and regulation of synaptic transmission in both inhibitory and excitatory synapses (Lerma 2003). Moreover, knowledge of KAR functions has been provided by their implications in several neurological disorders, including AD, HD, schizophrenia and related disorders such as bipolar disorder (Lerma 2006). An increasing understanding about the function of KARs and signaling mechanisms in neurons may help to better understand various neurological disorders accompanied by excitotoxic damage.

GluR6

As shown in GluR6 deficient mice, the GluR6 receptors are important for the KA-mediated epileptiform seizure and cell death in the CA3 area of the hippocampus (Mulle *et al.*, 1998). Despite this, no clear genetic association for GluR6 and epilepsy has been found in humans. However, polymorphisms in the *GluR6* gene has been linked to a number of mental disorders, for instance, schizophrenia and bipolar disorder (Beneyto et al. 2007, Blackwood et al. 2007). Levels of GluR6 were shown to be elevated in schizophrenia (Bullock et al. 2008), and downregulated in bipolar disorder, especially during manic symptoms (Dick et al. 2003, Shaltiel et al. 2008, Malkesman et al. 2009). Moreover, GluR6 KO mice exhibited specific behavioral features resembling those seen in mania, including hyperactivity, aggressiveness, decreased anxiety, and sensitivity to psychostimulants, such as amphetamine (Shaltiel et al. 2008). In addition, several studies have implicated GluR6 polymorphism association in autism (Strutz-Seebohm et al. 2006, Jamain et al. 2002). Linkage of a mutation in *GluR6* gene to autosomal recessive mental retardation in a consanguineous Iranian family provided proof of GluR6 being indispensable in higher cognitive functions in humans (Motazacker et

al. 2007). Some evidence has also been provided of GluR6 polymorphism affecting the age-of-onset in HD (Rubinsztein et al. 1997).

As other glutamate receptors, the KARs at the postsynaptic density are regulated by binding to PSD proteins, including PSD95, GRIP and PICK1 (Hirbec et al. 2003). The activity of KARs is also regulated by modification of the polypeptide, for instance, by phosphorylation. GluR6 has been indicated in ischemic neuronal death via activation of the JNK pathway through GluR6-PSD95-MLK3 signaling module (Pei et al. 2004, Pei et al. 2005, Chen et al. 2009). Activation of p38 resulted from signaling cascade involving MLK3 (Mixed lineage kinase 3; mitogen activated protein kinase kinase kinase (MAPKKK)) and downstream MAPKKs MKK3/MKK6/MKK4 (Chen et al. 2009). MAP kinases are involved in multiple biological responses from proliferation to cell death and are activated by various extracellular signals (Irving, Bamford 2002). Previously, CaMKII, being the most abundant signaling protein at the PSD, was implicated in GluR6 phosphorylation during ischaemia (Hao et al. 2005). CaMKII is activated by autophosphorylation during NMDAR activation and travels to plasma membrane where it can activate a myriad of substrates, including the AMPAR, GluR1 (Tan, Wenthold & Soderling 1994). In physiological conditions, CaMKII is essential in hippocampal learning and LTP formation (Soderling 1993). Recently, it was shown that GluR6 phosphorylation by CaMKII occurred via GluR6-PSD95-CaMKII signaling module formation during ischaemia injury (Xu et al. 2010). This resulted in JNK activation and could be inhibited by CaMKII inhibitor KN-93. Despite these, it is likely that several other kinases in addition to the aforementioned, play a role in GluR6 modulation.

Apart from phosphorylation, proteins can be posttranslationally modified by various other tags, including ubiquitin, SUMO (small ubiquitin-like modifier), nitrosyl- and acetyl-groups. The actin-binding protein present in neuronal PSDs, actinfilin, was shown to bind and link GluR6 to the UPS-mediated degradation via presentation of GluR6 to the ubiquitin E3 ligase Cullin 3 (Salinas et al. 2006). Moreover, SUMOylation was shown to take part in kainate-mediated GluR6 endocytosis and resulting in decrease in postsynaptic currents (Martin et al. 2007). In relation to ischemic injury, it was also represented that nitrosylation, the addition of NO group, takes part in GluR6 modulation (Yu et al. 2008). This modification was presumed to facilitate the GluR6-PSD95-MLK3 signaling module formation. Among others, the S-nitrosylation pathway presents a possible target for drug develoment in ischaemia since inhibition GluR6 nitrosylation attenuated the GluR6-PSD95-MLK3 signaling module activity (Yu et al. 2008).

1.5.2.5 Metabotropic glutamate receptors

Metabotropic glutamate receptors are coupled to G-proteins and their activation leads to regulation of cAMP levels and calcium release from intracellular stores. Contrary to ionotropic glutamate receptors, mGluRs mediate mostly slow synaptic responses and modulate the activity of the glutamatergic circuits (Conn, Pin 1997). The categorizations of mGluRs to groups I-III are based on their pharmacological properties, sequence similarity, and signal transduction mechanisms. Group I mGluRs are usually located in the post-synaptic neuron and regulate ion channels and NMDA and AMPA receptor, for instance, by potentiating NMDA receptor activation and thus calcium influx (Bruno et al. 1995). Group I

mGluRs are linked to phopspholipase C activation and downstream inositol triphosphate (IP_3) production that can induce intracellular calcium movements thus providing means for NMDA/AMPA receptor activation (Abe et al. 1992, Aramori, Nakanishi 1992). Group II and III mGluRs are often presynaptic and inhibit certain calcium channels and neurotransmission via regulation of neurotransmitter release.

1.5.3 Mechanisms of excitotoxicity

Excitotoxicity has been proposed as a separate form of cell death (Kroemer et al. 2005); however, it seems to involve a mixture of cell death pathways such as apoptosis and necrosis depending on the intensity of the stimulus and was not coined as one (Kroemer et al. 2009). As mentioned above, excitotoxicity can be triggered both in acute hypoxic and ischemic conditions as well as chronically in neurodegenerative diseases, where the neurons are exposed to excitotoxic signals over a long period of time. In chronic conditions, the damage accumulates and the symptoms emerge gradually, e.g. gradual memory loss in AD. Despite the difference in exposure, both acute and chronic excitotoxicity are caused by prolonged excitation by glutamate and share similar molecular mechanisms.

During ischemic stroke, for instance, the blood flow to certain brain areas seizes and results in glucose and oxygen deprivation (Ames et al. 1968). In low energy (ATP) conditions neurons are unable to maintain the ionic gradients across their cell membrane, which leads to depolarization and release of neurotransmitter vesicles. As a consequence, also the neurotransmitter uptake machinery is stunned and more neurotransmitters accumulate to the extracellular space. Although the precise molecular mechanisms in excitotoxicity are still somewhat unclear, it is clear that rise in intracellular calcium plays a key role in subsequent events. Other events taking place during excitotoxicity include organelle dysfunction, and increased nitric oxide and free radical production as well as activation of proteases and kinases. For instance, excitotoxicity involves activation of calpains (Wu, Lynch 2006) as previously described.

Calcium has numerous important functions in normal cellular homeostasis and signaling. Involvement in toxicity was noted in the late 1970s when several toxins were found to effectively kill cells in the presence of calcium but not without it (Schanne et al. 1979). Cells are surrounded by calcium-rich extracellular fluid with a concentration of approximately 2 mM. To control calcium-dependent proteins and processes, the concentration of calcium in the cytoplasm is maintained in 10-100 nM range by actively pumping out Ca^{2+} from cytosol to extracellular space, or intracellular organelles, including the endoplasmic reticulum, Golgi apparatus and mitochondria. Certain signals can trigger opening of calcium channels and pumps at the plasma membrane or the ER leading to sudden rise in intracellular calcium. Excitotoxicity often results in excessive calcium rise and activation of cell death pathways. It has been shown that the route of calcium entry is the main determinant in calcium-induced neurotoxicity (Sattler et al. 1998) since different micro- and nanodomains around the specific receptors or channels contain different proteins that may induce totally diverse signaling pathways. Thus, calcium entry via voltage-gated Ca^{2+}-channels did not alter cell morphology even with high calcium load. However, glutamate-induced NMDA receptor opening has a calcium threshold above which calcium readily induces cell death (Sattler et al. 1998).

One toxic function of excessive calcium is the induction of free radical production from mitochondria (Dykens 1994). The mitochondria are responsible for most reactive oxygen species (ROS) produced in cells and taken into account the post-mitotic nature of neurons it is of outmost importance to clear these harmful species. Calcium increase in excitotoxicity has several consequences that induce ROS production; activation of phospholipase A_2, nNOS (neuronal nitric oxide synthase) and xanthine oxidase, that further induce acide arachidonic cascade (Emerit, Edeas & Bricaire 2004).

NMDAR activation and subsequent calcium flow has been shown to regulate nitric oxide (NO) production via coupling of PSD95 and nNOS (Sattler et al. 1999). NO toxicity was reduced by PSD95 antisense downregulation and was specific for only NMDAR stimulation since KA-induced toxicity was unaffected in PSD95 -depleted cells. Thus, PSD95 is a potential target in treatment of excitotoxic diseases involving NMDAR-mediated toxicity. Like ROS, NO acts as a rective nitrogen species (RNS) and together with superoxide anion (O'^{-}_{2}), NO forms peroxynitrite ($ONOO^-$) that is highly reactive and harmful for cell functions (Emerit, Edeas & Bricaire 2004).

1.6 KA model to study excitotoxic cell death

Systemic or intracerebral injection of KA has been long used as a model of temporal lobe epilepsy (TLE) (Ben-Ari 1985, Nadler 1981). Epilepsy in general is a common neurological disorder characterized by spontaneous recurrent seizures consisting of excessive synchronized neuronal activity. TLE is a common form of epilepsy in humans manifesting recurrent partial seizures originating from the hippocampus. In some cases the epileptiform activity can propagate also to other structures of the limbic system, such as amygdala and medial entorhinal cortex inducing secondary generalized tonic-clonic seizures (Lothman, Bertram & Stringer 1991). A hallmark of TLE is a specific type of hippocampal damage, called hippocampal sclerosis, characterized by extensive neuronal loss (>50%) in the CA3 and CA1 areas and the dentate hilus (Engel 1996).

KA provokes acute seizures in model animals originating in the hippocampal CA3 region (See fig 6) and selectively destroys these neurons along some other regions depending on the route of KA administration. Systemic (intraperitoneal, intravenous or subcutaneous) administration of KA triggers limbic seizures lasting for several hours. After a few days to weeks the animals usually exhibit a chronic phase of spontaneous limbic seizures that increase in frequency without remission (Ben-Ari 1985). KA-provoked seizures produce brain damage closely resembling the neuropathology characteristic of TLE, involving loss of CA1 and CA3 pyramidal neurons and interneurons of the dentate hilus (Fig 6). Intraventricular KA injections mainly cause degeneration of the CA3 pyramidal neurons while intraperitoneal administration is often accompanied with CA1 degeneration (Nadler, Perry & Cotman 1978, Sperk 1994). Besides region-selective pattern of damage, age of the model animal contributes greatly to the degenerative effects of KA. For instance, P9 rats show no clear degeneration of the hippocampal CA1 or CA3 areas while P21 and older rats show damage of the CA1 and CA3 (Holopainen 2008) as described earlier. Postnatal rat brain shows also age-dependent differences in microglial activation and inflammatory processes (Holopainen 2008).

KA model was developed well before the identification of KARs. Subsequent studies revealed KA binding sites in the CNS, especially enriched in the hippocampal CA3 area, consistent with the low dose of KA triggering epileptic activity in hippocampal slice preparations (Monaghan, Cotman 1982, Robinson, Deadwyler 1981). The CA3 pyramidal neurons are most vulnerable, exhibiting 10- to 30-fold higher sensitivity to KA as compared to CA1 pyramidal cells. Furthermore, concentrations below 3μM (20mg/kg) selectively activate KARs containing the GluR6 subtype in the CA3 neurons, while higher concentrations also activate other KARs and AMPARs (Mulle et al. 1998). As reviewed earlier, the GluR6 subtype of KARs was shown to be responsible for the epileptogenic effect of KA on CA3 pyramidal cells when applied at small concentrations (<3μM) (Mulle et al. 1998). The CA3 pyramidal neurons are also vulnerable to repetitive high-frequency stimulation and network hyperactivity *per se*.

KA-mediated synaptic responses have been characterized for GluR6 and KA2 –containing synapses between CA3 pyramidal cells and Mossy fibers (Castillo, Malenka & Nicoll 1997, Mulle et al. 1998) (Fig 6). Postsynaptic KARs seem to have restricted and specific subcellular localization patterns, since many other areas, including CA1 pyramidal neurons fail to fire KAR-mediated EPSCs despite expressing functional KARs (Bureau et al. 1999).

The chronic phase of epileptogenic activity has been proposed to arise from axonal sprouting of the dentate granule cells. The mossy fibers have been shown to form new synapses to aberrant targets, including CA3 pyramidal cells and granule cells, thus recreating recurrent, synchronized excitatory circuits that include KAR EPSCs and exhibit reduced seizure threshold (Epsztein et al. 2005).

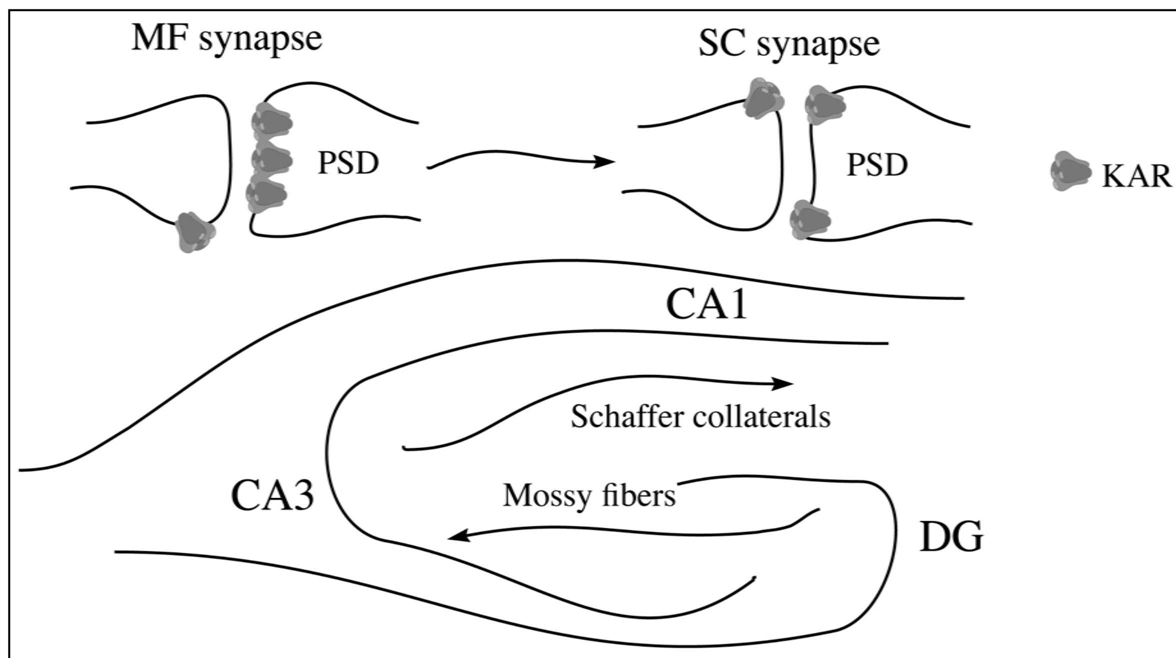


Figure 6 Hippocampal connections. KARs at the MF synapses are often located to the post-synaptic density while SC synapses have been found to contain extrasynaptic KARs. In addition, KARs can be found from the presynaptic areas where they might function as autoreceptors. The lower figure shows the Mossy fiber and Scaffer collateral pathways. Abbreviations: CA: Cornu Ammonis; DG: Dentate Gyrus; KAR: Kainate receptor; MF: Mossy fiber; PSD: Post-synaptic density; SC: Schaffer collateral.

Apart from its epileptogenic properties KA can be considered as a model for excitotoxicity. KA-induced excitotoxicity triggers at least apoptotic and necrotic cell death as well as PCD and autophagy (Wang et al. 2005). The type of cell death and damage caused by KA is dose-dependent as well as influenced by the extent and duration of seizures (Tokuhara et al. 2007). Higher doses tend to induce necrosis while lower doses are characterized by PCD without apoptotic morphology. Moreover, kainate has been shown to mediate PARP-mediated cell death, parthanatos, in rat striatum and spinal cord neurons (Kuzhandaivel, Nistri & Mladinic 2010, Cosi et al. 2000). As mentioned earlier, AIF has been implicated in KA-mediated neuronal injury in the hippocampus. "Harlequin" (*Hq*) mice with reduced expression of AIF were studied for neuroprotection against NMDA- and KA-excitotoxicity (Cheung et al. 2005). AIF was shown to be involved in both NMDA- and KA-mediated cell death in cortical neurons and in hippocampal CA3 area *in vivo* as Hq mice showed increased resistance against hippocampal damage (Cheung et al. 2005). These data indicate strong involvement of AIF-mediated cell death pathway in KA excitotoxicity. Table 5 summarizes the involvement of certain cell death mediators in KA-induced excitotoxic cell death in the hippocampus.

Table 5 Selected cell death effectors and modulators in the KA model. The cell death mediators and effects studied in the hippocampal CA3 area unless otherwise stated.

Apoptotic effectors	**Mediator**	**Effect of KA *in vivo***	**Functional significance/ other remarks**	**Reference**
Pro-apoptotic	Caspase-3	Cleaved/activated 4-48h after KA in the hippocampus	Execution of apoptosis, colocalization with TUNEL positive neurons	(Henshall, Chen & Simon 2000, Korhonen, Belluardo & Lindholm 2001)
	AIF	Translocates to the nucleus	Reduction of AIF reduces hippocampal damage in Hq mice	(Cheung et al. 2005)
	Cytochrome c	Released from mitochondria 2-24h after KA	Activation of caspase cascade	(Henshall, Chen & Simon 2000, Henshall et al. 2001a)
	Caspase-2	Cleaved/activated immediately after KA in the hippocampus	Linkage to surface death receptor?	(Henshall et al. 2001)
	RAIDD	Increased after KA	Caspase-2 interacting death receptor adaptor	(Henshall et al. 2001)
	Caspase-8	Cleaved/activated after KA similar to caspase-2	Extrinsic apoptotic pathway Caspase-8 inhibitor reduced caspase-8, -9 and -3 activites	(Henshall et al. 2001, Henshall et al. 2001b)
	Fas	Upregulated after 4-24h KA	Death receptor pathway	(Henshall et al. 2001b)
	FADD	Upregulated after 4-72h KA	Fas/caspase-8 adaptor	(Henshall et al. 2001b)
	Bid	Cleaved immediately after KA (upto 72h)	Caspase-8 mediated cleavage – integration to cytochrome c release	(Henshall et al. 2001b)
	Caspase-9	Cleaved/activated 4-72h after KA	Initiator caspase, activated by the apoptosome	(Henshall et al. 2001a)
	Apaf-1	Binds to cytochrome c after KA, 0-24h	Apoptosome formation and caspase-9 activation platform	(Henshall et al. 2001a)
	Caspase-6	Cleaved/activated after 2-72h KA	Executor caspase, proteolyses e.g. lamin A	(Henshall et al. 2002)

	Bad	Dissociates from the molecular chaperone 14-3-3 after KA	Free Bad binds Bcl-X_L that binds and neutralizes Bax in unstressed conditions	(Henshall et al. 2002)
	Bax	Relocalizes to mitochondrial membrane after KA, increased after KA	Facilitation of cytochrome c release	(Henshall et al. 2002, Korhonen et al. 2003)
	Bim	Upregulated 4-8h after KA-induced seizure/ downregulated 12h after KA (Korhonen et al. 2003)	Binds the anti-apoptotic Bcl-w	(Shinoda et al. 2004, Korhonen et al. 2003)
	Hrk/DP5	Reduced after KA	Interacts with Bcl-2 and Bcl-X_L	(Korhonen et al. 2003)
	Puma	Inreased after 1h KA	Binds anti-apoptotic Bcl-2 binds and activates Bax. Deletion of *puma* protects against KA-induced hippocampal damage	(Engel et al. 2010)
	JNK, p38 MAPKs	Increased activation in the early time points upto 2h after KA	MAPK family kinases involved in cell death	(Jeon et al. 2000)
Anti-apoptotic	Akt	Phosphorylated/activated in the cortex	May induce Bad phosphorylation and sequestration by 14-3-3, providing protection in surrounding tissue	(Henshall et al. 2002)
	Bcl-w/Bcl2L2	Decrease after 6-24h KA	Pro-apoptotic BH3-only Bim binds and inactivates Bcl-w	(Murphy et al. 2007, Korhonen et al. 2003)
	Bcl-X_L	Downregulated after 6-24h KA	Normally binds and neutralizes pro-apoptotic Bcl-2 family proteins	(Korhonen et al. 2003)
	Bcl-2	Increased levels and phosphorylation after KA	Anti-apoptotic, phosphorylation inactivates anti-apoptotic functions	(Korhonen et al. 2003)
	XIAP	Biphasic regulation by KA; initial increase in CA3 after KA, absence from dying neurons	Protect against caspase-3, -7 and -8 activation	(Korhonen, Belluardo & Lindholm 2001)
	c-IAP-1/RIAP-2	Biphasic regulation by KA; initial increase in CA3 after KA, absence from dying neurons	Protect against caspase-3, -7 and -9 activation	(Belluardo et al. 2002)
	Bruce/Apollon	Downregulated by KA	Protects against cell death and caspase-3 activation	(Sokka et al. 2005)
	BDNF	Increased production after KA	Neuroprotective effect	(Castren et al. 1998)
	ERK1, 2	Increased activation upto 6h after KA	MAPK family kinases involved in survival	(Kim et al. 1994)

Abbreviations: AIF: Apoptosis Inducing Factor; Apaf-1: Apoptotic Protease-Activating Factor-1; Bad: Bcl-2 antagonist of cell death; Bax: Bcl-2-Associated X protein; Bcl-2: B cell lymphoma-2; Bcl-w/Bcl2L2: Bcl-2 like 2; Bcl-XL: Bcl-extra long; BDNF: Brain Derived Neurotrofic Factor; Bid: BH3 interacting domain death agonist; Bim: Bcl-2 interacting mediator of cell death; CA3: cornu ammonis 3; c-IAP-1/RIAP-2: Cellular Inhibitor of Apoptosis Protein-1/Rat Inhibitor of Apoptosis Protein-2; ERK: Extracellular signal-Regulated Kinase; FADD: Fas-associated protein with death domain; Hrk/DP5: HaRaKiri; JNK: c-Jun N-terminal Kinase; MAPK: Mitogen-Activated Protein Kinase; Puma: p53 promoter Upregulated Modulator of Apoptosis; RAIDD: Receptor interacting protein (RIP)-associated Ich-1/CED-3 homologous protein; TUNEL: Terminal deoxynucleotidyl transferase-mediated biotinylated UTP Nick End Labeling; XIAP: X-linked Inhibitor of Apoptosis Protein.

1.7 Cdk5 and cell death

Cyclin-dependent kinases (Cdk1-9) are a family of nine small (30-35kDa) serine/threonine kinases (Morgan 1997). Their functions are vast and diverse; however, most of the Cdks are closely related to cell cycle control. Dysregulation of Cdk function in proliferating cells can induce tumor formation, while terminal differentiation of neuronal cells is associated with Cdk inhibition and disappearance (Okano, Pfaff & Gibbs 1993). Distinctive features of Cdk activity include dependence on association with regulatory proteins, called cyclins, as well as regulatory phosphorylations and dephosphorylations (Morgan 1997).

Unlike most Cdks, Cdk5 (alternatively called neuronal cdc2-like kinase, tau protein kinase II, PSSALRE) is not directly involved in cell cycle; on the contrary, it has a myriad of functions in differentiated cells, especially in neurons (Dhariwala, Rajadhyaksha 2008). As other Cdks, Cdk5 activity is dependent on regulatory subunits. These activator proteins are, however, not cyclins, but neuronally expressed activator proteins, p35 and p39 (Tsai et al. 1994, Tang et al. 1995). In addition, truncated forms of the activator proteins, produced by calpain, p25 and p29 are strong Cdk5 activators (See chapter 1.7.5. Deregulation of Cdk5). Furthermore, as other Cdks, Cdk5 can be phosphorylated at Tyr-15 resulting in several-fold increase in the activity of Cdk5 (Zukerberg et al. 2000), however, this is not a functional necessity as for other Cdks. Active Cdk5 phosphorylates serine/threonine residues located just N-terminal to a proline-residue (belonging to a kinase family of proline-directed serine/threonine protein enzymes) in its substrates.

Although Cdk5 seems to have widespread expression, p35 and p39 show complementary spatial and temporal expression in the CNS (Zheng, Leung & Liem 1998) thus governing the prominent activity of Cdk5 in the CNS. p35 appears to be essential in the cerebral cortex, governing the laminar organization of neurons, while p39 has important roles in the cerebellum (Ko et al. 2001). In addition, p35 can substitute the absence of p39 whereas p39 can only compensate for some functions of p35. On the contrary, deletion of Cdk5 results in perinatal lethality, abnormal corticogenesis and cerebellar defoliation (Ohshima et al. 1996). Studies using conditional knockout of Cdk5 have provided more insights into Cdk5 functions in the adult CNS and indicated Cdk5 essential in neuronal migration in certain brain areas, including the cortex and cerebellum (Hirasawa et al. 2004).

Subcellular localization of Cdk5 and its activators contribute essentially to the substrate pool and, interestingly, p35 and p39 possess N-terminal myristoylation sites that link Cdk5 to the membranes (Asada et al. 2008). In addition, lysine cluster in the N-terminal p10 regions was also shown to act as a nuclear localization signal (NLS) when p35 and p39 are not myristoylated. Importantly, p35 and p39 exert major regulatory influence on Cdk5 activity due to their short half-lives of approximately 20-30 min in neuronal cultures (Patrick et al. 1998). p35 and p39 are degraded by the UPS and degradation involves an autoregulatory function mediated by Cdk5 kinase activity. Inhibition of Cdk5 activity by roscovitine, proteasome blockers or phosphorylation mutant of p35 increased the half-life of p35 underlining the importance of Cdk5 feedback loop (Patrick et al. 1998). Phosphorylation of p35 has been shown to affect the membrane association of Cdk5/p35 complex (Sato et al. 2007). Another important aspect of membrane association is that membrane-bound Cdk5/p35

complex seems to be inactive while cytosolic form is the active form (Zhu et al. 2005). In addition, membrane association appears to facilitate p35 and p39 degradation (Minegishi et al. 2010). On the contrary, p35 expression can be induced by neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Harada et al. 2001, Bogush et al. 2007, Ledda, Paratcha & Ibanez 2002).

1.7.1 Cdk5 in the synapse

During development, Cdk5 has been proposed to have pro-differentiation and anti-cell cycle roles (Cicero, Herrup 2005). Mechanistically, it appears that Cdk5 activity and/or Cdk5 subcellular localization are important for preventing re-activation of the cell cycle in postmitotic neurons. However, in the adult animals, the kinase has diverse roles in the synapses affecting a myriad of synaptic proteins (Rosales et al. 2000, Fu et al. 2001, Fu et al. 2005, Fu et al. 2007, Morabito, Sheng & Tsai 2004, Tomizawa et al. 2002, Tomizawa et al. 2003, Zhang et al. 2008, Hawasli et al. 2007). In higher level, Cdk5 has been proposed to control synaptic plasticity, learning, and memory (Hawasli, Bibb 2007, Lai, Ip 2009). In physiological conditions, Cdk5 regulates numerous proteins both at the pre- and postsynaptic parts of the synapses and, for instance, the cholinergic and the glutamatergic neurotransmitter systems (Fu et al. 2001, Hawasli et al. 2007). Cdk5 deregulation has been linked to neurodegenerative diseases such as AD (Weishaupt, Neusch & Bahr 2003, Kanungo et al. 2009).

Presynaptic regulation by Cdk5

The presynaptic terminal is the neuronal output station, where neurotransmitters are packed to synaptic vesicles, released to the synaptic terminal by exocytosis of the vesicles followed by endocytosis of the vesicles for local recycling. Cdk5 has been implicated as a major controller of the neurotransmitter release and determinant of silent synapses since silencing the synapse severely depletes Cdk5 levels (Kim, Ryan 2010). Endocytosis of synaptic vesicles is triggered by dephosphorylation of a group of phosphoproteins at the nerve terminals, collectively called the dephosphins (Cousin, Robinson 2001). This is accomplished by a single phosphatase, the Ca^{2+}-calmodulin dependent phosphatase, calcineurin (CaN). The reversal, namely phosphorylation of dephosphins, is regulated by Cdk5 that phosphorylates at least dynamin 1, synaptojanin, and phosphatidylinositol phosphate kinase Iγ (PIPK Iγ) (Tomizawa et al. 2003, Lee et al. 2005, Lee et al. 2004). This generates a cytosolic pool of dephosphins and prevents premature interactions with the plasma membrane (Cousin, Robinson 2001). Thus, phosphorylation of dephosphins by Cdk5 is needed to repress endocytosis and necessary for continued vesicle recycling and neurotransmitter release, in other words, resetting the mechanism for new rounds of activity (Tan et al. 2003).

In addition, synapsin I and MUNC18 have been characterized as Cdk5 substrates thus broadening Cdk5-dependent regulation of presynaptic nerve terminal to the control of exocytosis (Matsubara et al. 1996, Shuang et al. 1998).

Postsynaptic regulation by Cdk5

The dendritic spines make contacts with axonal terminals forming the input station or postsynaptic density at the synapse. The postsynaptic density is involved in postsynaptic potential formation and is composed of neurotransmitter receptors, ion channels, signaling, scaffolding, as well as cytoskeletal proteins facilitating in the process of neurotransmission. Cdk5 signaling has been implicated in numerous signaling pathways involved in synaptic plasticity in the dendritic spines (Barnett, Bibb 2011).

The postsynaptic density is an insoluble intracellular matrix rich in scaffolding proteins, such as PSD95, Shank, GKAP and Homer, involved in anchoring and clustering neurotransmitter receptors and modulation of the structure of the PSD (Sheng, Hoogenraad 2007). Cdk5 has been identified to phosphorylate PSD95 resulting in inhibition of ion channel and NMDA receptor multimerization and clustering (Morabito, Sheng & Tsai 2004). Cdk5-mediated phosphorylation suppressed PSD95 multimerization thus affecting the composition of the PSD. Recently, reduced Cdk5 activity was shown to induce PSD95 monoubiquitination and interaction with β-adaptin AP-2/clathrin adaptor complex (Bianchetta et al. 2011). PSD95 ubiquitination has been implicated to regulate NMDAR-induced AMPAR endocytosis, interaction of PSD95 with AP-2 providing a previously missing link in AMPAR regulation. In addition, these results link Cdk5 in AMPAR internalization and synaptic plasticity. Furthermore, Cdk5 was shown to regulate AMPA receptors via phosphorylation of the neuronal adherens junction protein δ-catenin (Poore et al. 2010), which is involved in cadherens-cytoskeletal interaction at the plasma membrane (Lu et al. 1999). Cdk5-mediated phosphorylation of δ-catenin affected its cellular localization and morphology of the synapse while inhibition of Cdk5 or expression of phosphorylation mutants of δ-catenin resulted in increase in GluR2 membrane localization and AMPAR/NMDAR ratio.

Apart from AMPAR regulation, Cdk5 is involved in NMDAR modulation. Cdk5 was shown to facilitate NR2B degradation by calpain while conditional Cdk5 knockout mice showed improved learning and plasticity resulting from increase in synaptic NR2B levels (Hawasli et al. 2007). The mechanism was further studied to involve PSD95-Src kinase interaction. Src-mediated phosphorylation of NR2B was shown to decrease its binding to AP-2 and inhibit endocytosis of NR2B-containing NMDARs (Zhang et al. 2008). Moreover, the NR2A subunit, important mediator of LTP, is a direct target for Cdk5 (Li et al. 2001a). Inhibition of Cdk5-mediated NR2A phosphorylation reduced LTP formation and inward currents induced by NMDA. In another study, NR2A phosphorylation by Cdk5 was shown to enhance NMDAR currents and inhibition of Cdk5 protected against ischemic insults decreasing ischaemia-induced EPSCs (Wang et al. 2003).

Furthermore, the type I mGluRs, mGluR1 and mGluR5, have been shown to be phosphorylated by Cdk5 (Orlando et al. 2009). The localization of mGluRs in the periferal parts of PSD is managed by their interaction with the scaffolding Homer –proteins (Fagni, Worley & Ango 2002). Cdk5 phosphorylates the Homer-binding domains of mGluR1 and -5 and this phosphorylation enhances their binding to Homer thus increasing their localization at the PSD and Homer-regulated trafficking (Orlando et al. 2009).

Another important protein present in the postsynaptic density is the Ca^{2+}/calmodulin-dependent kinase (CaMKII) that has been implicated as a Cdk5 target (Hosokawa et al. 2006). CaMKII is an important mediator of LTP and decreased Cdk5 activity was shown to facilitate CaMKII activation and long-term potentiation.

Cdk5 phosphorylates protein phosphatase inhibitor-1 (PPI) as well as its homolog DARPP32 that are activated by cAMP/PKA (Bogush et al. 2007, Bibb et al. 2001). Recently, Cdk5 was shown to regulate the cAMP signaling cascade and synaptic plasticity via modulation of cyclic nucleotide phosphodiesterase (PDE) that catalyzes the conversion of cAMP and cGMP to AMP and GMP, respectively (Guan et al. 2011). In this study, conditional knockout of Cdk5 in mice resulted in upregulation of PDE isoforms and inhibition of cAMP pathway. The deficiencies were, however, rescued by treating the mice with a PDE4-specific inhibitor, Rolipram, indicating an important role for Cdk5-dependent modulation of PDEs.

Taken together, Cdk5 regulates numerous receptors at the post-synaptic neuron and presents an essential control switch in synaptic function.

1.7.2 **Cdk5 in the nucleus**

Besides its synaptic functions, Cdk5 has been implicated in nucleus. The first nuclear substrate of Cdk5 was found to be the pro-survival transcription factor myocyte enhancer factor 2 (MEF2) (Gong et al. 2003) that is involved in reduction of dendritic spines (synaptic pruning) in interneurons in activity-dependent fashion (Flavell et al. 2006). Importantly, excitotoxicity-induced Cdk5 deregulation results in hyperphosphorylation of MEF2, which in turn induces its calpain-dependent cleavage followed by neuronal death (Gong et al. 2003). Furthermore, MEF2 inhibition by Cdk5/p25 has been proposed to mimic the autistic phenotype of Fragile X syndrome, an inherited disorder causing autism and intellectual disability, where loss of fragile X mental retardation protein (FMRP) is associated with reduction of MEF2-dependent synaptic pruning (Barnett, Bibb 2011).

Another nuclear target for Cdk5 is the histone deacetylace 1 (HDAC1) that catalyzes acetylation of histones thereby altering chromatin and access of transcription factors (Brehm et al. 1998). Cdk5/p25 expressing transgenic mice show inactivation of HDAC1 and cell cycle re-entry in combination of double-strand DNA breaks (Kim et al. 2008). On the contrary, gain-of-function of HDAC1 was neuroprotective against p25-induced neurotoxicity.

1.7.3 Deregulation of Cdk5

Activation of calpain occurs when intracellular calcium levels increase above controllable levels, for instance, after overactivation of glutamate receptors or by exposure to Aβ (Rami, Ferger & Krieglstein 1997, Saito et al. 1993b). Active calpain cleaves the Cdk5 activator proteins p35 and p39 generating C-terminal p25 and p29 peptides, respectively, that lack the myristoylation sites (Kusakawa et al. 2000, Patzke, Tsai 2002, Lee et al. 2000). These new forms of activators have distinct properties as compared to their full-length precursors, mainly, longer half-lives, stronger association with Cdk5 and resulting increased activity of Cdk5/p25 (or Cdk5/p29) complex (100% vrs 80% for p25 and p35 complexes, respectively) (Patrick et al. 1998, Amin, Albers & Pant 2002). Interestingly, p35 has three domains

involved in Cdk5 interaction and inactivation of any of these domains inhibited Cdk5 kinase activity (Amin, Albers & Pant 2002). Furthermore, p25 and p29, lacking the myristoylation sites, distribute to different subcellular sites in the cell, from periphery to the more perinuclear regions (Kusakawa et al. 2000, Asada et al. 2008, Patrick et al. 1999). Although certain Cdk5 substrates, such as tau, are increasingly phosphorylated in p25-transgenic (overexpressing) mice, the phosphorylation of the physiological substrates of Cdk5 is not increased (Cruz et al. 2003). In addition, mice expressing p25 for long periods showed impairment of hippocampal long-term potentiation (LTP) and memory deficits combined with neuronal loss (Cruz et al. 2003). These effects were reversed by transient expression of p25 in mice actually facilitating learning and memory (Fischer et al. 2005). Taken together, prolonged activity of Cdk5/p25 seems to underlie the switch from physiological to pathological function.

1.7.4 Cdk5 in neuronal cell death

Cdk5 has been implicated in cell death in AD as responsible for, for instance, hyperphosphorylation of the microtubule-associated protein (MAP) tau (Baumann et al. 1993). Hyperphosphorylation of tau causes its detachment from microtubules and accumulation in the cytoplasm as filaments and tangles (Alonso et al. 2001). An interesting link between Cdk5, tau phosphorylation and calcium transfer from the ER to mitochondria was made in a study showing that Cdk5-mediated phosphorylation of tau at T231 during ceramide neurotoxicity enhanced its detachment from microtubules resulting in microtubule-dependent clustering of the ER and mitochondria in the centrosome region (Darios et al. 2005). Close contacts between the organelles allowed calcium transfer from the ER to mitochondria resulting in neurotoxic levels and cell death activation (Darios et al. 2005). This study showed a In addition, p25 has been shown to accumulate in AD brains (Patrick et al. 1999). In PD the Lewy bodies have been shown to contain Cdk5 and p35 (Nakamura et al. 1997). Cdk5 deregulation has been shown to occur in HD; in mutant Hdh^{Q111} mice as well as human postmortem brain (Paoletti et al. 2008). Importantly, inhibition of Cdk5 was neuroprotective against neuronal loss in mutant Hdh^{Q111} mice (Paoletti et al. 2008).

Cdk5 is a major regulator of cell survival and cell death. Inhibition of Cdk5 during development results in failure to neuronal differentiation and migration (Cicero, Herrup 2005). In addition, these cells, despite having neuronal morphology, expressed both cell cycle and cell death markers indicating that Cdk5 deficiency accompanies failure to exit the cell cycle. Thus, Cdk5 seems to play both the roles of maintenance of mitotic arrest and progression of neuronal differentiation. The mechanism by which Cdk5 regulates cell cycle has been linked to its binding of several cyclins and the retinoblastoma protein (Lopes, Oliveira & Agostinho 2009).

Cell cycle reentry has been observed in several neurological disorders, including AD, ALS, and stroke (Andorfer et al. 2005, Ahn et al. 2008, Nguyen et al. 2003, Wen et al. 2005). Excitotoxicity, oxidative stress and DNA damage are linked to re-expression of cell cycle proteins (Lopes, Agostinho 2011) and it remains to be determined how powerful player Cdk5 is in all these diseases and cell death mechanisms. One link might be previously described Cdk5-mediated HDAC1 inactivation in p25-transgenic mice (Kim et al. 2008) since p25

accumulation coincides many neurodegenerative disorders. Moreover, for instance, in a mutant SOD1 mouse model of ALS, Cdk5 was linked to increased Cdk4 expression via induction of Aβ, while the changes were prevented when Cdk5 was inhibited (Nguyen et al. 2003).

1.7.5 Inhibition of Cdk5 as a therapeutic tool

Taken its important and broad functions in neurons, Cdk5 has been a subject of a number of studies aiming to modulation of its activity. Many investigations are based on pharmacological or genetic inhibition of Cdk5. Generally used inhibitors, Roscovitine and Olomoucine resemble ATP and antagonize its binding to Cdks, especially Cdk2, Cdk1 and Cdk5 (Schmid, Strosznajder & Wesierska-Gadek 2006). In neurons, where expression and function of cell cycle-related Cdks are minimal roscovitine can be used to primarily target Cdk5. However, for *in vivo* studies, more drugs selectively targeting Cdk5 are required. Despite this, roscovitine has shown potential neuroprotective effects in *in vitro* (Maas et al. 1998) and *in vivo* studies of tauopathies and stroke (Wen et al. 2007, Menn et al. 2010). Indolinone A is another Cdk inhibitor implicated in neuroprotection via prevention of mitochondrial dysfunction (Weishaupt et al. 2003). Indolinone-mediated Cdk5 inhibition was effective against various cell death models, including growth factor withrawal and staurosporine treatment (Weishaupt et al. 2003).

Genetic inhibition of Cdk5 includes antisense and siRNA-based methods as well as expressing dominant negative mutant of Cdk5 (DN-Cdk5) (Tsai et al. 1994, van den Heuvel, Harlow 1993). Furthermore, inhibition of Cdk5/p25 complex by an inhibitory peptide (CIP; Cdk5 inhibitory peptide) has shown promising results in cultured neurons and is also studied for *in vivo* applicability (Kanungo et al. 2009, Zheng et al. 2002).

1.8 Huntington’s disease and cell death

HD is a progressive neurodegenerative disorder characterized by motor, cognitive and psychiatric disturbances, first described by George Huntington in 1872 (reprinted in Huntington 2003). HD is caused by an unstable CAG expansion in the exon-1 of the *huntingtin* gene (*HTT, IT-15*) inherited in an autosomal dominant manner. In humans, the non-disease causing number of CAG repeats varies between 6 and 35 whereas in HD patients the number raises usually over 40 repeats (The Huntington's Disease Collaborative Research Group 1993). Due to the unstable nature of the mutation, the disease progression, age of onset, and severity varies greatly between individuals but correlates inversely to the size of the expansion. The size explains approximately 70% of the variance of the age of onset (Brinkman et al. 1997) and several other genes have been proposed to modulate the disease onset (Li et al. 2003, Wexler et al. 2004). Analysis of postmortem brains of HD patients shows neuropathological changes particularly in the striatum (Reiner et al. 1988) but also in other brain areas, such as the cortex, thalamus and cerebellum (Walker 2007).

The *HTT* gene encodes for a 350kDa protein, huntingtin (Htt), that has several subdomains and functions, although the precise physiological role of the protein remains unclear (Bano et al. 2011) (Fig 7). Htt is ubiquitously expressed, however, particularly in mature postmitotic neurons (Strong et al. 1993). In neurons, Htt is found in the nucleus, Golgi, ER, neuronal

body, synapses and dendrites (DiFiglia et al. 1995). Characterization of the structure and function of huntingtin and mutant huntingtin have revealed important functional properties that underlie the disease pathogenesis. In humans, the CAG repeat encoding for a polyglutamine (polyQ) stretch has a potential to associate with membranes (Atwal et al. 2007). In addition, the polyQ stretch associates with the ER and Htt was predicted to be released from the ER in response to ER stress (Atwal et al. 2007). The polyQ-domain is followed by a polyproline sequence that has a stabilizing function in the protein structure. Furthermore, the N-terminal part of the protein contains three clusters of HEAT repeats (acronym for proteins that include the domain: Huntingtin, Elongation factor 3 (EF3), Protein phosphatase 2A (PP2A), and the yeast PI_3-kinase TOR1) that interact with other proteins and are involved in intracellular transport (Fig 7).

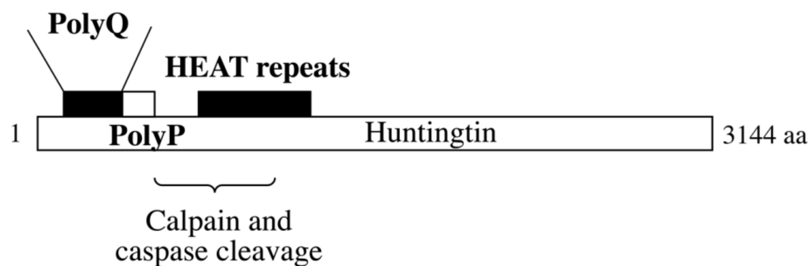


Figure 7 Schematic presentation of huntingtin domains and sites for calpain and caspase cleavage. Abbreviations: HEAT: Huntingtin, Elongation factor 3, Protein Phosphatase 2A, yeast PI3-kinase TOR1; polyQ: polyglutamine repeat, polyP: polyproline repeat.

The normal function of Htt has been studied, for instance, by deleting the gene in mice. Full deletion results in embryonic lethality due to extensive apoptosis indicating an anti-apoptotic function for Htt (Nasir et al. 1995). In addition, heterozygous knockout animals are characterized by increased apoptosis of neurons in the basal ganglia combined with cognitive deficits (Nasir et al. 1995). Later it was shown that Htt binds and inhibits caspase-3 and also activates the pro-survival pathway mediated by the serine/threonine kinase Akt (Zhang et al. 2003, Humbert et al. 2002).

The expansion of the polyQ stretch alters the properties of Htt. This mutation is a characteristic gain-of-function mutation, since the mutant Htt (mHtt) downregulates the wild-type Htt functions. Why has the polyQ expansion evolved in the first place? Evidence for evolutionary positive selection for expansion of the polyQ stretch came from studies where the murine *Htt* gene carried only seven CAG repeats (Clabough, Zeitlin 2006). These mice exhibited slight memory and learning deficits combined with alterations in mitochondrial energy metabolism, indicating that elongation of the polyQ stretch is essential for the protein activity.

Pathological elongation of the CAG repeat in the *Htt* gene renders the mHtt protein aggregation-prone and HD is characterized by intracellular inclusions containing mostly N-terminal fragments of the mHtt protein (DiFiglia et al. 1997). These inclusions intriguingly affect mostly the GABAergic medium spiny neurons of the striatum (Martindale et al. 1998, Ferrante et al. 1985). Degeneration of these neurons is thought to underlie the development of uncontrolled movements during the disease progression. The biochemical properties of the

medium spiny neurons make them vulnerable to cell death in HD; in addition to GABA, these neurons express, for instance, NMDA, AMPA and KA receptors and receive glutamatergic inputs from the motor cortex and thalamus (Mori et al. 1994, Stefani et al. 1998). Consequently, one of the contributors of the disease pathogenesis is thought to be excitotoxicity, as will be explained in more detail.

1.8.1 Cell death mechanisms and contributors of HD pathogenesis

Several mechanisms have been proposed to mediate cell death in HD. These include, among others, excitotoxicity, disturbances in the function of the UPS and mitochondrial energy production, autophagy, inflammation, defective trophic factor support and ER stress.

Before the underlying cause of HD was characterized, one of the hypotheses behind the disease was proposed to be excitotoxicity (Coyle, Schwarcz 1976, McGeer, McGeer 1976). Treatment of rats or mice with KA developed motor abnormalities and memory deficits accompanied with striatal lesions resembling those seen in human HD patients (Sanberg, Lehmann & Fibiger 1978, Mason, Fibiger 1979). Later, the excitotoxin quinolinic acid was found to produce even more resembling characteristics of HD (Beal et al. 1986). Despite the errorneous assumptions of these excitotoxins being the cause of HD, glutamate excitotoxicity is still considered a major mechanism in the course of HD pathogenesis.

As previously explained, excitotoxicity induces calcium influx that can further activate calpain. Calpain has been shown to proteolyse Htt into a series of fragments (Gafni, Ellerby 2002). It was also noted that the cleavage of Htt is dependent on the polyQ repeat length. These fragments, along others produced by caspases (Wellington et al. 2002) and matrix metalloproteinase (MMP) (Miller et al. 2010) are found in the intracellular inclusions in the affected neurons (Bano et al. 2011). However, the more toxic functions of these fragments are thought to be produced during their monomeric states and the aggregates serve more likely as to scavenge the toxic oligomers.

The ubiquitin-proteasome system (UPS), being responsible for protein degradation of numerous proteins, especially, those abnormally assembled, was found to be dysfunctional in HD (Bence, Sampat & Kopito 2001). Cells expressing high levels of a Htt construct encoding a polyQ stretch of 103Q accumulated high molecular weight ubiquitin conjugates indicating inhibition of the proteasomal machinery (Bence, Sampat & Kopito 2001). One reason for this is the scavenging properties of the inclusion bodies, found to contain ubiquitin, other components of the UPS, in addition to molecular chaperones, cytoskeletal proteins and even transcription factors (DiFiglia et al. 1997, Davies et al. 1997). Thus, dysfunction of the UPS and accumulation of proteins that normally would be degraded by the proteasome definitely influence the pathogenesis of HD. Furthermore, the activity of the UPS slows down simply during ageing (Keller, Hanni & Markesbery 2000, Keller, Huang & Markesbery 2000) and might thus contribute to the age of onset of the disease.

Mitochondrial deficiency has been implicated in HD. Investigations have revealed that the aggregates are associated with organelles, especially, with mitochondria resulting in disruption of the organelle homeostasis (Li et al. 2001). Dysregulated mitochondria show

reduction in Ca^{2+} uptake (Panov et al. 2002) possibly enhancing the activation of calpains and other calcium-regulated pathways.

Neurotrophic factor support is an essential survival mechanism for neurons and has been considered as a treatment for neurological diseases, including HD (Sari 2011). Mutant Htt has been shown to disrupt BDNF signaling both in transcriptional level as well as via altered axonal transport (Zuccato et al. 2001, Gauthier et al. 2004). BDNF transport from cortical afferents to the striatum is impaired in HD and, thus, considered as one important mediator of striatal cell death in HD.

Despite enormous efforts made to clarify the mechanisms behind HD pathogenesis, it remains to be shown how all the participating factors contribute to the disease. Although the medium spiny neurons of the striatum are the most clearly affected, they are not the only ones. Other areas of the brain might be better protected due to the lack of, for example, receptor proteins that might contribute to the excitotoxic effects of neurotransmitters. In fact, it was hypothesized that striatal neurons are vulnerable due to a striatal-specific processing machinery (Li et al. 2000). Furthermore, the expression of Htt is high in the striatum and in the cortico-striatal pathway and is likely especially important for these particular neurons.

2 Aims

The ***general aim*** of this study was to characterize the molecular mechanisms of organelle specific cell death initiation. Special attention was paid to the roles played by the ER and involvement of Cdk5 in signalling pathways. The ***specific aims*** were:

- To study the role of the ER in KA-induced excitotoxic cell death (I)
- To characterize synaptic degeneration and regulation of GluR6 in KA-induced excitotoxic cell death and the role of Cdk5 in regulation of these processes (II)
- To study the role of the ER in a cell model of Huntington's disease (III)

3 Materials and methods

3.1 Animals and kainic acid injections (I-II)

The animal experiments were approved by the ethical committee and carried out in accordance with the European Communities Council Directive (86/609/EEC). Adult male Wistar rats weighing 200-300 g (B&K, Hull, UK) were used in experiments. Prior to KA injections the rats were anaesthetized with ether and placed in a David Kopf stereotaxic apparatus using coordinates AP=-0.85, L=4.3 from the Bregma. Status epilepticus was induced by injecting 0,175 µg KA (0.35 µg/µl in saline; Sigma, Finland) into the lateral ventricles of the rat in a total volume of 0.5 µl per side. The rats were subsequently killed by decapitation in deep anesthesia at time-points ranging from 3 to 72 h. Control rats received equal volume of saline. All experimental groups consisted of at least quadruplets. The brains were rapidly dissected, frozen in isopentane, cooled in liquid nitrogen and stored at −70 °C until analysis. The right side of the brain was used for histology and the left for Western blot analyses.

In some experiments, Salubrinal (Sal) was given prior to KA injections (I). Sal (Calbiochem) was dissolved in dimethylsulfoxide (DMSO; Sigma, Finland) and diluted in saline. Sal was given either intracerebroventricularly (1 µl) with a 75 µM solution or intraperitoneally (0.1 ml) with 1 mg/kg. Control groups were given equal amounts of DMSO.

3.2 Hippocampal neuronal cultures, treatments and transfections (I-II)

Hippocampal neurons were prepared from embryonic, E17-old Wistar rats (Harlan, Holland). Pregnant rats were anaesthetized by carbon dioxide and killed by cervical dislocation or decapitation. The embryos were immediately collected to sterile phosphate buffered saline (PBS) and placed on ice. The hippocampi were isolated from the brains and incubated with papain and cystine (0,5 mg/ml and 0,2 mg/ml respectively; Sigma; in PBS containing 1 mg/ml BSA) at +37 °C for 15 min. The cells were gently triturated in RT in the presence of 20 µg/ml DNase (Sigma), pelleted by centrifugation and resuspended in Neurobasal medium (Invitrogen) containing 0.5 mM GlutaMAX (Invitrogen), 1 % PenStrep and 2 % B27 supplement (Gibco). The initial plating medium contained 25 µM glutamate to induce neurite outgrowth and was replaced with normal medium after 4-6 h. The neurons were plated on poly-ornithine (Sigma) coated 6-well plates at a density of 1.8×10^6 cells or 150-180x10^3 cells on poly-ornithine coated cover slips on 24-well plates, and cultured for 7 days.

KA treatment. Neurons were treated with different concentrations of KA for varying periods of times as indicated (I-II). Cell death was induced preferentially with 100 µM KA that induces death of approximately 40-50 % of the neurons within 24 h.

Treatments with other chemicals. I: In addition to KA, neurons were in some cases stimulated with glutamate (75-300 µM; Sigma) for various time periods. In some experiments the ER stress inhibitor Sal (50 µM), the glutamate receptors inhibitor, Kynurenic acid (1 mM; Sigma), and the NMDA receptor blocker MK801 (10 µM; Sigma) were added to cells 30 min before KA. To reduce calcium increase after KA 50 and 20 µM BAPTA-AM (1,2-bis(o-

aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester; Sigma) was added 2 h and 30 min before KA, respectively. In some experiments, Brefeldin A (0,5 μg/ml, 1h; Sigma) was used as a positive control for ER stress. II: In some experiments, 1-10 μM BAPTA-AM, 2 mM EGTA (glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; Sigma), the Cdk5 inhibitor roscovitine (20 μM; Sigma), the calpain inhibitor XI (20 μM; Z-Leu-Abu-CONH(CH2)3-morpholine; Calbiochem) were added 10-60 min before KA.

Modulation of Cdk5 in neurons (II). To downregulate Cdk5, 5×10^6 neurons were transfected with 100 nM siRNA constructs against Cdk5 (On TargetPlus Smartpool, Dharmacon) using the Amaxa Rat Neuron Nucleofector system (Amaxa GmbH, Germany) according to manufacturer's protocol. As control, a negative control siRNA (Mission® siRNA; Sigma) was used. Cdk5 activity was alternatively inhibited using expression plasmids containing dominant-negative construct of Cdk5, DN-Cdk5, with a mutation in the active site (D144N) and linked to HA (hemagglutinin) epitope (van den Heuvel and Harlow, 1993; Addgene) or to EGFP (enhanced green fluorescent protein; courtesy by Dr Tsai; Addgene). The DN-Cdk5-HA or DN-Cdk5-EGFP were transfected into hippocampal neurons as above, and the vectors containing HA- or EGFP were used as controls. siRNA-treated neurons were cultured for 3 d, while DN-Cdk5 –transfected neurons were cultured for 5-7 d and stimulated with KA.

3.3 PC6.3 cell cultures, transfections and treatments (III)

PC6.3 is a neuronal-like subline of rat PC12 pheochromocytoma cell line that can be differentiated by and made dependent on NGF. PC6.3 cells were cultured on Nunc dishes in RPMI-1600 (Biochrom) medium supplemented with 10 % horse serum and 5 % fetal calf serum, 7.5 % $NaHCO_3$, 100 mM GlutaMAX (Gibco) and 100 mM penicillin-streptomycin (Gibco).

Transfections. Cells were transfected with expression vectors encoding for different CAG-repeat lengths (18, 39, 53 or 120) of huntingtin exon-1 fused to EGFP (Hasholt et al. 2003). Transfectin reagent (BioRad) was used following the instructions provided by the vendor using 0.5 μg DNA per 24 well plates, and 4 μg per 6 well plates. Controls were transfected with EGFP expression plasmid (Clontech) and cells were incubated for 24–30 h prior to analysis. In some experiments 5 μM Sal was added 2 h after transfection. In addition, 200 ng/ml cycloheximide (CHX, Calbiochem), inhibiting protein synthesis was used in certain experiments. For positive controls, ER stress was induced by 0.5 μg/ml Brefeldin-A (Sigma) or cellular stress with the general kinase inhibitor Staurosporine (1 μM; STS, Sigma).

3.4 Calcium imaging (I, II)

I: Transient Ca^{2+} responses were studied with conventional wide-field fluorescence microscopy integrated in CellR Olympus (Tokyo, Japan) microscopy station. Cells were pretreated with vehicle (1% dimethylsulfoxide) or 50 μM Sal for 30 min (short-term treatment) or for 20–24 h (long-term treatment). After washing the cells were loaded with fura-2 acetoxymethyl ester (2 μM; Sigma) for 40 min at RT. 50 μM KA or 50 μM glutamate was applied for 1 min through a bath perfusion system. Ca^{2+} transient responses were measured after background subtraction as the ratio between the fluorescence intensity at 340 and 380 nm, respectively. Plots were made using Microcal (Northampton, MA) Origin 6.0

software. Peak amplitude of Ca^{2+} responses was calculated as mean amplitude of the five time points after agonist application.

II: Hippocampal neurons were loaded with 4 μM fura-2 acetoxymethyl ester (Invitrogen, Carlsbad, CA, USA) for 20 min at 37 °C in Hepes-buffered medium (HBM: 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 10 mM glucose, 20 mM Hepes, pH7.4), rinsed once, and studied immediately. Ca^{2+} measurements were performed at 35 °C using Nikon TE2000 fluorescence microscope (20×/0.75 air objective) and Andor iXon 885 EM-CCD camera under the control of Nikon NIS Elements AR software with 6D extension. For Ca^{2+} imaging, the cells were excited with alternating 340 and 380 nm light (Sutter DG4 Plus) and the emitted light collected through a 400 nm dichroic mirror and a 450 nm long-pass filter. DN-Cdk5-EGFP transfected cells were selected based upon GFP-fluorescence. Different concentrations of KA were added by constant perfusion (HBM). Regions of interest (cell bodies) were defined in NIS software and the data extracted to Microsoft Excel for visualization and quantitation. 19-70 cells were measured in each experiment, and each experiment was repeated altogether 4 times or more.

3.5 Antibodies (I-III)

Target protein	Species	Dilution	Origin
Actin (I-III)	Rabbit	1:2000-5000 (WB)	Sigma
AIF (I, III)	Goat	1:1000	Santa Cruz Biotechnologies
ATF6 (I, III)	Mouse	1:100 (ICC)	Pierce
BiP (I, III)	Mouse	1:1000 (WB); 1:100 (ICC)	BD Biosciences
Caspase-12 (I, III)	Rabbit	1:1500	Chemicon
Caspase-3 (I, III)	Rabbit	1:400 (WB)	Cell Signaling
Cdk5 (II)	Mouse	1:2000 (WB)	Millipore/Upstate
Chop (I, III)	Mouse	1:250-500 (WB); 1:100-200 (ICC)	Santa Cruz Biotechnologies
Cleaved (active) caspase-3 (I, III)	Rabbit	1:150-200 (ICC)	Cell Signaling
Cytochrome c (III)	Mouse	1:1000 (WB); 1:200 (ICC)	BD Biosciences
eIf2α (I, III)	Rabbit	1:1000 (WB)	Cell Signaling
GFP (III)	Mouse	1:5000 (WB)	Roche
GluR6/7 (II)	Rabbit	1:1000 (WB); 1:100 (ICC)	Millipore/Upstate
Hsp60 (III)	Mouse	1:1000	BD Biosciences
NeuN (II)	Mouse	1:100 (ICC)	Millipore/Chemicon
p-c-jun (I, III)	Rabbit	1:1000	Cell Signaling
p-Cdk5 (II)	Goat	1:1000 (WB); 1:100 (ICC)	Santa Cruz Biotechnologies
p-eif2α (I, III)	Rabbit	1:1000 (WB); 1:200 (ICC)	Cell Signaling
p-JNK (III)	Rabbit	1:250 (WB)	Cell Signaling
p-PERK (I, III)	Rabbit	1.500 (WB); 1:100 (ICC)	Santa Cruz Biotechnologies
p35/25 (II)	Rabbit	1:1000 (WB)	BD Biosciences

PARP (III)	Rabbit	1:2500	Cell Signaling
PERK (I, III)	Rabbit	1:500	Santa Cruz Biotechnologies
PSD95 (II)	Mouse	1:2000 (WB); 1:500 (ICC)	BD Biosciences
Synapsin I (II)	Rabbit	1:500 (ICC)	Nordic Biosite/Covance
α-Spectrin (II, III)	Mouse	1:2000 (WB)	Chemicon

3.6 Western Blotting (I-III)

For Western blotting, hippocampal tissue, neurons or PC6.3 cells were rinsed twice with ice-cold PBS and lysed in RIPA buffer (150 mM NaCl, 1 % Triton-X-100, 0.5 % sodium deoxycholate, 1 % SDS, 50 mM Tris-HCl pH 7.4) supplemented with protease inhibitor cocktail (Roche, Espoo, Finland) and phosphatase inhibitors (PhoStop; Roche, Espoo, Finland). Equal amounts of protein were subjected to SDS-PAGE and blotted onto nitrocellulose filters (Amersham, Helsinki, Finland), which were incubated for 1 h in 5% skimmed milk or BSA, in TBS-T (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 % Tween 20), and then with primary antibodies overnight at 4 °C. After washing the filter was incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,500; Jackson ImmunoResearch Laboratories, Espoo, Finland), followed by detection using enhanced chemiluminescence (Pierce, Helsinki, Finland). Quantifications were done using the ImageJ software (version 1.41o).

3.7 Immunochemistry (I-IV)

In vivo: 10 μm rat brain sections were fixed for 10 min at −20 °C using acetone-methanol (1:1). Slides were incubated for 1 h with phosphate-buffered saline (PBS)-5 % Bovine Serum Albumin (BSA; Sigma) -0.1 % Triton-X-100 at room temperature, and then overnight at +4 °C with primary antibodies. The nuclei were counterstained with bisbenzimide/Hoechst 33258 (Sigma).

In vitro: Neurons and PC6.3 cells were fixed for 20 min using 4 % paraformaldehyde, incubated for 1 h using PBS-5 % BSA-0.1 % Triton-X and stained as above with primary and secondary antibodies followed by counterstaining of the nuclei with Hoechst 33258. Stainings were visualized by fluorescent microscope (Leica DM4500B, Espoo, Finland).

3.8 Cell degeneration assays (I-III)

In vivo:

(I-II) *Fluoro-Jade staining*. Frozen 10 μm sections were immersed for 5 min in 1 % sodium hydroxide/80 % ethanol, rinsed for 2 min in 70 % ethanol, and then rinsed in distilled water. Potassium permanganate solution (0.06 %) was added for 10 min, followed by a 10 min incubation in 0.0001 % Fluoro-Jade C (Chemicon). After washing, the sections were air dried for 10 min at 50 °C, cleared in xylene, and mounted in dibutyl phthalate xylene (Sigma).

(I) *Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining*. Frozen sections were fixed for 10 min with acetone : methanol (1:1) at −20 °C and permeabilized with 0.1 % Tween-20/1 % bovine serum albumin/phosphate-

buffered saline on ice. Fluorescein-labelled UTP (Roche, Germany) was added and sections incubated for 1 h at +37 °C, washed and studied under a microscope.

In vitro:

(I-II) *Hoechst staining.* Neuronal cell death after 24 h treatment with 100 μM KA was determined by staining hippocampal neurons with Hoechst blue (Sigma) and counting nuclei with fragmented/condensed nuclei. Similarly, neurons expressing DN-Cdk5-EGFP or EGFP were studied for nuclear condensation/fragmentation (II). Alternatively, neurons were stained with propidium iodide (Sigma), which penetrates cells with plasma membrane breaks (I).

(I) MTT assay. In some experiments, MTT (Sigma) assay was used for cell viability. Cells on 96-well plates were incubated for 2 h at +37 °C in the presence of MTT solution (Thiazolyl blue tetrazolium bromide; Sigma) after which the medium was removed. 40 mM HCl in isopropanol was added to solubilize the dye absorbed by the cells. The amount of the dye was measured by absorbance at 560 nm with Labsystems Multiskan MS spectrophotopmeter. The absorbance was linear to cell viability.

(III) *PC6.3 cells transfected with huntingtin exon-1 –EGFP contructs.* Cells were examined under a Zeiss fluorescence microscope and the number of transfected cells with fragmented or condensed nuclei was counted as an estimate of cell death. In each experiment, approximately 100 cells in four different fields per well using triplicates cultures were counted, and experiments were repeated at least three times. Results are expressed as percentage of mock-transfected controls.

3.9 Confocal imaging (II-III)

Neurons were analyzed at RT with a confocal microscope (LSM 510 Meta; Carl Zeiss, Inc.) using LSM AIM software and 63×/1.4 Plan-Apochromat oil objective. Images were processed using ImageJ and Adobe Photoshop CS softwares.

3.10 PCR and Quantitative PCR (I, III)

RNA was prepared from hippocampal neurons and cDNA synthesized using 50 U of SuperScript II reverse transcriptase and components given by the vendor (Invitrogen). Quantitative PCR was performed using LightCycler (Roche) and the following primers:

BiP (I, III):	forward 5′-AAG GTG AAC GAC CCC TAA CAA A-3′,
	reverse 5′-GTC ACT CGG AGA ATA CCA TTA ACA TCT-3′;
Chop (I, III):	forward 5′-GCC TTT CGC CTT TGA GAC AGT-3′,
	reverse 5′-TGA GAT ATA GGT GCC CCC AAT T-3′;
ATF4 (I):	forward 5′-CTA CTA GGT ACC GCC AGA AG-3′,
	reverse 5′-GCC TTA CGG ACC TCT TCT AT-3′;
β-actin (I, III):	forward 5′-CAC ACT GTG CCC ATC TAT GA-3′,
	reverse 5′-CCA TCT CTT GCT CGA AGT CT-3′.

Amplification was performed using an initial 10 min step at 95 °C, followed by 50 cycles with 15 s at 95 °C, 5 s at 60 °C, and 4–12 s at 72 °C, and with a final extension for 10 min at

72 °C. Specificity of the product was confirmed by melting-curve analysis. Quantification was done from data on the reaction kinetics, and expression levels were related to β-actin. To study splicing of *XBP*, reverse transcription-PCR and the following primers were used: forward 5′-AGAGTAGCAGCACAGACTGCGCG-3′; reverse 5′-GGAACTGG-GTCCTTCTGGGTA-3′.

3.11 Analysis of ER fragmentation (I)

Hippocampal neurons were loaded for 30 min with 100 nM Blue-white ER-tracker (Invitrogen, Espoo, Finland). Different concentrations of KA or glutamate were added for 60 min after medium change. Cells were studied under a fluorescent microscope (Leica, Espoo, Finland) using UV filters. In addtion, cells were also stained using an anti-inositol-3-phosphate receptor (IP_3-R) antibody as described in immunochemistry (1:100; Chemicon).

3.12 Solubility assay (III)

Cells were lysed in buffer containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 3 mM EGTA, 0.5 % Triton X and protease inhibitors (Roche) and kept for 5 min on ice and suspended in 3 volumes of SDS loading buffer to obtain the total cell lysate as described previously. In order to detect high molecular weight forms of the huntingtin fragment proteins, we also blotted the stacking gel after the run by which protein aggregates in cells can be resolved.

3.13 Subcellular fractionation (III)

PC6.3 cells were harvested by scraping, washed twice in ice-cold PBS, and resuspended in 600 μl of homogenization buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM NaEDTA, 1 mM NaEGTA, and 1 mM dithiothreitol) containing 250 mM sucrose and a mixture of protease inhibitors (Roche). After 30 min of incubation on ice, cells were homogenized using a Dounce homogenizer (type B pestle, 30 strokes). Unbroken cells, and nuclei were collected by low speed centrifugation, and the resulting supernatant was centrifuged at for 20 min at 10,000 g to obtain mitochondria, followed by centrifugation of the supernatant for 1 h at 100,000 ×g. The membrane fraction obtained called P3 contains the ER and other cell membranes, and the remaining supernatant represents the cytosolic fraction. The mitochondrial and P3 membrane fractions were washed three times in homogenizing buffer, and then solubilized in a buffer containing 10 mM Tris acetate, pH 8.0, 0.5 % Nonidet P-40, 5 mM $CaCl_2$ and protease inhibitors. The purity of cell fractions was analyzed using antibodies against Hsp60 as a marker for mitochondria, and against inositol- (1,4,5) triphosphate receptor (IP_3-R) as a marker for ER.

3.14 Surface biotinylation assay

Cell surface proteins were studied using EZ-Link® Sulfo-NHS-SS-Biotin (Pierce) label. Hippocampal neurons were treated with KA and in some experiments roscovitine or transfected with DN-Cdk5. Treated neurons were transferred on ice to stop endocytosis. After washing with PBS, the cells were biotinylated with freshly prepared, membrane impermeant Sulfo-NHS-SS-Biotin (0,2 mg/ml in PBS) for 1 h. Excess Sulfo-NHS-SS-Biotin was quenched by washing three times in 50 mM Tris, pH 8,0. Cells were then lysed in extraction buffer containing 10 mM Tris-HCl, pH 7,5, 10 mM EDTA, 1 % Triton X-100, 0.1 % SDS, supplemented with protease and phosphatase inhibitors (Roche). After brief sonication and

centrifugation (14 000 rpm, 10 min at +4 °C) the supernatants were collected. Equal amounts of proteins were incubated 1 h with streptavidin beads (Pierce) to pull down biotinylated proteins. After washing extensively with extraction buffer, reducing sample buffer was added to the streptavidin beads and boiled for 5 min to elute bound proteins. The samples were analysed by Western blotting with antibodies for GluR6/7. GluR6/7 levels were compared to the levels of GluR6/7 vs Actin in total samples. All experiments were repeated > 4 times.

3.15 Statistical analyses (I-III)

Statistical analyses were performed using one-way ANOVA followed by Bonferroni or Tukey's (> 5 groups) post-hoc test. Student's t-test was used for experiments with two groups. For all experiments n= > 3 and $p \leq 0.05$ was considered significant. Data from Western blots were quantified by ImageJ software. All values are expressed as percentage of controls as mean ± SEM.

4 Results

4.1 KA induces neuronal degeneration in hippocampal neurons (I-II)

4.1.1 In vivo

KA treatment in rodents has been used as model of temporal human lobe epilepsy as well as excitotoxicity. KA induces excitotoxicity via prolonged excitatory activation of KAR subtype of glutamate receptors that are especially abundant in the hippocampal CA3 area. The CA3 pyramidal neurons are extremely vulnerable to excitotoxicity due to high density of postsynaptic KARs and network-like assembly of glutamatergic collaterals able to synchronize their discharges (Ben-Ari, Cossart 2000). We studied the effect and extent of degeneration caused by intraventricular injections of KA in the rat, known to preferentially target the CA3 neurons with little effect on the CA1 area (Nadler, Perry & Cotman 1978).

Fluoro-Jade, a fluorescent compound that penetrates and stains specifically degenerating neurons, was used to assess the extent of degeneration in the hippocampus (I-II). After 24 h of KA injection, the CA3 area of treated rats showed degenerating neuronal somas in the CA3 area (I: Fig 2a and 5d, II: Fig 1a). In addition, the method was able to stain also degenerating neurites (II: Fig 1a). TUNEL staining indicated similar cell death pattern that was seen with Fluoro-Jade staining (I: Fig 5e).

To further assess the cell death in the hippocampus, we studied activation of the major execution caspase, caspase-3, by immunostaining the brain slices of KA-treated rats with an antibody recognizing the cleaved (activated p17) form of caspase-3. Caspase-3 activation was present in neuronal nuclei at the CA3 area similar to Fluoro-Jade and TUNEL stainings (I: Fig 5e). The number of TUNEL and activated caspase-3 positive neurons at 24 h after KA treatment were quantified and exhibited similar pattern, being around 65 % for both at this time-point (I: Fig 5e).

Early signs of neuronal cell death include protraction and degeneration of neurites that can propagate the injury further to the whole cell. In addition, axonal degeneration is a hallmark of neuronal death in some neurological disorders, such as ALS (Dadon-Nachum, Melamed & Offen 2011). Dendritic spine loss occurs, for instance, in AD (Spires-Jones, Knafo 2012). We studied the effects of KA treatment on the post-synaptic neurons (II). The major constituent of the post-synaptic density is PSD95, a scaffold protein involved in correct targeting and clustering of ion channels and neurotransmitter receptors at the postsynaptic neuron. As early as after 3 h post treatment, KA had induced loss of PSD95 in the neurites of the CA3 region, innervated by the Mossy fiber axons (II: Fig 1b). Whole hippocampal lysates of KA-treated rats indicated that initially (12 h KA) PSD95 is dramatically decreased, however, the levels were somewhat recovered after longer periods post treatment (II: Fig 1c), possibly due to newly synthesized protein. In addition, the lysates included the whole hippocampus, making it possible that the downregulation of PSD95 seen by WB was an understatement of the localized situation at the CA3 area, as immunolabeling showed a specific effect on PSD95 levels at the neurites of the CA3 area (II: Fig 1b).

4.1.2 In vitro

To more specifically study the effects of KA at molecular level, we used hippocampal neurons isolated from E17 rats. Isolated neurons develop networks when cultured *in vitro* and can be used to study neuronal functions in more detail (Korhonen, Belluardo & Lindholm 2001, Banker, Cowan 1977). Importantly, cultured hippocampal neurons abundantly express the KAR subtype GluR6 (II: Fig 2c-d). We first tested whether the KA concentration of 100 μM that is known to cause delayed neuronal degeneration (Korhonen, Belluardo & Lindholm 2001) is suitable for our studies. The concentrations from 25 μM up to 200 μM had little effect on plasma membrane in 1 h as assessed by propidium iodide (PI) staining and excluded the possibility for rapid necrotic effects of KA (I: Supplementary fig 1b). For comparison, high levels of glutamate (300 μM) increased PI positive neurons in cultures (I: Supplementary fig 1b). We then studied the levels of cell death caused by 100 μM KA on hippocampal neurons. The number of condensed or fragmented nuclei, indicative of ongoing cell death, in KA-treated hippocampal neurons was around 45 % at 24 h post treatment as compared with basal level of cell death of around 10 % seen in cultures (I: Fig 5a). Respectively to the *in vivo* assessment of cell death, hippocampal neurons positive for active caspase-3 were determined. Active caspase-3 was found in around 40% of neurons following the pattern of cell death determined by nuclear morphology (I: Fig 5c).

The levels of PSD95 in cultured hippocampal neurons decreased rapidly in response to KA (II: Fig 2b). In addition, the amount of synaptic networks declined already in 3 h in KA-treated cultures indicative of synaptic degeneration (II: Fig 2a). Double labeling with PSD95 and synapsin I, a presynaptic marker, displayed reduction in co-labeling and thus in amount of synaptic contacts in KA treated cultures in a time-dependent mechanism (Fig 8).

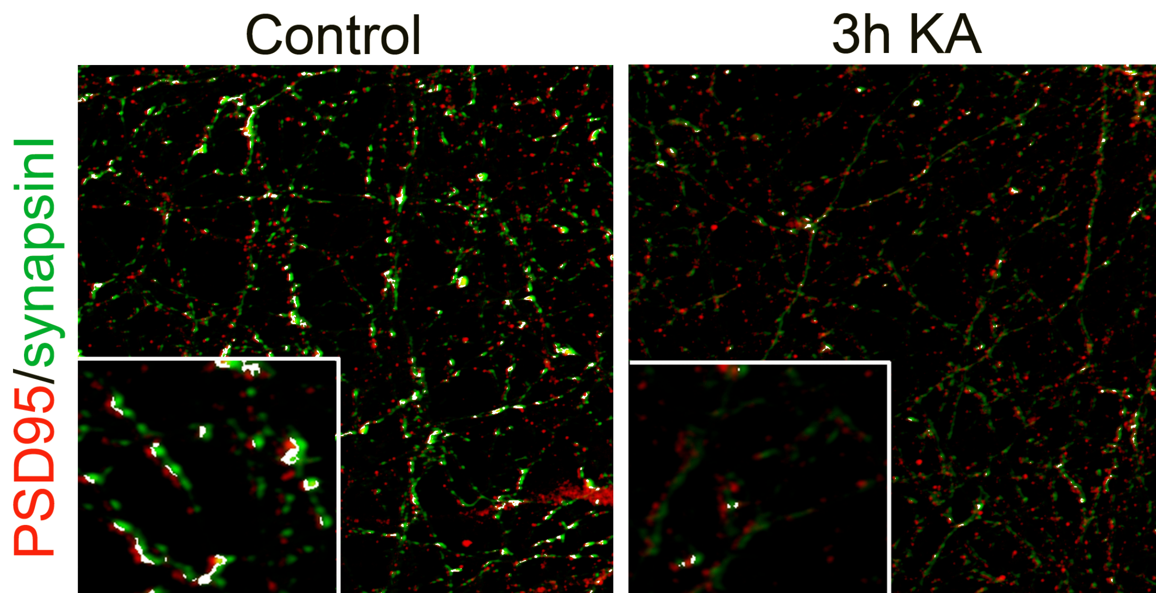


Figure 8 Double labeling of PSD95 and synapsin I indicating sites of contacts. Neurons were cultured for 7 div, treated with KA, fixed and stained as described in Materials and methods. Confocal images were captured from areas with average density of neurites using the same adjustments for different treatments. Images of PSD95 (red) and synapsin I (green) stainings were deconvoluted and analyzed with a Colocalization finder –plugin using ImageJ. Colocalized areas are shown in white. KA treatment showed a reduction in the amounts of synaptic contacts.

4.2 KA induces ER stress in hippocampal neurons *in vitro* and *in vivo* (I)

ER stress involvement was indicated in neurological disorders, including PD and ALS, in the middle 2000s (Wootz et al. 2004, Lindholm, Wootz & Korhonen 2006). Apart from being an indispensable factory of proteins and lipids, the ER is also a major regulator of calcium homeostasis (Sammels et al. 2010). One of the most acknowledged consequences of excitotoxicity is increased calcium flow from extracellular space but also from intracellular organelles. Since ER stress was implicated in neurological disorders involving protein accumulation it was an interesting and presumable outcome for a disease model involving disturbances in calcium homeostasis. Thus, we studied the involvement of the ER in KA-induced excitotoxicity as little was known of the ER responses in this type of brain injury.

First, we studied whether KA (100 μM) had effects on the ER by loading cultured hippocampal neurons with an ER-tracker dye prior to KA-treatment. In addition, KA-treated neurons were stained with an antibody for an ER resident protein and calcium channel, IP_3-R (inositol triphosphate receptor). In healthy cells, both the ER-tracker staining and IP_3-R immunolabeling appeared continuous in the cell soma as well as in the neurites (I: Fig 1). KA-treatment induced disintegration of the ER and by 1h after KA treatment the staining of IP_3-R was dramatically decreased and discontinuous in the neurites (I: Fig 1). Likewise, ER networks appeared fragmented after KA in neurons treated with the ER-tracker (I: Fig 1 and supplementary fig 1a).

During stress, the ER responds by activating the UPR consisting of three conserved pathways (PERK, IRE and ATF6) aiming to restore homeostasis. Encouraged by our finding of ER disintegration by KA, we studied the activation of these pathways *in vivo* and *in vitro*.

4.2.1 KA-induced ER stress response *in vivo* (I)

The ER chaperone BiP is involved in binding and guarding all unfolded proteins in the ER as well as the stress sensors found at the ER membranes. Accumulation of unfolded proteins or disruption of calcium homeostasis triggers release of BiP from the membranes and stress sensors thereby activating the UPR cascade. Key events also include upregulation of BiP to overcome the growing demands for the chaperone.

We first studied the levels of BiP in hippocampal lysates from KA treated rats. BiP levels increased in 12 h up to 24 h (> 1,5 fold) from KA-treatment but decreased after longer time points (I: Fig 2b). Immunostaining of KA-treated rat brains showed increase in BiP levels in surviving neurons in the CA3 region after 24 h post treatment (I: Fig 2c). Next, we analyzed the activation of the stress sensors, ATF6, PERK and IRE1 (See fig 3: ER stress pathways). The ATF6 pathway was activated in the CA3 region as shown by translocation of ATF6 immunolabeling from the cytosol to the nucleus (I: Fig 2e), consistent with the generation of the transcription factor N-ATF6. Second, the PERK pathway activation was investigated by studying its phosphorylation status with an antibody specific for the phosphorylated form of PERK (p-PERK). Accumulation of p-PERK in the CA3 region was observed at 3 h post KA-treatment (I: Fig 3c and supplementary fig 2a). The observation was transient, perhaps an initial activity boost, since p-PERK immunolabeling diminished in longer time-points (I: Supplementary fig 2a). PERK activation is responsible for phosphorylation of eIF2α that in

turn results in generalized inhibition of translation. p-eIF2α was observed in the CA3 area after 3 h of KA treatment and continued with a slight reduction in longer time points (24-48 h) possibly due to switch of the UPR to apoptosis-promoting mode (I: Fig 3c and supplementary fig 2a). To assess the IRE1 pathway activation we studied expression of p-c-jun that is induced downstream of JNK activation. As expected, p-c-jun was increased in CA3 regions of KA-treated hippocampi (I: Supplementary fig 2b).

Chop, the ER stress-inducible leucine zipper-containing transcription factor, was also induced *in vivo* at 24 h post KA treatment in hippocampal lysates (I: Fig 2b). In addition, immunostaining of the proapoptotic Chop was induced and localized to the nuclei of CA3 neurons (I: Fig 2c), indicating that prolonged UPR was aiming toward apoptosis.

4.2.2 KA-induced ER stress response *in vitro*

As for *in vivo*, we studied the activation of ER stress pathways in cultured hippocampal neurons at 7 div. Consistent with the situation *in vivo*, BiP levels increased after 100 μM KA treatment (I: Supplementary fig 3). Furthermore, the increase was seen clearly also with lower KA doses (25 μM) (I: Supplementary fig 3). Levels of BiP mRNA were also studied with quantitative PCR that demonstrated time-dependent increase for up to 24 h of KA treatment (100 μM) (I: Fig 2d). The stress sensor pathways indicated similar activation as *in vivo*. ATF6 immunostaining showed translocation of ATF6 from cell body to the nuclei already in short KA stimulations (90 min) (I: Fig 2e). Both PERK and eIF2α phosphorylation was observed after short KA treatments (from 15 min onwards) with a transient pattern showing reduction in longer time points (by 3 h) (I: Fig 3a). There was also a clear increase in immunostaining of both p-PERK and p-eIF2α (I: Fig 3b and supplementary fig 3a). Furthermore, we studied the specificity of KA activity on KARs by treating neurons with NMDA receptor blocker MK801 (10 μM) as well as a general glutamate receptor blocker Kynurenic acid (Kyn; 1 mM) and saw that Kyn but not MK801 could block eIF2α phosphorylation (I: Fig 3f). The translational block exerted by eIF2α phosphorylation overcomes several mRNAs, one being the transcription factor ATF4 that in turn can induce translation of pro-survival as well as pro-apoptotic genes, including the aforementioned Chop. We found by quantitative PCR that ATF4 mRNA levels increased upto 4-fold (I: Fig 3d). The IRE1 pathway activation was studied by PCR of the XBP1 mRNA around the splicing site (I: Fig 3e). Therefore, the splicing of the XBP1 mRNA could be seen by the appearance of a second, shorter band in addition to the full-length band. As a control, we used Brefeldin A-treatment known to induce ER stress. The spliced isoform was readily seen in the control Brefeldin A-treated neurons (0,5 μg/ml). With the exception of the 16 h time point, in KA-treated neurons the spliced isoform was harder to detect, although the mRNA level of XBP1 increased in longer time points (I: Fig 3e), which can be a result of ATF6-induced transcription. The absence of the spliced isoform can be a result of rapid translation of XBP1. Furthermore, the splicing could occur at even earlier time points as the ones studied here (from 2 h upwards). Thus, the activation of IRE1 pathway cannot be overruled.

4.3 KA-induced ER stress mediates cell death via activation of ER resident caspase-12 *in vivo* and *in vitro* (I)

The continuation of ER stress activates several mediators promoting cell death, including Chop and the JNK pathway. In addition, the ER resident caspase-12 has been shown to be activated by ER stress and promote apoptosis (Nakagawa et al. 2000). We studied rat hippocampi treated with KA and found that caspase-12 is upregulated and cleaved after 12 h KA treatment (I: Fig 4a). Furthermore, immunostaining revealed the presence of caspase-12 in neuronal nuclei in the CA3 region (I: Fig 4b). In cultured hippocampal neurons the cleavage of caspase-12 was present rapidly, already after 15min of KA treatment (I: Fig 4c). We suspected that this fast signal could be mediated by Ca^{2+} and used the Ca^{2+} chelator BAPTA-AM to block the Ca^{2+} -activated signaling pathways. Indeed, BAPTA-AM (50 μM and 20 μM, 2 h and 30 min prior to KA, respectively) could reduce the KA-induced formation of cleaved form of caspase-12 in neuronal cultures (I: Fig 4d).

4.4 Inhibition of ER stress by Salubrinal decreases cell death in hippocampal neurons *in vitro* and *in vivo* (I)

Salubrinal (Sal) inhibits the dephosphorylation of p-eIF2α and has been shown to reduce ER stress-induced cell death (Boyce et al. 2005, Wiseman, Balch 2005). To test whether Sal affects KA-induced cell death we studied its effects on cultured hippocampal neurons. Sal (50 μM) significantly reduced KA-induced cell death by a 70 % reduction in condensed and fragmented nuclei in Sal pretreated cultures (I: Fig 5a). In addition, we saw that Sal readily counteracted KA-induced increase in BiP levels, cleavage of caspase-12 as well as the number of active caspase-3 –positive cells in cultured neurons (I: Fig 5b-c). To exclude the possibility that Sal affects Ca^{2+} movements in neurons we studied calcium movements using the calcium-binding fluorescent dye, fura-2, reflecting the amounts of free calcium in the cells. The results indicated no differences between Sal-treated and -untreated neurons on KA and glutamate-mediated calcium movements (I: Supplementary fig 4).

Next, we studied if Sal was able to protect KA-induced cell death *in vivo*. Rats were given intracerebroventricular (icv) or intraperitoneal (ip) injections of Sal prior to KA-treatment as described in materials and methods. Both delivery methods significantly reduced KA-induced cell death in CA3 neurons (75 % for icv, 60 % for ip) shown by TUNEL and active caspase-3 staining (I: Fig 5e). Thus, Sal had a significant neuroprotective effect against neuronal cell death *in vivo.* These data represent a novel and important pathway to consider in developing drugs against excitotoxic brain damage.

4.5 KA-induced deregulation of Cdk5 (II)

It has previously been shown that Cdk5 is involved in various neurological disorders, including AD, where deregulation of Cdk5 has been shown to contribute at least to hyperphosphorylation of tau (Weishaupt, Neusch & Bahr 2003, Kanungo et al. 2009). Deregulation of Cdk5 is brought up via calpain-mediated truncation of the Cdk5 activator protein p35 to p25 (Kusakawa et al. 2000). Importantly, calpain-mediated p35 cleavage was detected in neurons treated with glutamate as well as the excitotoxins NMDA, AMPA and KA (Kerokoski et al. 2004). Cdk5 has been implicated in regulation of numerous neuronal functions including the clustering of certain NMDA receptor subtypes and the scaffolding

protein PSD95 (Morabito, Sheng & Tsai 2004, Zhang et al. 2008, Hawasli et al. 2007, Li et al. 2001a). In addition, Cdk5 was indicated in AMPA receptor regulation via δ-catenin (Poore et al. 2010). These studies intrigued us to investigate possible involvement of Cdk5 in regulating the KAR subtypes of glutamate receptors. GluR6, being the most important KAR in mediating KA excitoxicity in the hippocampus (Mulle et al. 1998), was chosen for more detailed studies.

First, we analyzed the expression of GluR6 in hippocampal neurons (II). We used an antibody for GluR6/7 recognizing both KAR subunits. Hippocampal expression of GluR7 is, however, extremely low and most immunolabeling represents the GluR6 subtype, especially in the CA3 area of the hippocampus (Bureau et al. 1999, Wisden, Seeburg 1993). Immunostaining of the rat hippocampus indicated specific and concentrated expression of GluR6/7 in the CA3 region in neurites from the pyramidal cell layer (II: Fig 1d). Analysis of hippocampi from KA-treated rats revealed massive reduction in GluR6/7 immunostaining that was evident after 12 h after KA and almost totally absent by 24 h (II: Fig 1d). Similarly, the levels of GluR6/7 were reduced in WB with lowest levels at 3-6 h and a rebound in longer times, possibly representing newly synthesized protein (II: Fig 1f).

Next, we studied the GluR6/7 levels in cultured hippocampal neurons and observed a dramatic and long lasting reduction of GluR6/7 without similar rebound as seen *in vivo* (II: Fig 2d-e). Immunostaining of GluR6/7 showed how the receptor initially disappears specifically from the neurites (II: Fig 2c). GluR6 has been shown to be modulated by phosphorylation, ubiquitination and sumoylation, among others, affecting its trafficking and function.

4.5.1 KA treatment induces activation of the calcium-dependent protease calpain (II)

As already described above, KA induces dose-dependent calcium movements in neurons. Calpain is a calcium-activated protease that has functions both in physiological as well as in pathological conditions (Goll et al. 2003). Calpain mediates proteolysis of a vast variety of substrates, including spectrin and the Cdk5 activator protein p35 (Kusakawa et al. 2000, Harris, Morrow 1988). We first studied the activation of calpain by KA-induced increase in intracellular Ca^{2+} (II: Fig 3c). Analysis of spectrin breakdown products by WB was done as described in materials and methods. KA treatment induced accumulation of calpain breakdown product of 150 kDa and 145 kDa, representing calpain activation (II: Fig 3d). Next, we studied the effects of KA on p35 using an antibody that recognizes both p35 and the cleaved form, p25. KA treatment induced rapid p35 cleavage in hippocampal neurons already in 15 min, with time-dependent increase in p25 levels and decrease in p35 levels (II: Fig 3a-b). The decrease in p35 levels were probably the result of Cdk5-dependent phosphorylation that enhances p35 degradation via the UPS as has been previously shown (Patrick et al. 1998). Furthermore, we wanted to verify the activation of calpain also *in vivo*. Western blotting experiments revealed p25 formation after 3 h KA treatment (II: Fig 4f-g). The levels of p25 increased and peaked at 12 h post treatment and decreased in longer time points (48-72 h) (II: Fig 4f-g). In contrast, p35 levels dropped only slightly 3-12 h after KA but were significantly reduced (by 50 %) in longer time points (24, 48 and 72 h after KA) (II: Fig 4f-g).

To investigate the mediators of calpain activation and p35 cleavage, we used calcium and calpain inhibitors to verify the KA-mediated calpain activation and resulting effects on p35. Both BAPTA-AM (1-10 μM), chelating intracellular calcium, and EGTA (2 mM), stripping the extracellular calcium, successfully inhibited KA-mediated cleavage of p35 to p25 (II: Fig 3e-h). Moreover, the calpain inhibitor XI (20 μM) also inhibited p25 formation providing evidence of its involvement in the proteolytic event (II: Fig 3g-h). The levels of p35 in these experiments, however, decreased, possibly reflecting to Cdk5-aided p35 degradation.

4.5.2 Cdk5 is phosphorylated after KA treatment (II)

Cdk5 can be phosphorylated at tyrosine 15 (Y15) and thereby achieve increased activity, although it is not a necessity for its function (Zukerberg et al. 2000). We investigated if KA-induced phosphorylation of Y15 in cultured hippocampal neurons. Specific antibody recognizing Cdk5 phosphorylated at Y15 (p-Cdk5) revealed that Cdk5 indeed was phosphorylated in response to KA. The level of p-Cdk5 was present after 30 min KA (II: Fig 4a-b). Immunostaining with p-Cdk5 antibody revealed intensified staining in KA-treated neurons within the soma as well as in the neurites (II: Fig 4c). The phosphorylation of Cdk5 occurred later than formation of p25 indicating that the Cdk5/p25 complex might achieve its full activation in longer time points (30 min onwards). The levels of Cdk5 remained unchanged even in longer KA treatments (II: Fig 4a) reflecting that its activity is mainly regulated by the activator proteins.

In addition, the presence of p-Cdk5 was studied *in vivo*. As in cultured neurons, the levels of Cdk5 remained almost constant (II: Fig 4f-g). We saw p-Cdk5 appear after 3 h KA treatment and peak at 12 h after KA (II: Fig 4f-g). The levels decreased by 24 h but showed a rebound in longer times (72 h), which was, however, not significant (II: Fig 4f-g). The phosphorylation was dependent on calcium as both calcium blockers, BAPTA-AM and EGTA prevented the KA-induced phosphorylation of Y15 in Cdk5 (II: Fig 4d-e).

4.5.3 Inhibition of Cdk5 in hippocampal neurons prevents KA-induced decreases in GluR6 and PSD95 (II)

Since many receptors and other proteins involved in glutamatergic signaling have been implicated as Cdk5 targets, we wanted to study if Cdk5 is involved in KA-induced GluR6 downregulation. Phosphorylation of PSD95 by Cdk5 inhibits its multimerization and regulates the clustering of cell surface receptors (Morabito, Sheng & Tsai 2004). In addition, Cdk5 has been implicated in receptor endocytosis and degradation (Hawasli et al. 2007, Poore et al. 2010). Since both NMDARs and AMPARs are Cdk5 targets, it is possible that KARs are modulated by this kinase as well.

Cdk5 activity can be modulated in neurons with the chemical inhibitor, Roscovitine, which also inhibits some other Cdks, however, these are mostly absent from postmitotic neurons. Thus, we studied the effect of Roscovitine pretreatment (20 μM, 1h) on KA-induced GluR6 downregulation. Moreover, we also studied the levels of known Cdk5-substrate, PSD95. The levels of GluR6/7 in neurons treated with Roscovitine did not decrease in response to KA (II: Fig 5a-b). Interestingly, the levels of PSD95 in Roscovitine treated neurons increased after KA (II: Fig 5a-b). This might indicate activity-dependent translation of PSD95 that normally

is regulated and stabilized by Cdk5. Immunostaining of Roscovitine-treated neurons showed increase in GluR6/7 staining intensity that was even sharper after KA-treament (II: Fig 5c). Next, we used dominant-negative Cdk5 (DN-Cdk5) construct that is defective for its kinase activity. Expression of DN-Cdk5 in neurons reduced KA-induced downregulation of GluR6/7 and PSD95 as seen with Roscovitine (II: Fig 5d-e). Moreover, we silenced Cdk5 expression in hippocampal neurons by siRNA construct and found that this also was successful in counteracting the effect of KA on GluR6/7 and PSD95 (II: Fig 5f-g). Taken together, inhibition of Cdk5 reduced the KA-mediated downregulation of GluR6/7 and PSD95 that could be due to ineffective endocytosis or degradation. In addition, it is not clear whether Cdk5 directly targets GluR6/7 or if the effect is indirect, via a regulatory protein or perhaps PSD95.

We hypothesized that stabilization of GluR6/7 protein levels and increase in staining intensity seen in Roscovitine-treated neurons could be the result of defective endocytosis of the receptor. Thus, using sulfo-NHS-biotin we labeled surface proteins at different time points after KA and/or Roscovitine. Labeled proteins were pulled down and assayed by WB as described in Materials and methods. KA treatment induced reduction in cell surface GluR6/7 while pre-treatment with Roscovitine counteracted this effect (Data not shown). Similar results were obtained with Cdk5 siRNA and DN-Cdk5-transfected neurons (See Fig 9 for results obtained using DN-Cdk5 construct). These data provided evidence of Cdk5 involvement in GluR6/7 endocytosis.

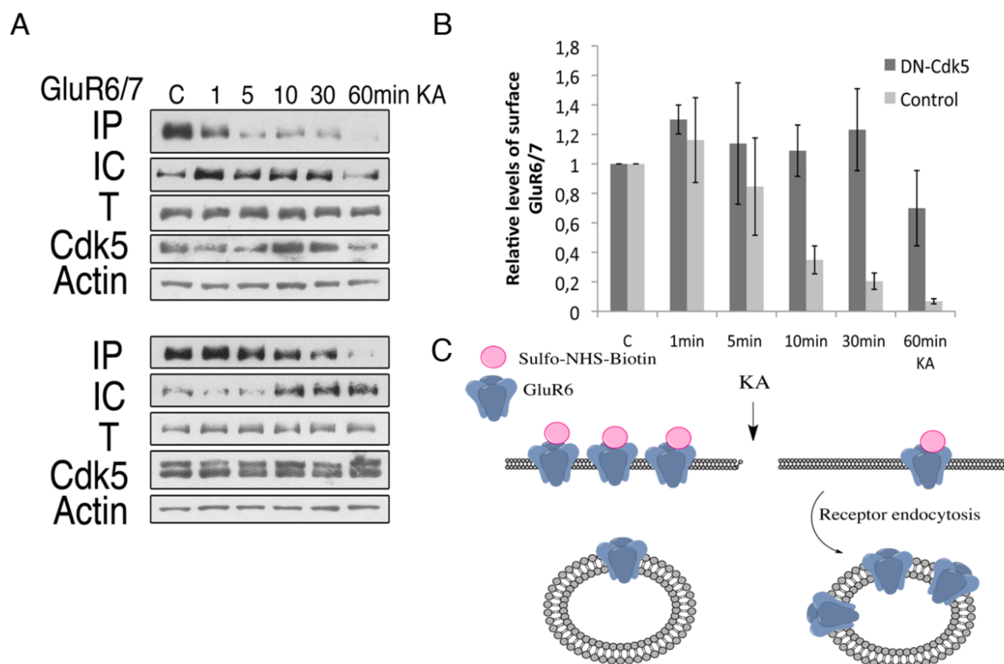


Figure 9 Biotinylation of cell surface GluR6/7 in hippocampal neurons. Hippocampal neurons were transfected with HA-DN-Cdk5 construct or empty plasmid. Neurons were treated with for 1-60min and biotinylated with Sulfo-NHS-Biotin reagent as described in materials and methods. Immunoprecipitated cell surface proteins (IP), unbound intracellular proteins (IC) and total samples (T) were studied by WB. A. Upper panel shows cell surface GluR6/7 decreasing after KA treatment in neurons transfected with empty plasmid. Lower panel shows how cell surface GluR6/7 levels decrease slower in HA-DN-Cdk5 expressing neurons. DN-Cdk5 is presented by another, higher molecular weigth band in the lower panel Cdk5. B. Quantification of data from biotinylation experiments, n=4. Notice high GluR6/7 levels in 10-60min after KA. C. Schematic presentation of receptor labeling with sulfo-NHS-biotin in normal (left) and KA-treated (right) conditions.

4.5.4 Inhibition of Cdk5 protects against cell death induced by KA *in vitro* (II)

Function of Cdk5 is essential for the development and proper functioning of the CNS (Ohshima et al. 1996). Deregulation of Cdk5, however, changes its physiological functions towards pathological and are associated with cell death. Studies have shown that inhibition of Cdk5/p25 can be protective in several pathological conditions (Kanungo et al. 2009). Roscovitine has been documented to have toxic effects in cell cultures during prolonged incubations and we also saw decrease in cell viability in MTT experiments with longer Roscovitine treatments (Data not shown). Thus, for further study of the effect of Cdk5 inhibition on cell death, we used the DN-Cdk5 construct. Neurons were transfected with DN-Cdk5-GFP constructs and control GFP vector. Cell death was assessed by calculating GFP-expressing neurons exhibiting condensed or fragmented nuclei as described in Materials and methods. The results indicated that DN-Cdk5 protected neurons from KA-induced cell death after 24 h (55 % cell death for GFP vrs 35 % for DN-Cdk5-GFP) (II: Fig 5h). The results were verified and in line with MTT experiments for the same conditions (Data not shown). This shows that catalytic activity of Cdk5 is involved in KA-induced cell death in hippocampal neurons and preventing its function has beneficial properties, making it a possible drug target in neuropathological conditions.

To investigate the possible mechanisms behind the protective effect of Cdk5 inhibition we decided to study ER stress markers. One study has shown that Cdk5/p25 translocates to the nucleus and promotes cell death in response to ER stress (Saito et al. 2007). We found that expression of DN-Cdk5 or Roscovitine upregulated BiP levels (unpublished data). Upregulation of BiP by an inducer compound, BIX, has been reported to protect against ER stress induced cell death (Kudo et al. 2008). On the other hand, phosphorylation of eIF2α was counteracted by Cdk5 inhibition. This might suggest that upregulation of BiP by Cdk5 inhibition has a protective, buffering effect on ER functions and, second, Cdk5 might take part in promoting excitotoxicity induced ER stress. It remains to be studied if Cdk5 mediates signaling from the receptor to the ER. In regards to calcium signaling, we also studied if Cdk5 inhibition affects KA-induced calcium movements since the expression of GluR6 on the cell surface was altered by Cdk5 inhibition. DN-Cdk5 expression or roscovitine treatment did not significantly alter KA-mediated calcium movements indicating that Cdk5 could be involved in signaling pathways leading to cell death and promotion of ER stress. One possibility is that the protective effect on cell death by Cdk5 inhibition occurs by decreasing ER stress.

4.6 ER stress is involved in a cell model of Huntington's disease (III)

Huntington's disease is a neurodegenerative disorder caused by the expansion of N-terminal poly-CAG repeat in the *huntingtin* gene. This results in translation of aggregation-prone huntingtin (htt) protein that selectively destroys striatal neurons in affected individuals. Cell death mechanisms in HD has been suggested to involve excitotoxicity, mitochondrial dysfunction and disturbed neurotrophic support. We suspected that accumulation of mutant htt could also induce ER stress as the ER is essentially involved in proper folding of proteins.

It has been shown that the bare N-terminal fragment of mutant htt is sufficient to induce cell death (Mangiarini et al. 1996). To study mutant htt-mediated effects on ER functions and cell

death, we transfected PC6.3 cells with N-terminal htt-EGFP constructs encoding for different polyQ lengths (18Q, 39Q, 53Q and 120Q) (Hasholt et al. 2003) as described in Materials and methods. Expression of different mHtt constructs was determined by Western blotting and immunofluorescence microscopy (III: Fig 1a, c-d). Cells expressing Htt fragment proteins with 39Q or more showed aggregates both in the nucleus and perinuclear regions (III: Fig 1c). Cell death was assessed by measuring nuclear condensation or fragmentation and revealed increase in polyQ length-dependent manner (III: Fig 1b).

Next, we studied the ER stress proteins in cells expressing Htt proteins. BiP protein levels were increased in cells expressing Htt constructs 24 h after transfection (III: Fig 2a). BiP mRNA levels were also increased in polyQ length-dependent manner (III: Fig 2b). Phosphorylation of eIF2α also took place in transfected cells, especially, in cells with 53Q- and 120Q-containing Htt fragment proteins (III: Fig 2a). Immunostaining of p-eIF2α showed increase in cells expressing 120Q-containing Htt fragment protein (III: Fig 2c). Moreover, ATF6 was shown to translocate to the nucleus in cells expressing the 120Q-containing Htt fragment protein (III: Fig 2c). To assess the cell death mediators activated by mutant Htt we studied proteins involved in ER stress-evoked cell death pathway, namely, Chop, JNK and caspase-12. Chop was induced in cells expressing the 53Q and 120Q-containing Htt fragment proteins (III: Fig 2c). The JNK-pathway was activated as well, shown by appearance of phosphorylated JNK in cells expressing 53-120Q-containing Htt fragment proteins (III: Fig 2a). Also the downstream transcription factor c-jun was phosphorylated (III: Fig 2a). In addition, caspase-12 was cleaved and translocated to the nucleus in cells expressing 53-120Q-containing Htt fragment proteins (III: Fig 2d-e).

Activation of ER stress and downstream cell death mediators led us to study further cell death mediators. Caspase-3, the key effector caspase, was activated in cells expressing the 53-120Q containing Htt fragment proteins shown by immunostaining and cleavage of the full-length protein by WB (III: Fig 3a-b). In addition, PARP, being a substrate for caspase-3 was cleaved in these cells (III: Fig 3c). As mitochondrial cell death pathway has been implicated in HD pathogenesis we then studied if it is involved in our cell model. Cytochrome c and AIF, released from mitochondria during MOMP, were studied by immunostaining, WB as well as fractionation of cells expressing Htt fragment proteins. We did not observe cytochrome c or AIF release in our cell model after 24-30 h transfection (III: Fig 4), indicating that ER stress is playing a prominent role in this model as the activator of caspase-3 pathway.

4.6.1 Inhibition of ER stress by Salubrinal reduces cell death and aggregation of mutant huntingtin fragment proteins (III)

ER stress inhibition by Sal was successful in decreasing KA-induced cell death in hippocampal neurons *in vitro* and *in vivo* (I). Therefore, we were intrigued to study its effects on our cell model of HD. Sal-treatment (5 μM) of PC6.3 cells 2 h after transfection with Htt fragment protein-constructs showed that inhibition of ER stress reduced cell death by 40 % caused by 39-120Q -containing Htt fragment proteins (III: Fig 5a). Calculating the number of aggregates in htt-expressing cells showed increase in polyQ length- dependent manner and was also significantly reduced in cells treated with Sal (III: Fig 5b). The level of aggregated proteins was additionally studied by immunoblotting the membrane fractions (P3) of cells

expressing 53Q and 120Q containing Htt fragment proteins. 120Q containing Htt fragment protein was clearly present in the P3 fraction and was reduced by Sal –treatment (III: Fig 5c). This was not due to reduction in total expression level of the 120Q-containing Htt fragment protein but could reflect to Sal-mediated inhibition of aggregate formation or breakdown of the aggregates (III: Fig 5d).

Furthermore, we studied the effect of Sal on ER stress proteins in Htt fragment protein-expressing cells. As expected, the levels of BiP and p-eIF2α were increased in 53Q-containing Htt fragment protein treated with Sal, as compared to Sal-untreated cells (III: Fig 5e). In addition, caspase-12 levels were increased by 53Q- containing Htt fragment protein expression but the cleavage was significantly reduced in Sal-treated cells (III: Fig 5e). These data clearly implicate ER stress involvement in the cell damage caused by mutant Htt fragment proteins. Inhibition of the PERK pathway by Sal greatly reduced cell death and might be beneficial also in postmitotic striatal neurons expressing mutant Htt.

5 Discussion and future prospects

5.1 KA induced excitotoxicity, cell death pathways and organelle dysfunction

Kainic acid is a well-known agonist for non-NMDA glutamate receptors targeting specific brain regions enriched in KA receptors *in vivo* (Bahn, Volk & Wisden 1994). The data obtained in this thesis with *in vivo* i.cv. injections of KA are consistent with previous studies (Ben-Ari 1985, Nadler, Perry & Cotman 1978, Bahn, Volk & Wisden 1994) showing localized degeneration of neurons in the CA3 area of the hippocampus (I-II).

The cell death triggered by KA excitotoxicity appears to be a continuum of different cell death modalities, including necrosis, apoptosis, and autophagy, depending on the duration and extent of the seizures (Vincent, Mulle 2009, Wang, Qin 2010). For *in vitro* experiments, we used a concentration of KA shown to induce delayed neuronal degeneration with approximately 45-50 % cell death in 24 h (I) and (Korhonen, Belluardo & Lindholm 2001). This type of treatment also excluded the induction of necrosis in the early time points after KA (I).

The cell death proteins involved in neuronal degeneration after KA treatment have previously been shown to involve Bcl-2 and IAP family proteins (Korhonen, Belluardo & Lindholm 2001, Belluardo et al. 2002, Sokka et al. 2005, Korhonen et al. 2003). KA-induced excitotoxicity was shown to accompany upregulation and punctate staining indicative of mitochondrial localization of the pro-apoptotic Bax as well as increased phosphorylation of the anti-apoptotic Bcl-2, which abrogates its normal functions (Korhonen et al. 2003). Unlike Bax, the pro-apoptotic BH3-only proteins Bim and Hrk/DP5 showed decrease after KA (Korhonen et al. 2003). This indicates that Bax is an important pro-apoptotic mediator in KA excitotoxicity, however, other factors regulating KA-induced cell death remain to be studied. The inhibitor of apoptosis proteins XIAP, RIAP-2 and BRUCE, normally antagonizing cell death mediators, are downregulated by KA specifically in areas vulnerable for KA (Korhonen, Belluardo & Lindholm 2001, Belluardo et al. 2002, Sokka et al. 2005). As also previously shown, KA induces cleavage and activation of the executor caspase-3 *in vivo* and *in vitro* (I) and (Korhonen, Belluardo & Lindholm 2001, Becker et al. 1999). Inhibition of caspase-3 has neuroprotective effects against KA toxicity (Becker et al. 1999) and decreases degradation of XIAP and BRUCE (Korhonen, Belluardo & Lindholm 2001, Sokka et al. 2005). According to their neuroprotective roles, it would an intriguing possibility to prevent neuronal death by overexpressing or preventing the degradation of IAP proteins.

The main trigger of cell death pathways in KA excitotoxicity is thought to be calcium influx via ionotropic glutamate receptors and secondarily through voltage-gated channels activated by depolarization (Koh et al. 1990). We also observed calcium movements caused by KA in cultured hippocampal neurons and saw that blocking calcium movements by BAPTA-AM or EGTA inhibited activation of caspases and calpains indicating that calcium mediates the primary neurotoxic effects by KA.

Apart from the influx from extracellular spaces, calcium movements are also regulated by intracellular organelles. The main calcium store in cells is found in the ER. In addition, mitochondria and the Golgi apparatus play roles in calcium regulation. The ER contains numerous Ca^{2+} -binding proteins and Ca^{2+} pumps, including sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA), ryanodine reseptor (RyR) and IP_3R, transporting Ca^{2+} in to the ER (SERCA) or outwards to the cytosol (RyR and IP_3R) (Stutzmann, Mattson 2011). Importantly, apoptotic Ca^{2+} efflux was regulated by Bcl-2 and upregulation of Bcl-2 reduced thapsigargin-induced Ca^{2+} release (Stutzmann, Mattson 2011, Lam et al. 1994). Bcl-2 binds IP_3R and controls its Ca^{2+} release. Apoptotic activation of JNK, however, results in Bcl-2 phosphorylation and release of the IP_3R that apparently facilitates calcium release (Stutzmann, Mattson 2011). From the ER, calcium can be transferred to mitochondria at specific contact sites, called mitochondria associated membranes (MAMs) (Hayashi et al. 2009). Mitochondrial calcium overload in turn can induce permeability transition pore opening and release of apoptotic mediators to the cytosol (Giacomello et al. 2007). In ceramide-induced apoptosis, it was shown that Cdk5 regulates the clustering of the ER and mitochondria via phosphorylation of tau, thus inducing its detachment from the microtubules that favors retrograde transport of organelles (Darios et al. 2005). In addition, overexpression of BiP was protective against ischaemic injury in primary astrocytes by slowing the mitochondrial Ca^{2+} transfer (Ouyang et al. 2011). It remains to be shown if KA has the potential to induce similar Ca^{2+} transfer and how this contributes to overall cell death induced by KA.

Centrally involved in the secretory pathway, the Golgi also is a dynamic calcium store and contains, for instance, a Ca^{2+} ATPase SPCA1 (Secretory pathways Ca^{2+} ATPase) (Wootton et al. 2004), which is deactivated by ischaemia reperfusion injury (Lehotsky et al. 2009). In addition, several Ca^{2+} pumps also found in the ER are localized to the Golgi. These include SERCA and IP_3Rs (Pizzo et al. 2011). In keeping with this, it is understandable that any disturbances in calcium homeostasis in the cell affect the functions of the Golgi and vice versa. Indeed, we have observed a fairly rapid dose- and time-dependent fragmentation of the Golgi after KA treatment (unpublished data). Regarding the important role of calcium in cellular homeostasis and its regulation by intracellular organelles, we studied the involvement of the ER in KA excitotoxicity. In addition, it would be of importance to characterize the mediators and pathways involved in excitotoxic Golgi fragmentation.

5.2 ER stress in KA excitotoxicity

Studies concerning the ER showed that KA excitotoxicity involves activation of ER stress sensors PERK, ATF6 and IRE1 in combination with BiP upregulation. ER stress signaling was previously detected in neurological disorders, including ALS as well as in human TLE patients (Wootz et al. 2004, Yamamoto et al. 2006). In human TLE, expression of the ER chaperones BiP/GRP78, GRP94 and calnexin were upregulated in combination with localization of the pro-apoptotic Bid, Bim and caspase-3 to the ER fraction (Yamamoto et al. 2006). Adaptive changes including XIAP binding to caspase-7 was detected indicating that anti-apoptotic signaling minimizes seizure-induced cell death in TLE (Yamamoto et al. 2006). Evidence of ER stress signaling in increasing number of disorders and especially in neurological diseases involving KAR activity makes it a significant area of study for both

clinical means as well as as a target for drug development. Subsequently to our study, ER stress has been characterized after KA treatment also by others (Chihara et al. 2009, Murphy et al. 2008) and it was shown that in addition to hippocampal pyramidal neurons, also astrocytes show activation of ER stress markers such as BiP (Chihara et al. 2009).

Inhibition of ER stress with the small molecular compound, Sal, decreased cell death caused by KA treatment *in vitro* and *in vivo* (See fig 10). This occurred via decrease in activation of caspase -12 and caspase-3. These data indicated strong involvement of ER stress in KA-mediated excitotoxicity and proves that ER homeostasis is very important in normal neuronal functions. Sal, inhibiting the dephosphorylation of eIF2α (Fig 10) acts to prolong the levels of phosphorylated eIF2α and thereby halting protein synthesis (Boyce et al. 2005). This is accomplished by inhibition of the protein phosphatase 1 (PP1) in a complex with the growth arrest and DNA damage gene (GADD34) that dephosphorylates eIF2α during ER stress giving negative feedback for the PERK pathway (Novoa et al. 2001). It has also been shown that Sal binds Bcl-2 and protects cells from pro-apoptotic and pro-autophagic effects mediated by Bcl-2 inactivation (Kessel 2006). Bcl-2 inactivation occurs also after KA treatment (Korhonen, Belluardo & Lindholm 2001). PD and α-synucleinopathies involving mutant α-synuclein (αS) accumulation was recently shown to involve ER stress in transgenic mouse models with A53T mutant human αS (*A53TαS*) (Colla et al. 2012a, Colla et al. 2012b). Moreover, Sal was used to inhibit ER stress pathways and found to reduce disease manifestation, αS accumulation (Colla et al. 2012a, Colla et al. 2012b) as well as to extend the lifespan of the *A53TαS* Tg mice and decrease Golgi fragmentation of dopaminergic neurons (Colla et al. 2012a). Interestingly, we have also seen a decrease in levels of fragmented Golgi in KA treated hippocampal neurons when pretreated with Sal (data not shown). Sal was also used in a study with mouse model of familial ALS (FALS) where the vulnerable motor neurons manifest with ER stress (Saxena, Cabuy & Caroni 2009). Superoxide dismutase-1 (SOD1)-mutant/FALS mice treated with Sal showed reduction in loss of muscle force and extension of their lifespan as well as reduced axon pathology and denervation (Saxena, Cabuy & Caroni 2009). Sal was neuroprotective both when applied in early and later time points of the disease (Saxena, Cabuy & Caroni 2009) with the indication that attenuating ER stress would greatly benefit ALS patients. Previously, Aβ neurotoxicity and microglial activation were shown to be attenuated by Sal via activation of NFκB pathway (Huang et al. 2012). These data enlighten the mechanisms by which Sal protects neuronal cells from injury and make it an interesting drug to study in more detail for neurological disorders. However, Sal has also been implicated to promote apoptosis in certain cancer cells when used in high doses bringing up the possibility for its use as anti-cancer drug (Park et al. 2011). Furthermore, Sal was shown to potentiate ER stress caused by fatty acids in pancreatic beta cells, indicating that different cell types might have very different responses to this drug (Cnop et al. 2007). Taken together, Sal shows promising effects against neuronal cell death and it would be interesting to study if it has protective effects in other neuronal diseases involving excitotoxic damage, such as ischaemia.

BiP/GRP78 is an important protective chaperone in regards to ER stress and its depletion under stressful conditions sets the ER vulnerable for prolonged ER stress and subsequent cell

death activation. Upregulation thus occurs as a first aid mechanism, however, other signals can overcome the protective effect of BiP. It was recently found that a new compound, named BiP inducer X (BIX), is able to induce BiP via the ATF6 pathway (Kudo et al. 2008). BIX-mediated induction of BiP protected neuroblastoma cells against ER stress *in vitro* and reduced cell death in mice after focal cerebral ischaemia (Kudo et al. 2008). Compounds inducing BiP could therefore be of therapeutic relevance. In regards to our studies with Cdk5, we observed upregulation of BiP in neurons when Cdk5 was downregulated (unpublished data). Cdk5 inhibition also reduced the PERK pathway -mediated phosphorylation of eiF2α that occurs after KA in mock cells (data not shown). ER stress has been shown to induce calpain-mediated cleavage of p35 to p25 and deregulation of Cdk5 that promoted cell death (Saito et al. 2007). This study also pointed out that inhibition of Cdk5 protected against ER stress-induced cell death (Saito et al. 2007). It remains unclear, if Cdk5 also plays other roles directly in ER stress and if Cdk5 regulates ER-to-mitochondria Ca^{2+} transfer during KA excitotoxicity as was shown in ceramide-induced cell death (Darios et al. 2005). In future studies it should be verified whether also Cdk5 could be a target for excitotoxic cell death involving ER stress as Cdk5 inhibition has shown promising neuroprotective effects in several neurological disorders.

In addition, ER stress and Golgi fragmentation induced by Brefeldin A treatment has some intriguing properties and these organelles might have more common factors causing organelle dysfunction that spreads via cross talk of the organelles. Brefeldin A targets the Golgi-localized guanine nucleotide exhange factors (GEFs) for the ADP-ribosylation factor 1 (Arf1) that is involved in coat protein recruitment and transport vesicle formation at the Golgi (Klausner, Donaldson & Lippincott-Schwartz 1992, Donaldson, Finazzi & Klausner 1992). These GEFs include three large factors that are localized to different Golgi compartments: Golgi-specific BFA resistance factor 1 (GBF1), BFA-inhibited GEF (BIG1) and BIG2 (Zhao, Lasell & Melancon 2002). Interestingly, siRNA depletion of GBF1 resulted in Golgi dispersion and UPR induction that resulted from mixing of the Golgi membranes with the ER (Citterio et al. 2008). BFA has been shown to cause similar redistribution of Golgi membranes with the ER (Klausner, Donaldson & Lippincott-Schwartz 1992). GBF1 depletion caused relocation of the site 2 protease (S2P) that is responsible for ATF6 cleavage during the UPR thus resulting in activation of the ATF6 pathway and increase in ER chaperones (Citterio et al. 2008). The close relationshop of Golgi and ER makes it a fascinating target of study how the organelles talk together in different cellular stress conditions. It remains to be studied, for instance, how KA affects these organelles, which is affected first or are the insults simultaneous.

Deletion of caspase-12 from mice resulted in resistance to ER stress induced apoptosis, for instance, triggered by Aβ toxicity (Nakagawa et al. 2000). The absence of caspase-12 from humans suggests that it might have evolved to prolong the survival of human cells from ER stress. This also might underlie the development of degenerative diseases involving accumulation of un- or misfolded proteins with defective clearance of affected cells. However, in light of neuronal irreplaceable nature it might have been important to save as many as possible. Humans that have been found to have functional caspase-12 have not been

reported to have more neurodegenerative diseases, although they are more susceptible for sepsis (Saleh et al. 2004).

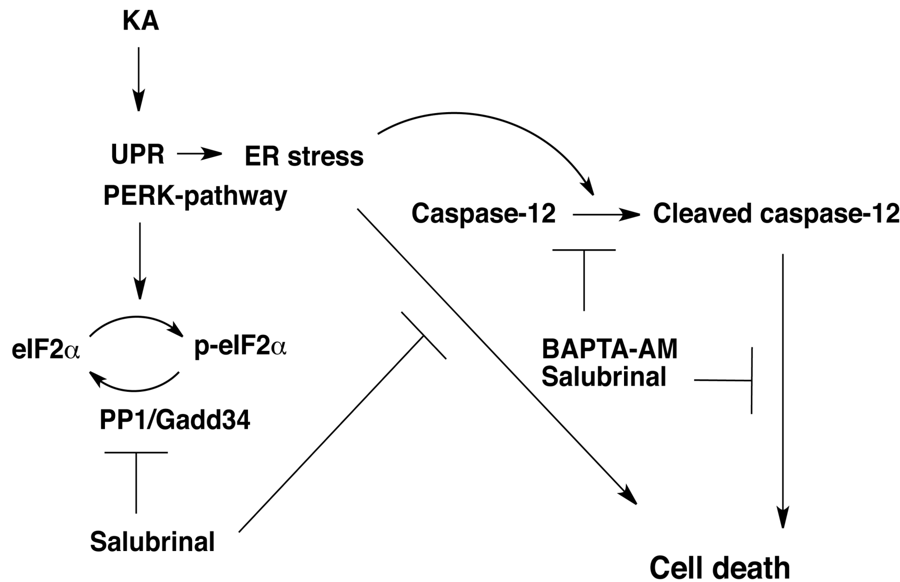


Figure 10 Summarizing figure of I.

5.3 KA-induced activation of Cdk5 and cell death

KA induced fast deregulation of Cdk5 in hippocampal neurons that was also apparent *in vivo.* Deregulation of Cdk5 was verified by the appearance of calpain cleavage product of the Cdk5 activator protein p35 (See fig 11). Modification of p35 occurs in neurological disorders including AD and HD (Paoletti et al. 2008, Kobayashi et al. 1993, Noble et al. 2003). Modification p35 to p25 results in detachment from membranes due to the loss of the myristoylation site at the N-terminal part of p35 (Kusakawa et al. 2000). Apart from relocalization of the activator protein, p25 achieves increased stability and activity due to the loss of Cdk5 phosphorylation site responsible for p35 degradation (Patrick et al. 1998, Patrick et al. 1999). KA readily induced p25 formation indicating that deregulated Cdk5/p25 complex is involved in KA excitotoxicity. In addition to the modification of the activator protein, Cdk5 activity can be increased by phosphorylation of the tyrosine residue Y15 (Zukerberg et al. 2000). We saw phosphorylation of this residue occurring in cultured neurons after KA treatment after p25 appearance. Moreover, phospho-Cdk5 was also present *in vivo* after KA treatment. Cdk5 phosphorylation is mediated at least by Src, c-Abl, Fyn and semaphorin A (Zukerberg et al. 2000, Qiao et al. 2008, Sasaki et al. 2002). It would be interesting also to characterize the kinase involved in KA-mediated Cdk5 phosphorylation as it was not studied here.

Cdk5 activation has also been implicated in ischaemic injury (Wen et al. 2007, Menn et al. 2010). Inhibition of Cdk5 by the chemical compound Roscovitine was neuroprotective in animal models of ischaemia and tauopathies (Wen et al. 2007, Menn et al. 2010, Zhu et al. 2007). It remains to be determined, whether Roscovitine exerts protection against KA excitotoxicity or other excitotoxic events. We used the dominant negative Cdk5 construct to determine the effect of Cdk5 inhibition on KA induced cell death *in vitro*. DN-Cdk5

decreased KA-induced cell death significantly indicating that Cdk5 plays an important role in mediating cell death after KA treatment (Fig 11).

The GluR6 KA receptor has been implicated as the mediator of hippocampal damage in rodents treated with KA (Mulle et al. 1998). We saw robust reduction in GluR6 levels accompanied with reduction in the scaffold protein PSD95 in the Mossy fiber area of the CA3 of the hippocampus after KA. This reduction was apparent also *in vitro.* We found that inhibition of Cdk5 greatly affected the levels of these proteins (Fig 11). PSD95 has been previously reported as a target of Cdk5 regulating PSD95 multimerization and clustering abilities of neurotransmitter receptors (Morabito, Sheng & Tsai 2004, Zhang et al. 2008, Bianchetta et al. 2011). The effect of Cdk5 inhibition on KARs has not been studied before and this study sheds light for Cdk5 being a grand modulator of even larger variety of glutamate receptors, as it has been previously implicated in NMDAR, AMPAR and mGluR regulation (Morabito, Sheng & Tsai 2004, Zhang et al. 2008, Poore et al. 2010, Wang et al. 2003, Orlando et al. 2009). It remains to be studied how Cdk5 exerts its effect on GluR6 levels; via PSD95 or directly phosphorylating GluR6. It is of notice that GluR6 has a predicted Cdk5 site in its amino acid sequence. However, this site localizes in the extracellular S2 domain excluding the possibility of Cdk5 phosphorylating GluR6 localized at the cell membrane. It still might have other functions, for instance, regulating the surface translocation of stored GluR6. We saw increased surface localization of GluR6 both by immunostaining and using a surface biotinylation assay in neurons treated with Roscovitine or expressing DN-Cdk5. A hypothesis could state that in cells with normal or deregulated Cdk5 activity (caused by KA) GluR6 levels are regulated by Cdk5 phosphorylation that inhibits excess GluR6 targeting to the cell surface. Inhibition of Cdk5 could therefore result in free movement of GluR6 from intracellular sites to the cell surface. However, we also saw that the endocytosis of GluR6 was reduced with prolonged surface expression as compared to control. This might be due to another mechanism involving other Cdk5 targets. As earlier mentioned, Cdk5 was recently linked closely to regulation of endocytosis via phosphorylation of dephosphins that are essential for continued vesicle recycling (Tan et al. 2003). It cannot be overruled, however, that GluR6 has a Cdk5 phosphorylation site in its C-terminal domain that in many GluRs contains binding sites for signaling proteins. Moreover, as likely is that general Cdk5 inhibition affects numerous other proteins in neurons as has been reviewed earlier, and that multiple mechanisms exist for GluR6 modulation by Cdk5, one possible modulator being PSD95. We were also curious about the prolonged surface localization of GluR6 in neurons treated with roscovitine or expressing DN-Cdk5 and asked if this affects the calcium movements after KA treatment. We concluded that there was no significant effect of Cdk5 inhibition on KA-induced calcium movements.

Cdk5 has been implicated in Golgi fragmentation with deregulation of Cdk5 inducing robust Golgi disassembly after glutamate or Aβ treatment (Sun et al. 2008). Moreover, the Golgi matrix protein, GM130 (Golgi matrix protein of 130kDa) was found to be a substrate for Cdk5 and the phosphorylation of GM130 by Cdk5 may be a key link in Golgi fragmentation (Sun et al. 2008). As Cdk5 has also been linked to ER stress it would be interesting to clarify

its overall involvement in cell death pathways involving different organelles and pathways controlling organelle dysfunction.

As the most dangerous effect of Cdk5 is exerted by its association with p25, it is an intriguing target for drug development. Optimized targeting would save normal Cdk5/p35 functions important for the cell. Furthermore, drugs targeting Cdk5, such as Roscovitine, have toxic effects possibly due to inhibition of Cdc2 and Cdk2 indicating that there is a need for more specific drugs. A Cdk5 inhibitory peptide (CIP) was recently developed that specifically targeted the Cdk5/p25 formation, however, this method is presently not tested in vivo although has shown promising results *in vitro* (Kanungo et al. 2009, Zheng et al. 2002). The authors have recently constructed a smaller 24-residue peptide, p5, that is efficient inhibitor of Cdk5/p25 with no effect on Cdk5/p35 and also has potential for therapeutic trials in animal models (Zheng et al. 2010). It will be interesting to see if this approach develops to be of clinical applicability.

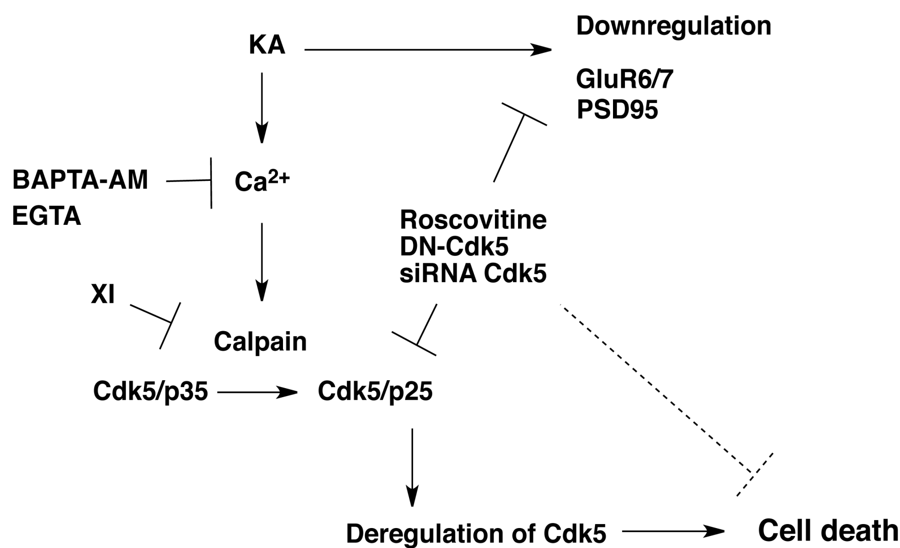


Figure 11 Summarizing figure of II.

5.4 Huntington's disease, ER stress and cell death

HD is a debilitating inherited disorder caused by an expansion of the N-terminal CAG repeat in the *huntingtin* gene (The Huntington's Disease Collaborative Research Group 1993). It has been shown that the bare N-terminal fragment of huntingtin protein with an enlarged polyQ stretch is enough to induce cell death and that aggregates seen in HD pathology mainly consists of N-terminal fragment (DiFiglia et al. 1997). Thus, we studied HD pathogenesis with Htt constructs consisting of different polyQ repeats; 18Q, 39Q, 53Q and 120Q (Hasholt et al. 2003). We saw that cell death was induced in PC6.3 cells expressing these Htt fragment proteins in a polyQ length dependent fashion. Moreover, as HD involves aggregation of the mutant protein we hypothesized that the ER of mutant Htt expressing cells might exhibit stress reactions. As assumed, ER stress pathways were induced in our HD cell model with the activation of caspase-12 and caspase-3. Previously, it has been shown that caspases in addition to calpains are involved in cleaving Htt (Gafni, Ellerby 2002, Wellington et al. 2002). It would be of interest to see if caspase-12 is also able to cleave mutant Htt. Later it has been

shown also by others that ER stress indeed plays an important role in HD (Duennwald, Lindquist 2008) and it presents an important target for drug development for HD.

Excitotoxicity was implicated early in HD pathogenesis. Although it does not have a direct role in our cell model it is a real event in human patients and can also be studied in animal models of HD. Striatum is rich in KARs and thus our findings with the KA model might share similar cell death mechanisms.

We also studied if Sal mediates protection against cell death caused by Htt in PC6.3 cells. Sal reduced cell death in mutant Htt-expressing cells, although the protection was partial and could mean involvement of other cell death mechanisms. Moreover, Sal-treated cells exhibited less aggregation of mutant Htt suggesting that it mediates either more efficient breakdown of the proteins or inhibition of aggregate formation since the overall translation of Htt proteins was not significantly reduced. These data further indicate that Sal or other compounds targeting ER stress have possible therapeutic value in neurological disorders.

5.5 Therapeutic considerations on Salubrinal

Since its discovery, Salubrinal has been studied in multiple conditions involving ER stress. As earlier mentioned, Sal has exerted both protective effects against ER stress-induced cell death but also potentiating ER stress in certain cell types. At the moment its therapeutic potential in healing bone wounds is under investigations (Zhang et al. 2012).

Sal has been shown to protect against cell death in several *in vivo* –studies involving neuronal damage. These include our study on KA excitotoxicity (I), mouse models of PD and α-synucleinopathy, and familial ALS (Colla et al. 2012a, Colla et al. 2012b, Saxena, Cabuy & Caroni 2009), described earlier. On the contrary, in an *in vivo* study of prion neurodegeneration, Sal treatment accelerated the disease progression and the opposite approach, sustaining the translational capacity of the neurons was protective (Moreno et al. 2012). Interestingly, in this study a reduction in synaptic proteins caused by prions was even aggravated when Sal was used, causing further repression of translation (Moreno et al. 2012).

Salubrinal was indicated as a potential cancer drug in a study on multiple myeloma where the proteasome inhibitor bortezomib (Velcade) has been a successful therapy (Schewe, Aguirre-Ghiso 2009). Some multiple myeloma cells treated with bortezomib have been shown to enter quiescence and survive by attenuating eIF2α phosphorylation, resulting in recurrence of the disease. Thus, using a combinatorial approach of bortezomib and Sal to counteract the dephosphorylation of eIF2α in these cells, the authors could minimize the survival of multiple myeloma cancer cells (Schewe, Aguirre-Ghiso 2009). Another study, investigating Sal as a possible protective agent against renal damage induced by the tumour drug, Cisplatin, showed that Sal actually enhanced Cisplatin-induced oxidative stress and nephrotoxicity in mouse (Wu et al. 2011).

Taken together, Sal might prove a possible target for certain disorders involving ER stress. However, detailed studies are needed to determine its suitability in each condition, as there is a possibility for bidirectional effects depending on cell types and stress pathways. In addition,

it should be determined whether Sal is suited better for acute or more chronic neuronal damage.

6 Conclusions

This thesis is based on three individual studies aimed to clarify cell death involved in excitotoxicity and in a cell model of Huntington's disease with a focus on organelle specific mechanisms. The studies showed that ER stress plays an important role in both KA-induced excitotoxic damage as well as in the HD model. Inhibition of ER stress by Sal was protective against cell death caused by KA and mutant Htt fragment proteins. Furthermore, we saw that Cdk5 is involved in KA excitotoxicity and is deregulated by calpain-mediated cleavage of the Cdk5 activator protein p35. Inhibition of Cdk5 by different means was able to reduce KA-induced GluR6 downregulation and cell death.

These studies strongly support the dysfunction of the ER and other organelles in neuronal cell death. Moreover, the multitasking protein, Cdk5, was here for the first time indicated in modulation of a KA receptor expression. The results show two relevant therapeutic approaches against neuronal cell death, namely inhibition of ER stress and inhibition of Cdk5. More studies are needed to verify and pinpoint the temporal applicability of these possible approaches in order to continue with further drug design and development.

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