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Neuronal changes under excitotoxic conditions
- the role of 26S proteasome

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"Imagination is more important than knowledge"

- Albert Einstein

"If you are not part of the solution, you are part of the precipitate"

- Henry J. Tillman

Cover illustration: Hippocampal neurons transfected with the Ub^{G76}-GFP construction

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ABBREVIATIONS

$[Ca^{2+}]_i$	Intracellular calcium concentration
AIF	Apoptosis Inducing Factor
AMC	7-amino-4-methylcoumarin
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	AMPA receptors
ANOVA	Analysis of variance
APV	2-amino-5-phosphonovalerate
ASIC	Acid-sensing ion channel
ATP	Adenosine triphosphate
BCA	Bicinchoninic Acid
BDNF	Brain-derived neurotrophic factor
BSS	Balanced salt solution
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
CLAP	Chymostatin, Leupeptin, Antipain and Pepstatin
CNS	Central nervous system
CP	Catalytic particle
DAG	Diacylglicerol
DG	Dentate gyrus
DIV	Days *in vitro*

DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
ECF	Enhanced chemifluorescence
EEAT	Excitatory amino acid transporter
GFP	Green fluorescent protein
GKAP	Guanylate-kinase associated proteins
HBSS	Hank´s balanced salt solution
HECT	Homologous to E6-AP carboxylterminus
iGluR	Ionotropic glutamate receptors
IP3	Inositol-1,4,5-triphosphate
KA	Kainate
LDH	Lactate dehydrogenase
LTP	Long-term potentiation
LTD	Long-term depression
MCAO	Middle cerebral artery occlusion
Mdm2	Murine double minute 2
Mib2	Mind bomb-2
MK-801	5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate
NCX	Sodium-calcium exchanger
Nedd4	neuronal-precursor cell-expressed developmentally downregulated gene 4
NF-kB	Nuclear-factor kappa B

NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptors
nNOS	Neuronal nitric oxide synthetase
NO	Nitric oxide
NT-3	Neurotrophin-3
OGD	Oxygen and glucose deprivation
OUT	Ovarian tumour proteases
PBS	Phosphate-buffered saline buffer
PC	Pre-conditioning
PMCA	Plasma membrane Ca^{2+} ATPase
polyUb	Polyubiquitin
Pru	Pleckstrin-like receptor for ubiquitin
PSD	Post-synaptic density
PSD-95	Post-synaptic density protein-95
PMSF	Phenylmethanesulfonyl fluoride
PVDF	Polyvinylidene difluoride
RING	Really interesting new gene
ROS	Reactive oxygen species
RP	Regulatory particle
SBDP	Spectrin breakdown products
TBI	Traumatic brain injury
TIA	Transient ischemic attack
TRP	Transitory receptor potential
UBA	Ubiquitin associated domain

Ub-Ald	Ubiquitin aldehyde
UBC	Ubiquitin conjugating domain
UBL	Ubiquitin-like domain
UCH	Ubiquitin C-terminal hydrolases
UCH-L1	Ubiquitin C-terminal hydrolase L1
UFD	Ubiquitin fusion domain
UIM	Ubiquitin interacting motif
UPS	Ubiquitin-proteasome system
USP	Ubiquitin specific protease
VGCC	Voltage-gated calcium channels
VRAC	Volume-regulated anion channels

RESUMO

Os neurónios são um tipo celular altamente especializado e em condições fisiológicas normais a transmissão sináptica está envolvida em processos homeostáticos essenciais, nomeadamente em fenómenos de aprendizagem e memória. No entanto, durante um episódio isquémico os níveis de ATP decrescem para níveis abaixo do limiar necessário para os neurónios manterem os seus gradientes iónicos. Como resultado, o neurotransmissor glutamato é massivamente libertado e acumulado no espaço extracelular. O excessivo influxo de Ca^{2+} para a célula pós-sináptica devido à sobreactivação dos receptores do glutamato é responsável por iniciar diversas cascadas responsáveis por induzir morte celular.

Em condições fisiológicas normais o sistema ubiquitina-proteossoma (UPS) é responsável pela reciclagem de numerosas proteínas, assim como pela degradação de proteínas danificadas. O UPS é o principal sistema de proteólise intracelular em células eucarióticas. Antes de as proteínas serem degradadas no UPS são primeiro marcadas com quatro (ou mais) ubiquitinas, por acção de ligases de ubiquitina (E3), sendo posteriormente enviadas para degradação no proteossoma 26S, um grande complexo dependente de energia composto por uma subunidade reguladora (19S) e por uma subunidade catalítica (20S). Tem sido demonstrado em diversos modelos de isquémia transiente global que o proteossoma 26S é desmontado nas suas subunidades constituintes, 19S e 20S, sugerindo o seu comprometimento após isquemia.

Tendo em conta o papel desempenhado pela activação excessiva dos receptores do glutamato na morte neuronal na isquemia cerebral, neste trabalho foi estudado o efeito da excitotoxicidade na actividade do proteossoma em culturas de neurónios do hipocampo. Verificou-se uma redução transitória da actividade de quimiotripsina do

proteossoma, em cerca de 50%, 4 h após uma breve estimulação com glutamato (125 µM, 20min), tendo-se observado uma redução da viabilidade celular nas mesmas condições experimentais. A inibição do proteossoma em neurónios do hipocampo submetidos a condições de excitotoxicidade foi confirmada usando um repórter de actividade do proteossoma Ub^{G76V}-GFP. Foi observada um aumento de cerca de 2.67 vezes na acumulação de Ub^{G76V}-GFP em neurónios expostos a condições de excitotoxicidade quando comparado com o controlo. Ao contrário do estímulo excitotóxico que reduziu a actividade do proteossoma e induziu morte celular num período de 4 h após a lesão, a incubação dos neurónios do hipocampo com o inibidor do proteossoma MG132 durante 5 h não teve qualquer efeito sobre a viabilidade celular. Só foi observada morte celular em resposta a incubações prolongadas (superiores a 8 h) com 0.05 µM e 1 µM do inibidor do proteossoma β-lactona. Estes resultados sugerem que a inibição do proteossoma não desempenha um papel relevante na morte neuronal em condições de excitotoxicidade.

Neste trabalho estabelecemos ainda um protocolo para OGD em culturas de neurónios corticais. Foi observado um aumento da morte celular em cerca de 13,8% após um período de OGD de 2 h, seguido da incubação em meio de cultura condicionado, durante 12 h. Demonstrámos ainda a formação rápida de produtos de clivagem da proteína do citoesqueleto α-espectrina, com 145 e 150 kDa, 30min após o estímulo de OGD. A formação destes fragmentos sugere que as calpaínas são activadas rapidamente após a lesão isquémica *in vitro*.

No conjunto, estes resultados sugerem que em condições excitotóxicas, características da isquemia cerebral por exemplo, ocorrem alterações bioquímicas que levam à desregulação do proteossoma e que, durante OGD há clivagem de proteínas do citoesqueleto que sugerem uma activação rápida das calpaínas. A prevenção de todas

estas alterações bioquímicas pode ser encarada com uma potencial estratégia neuroprotectora que valerá a pena ser explorada.

Palavras-chave: OGD; excitotoxicidade; receptores do glutamato; ubiquitina; proteossoma

ABSTRACT

Neurons are a very specialized cell type and proper synaptic transmission is involved in several important homeostatic mechanisms such as learning and memory. However, during an ischemic episode, ATP levels drop below the threshold required for normal cellular activity and ion gradients are dissipated. As a result, the neurotransmitter glutamate is massively released and accumulated in the extracellular space, and the excessive activation of glutamate receptors induces a Ca^{2+} overload into postsynaptic neurons which initiate cell death cascades.

Under normal physiological conditions the ubiquitin-proteasome system (UPS) is responsible for the turn-over of proteins and for the degradation of damaged proteins. The UPS is the major proteolytic system in eukaryotic cells, and before degradation the substrate proteins are first tagged with four (or more) ubiquitin molecules by E3 ligases. Ubiquitinated proteins are then delivered to the 26S proteasome, a large energy-dependent entity consisting of a 20S catalytic core and a 19S regulatory particle, where they are degraded. The 26S proteasome was shown to be disassembled into the 20S and 19S particle in several models of brain ischemia, suggesting that is may be impaired.

Given the role of excessive activation of glutamate receptors in neuronal death in brain ischemia, we investigated the effect of excitotoxicity on the proteasome activity in cultured hippocampal neurons. The chymotrypsin-like activity of the proteasome decreased by 50% when determined 4 h after excitotoxic stimulation with glutamate (125 µM glutamate; 20 min), and these results were correlated with a decrease in cell survival. Inhibition of the proteasome following excitotoxic stimulation with glutamate was confirmed by using the proteasome activity reporter Ub^{G76}-GFP, which showed an increased accumulation (2.67 fold) in hippocampal neurons exposed to glutamate when

compared with the control. In contrast with the effect of excitotoxic stimulation which decreased proteasome activity and induced cell death within 4 h after the insult, incubation of hippocampal neurons with the proteasome inhibitor MG132 for 5 h did not affect cell viability. Proteasome inhibition with 0.05 and 1 μM β-lactone was induced hippocampal cell death only after incubation for more than 8 h. These results suggest that proteasome inhibition is not the major effector in excitotoxic neuronal death.

We have also established a new OGD protocol to use in cultured cortical neurons. Exposure of cortical neurons to OGD for 2 h followed by incubation in culture conditioned medium for 12 h increased cell death by 13.8% when compared to the control. Cleavage of the cytoskeletal protein α-spectrin into its major 150 kDa and 145 kDa breakdown products was an early event after OGD, being observed at 30 min after the insult. The formation of these α-spectrin cleavage products suggests that calpains are rapidly activated under the experimental conditions used in this work.

Taken together, the results suggest that excitotoxic glutamate application induces biochemical changes in the proteasome resulting in its downregulation, and OGD induces breakdown of cytoskeleton proteins prompting its disorganization. Preventing these biochemical changes can be a potential neuroprotective strategy to be exploited.

Key words: OGD; excitotoxicity; glutamate receptors; ubiquitin; proteasome

1.1- The Glutamatergic Synapse

Glutamate, the major excitatory neurotransmitter in the central nervous system (CNS), acts on synaptic transmission primarily through activation of ionotropic and metabotropic glutamate receptors present in the postsynaptic membrane. Excitatory synapses are characterized by the presence of an electro-dense ~30-40 nm thick structure in the postsynaptic site, termed the postsynaptic density (PSD) (Figure 1). This large structure is build-up of several membrane-bound receptors, cytoskeleton and scaffold proteins and also signaling molecules. Numerous scaffolding proteins have been described such as the postsynaptic density protein 95 (PSD-95), guanylate-kinase associated proteins (GKAP) and Shank protein family members. Signaling molecules such as Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) and neuronal nitric oxide synthase (nNOS) are also present in the PSD (Figure 2) (Sheng et al., 2007).
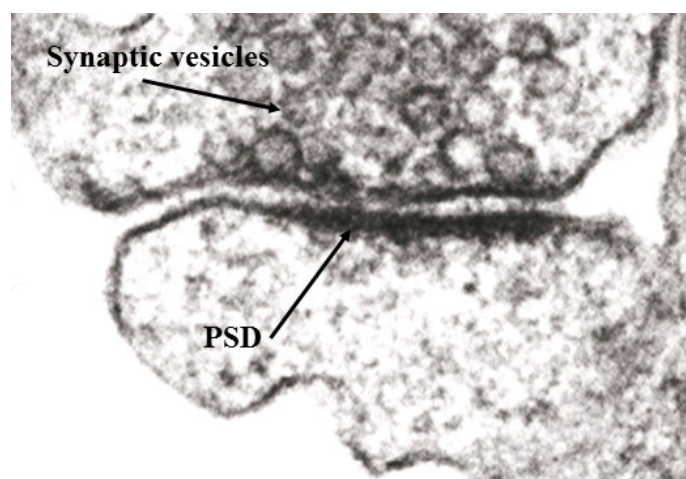

Figure 1- Electron microscopy photograph of an excitatory synapse. The presynaptic terminal contains synaptic vesicles loaded with glutamate, facing the postsynaptic density (PSD) located on the tip of the dendritic spine (adapted from Kaeser et al., 2011).

Three classes of ionotropic glutamate receptors (iGluR) have been described, the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (KA) receptors (Figure 2). Activation of these receptors with glutamate increases the permeability to Na^+ and some of the iGluR are also Ca^{2+} permeable. Unlike the ionotropic receptors, the metabotropic receptors (mGluRs) are G-protein coupled receptors and act intracellularly by elevating several second messengers such as inositol-1,4,5-triphosphate (IP_3), Ca^{2+}, diacylglicerol (DAG) (group I mGluRs) or decreasing the cyclic adenosine monophosphate (cAMP) (groups II and III mGluRs) (Niciu et al., 2012).

NMDA receptors (NMDARs) are composed of four subunits, two obligatory GluN1 subunits, which contain the binding sites for the co-agonist glycine (or D-serine), and two GluN2 subunits (GluN2A-D), responsible to bind glutamate. In addition, NMDARs can also have in its composition GluN3A-B subunits. Under non-excitable conditions the NMDAR channel pore is blocked by Mg^{2+} (Lau et al., 2010). In contrast, AMPA receptors (AMPARs) are made up of four subunits (GluA1-4) and mediate fast excitatory neurotransmission in the CNS. While the GluA1, 3 and 4 subunits are permeable to Ca^{2+}, GluA2-containing AMPARs are generally Ca^{2+} impermeable. AMPARs activation by glutamate depolarizes the post-synaptic neuron due to the influx of Na^+, removing the Mg^{2+} ion blockade from NMDAR channels and allowing the influx of Ca^{2+} (Santos et al., 2009; Niciu et al., 2012). The NMDARs can be found in synaptic sites (adjacent to the presynaptic terminal) or in extrasynaptic sites. While synaptic receptors are associated with neuronal survival, neuroprotection and with the molecular mechanism of learning and memory, such as LTP (long-term potentiation) and LTD (long-term depression), the NMDA receptors located in extrasynaptic sites are often

associated with cell death mechanisms (Hardingham et al., 2002; Hardingham et al., 2010).

The glutamate released from the nerve terminal is then taken up mainly by astrocytes, thereby preventing an excessive activation of its receptors present at the synapse. Extracellular glutamate is cleared by the action of excitatory amino acid transporters (EEAT1-5). In the CNS, both neurons and glial cells express the EEAT2 subtype, the most abundant form of these transporters. Glutamate is co-transported with three Na^+ and one H^+, and the counter transport of one K^+ and one OH^- (Chao et al., 2010; Niciu et al., 2012).
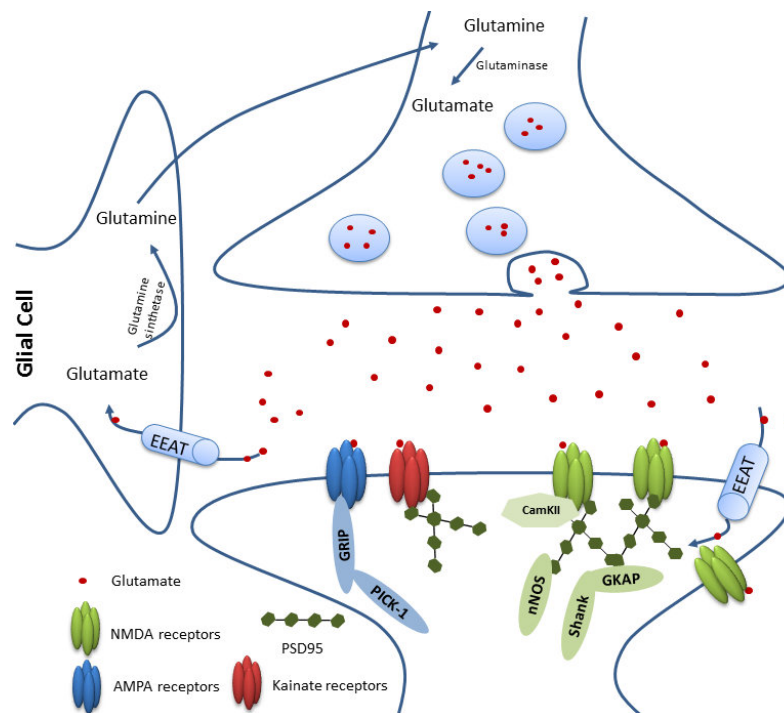
Figure 2- Overview of the glutamatergic synapse. Upon an action potential arrival in the pre-synaptic terminal, glutamate containing synaptic vesicle are release to the synaptic cleft. Under physiological conditions, glutamate acts on ionotropic (NMDA, AMPA and KA receptors) or metabotropic glutamate receptors (not shown) located at the synapse. Glutamate present in the synaptic cleft is uptaken mainly by glial cells and then converted to glutamine by the glutamine synthetase. Glutamine can be further converted to glutamate in the presynaptic neurons by glutaminase and stored in synaptic vesicles (glutamine-glutamate cycle). All the synaptic components are embedded in a network of scaffolding proteins essential to maintain proper neurotransmission.

1.2- Brain Ischemia

Due to its high energy demand, the brain is the major oxygen consuming organ in the entire human body. This oxygen is used to generate adenosine triphosphate (ATP) and a proper blood flow is thus crucial also to provide glucose, amino acids and other trophic factors. Consequently, it is highly conceivable that an interruption of the blood flow in the brain (from hereafter stroke) can be very deleterious to the tissue. After focal ischemia, ATP levels drop abruptly and cell death occurs very rapidly in the central part that is deprived from blood supply (ischemic core). However, in the vicinity, where collateral blood flow is not fully restricted (ischemic penumbra), neuronal damage is often less severe and this region can be potentially salvageable (Iadecola et al., 2011; Hossmann et al., 2012).

Stroke can be subdivided in two major categories: ischemic or hemorrhagic. Ischemic strokes (Figure 3A) are the most prevalent ones, making up approximately 87% of all cases and are the result of an obstruction within a blood vessel supplying blood to the brain. The hemorrhagic stroke (Figure 3B) takes account of 13 % of all cases and results from a weakened vessel that ruptures and bleeds into the surrounding brain. A third type of stroke, transient ischemic attack (TIA), has also been suggested. The TIA (Figure 3C) is a mini-stroke caused by a clot that can dissolve by itself with a question of minutes, and is the so called "warning stroke" (Doyle et al., 2008; http://www.strokeassociation.org).

Figure 3- Types of stroke. A) In the ischemic stroke, the vessel is blocked by a clot stopping the blood flow to the surrounding tissue; **B)** In the hemorrhagic stroke, the vessel cannot cope with the high pressure of the blood and eventually collapse; **C)** The transient ischemic attack is a less severe type of stroke when compared with the ischemic stroke. The size of the clot is smaller and therefore blocks transiently the blood vessel (adapted form www.strokeassociation.org).

1.3- Excitotoxicity

1.3.1- Role of Glutamate in excitotoxicity

The term excitotoxicity was first coined in the 60´s by John Olney. He discovered that when applied at higher concentrations, excitatory neurotransmitters such as glutamate induce neuronal death (Olney et al., 1969). Excitotoxicity is a common feature in several disorders of the nervous system, including stroke, traumatic brain injury (TBI), epilepsy and in neurodegenerative disorders (Lau et al., 2010).

Neuronal deprivation both from glucose and oxygen causes a reduction in ATP. Consequently, the Na^+ and K^+ gradient is lost due the failure of the Na^+/K^+ ATPase, which is responsible to maintaining higher concentrations of Na^+ and K^+ outside and inside the cell, respectively. Thus, neurons become highly depolarized and glutamate is massively released by the Ca^{2+}-dependent exocytotic mechanism (Grewer et al., 2008).

However, glutamate can also be released by a Ca^{2+}-independent mechanism mediated by reversal of the EAAT in response to alterations in the electrochemical Na^+ gradient. This is the case under conditions of energy deprivation, which favor the release of glutamate and do not allow the uptake of extracellular glutamate (Grewer et al., 2008). The depletion of extracellular divalent ions such as Ca^{2+}, Mg^{2+} and others, due to ionic unbalance, also promotes the release of glutamate through astrocytic hemichannels (Ye et al., 2003).

1.3.2- Role of Ca^{2+} in excitotoxicity

One of the first studies showing that an intracellular calcium overload may play a role in neuronal death was published by Dennis Choi back in the late 80´s. He found that glutamate excitotoxicity depends on two temporal components. The first component, the acute one, induces neuronal disintegration and is dependent of extracellular Na^+, while the second, occurring at a late phase, is Ca^{2+}-dependent (Choi et al., 1987). Further studies have shown that upon oxygen and glucose deprivation, glutamate is massively released by neurons, thereby increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Goldberg et al., 1993). Ca^{2+} uptake and neuronal cell death were prevented by the NMDAR antagonist D-aminophosphonovalerate (APV), indicating that NMDARs are the link between glutamate, Ca^{2+} and cell death (Goldberg et al., 1993). However, this rise in $[Ca^{2+}]_i$ is not permanent, and neurons are able to buffer their $[Ca^{2+}]_i$. Thus, after stimulation of hippocampal neurons with 500 µM glutamate for 5 min there is a rapid increase in the $[Ca^{2+}]_i$ by ~2.5 fold, which then returns to basal levels 20 min after stimulation (Dubinsky et al., 1993). Studies performed in a similar model showed three

different phases of $[Ca^{2+}]_i$ overload following stimulation of cultured hippocampal neurons with 100 µM glutamate for 5 min. An initial phase, lasting from 5 to 10 min, was followed by a decrease to basal levels during a 2 h period and a final increase in the $[Ca^{2+}]_i$ was often associated with increased cell death (Randall et al., 1992). There are additional routes for Ca^{2+} entry, besides the activation of ionotropic glutamate receptors (excitotoxic mechanisms), which may also contribute to the neuronal damage. Transient receptor potential melastatin (TRPM) channels, volume-regulated anion channels (VRAC), acid-sensing ion channels (ASICs; sensitive to the cytosolic pH acidification during excitotoxic glutamate application), voltage-gated calcium channels (VGCC) and others, have all been proposed to mediate Ca^{2+} influx (non-excitotoxic mechanisms) under excitotoxic conditions (Hartley et al., 1993; Besancon et al., 2008; Stanika et al., 2012).

Ca^{2+} overload can also contribute to the formation of free radicals due to a persistent mitochondrial depolarization, leading to a depletion of anti-oxidant defenses and a consequent accumulation of reactive oxygen species (ROS) (Schubert et al., 2001). Moreover, upon brain ischemia there is an increased interaction between cytosolic neuronal nitric oxide synthetase (nNOS) and the post-synaptic protein PSD-95. The formation of the complex nNOS-PSD-95 upregulates the production of nitric oxide (NO), which gives rise to peroxynitrite, another potent oxidant agent that is capable of causing protein nitration, protein oxidation, lipid peroxidation and direct DNA damage (Lau et al., 2010; Zhou et al., 2010). Furthermore, the $[Ca^{2+}]_i$ overload induces several potentially harmful cascades, which contribute to cell death, including activation of ATPases, lipases, DNAases and proteases (Figure 4) (Besancon et al., 2008).
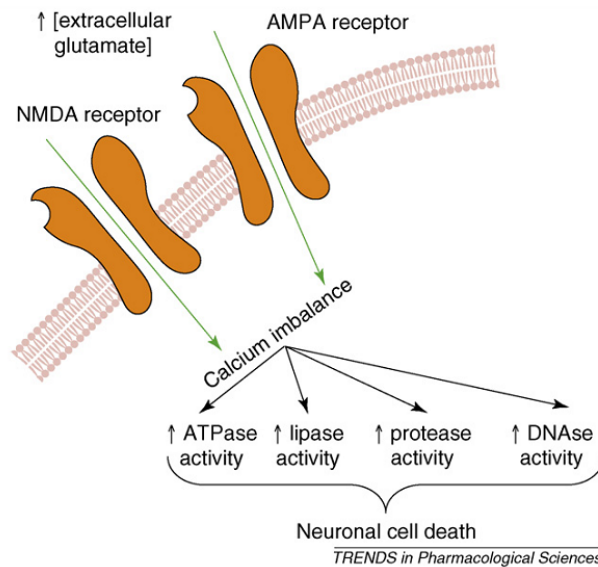
Figure 4- Mechanisms activated by Ca^{2+} ionic unbalance. During an ischemic episode, ATP depletion leads to a massive Ca^{2+} influx in the post-synaptic cell. Abnormal $[Ca^{2+}]_i$ can activate a wide range of cell death executioners such as ATPases, lipases, proteases and DNAses (Besancon et al., 2008).

1.4- Proteases and excitotoxicity

1.4.1- Calpains

Calpains belong to the family of calcium-regulated cysteine proteases and the best characterized brain calpains are the isoforms μ- and m-calpain. Activation of μ-calpain requires ~100 fold less Ca^{2+} when compared to the m-calpain, and several studies have shown a role for calpain activation in cell death induced by excitotoxic insults (Bevers et al., 2008). Studies performed in transgenic mice overexpressing human calpastatin (hCAST), an endogenous inhibitor of μ- and m-calpain, showed a decrease in kainate-induced excitotoxic cell death in the CA1 region of the hippocampus when compared

with wild type mice. This was mainly attributed to the maintenance of the integrity of cytoskeleton organization when compared with non-trangenic mice (Higuchi et al., 2005). Moreover, calpain inhibition due to calpastatin overexpression in these mutant mice was found to suppress the nuclear translocation of the apoptosis-inducing factor (AIF) and endonuclease G, which induce DNA fragmentation and promote cell death (Takano et al., 2005).

Several important proteins involved in the $[Ca^{2+}]_i$ homeostasis are also cleaved by calpains, contributing to the triphasic regulation of $[Ca^{2+}]_i$ following the excitotoxic insult. While the first increase in Ca^{2+} is directly arises from the activation of NMDA receptors (excitotoxic mechanism), the second increase is mainly due to the inability to extrude Ca^{2+} (non-excitotoxic mechanism) (Figure 5). The Na^+/Ca^{2+} exchanger (NCX) and the plasma membrane Ca^{2+}ATPase (PMCA) are the best characterized calpain substrates, and their truncation prevents the cell from extruding Ca^{2+}, thereby contributing to neuronal cell death (Bevers et al., 2008). On the other hand, calpains also generate an active proteolytic fragment of the proapoptotic Bcl-2 family member Bid, and the truncated protein (tBid) induces cytochrome-c and AIF release from the mitochondria, initiating an apoptotic signaling cascade by caspase-dependent and caspase-independent mechanisms, respectively (Takano et al., 2005).
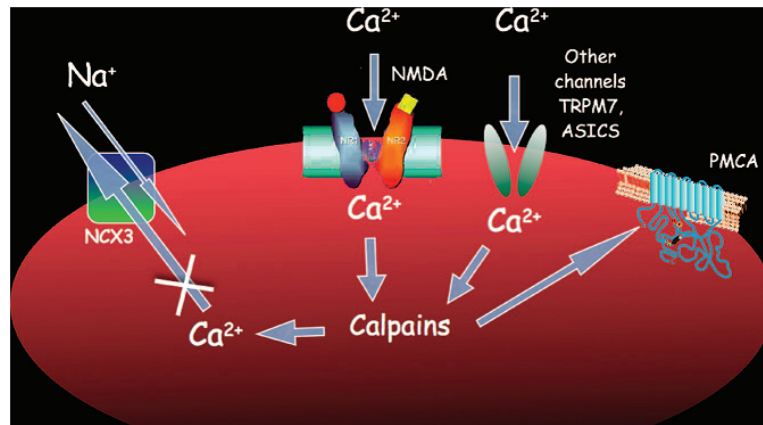
Figure 5- Routes for Ca²⁺ entry during excitotoxic and non-excitotoxic conditions. Several mechanisms can contribute to the $[Ca^{2+}]_i$ overload during an excitotoxic episode. Ca^{2+} enters primarily through NMDA receptors, and secondary waves can be generated due to the activation of the transitory receptor potential melastatin (TRPM) ion channels and acid-sensing ion channels (ASICs). Activation of calpains during excessive Ca^{2+} influx can contribute to the cleavage of Ca^{2+}-extruding pumps, such as the Na^+/Ca^{2+} exchanger (NCX3) and plasma membrane Ca^{2+}-ATPase (PMCA) (Bano et al., 2007).

1.4.2- Caspases

Caspases are cysteine proteases that cleave the substrates after aspartic residues and are responsible for the control of cell death and differentiation processes (Wang et al., 2000). They can be further subdivided in initiator (caspase 2, 8, 9, and 10) and effector caspases (caspase 3, 6, and 7). The effector caspases are activated through controlled proteolysis by the initiator caspases. Caspase-3 is a key player in regulating cell death (Zille et al., 2012), sharing common substrates with calpains, namely α-spectrin, calpastatin and CaMKII (Wang et al., 2000).

Excitotoxic stimulation of cultured hippocampal neurons with glutamate (15 min stimulation) was shown to induce apoptotic-like cells death when determined 7 h after the insult (Almeida et al., 2005). In addition, active (cleaved) caspase-3 was increased by ~4-fold 5 h after the insult, when compared with non-stimulated neurons. Although the time

window between activation of caspase-3 and initiation of cell death was not investigated in this work, caspase inhibitors such as zVAD-fmk and zDEVD-fmk, a pan-caspase inhibitor and a caspase-3 inhibitor, respectively, were shown to protect hippocampal neurons subjected to excitotoxic stimulation (Glazner et al., 2000; Almeida et al., 2005). Moreover, *in vivo* studies performed in rats subjected to 5 min of transient forebrain ischemia showed an accumulation of the p53 protein in the mitochondrial fraction after 8 h of reperfusion, and this translocation activates the initiator caspase-9 (Endo et al., 2006). These data suggest that caspases play an important role in cell death (directly or indirectly) under excitotoxic condition and the use of caspase inhibitors can be a potential therapeutic target to prevent cell death during brain ischemia.

1.5- Neuroprotective strategies

Since overactivation of NMDARs leads to excessive Ca^{2+} influx which lead to neuronal death, it is attractive to think that blockade of these receptors can be a potential neuroprotective strategy. In fact, the NMDA receptor antagonist APV provides some neuroprotection even when added after excitotoxic stimulation with glutamate (Choi et al., 1988). Similar results were also obtained with the non-competitive NMDAR antagonist 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801). MK-801 showed robust neuroprotective effects in the CA1 region of the hippocampus even if added 1 h after transient global ischemia (Buchan et al., 1990). The use of neurotrophic factors can also be a neuroprotective strategy. Pre-incubation for 24 h with brain-derived neurotrophic factor (BDNF) or with neurotrophin-3 (NT-3) partially protected cultured hippocampal neurons from glutamate-induced death of

hippocampal neurons (Almeida et al., 2005). A small molecule domain derived from BDNF was also shown to be neuroprotective in rats subjected to TBI (Massa et al., 2010).

Although the use of NMDA receptors antagonists in the clinic can be neuroprotective after an ischemic episode, by halting cell death pathways, their broad and unspecific inhibition can also interfere with cell survival and homeostatic mechanisms. In fact, there are reports showing that NMDA receptors antagonists can induce side effects such as psychosis, nausea, vomiting, memory impairment and neuronal cell death (Gardoni et al., 2006). One way to surpass this is to specifically target cell death pathways that are activated upon excessive Ca^{2+} influx. Accordingly, siRNA against PSD-95 was shown to protect cultured cortical cultures against NMDA toxicity (Cui et al., 2007) and, more specifically, disruption of nNOS-PSD-95 interaction, even after 3 h of an ischemic episode, can offer significant neuroprotection without affecting other neuronal functions (Zhou et al., 2010).

Preconditioning (PC) has also been proposed as a protective mechanism against stroke. The term PC is used when a certain stimuli applied to the brain, below the threshold of damage, is capable of protecting the organ against a severe stimuli (Dirnagl et al., 2003). There are two accepted time windows for PC: a rapid time window has been shown to exist 30 min to 1 h following PC, which is regulated by protein degradation mechanisms (further discussed in section 1.9). In contrast, delayed tolerance is induced 24-72 h following PC, by a mechanism that is sensitive to protein synthesis inhibitors and characterized by an upregulation of anti-apoptotic proteins such as Bcl-2 (Meller et al., 2005; Meller et al., 2006).

1.6- Experimental models of ischemia

A number of experimental *in vivo* and *in vitro* models are currently used to study brain ischemia. *In vivo* studies have the advantage of preserving neuronal circuitry, keeping the cells in their native environment and preserving the cross-talk between neurons and other cell types. In contrast, *in vitro* studies are more targeted to assess molecular and biochemical changes in the cell, taking advantage of cell cultures as experimental models.

1.6.1- *In vivo* models

A global ischemic insult to the brain consists of a brief but nearly complete cessation of cerebral blood flow resulting from a permanent occlusion of the vertebral arteries and transient occlusion of the common carotid arteries (the four vessel occlusion, 4-VO, in rats), or by transient occlusion of the common carotid arteries (the two-vessel occlusion, 2-VO, in gerbils and mice), followed by reperfusion (Small et al., 2000).

Focal ischemia is the animal model that most closely mimics stroke or cerebral infarction in humans (Nagasawa et al., 1989), and is produced by occlusion of the middle cerebral artery (MCAO) (McAuley et al., 1995). Arterial occlusion can be permanent, i.e. the arterial blockade is maintained throughout the experiment, or temporary, when occlusion is performed up to 3 h and is followed by reperfusion.

1.6.2- *In vitro* models

Incubation of dissociated cell cultures or organotypic cultures under conditions of oxygen and glucose deprivation (OGD) provides an *in vitro* model of global ischemia (Goldberg et al., 1993). *In vitro* OGD is performed in primary cultures of neurons from the neocortex, hippocampus, cerebellum and hypothalamus of embryonic mice. At 7-14 days *in vitro* (DIV) the culture medium is exchanged with a deoxygenated and glucose-free salt solution to induce OGD. The cultures are deprived of oxygen and glucose for a certain period of time and then transferred to the original culture medium in normoxic conditions. Similar experiments have been performed using hippocampal organotypic slice cultures (Gerace et al., 2012) which mimic more closely the cellular diversity found in the hippocampus and preserves the neuronal circuits.

1.7- The ubiquitin proteasome system (UPS)

1.7.1- Protein homeostasis

Cells are constantly challenged by environmental and physiological stress conditions that can ultimately lead to protein misfolding. Similarly, protein synthesis is not a highly efficient process and can also contribute to increase the amount of misfolded proteins, particularly when proper folding is unsuccessful. In an early attempt to quantify the amount of newly synthetized proteins that contains errors, Schubert and co-workers found that more than 30% of them are defective (Schubert et al., 2000).

Unfolded proteins can be highly toxic to cells mainly because of their tendency to form intracellular aggregates, and this has been attributed to the exposure of certain hydrophobic residues (Kriegenburg et al., 2012). Protein misfolding is counteracted by protective mechanisms based on molecular chaperones. They are responsible to identify unfolded proteins, and refold them to their native state (Kriegenburg et al., 2012). When protein misfolding reaches a certain threshold, and molecular chaperones are no longer capable to refold unfolded proteins, proteins are targeted for degradation.

Cells rely on two major proteolytic systems to eliminate unstable proteins or those that need to be turned-over, the Ubiquitin-Proteasome System (UPS) and the lysosomal system. In the former mechanism, substrate proteins are targeted to the 26S proteasome by the covalent attachment of multiple ubiquitin proteins (Hershko et al., 1998) whereas in the lysosomal system portions of cytoplasmic content are degraded in the lysosomal lumen by hydrolases (Wong et al., 2010).

1.7.2- Ubiquitin

Ubiquitin is a small, heat-stable and highly conserved 76-amino acid protein which can be covalently attached to other proteins (Hershko et al., 1998; Fang et al., 2004; Ikeda et al., 2008). This ~8.5 kDa protein, expressed in eukaryotic cells is considered a post-translational modifier (Nalepa et al., 2006), and provides the advantage of regulating a protein function, in a spacial and temporal manner, without affecting transcription.

The seven lysine (Lys) residues contained in ubiquitin (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) and the C-terminal Glycine (Gly) (Figure 6) are

essential for polyubiquitin chain formation (Grabbe et al., 2011), and to control the myriad of different biological functions that ubiquitin possesses (Meller et al., 2009).
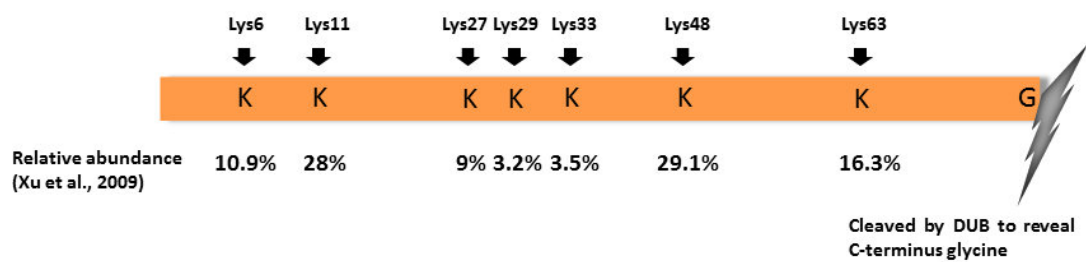

Figure 6- Ubiquitin structure. Ubiquitin is synthesized as a large precursor protein, whose C-terminus is cleaved by a deubiquitinating enzyme (DUB) to reveal a C-terminus glycine residue. Seven lysine residues are present in the primary amino acid structure of ubiquitin, which allows multiple potential polyubiquitin chain linkages. From all type of polyubiquitin chains, K48 (29.1%), K11 (28%) and K63 (16.3%) are the most abundant (adapted from Xu et al., 2009).

A single ubiquitin molecule can be added to the substrate protein on one (monoubiquitination) or multiple (multi- monoubiquitination) sites (Ye et al., 2009). Furthermore, ubiquitin can also be repeatedly attached to ubiquitin molecules, through a single type of lysine residue (a process termed homotypic polyubiquitylation), or through several distinct lysines in the ubiquitin monomers thereby forming mixed chains (Figure 7) (Ikeda et al., 2008; Kulathu et al., 2012). All the countless possibilities to add ubiquitin to a protein offers the possibility to increase the variety of ubiquitin-mediated post-translational modifications (Glickman et al., 2002), thus regulating, in a spatial and temporal manner, the physiological role of a specific protein.
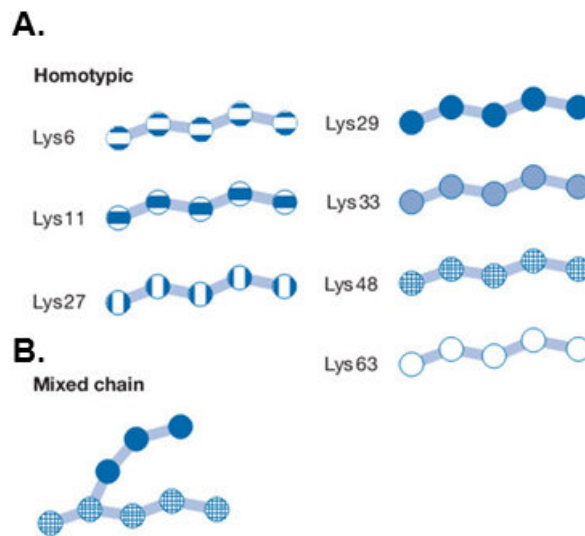
Figure 7- A schematic model of possible ubiquitin chains on a target protein. **A)** Homotypic polyubiquitin chains with lysine 6 (Lys 6)-, Lys 11-, Lys 27-, Lys 29-, Lys 33-, Lys 48- or Lys 63-linked ubiquitin molecules. **B)** Mixed-linkage atypical chains are formed by the use of different lysines for sequential ubiquitin conjugation, leading to the formation of bifurcated chains (adapted from Ikeda et al., 2008).

Modification by monoubiquitination is involved in histone regulation and in targeting membranar proteins to the lysosome (Hicke et al., 2001). In contrast, formation of polyubiquitin (polyUb) chains through the Lys48, which have been coined as the "canonical" ubiquitin chains, target proteins for proteasomal degradation in a process that requires at least four ubiquitin molecules (Fang et al., 2004; Ye et al., 2009; Xu et al., 2009; Lander et al., 2012). Non-canonical polyUb chains, such as Lys63 linked chains, is associated with non-proteolytic functions such as DNA repair, kinase activity modulation (Pickart et al., 2004), regulating plasma membrane protein internalization through the endocytic pathway, and deliver of internalized proteins for lysosomal degradation (Grabbe et al., 2011; Wagner et al., 2011). The importance of Lys11-linked polyUb chains in regulating cell cycle has been recently updated (Wickliffe et al., 2011), and along with Lys6, Lys27 and Lys29, Lys33-linked polyUb chains can also direct proteins

for proteasomal degradation (Xu et al., 2009; Kulathu et al., 2012). Surprisingly, it has been shown that mono-and multi-monoubiquitination can also be proteasomal degradative signals (Guterman et al., 2004; Boutet et al., 2007; Dimova et al., 2012).

1.7.3- The Ubiquitination Machinery

As a post-translational modification, the ubiquitination process, i.e., the process of adding ubiquitin to a substrate protein, and the removal of these ubiquitin molecules at later point is a finely tuned process governed by a cascade of ubiquitination enzymes and by deubiquitinating enzymes (DUBs), respectively. Almost 3% of the human genome is devoted to the ubiquitination machinery showing the importance of this process in cell survival and homeostasis.

Protein ubiquitination occurs through a sequential action of three different classes of enzymes, E1 or ubiquitin activating enzyme, E2 or ubiquitin conjugating enzyme and E3 or ubiquitin ligases (Figure 8). The human genome encodes for 2 E1s, ~40 E2s and ~650 E3s (Ye et al., 2009; de Bie et al., 2011).

E1 is the first enzyme involved in the ubiquitination cascade; it binds to Mg^{2+}-ATP and subsequently to ubiquitin in order to activate its C-terminal Gly residue. ATP hydrolysis generates an ubiquitin adenylate, followed by ubiquitin transference to a Cys residue of E1 through a thiol-ester linkage, with the release of adenosine monophosphate (AMP) (Hershko et al., 1983). Since this activating step is sequentially repeated, each fully loaded E1 carries two molecules of ubiquitin, one as a thiol-ester and the other as an adenylate (Fang et al., 2004). Activated ubiquitin is then transferred to a conserved core domain of ~150 residues (ubiquitin-conjugating (UBC) domain) of an E2 conjugating

enzyme, that includes an invariant cysteine responsible for accepting ubiquitin from E1 (Fang et al., 2004; Ye et al., 2009). In the third step, ubiquitin is transferred, specifically, to a substrate protein by an E3 ligase. Two different classes of E3 ligases interact with E2 conjugating enzymes and can serve either as catalytic intermediate, or mediate the direct transfer of ubiquitin to the substrate. Homologous to E6-AP Carboxyl Terminus (HECT) E3 ligases serves as intermediate ubiquitin acceptors through the formation of a thiol-ester linkage between ubiquitin and the Cys residue in the HECT domain before ubiquitin is attached to the substrate (Fang et al., 2004; de Bie et al., 2011). On the other hand, Really Interesting New Gene (RING) E3 ligases act only as a "scaffold" proteins forming an E2-substrate protein complex allowing the direct transfer of ubiquitin (Fang et al., 2004; de Bie et al., 2011). Despite the differences in the mechanisms of ubiquitin transference to the substrate proteins, both types of E3 ligases culminate in the formation of an isopeptide bond between the C-terminal Gly of ubiquitin and a ε-NH$_2$ group of an internal Lys of the substrate. However, it was recently found that ubiquitin can also be added to other residues (de Bie et al., 2011).
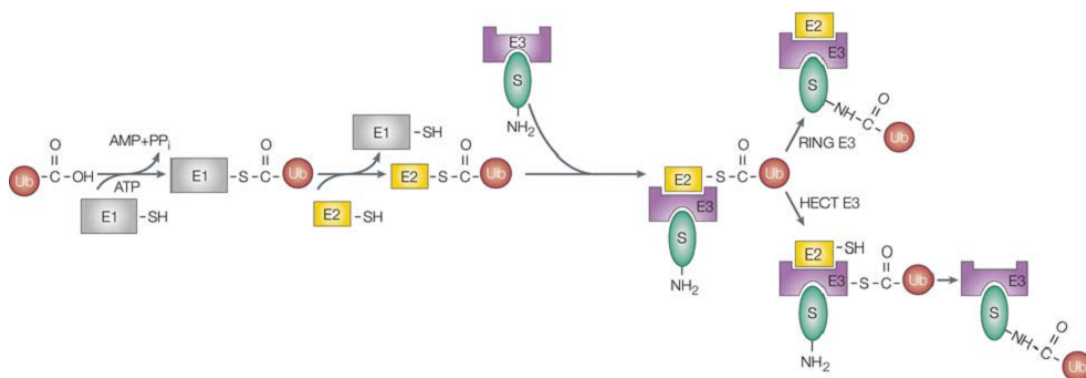
Figure 8- The ubiquitylation pathway. Free ubiquitin is activated in an ATP-dependent manner with the formation of a thiol-ester linkage between E1 (activating enzyme) and the carboxyl terminus of ubiquitin. Ubiquitin is transferred to an E2 (conjugating enzyme) cysteine (Cys) active site. The E3 ligase enzyme brings the protein to be ubiquitinated to the vicinity of a loaded E2 and mediates the transfer of ubiquitin to the protein. In the case of a RING ligase,

the ubiquitin-charged E2 binds to the E3 and transfers the activated ubiquitin moiety directly to the substrate that is also bound to the E3. In the case of HECT domain ligases, ubiquitin is transferred from the E2 to a Cys residue in the E3 and then to the substrate (Fang et al., 2004).

1.7.4- Deubiquitinating enzymes (DUBs)

The ubiquitination process can be counteracted by DUBs. Five major classes of DUBs have been described: Ubiquitin C-terminal hydrolases (UCHs), Ubiquitin-specific proteases (USPs), Machado-Joseph disesase protein domain proteases, ovarian tumour proteases and JAMM motif proteases (Love et al., 2007). Almost all DUBs are cysteine proteases, except the JAMM family which are metalloproteases (Todi et al., 2011). DUBs may be associated with the 26S proteasome, however, most of them have a cytosolic localization. Their overall function is to cleave ubiquitin-linked molecules after C-terminus of the last residue of ubiquitin (Gly76) being essential to i) maintaining monomeric ubiquitin pool, either by cleaving the ubiquitin precursor or by trimming polyUb chains; ii) rescue proteins targeted for degradation, allowing the cell to adapt quickly to physiological changes, and iii) prevent ubiquitin-proteasome dependent degradation (Guterman et al., 2004; Komander et al., 2009).

1.7.5- The 26S proteasome

The 26S proteasome is a 2.5 MDa multisubunit complex (Figure 9) responsible for controlled ATP-dependent degradation of polyubiquitinated proteins (Xie et al., 2010). It is composed of a catalytic 20S core particle (CP or 20S proteasome) associated with one (RP₁CP) or two (RP₂CP) 19S regulatory particles (RP or 19S proteasome) (Figure 9) that

are responsible for detecting, deubiquitylating and unfolding ubiquitinated proteins (da Fonseca et al., 2008; Djakovic et al., 2012). However, the physiological roles of RP_1CP and RP_2CP proteasomes are not fully understood.
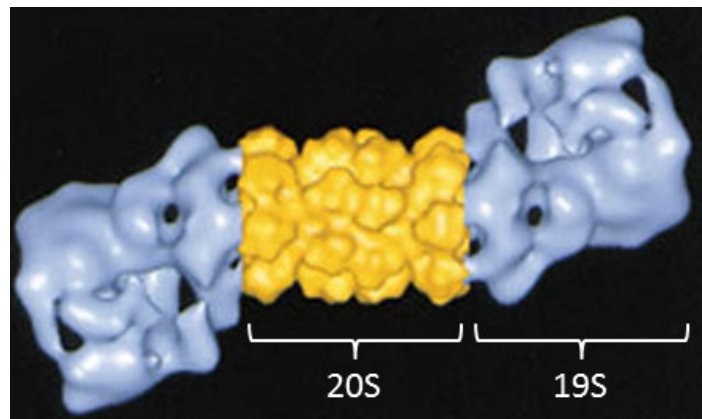
Figure 9- The 26S Proteasome, a catalytic 20S core particle (yellow) capped at both/one edges by a 19S regulatory particle (blue) (Baumeister et al., 1998).

The 26S proteasome consists of at least 66 resident subunits and associated proteins, e.g., E3 ligases, assembling factors, such as molecular chaperones and others (Leggett et al., 2002; Xie et al., 2010; Tai et al., 2010). In the cell the proteasomes are found both in cytoplasmic and nuclear fractions, and a constant trafficking between these two compartments seems to occur during cell cycle (Wojcik et al., 2003). Once a protein is committed for proteasomal degradation, with at least four ubiquitin moieties and, proper polyUb chain (Glickman et al., 2002; Lander et al., 2012), its binding and subsequent deubiquitination promotes the translocation and degradation by the proteasome (Figure 10). At the end, small peptides ranging from 2 to 20 amino acids and free ubiquitin are generated (Glickman et al., 2002).

Although recognition of ubiquitinated proteins is a stochastic process, several proteins such as Rad23 and Dsk2 (Husnjak et al., 2008) have been shown to act as shuttling factors and direct ubiquitinated proteins to the 26S proteasome (Figure 10). Binding to the proteasome is driven by the N-terminal ubiquitin-like domain (UBL), while the C-terminal ubiquitin associated domain (UBA) binds ubiquitin (Rosenzweig et al., 2012).
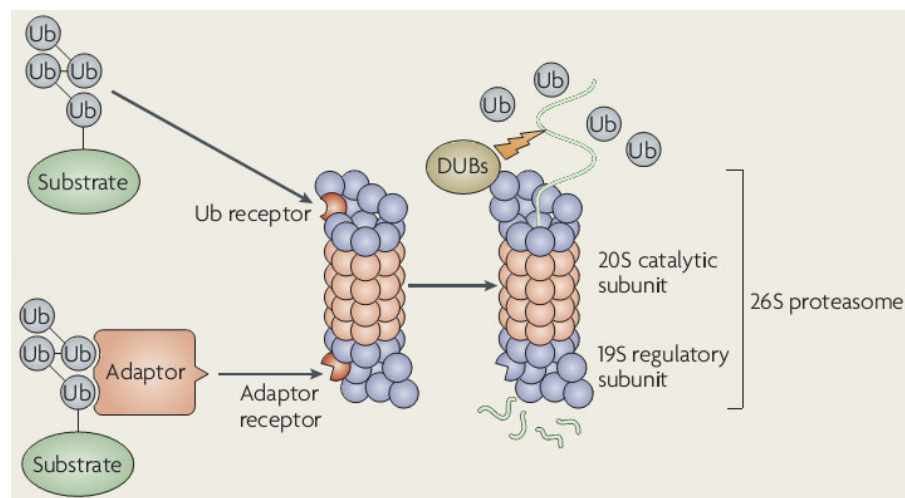
Figure 10- Overview of the Ubiquitin-Proteasome System (UPS). Polyubiquitinated proteins, with at least four ubiquitin moieties, most commonly joined through the Lys48 residue are recognized by the 19S regulatory particle and subsequently deubiquitinated. In addition, polyubiquitinated proteins can be directly targeted to the proteasome in association with several adaptor or shuttling factors (Rad23, Dsk2 and others). After polyubiquitin chain removal, the protein is unfolded by specific 19S RP unfoldases and degraded to small peptides by the 20S catalytic core. In the end, free ubiquitin is spared from degradation and is regenerated (Ravid et al., 2008).

1.7.6- The 20S proteasome

The 20S proteasome is a ~670 kDa barrel-shaped structure composed of 28 subunits arranged in a four stacked ring structure: two α_{1-7} inner rings and two β_{1-7} outer

rings (Figure 11A) (da Fonseca et al., 2008). In the center of the ring, there is a narrow pore where a protein targeted for degradation can enter in an unfolded state. In the free CP, this pore is closed by the N-terminus of the α-subunits, namely α2, α3 and α4 (Xie et al., 2010). However, the truncation of the α3 N-terminal is enough to keep the pore open and increases the degradation of small peptides (Peth et al., 2009).

Besides closing the pore, α-subunits are also responsible for compartmentalizing the catalytic β-subunits thereby preventing uncontroled cleavage of cytosolic proteins. The β ring also forms a central chamber where the peptidylglutamyl-like (cleaving after acidic residues), trypsin-like (cleaving after basic residues) and chymotrypsin-like (cleaving after hydrophobic residues) activities of the 20S proteasome are mediated by the β1, β2 and β5 subunits, respectively (Figure 11 B) (Groll et al., 1997).
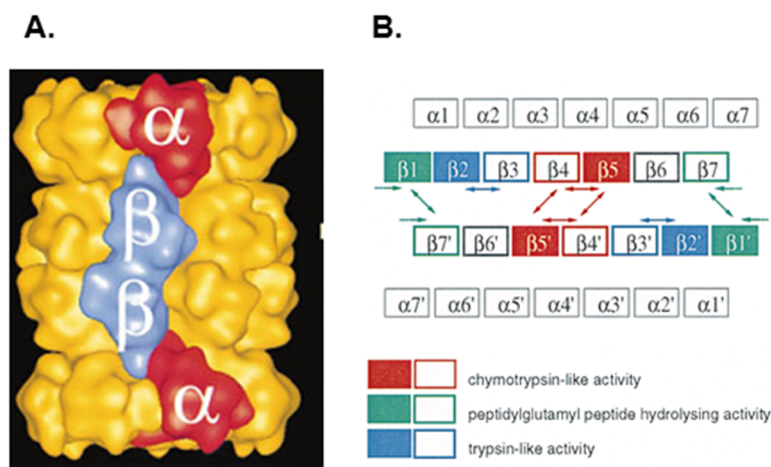
Figure 11- Structure of the 20S proteasome. A) The 20S proteasome consists in two inner β-rings and two outer α-rings juxtaposed, forming a barrel-shaped structure. Each ring is composed by seven subunits (α1-7 and β1-7). While the only function of the α-ring is to close the pore through its N-terminus region, the β-ring is responsible to the degradative function of this catalytic particle. **B)** The peptidylglutamyl-like, trypsin-like and chymotrypsin-like activities of the 20S proteasome are catalyzed by the β1, β2 and β5 subunits, respectively (adapted from Baumeister et al., 1998).

1.7.7- The 19S proteasome

Another component of the 26S proteasome is the 19S regulatory particle (RP), a ~700 kDa multisubunit complex composed of 19 subunits. The 19S proteasome serves to recognize ubiquitinated proteins, deubiquitinate and unfold the substrates in order to make them suitable to enter into the 20S catalytic pore. It is divided in two biochemically distinct sub-complexes: the lid and the base (Figure 12) (Hershko et al., 1998; Sakata et al., 2012). The lid consists of eight non-ATPase subunits, Rpn3, Rpn5-9, Rpn12 and Rpn11 (a DUB enzyme). The base contains six distinct AAA+ ATPases, Rpt1-6, and four non-ATPase subunits, Rpn1, Rpn2, Rpn10 and Rpn13 (Figure 12) (Lander et al., 2012).
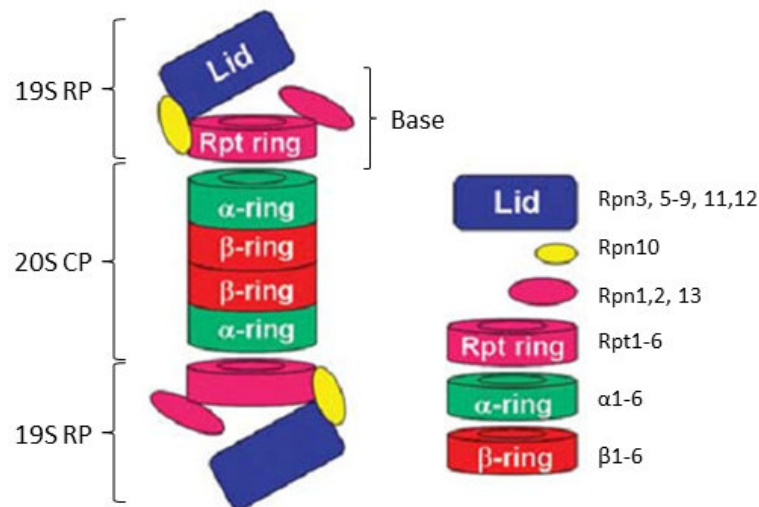
Figure 12- Schematic diagram of the 26S proteasome. While the 20S catalytic particle is responsible for protein degradation, the 19S regulatory particle (RP) possesses a more refined role in this degradative machine. The RP can be further subdivided in two distinct sub-complexes: the lid and the base. The lid is composed of eight non-ATPase subunits, Rpn3, Rpn5-9, Rpn11 and Rpn12, whereas the base is comprised by six distinct AAA+ ATPases, Rpt1-6, in addition to four non-ATPase subunits, Rpn1, Rpn2, Rpn10 and Rpn13 (Xie et al., 2010).

The interaction of polyubiquitinated proteins with RP base occurs on two intrinsic proteins, Rpn10 and Rpn13, located in the apical part (Sakata et al., 2012). Rpn10 binds ubiquitin conjugates through its C-terminus Ubiquitin-Interacting Motif (UIM), and a similar function is mediated by the Rpn13 subunit through a conserved amino-terminal region named pleckstrin-like receptor for ubiquitin (Pru) domain (Elsasser et al., 2004; Husnjak et al., 2008; Schreiner et al., 2008). The Pru domain is also important to attach Rpn13 to the proteasome (Schreiner et al., 2008).

Rpn1 and Rpn2 are large scaffold proteins of the lid. While Rpn1 is responsible for binding shuttling factors such as Rad23, Dsk2, Dd1 (Elsasser et al., 2002; Elsasser et al., 2004; Rosenzweig et al., 2012), and the non-obligatory deubiquitinating enzyme Ubp6/Usp14 (Elsasser et al., 2004; Rosenzweig et al., 2012), Rpn2 only binds the Rpn13 subunit (Schreiner et al., 2008; Rosenzweig et al., 2012).

The ATPase ring of the 19S proteasome is arranged in a spiral case and constitutes the driving force to unfold and pull the proteins targeted to be degraded inside the catalytic core (Lander et al., 2012). The Rpt2 subunit appears to be the only one required for CP opening and substrate entry (Kohler et al., 2001). Two independent studies attributed a role for the base ATPases, in preventing protein aggregation, by acting as chaperones, mediating protein refolding and not unfolding (Braun et al., 1999), and the Rpt5 subunit may also bind polyUb chains as shown by crosslinking studies (Lam et al., 2002).
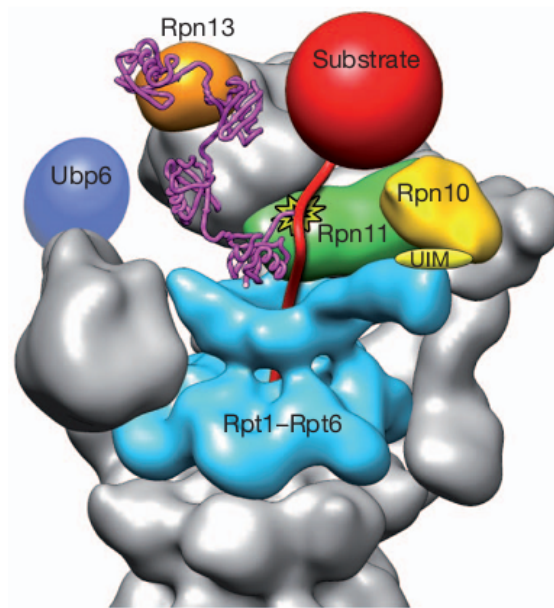
Figure 13- Overall model for the recognition, deubiquitination and unfolding of proteins promoted by the 19S regulatory particle. A K48-linked tetra-ubiquitin chain (magenta) is conjugated to the substrate (red) and bound to the pleckstrin-like receptor for ubiquitin (Pru) domain of the ubiquitin receptor Rpn13 (orange). The substrate is positioned for deubiquitination by Rpn11 (green), and its unstructured initiation region is engaged by the translocation machinery comprised by the Rpt1-6 subunits (cyan). A polyubiquitin chain could alternatively bind to the ubiquitin-interacting motif (UIM) of Rpn10 (yellow) or interact with both receptors simultaneously. The DUB Ubp6 is localized further from the central pore, in a position to trim excess ubiquitin chains or to prevent unintentionally degradation of ubiquitinated proteins (Lander et al., 2012).

1.8- The Role of the UPS in neuronal cells

Neurons are a post-mitotic and very specialized cell type, and several components of the UPS are present in both pre- and post-synaptic compartments. In the nervous system the UPS regulates several aspects of synaptic function, such as axon growth, synapse formation and elimination, presynaptic neurotransmission and LTP/LTD (Yi et al., 2007; Haas et al., 2008; Cajigas et al., 2010), being the major proteolytic system responsible for maintaining protein homeostasis. Rat cortical cell extracts possess a

higher proportion of 26S proteasomes (~57%), when compared to HeLa cell (~39%) and to rat liver/kidney cell extracts (~51%) (Tai et al., 2010). This points out the importance of the UPS in the nervous system, and suggests that selective protein degradation is a highly controlled process.

Synaptic activity can alter significantly the neuronal proteome within minutes after downstream receptor activation. In fact, several components of the UPS can also be regulated upon NMDAR activation. Bingol and others reported that increasing synaptic activity enhances proteasome activity and induces a redistribution of the 26S proteasome reporter Rpt1-GFP from dendritic shafts to dendritic spines (Bingol et al., 2006). These effects were prevented by CaMKIIα inhibition (Djakovic et al., 2009) and CamKII knockdown with siRNA (Bingol et al., 2010). Similarly, activation of NMDARs also activates the Ubiquitin C-terminal Hydrolase L1 (UCH-L1), increasing free monomeric ubiquitin levels (Cartier et al., 2009).

Postsynapticaly, the UPS is responsible for the regulation of the levels or localization of several synaptic components (Figure 14). For example, the NMDAR subunits, GluN1 and GluN2B, are regulated in an activity-dependent manner by the Fbx2 and Mind Bomb-2 (Mib2) E3 ligases, respectively (Kato et al., 2005; Jurd et al., 2008). The postsynaptic scaffold proteins PSD-95, Shank and GKAP undergo selective activity-dependent ubiquitination (Ehlers et al., 2003), and while the Murine-Double Minute 2 (Mdm2) is the putative E3 ligase for PSD-95 (Colledge et al., 2003), knockdown of endogenous TRIM3 E3 ligase increases the protein levels of GKAP and Shank (Hung et al., 2010), suggesting that these two proteins share a common E3 ligase. The UPS also mediate the internalization of several membrane-associated synaptic proteins. The initial studies showed that AMPA-induced internalization of AMPAR mediated by AMPA was abolished by the proteasome inhibitor MG132 but no ubiquitination of AMPA receptor

subunits was observed (Patrick et al., 2003). Later studies suggested that this internalization was due to a reduced stabilization of AMPA receptors into synaptic sites due to by PSD-95 degradation (Colledge et al., 2003). Recent studies showed that Nedd4 (neuronal-precursor cell-expressed developmentally downregulated gene 4) is the putative E3 ligase for GluA1 (Schwarz et al., 2010), and knockdown of Nedd4 prevented GluA1 C-terminal K868 residue ubiquitination, abolishing AMPARs internalization (Lin et al., 2011).
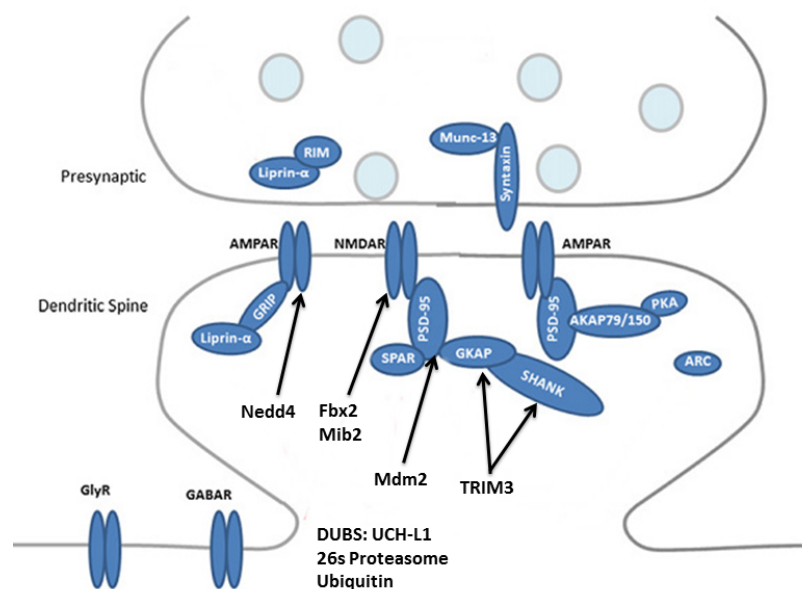
Figure 14- Examples of post-synaptic components regulated by the UPS. Several components of the UPS are present in the post-synaptic site such as deubiquitinating enzymes, 26S proteasomes, free ubiquitin and several E3 ligases. From the aforementioned components, Neuronal-precursor cell-expressed developmentally downregulated gene 4 (Nedd4), Fbx2, Mind Bomb-2 (Mib2) and Murine-Double Minute 2 (Mdm2) have been shown to physically interact and ubiquitinate GluA1, GluN1, GluN2B, PSD-95, respectively. The TRIM3 E3 ligase may regulate the levels of Shank and GKAP. Additionally, these components are negatively regulated by an increase in synaptic activity (adapted from Bingol., 2011).

1.9- Proteasome, excitotoxicity and neuroprotection

One of the major hallmarks in neurodegenerative diseases is the deposition of misfolded proteins in insoluble fractions. This is best described in Alzheimer´s disease, Parkinson´s disease, Creutzfeldt-Jakob disease, Polyglutamine expansion-related diseases, and is often correlated with mutations in several UPS components (Ciechanover et al., 2003).

As ATP depletion occurs in neurons upon brain ischemia, it is reasonable to expect and impairment of the UPS, an ATP-dependent proteolytic system. In fact, it was shown that after 10 min of transient forebrain ischemia the chymotrypsin-like activity of the 26S proteasome in the gerbil cortex was reduced by 50%, whereas after 30 min of recovery the activity of the 20S proteasome was increased (Kamikubo et al., 1996). Moreover, using the same model, other authors reported a recovery in proteasome activity in the cerebral cortex and dentate gyrus (DG) at later time points, while in certain vulnerable areas, namely the CA1 region of the hippocampus, showed a sutained decrease in proteasome activity. In fact, following a transient forebrain ischemia there is a disassembly of the 26S proteasome into its major components, RP and CP, and in the CA1 region, the 26S proteasome is not fully able to reassemble, indicating irreversible biochemical changes (Asai et al., 2002). Furthermore, recent results from our laboratory suggest that excitotoxic glutamate stimulation also promotes the dissociation of the 26S proteasome decreasing its overall activity. This effect is likely due to the activation of extrasynaptic NMDAR and is partly mediated by caspases/cathepsins (unpublished data).

In addition to proteasome dysfunction, brain ischemia also induces protein aggregation. Several reports have shown an increase in protein aggregates in the rat

hippocampal CA1 region aggregates 4 h after reperfusion, following 15 min of transient forebrain ischemia. In contrast, no protein aggregation was observed in the hippocampal DG under the same condition (Hu et al., 2000). Other studies also performed in rats, showed an accumulation of ubiquitinated proteins (ubi-proteins), chaperones, ribosomal proteins and the 26S proteasome in Triton-insoluble fractions during the reperfusion period, as well as a depletion of free ubiquitin (Hu et al., 2000; Hu et al., 2001; Asai et al., 2002; Liu et al., 2005).

Proteasome inhibition can also be used as a therapeutical strategy to reduce brain infarction after ischemia. Velcade is one of the available proteasome inhibitors used for relapsed, refractory multiple myeloma. In a rat model of focal cerebral ischemia, Velcade reduced the infarction volume even when applied 2 h after MCAO (Henninger et al., 2006), and similar results were observed with another proteasome inhibitor MLN519 (Williams et al., 2006). The neuroprotective effects observed with these proteasome inhibitors were attributed to a reduction in the activity of the Nuclear-Factor kappa B (NF-kB) pathway, a well-known transcription factor capable of regulating the inflammatory response (Wojcik et al., 2004). The UPS was also found to be involved in the rapid neuroprotective effects of ischemic tolerance, since proteasome inhibition with MG132 30 min after a non-harmful insult but prior to harmfull ischemia, obliterated the neuroprotective effect in a cortical cell culture (Meller et al., 2006). The authors also found that degradation of the pro-apoptotic Bcl-2 family member Bim is a key event for rapid ischemic tolerance (Meller et al., 2006).

CHAPTER 2 - OBJECTIVES

During brain ischemia, the interruption of blood flow to the brain leads to a massive release of the excitatory neurotransmitter glutamate, resulting in an excessive activation of ionotropic glutamate receptors. Overactivation of glutamate receptors promotes an intracellular Ca^{2+} overload, which leads to cell death, and this is termed excitotoxicity (Choi et al., 1987). During OGD, a well-established *in vitro* model for transient global ischemia, the excessive Ca^{2+} influx leads to the activation of several proteases, including calpains and caspases, which cleave several intracellular components leading ultimately to cell death (Goldberg et al., 1993; Takano et al., 2005). Additionally, the UPS has been shown to be downregulated and disassembled into its major components, 30 min after a 10 min period of transient forebrain ischemia in gerbils (Kamikubo et al., 1996; Asai et al., 2002). However, how overactivation of glutamate receptors observed in brain ischemia contributes to the downregulation of the UPS is not known. Taking all these points into account, the main goals of this work were: i) to investigate how excitotoxic stimulation affects the proteasome activity in primary cultures of hippocampal neurons, ii) to evaluate hippocampal cell death promoted by proteasome inhibition and iii) to establish and characterize a new protocol for OGD using cortical cell cultures to investigate the effect of in vitro ischemia on the UPS.

CHAPTER 3 - MATERIALS AND METHODS

Materials

Neurobasal medium, fetal bovine serum (FBS), horse serum (HS), gentamycin, trypsin and B27 supplement were acquired from GIBCO Invitrogen. Neurocult® SM1 supplement was bought from Stemcell Technologies. Minimum essential medium Eagle (MEM), kynurenic acid, dithiothreitol (DTT) as well as the proteases inhibitors phenylmethylsulfonyl fluoride (PSMF), Chymostatin, Leupeptin, Antipain and Pepstatin (CLAP, stock solution 1 mg/mL), and dimethyl sulfoxide (DMSO) were purchased by Sigma-Aldrich. 26S and 20S proteasome inhibitors, MG132 and *clasto*-Lactacystin β-lactone, respectively, were both acquired from Calbiochem (EMD Chemicals), while Ubiquitin Aldehyde (Ub-Ald), a deubiquitinating enzyme (DUB) inhibitor, was obtained from Boston Biochem. The enhanced chemifluorescence substrate (ECF) was purchased from GE Healthcare, the fluorescent mounting medium was from DAKO and Polyvinylidenedifluoride (PVDF) membranes were from Millipore. The fluorogenic peptide Suc-LLVY-AMC was from Peptide Institute. Protein quantification kits, namely Bicinchoninic Acid (BCA) and the BioRad kit were obtained from Pierce, as a part of Thermo Fisher Scientific, and from BioRad, respectively. The lactate dehydrogenase (LDH) kit was from Promega. All other chemicals/reagents were from Sigma-Aldrich, Merck, BioRad or Invitrogen.

Antibodies

The anti-green fluorescence protein (anti-GFP) polyclonal antibody was purchased from MBL International and the monoclonal anti-β-tubulin antibody was from Sigma. Anti-ubiquitin and anti α-spectrin were both from DAKO and Millipore,

respectively. The alkaline phosphatase-conjugated anti-rabbit and anti-mouse secondary antibodies, raised in goat, were obtained from GE Healthcare and from Jackson Immuno Research.

Experimental Procedures

Primary Hippocampal cultures

Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin (0.06%, 15 min at 37^0C Paisley, UK) in Ca^{2+}- and Mg^{2+}-free Hank´s Balanced Salt Medium (HBSS; 5.36 mM KCL, 0.44 mM KH_2PO_4, 137 mMNaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1mM sodium pyruvate, 10 mM HEPES and 0.001% Phenol Red). The hippocampi were then washed with HBSS containing 10% fetal bovine serum (GIBCO Invitrogen), to stop trypsin activity, washed with HBSS medium, to avoid the serum-dependent development of glial cells, and then transferred to Neurobasal medium (GIBCO Invitrogen) supplemented with B27 supplement (1:50 dilution, GIBCO Invitrogen), 25 μM glutamate, 0.5 mM glutamine and 0.12 mg/mL gentamycin. The cells were mechanically dissociated in this solution and plated in 6-well plates ($90x10^3$ cells/cm^2) or 24-well plates ($80x10^3$ cells/cm^2), depending on the type of experiment, coated with poly-D-lysine (0.1 mg/mL). The cultures were maintained in a humidified incubator with 5% CO_2/95% air, at 37^0C, for seven days, the time required for maturation of hippocampal neurons.

Primary cortical cultures

Primary cultures of rat cortical neurons were prepared from the cortices of E18-E19 Wistar rat embryos. Briefly, cortices where washed with ice-cold HBSS three and five times, prior and after trypsin (0.06%, 15 min at 37^0C) treatment, respectively. Cells were mechanically dissociated, no more than 10-15 times with HBSS. After ccounting, the cells were plated with Neuronal Plating Medium (MEM supplemented with 10% horse serum, 0.6% glucose and 1mM pyruvic acid) for 2-4 h in 6- or 24-well plates (94.7×10^3 cells/cm^2) coated with poly-D-lysine (0.1 mg/mL). After this period, the plating medium was removed and replaced by Neurobasal medium supplemented with SM1 supplement (1:50 dilution), without glutamate, 0.5 mM glutamine and 0.12 mg/mL gentamycin. After 2-3 days in culture, division of glial cells was halted by addition of 10 µM 5-FdU-NOAC (5-FDU) to the medium. The culture were maintained in a humidified incubator with 5% CO_2/95% air, at 37^0C, for 14-15 days, as mentioned in the figure captions.

Oxygen-Glucose Deprivation (OGD) assays

The protocols used for OGD were adapted from D. Choi and co-workers (Goldberg et al., 1993; Grabb et al., 2002) with some modifications. Cortical neurons (14-15DIV) were incubated for 2h in a balanced salt solution (BSS: 116 mMNaCl; 5.4mM KCL; 0.8 mM $MgSO_4$; 1.0 mM NaH_2PO_4; 26.2 mM $NaHCO_3$; 1.8 mM $CaCl_2$; 5mg/L phenol red, pH 7.3), and supplemented with 25 mM glucose (Sham condition) or with 25 mM sucrose (OGD condition). Prior to use, OGD medium was first gased with N_2 for 5 min, to remove residual oxygen, and maintained overnight in an oxygen

deprived chamber containing 5% CO_2, 7.5% H_2, 87.5% N_2. The sham medium was maintained overnight in a humidified incubator with 5% CO_2/95% air. Cells were exposed to Sham or OGD media for 2 h and then returned to the initial culture conditioned medium for the time mentioned in the figure captions, before preparation of cell extracts or LDH assay.

Excitotoxic stimulation with glutamate

Hippocampal neurons (7 DIV) were exposed to 125 µM glutamate for 20 min in Neurobasal medium and further incubated in culture conditioned medium for 4 o 8 h. Under control conditions neurons were not exposed to glutamate.

Transfection of hippocampal neurons with the calcium phosphate protocol

Ub^{G76V}-GFP(Dantuma NP et al., 2000, Nat Biotechnol, 18, 538; Addgene plasmid 11941) was recombinantly expressed in hippocampal neurons (DIV5) cultured at a high-density by transfection using the calcium phosphate coprecipitation method as described previously, with minor modifications (Gomes et al., 2011). Briefly, 10-15 µg of plasmid DNA were diluted in Tris-EDTA (TE) pH 7.3 and mixed with 2.5 M $CaCl_2$. This DNA/TE/calcium mix was added to 10 mM HEPES-buffered saline solution (270 mMNaCl, 10 mMKCl, 1.4 mM Na_2HPO_4, 11 mM dextrose, 42 mM HEPES), pH 7.2. The precipitates were allowed to form for 30 min, with vortex mixing every 5 min, protected from light, at room temperature to ensure that the precipitates had similar small sizes. Meanwhile, cultured hippocampal neurons were incubated with cultured conditioned medium with 2 mM kynurenic acid, a non-selective NMDA and

AMPA/kainate receptor antagonist. The precipitate was added drop-wise to each well and incubated at 37°C/ 5% CO_2, for 3 h. The cells were then washed with acidic 10% CO_2 equilibrated culture medium containing 2 mM kynurenic acid and returned to the 37°C/5% CO_2 incubator for 20 min. Finally, the medium was replaced with the initial culture-conditioned medium, and the cells were further incubated in a 37°C/5% CO_2 incubator for 48-72 h to allow protein expression.

Hippocampal neuronal cell extracts

Hippocampal neurons with 7 DIV were washed once with ice-cold phosphate-buffered saline buffer (PBS; 137 mM NaCl, 2.7 mM KCL, 1.8 mM KH_2PO_4, 10 mM $Na_2HPO_4.2H_2O$, pH 7.4). Cells werelysed with RIPA buffer, 100 μl/well (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS, pH7.5) supplemented with a cocktail of protease inhibitors (0.1 mM PMSF, CLAP: 1 μg/mL chymostatin, 1 μg/mL leupeptin, 1 μg/mL antipain, 1 μg/mL pepstatin; Sigma-Aldrich), 1 mM dithyothreitol (DTT). For Ub^{G76V}-GFP expression experiments, RIPA buffer was also supplemented with MG132 (25 μM), a reversible proteasome inhibitor. The extracts were then sonicated for 1 minute with 6 pulses of 5 seconds each and centrifuged at 16 100 x g for 10 min at 4°C. After sonication and centrifugation, total protein in the supernatants was quantified using the BCA method and then denaturated with 2× concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue), at 95°C for 5 min. The proteins of interest were then analyzed by Western Blot.

Preparation of cell extracts for proteasome activity-related experiments

For proteasome activity experiments, as well as for analysis of ubiquitinated-proteins, 7 DIV cultured hippocampal neurons were washed once in ice-cold PBS and lysed with Lysis Buffer (1mM EDTA, 10 mM Tris-HCl pH 7.5, 20% Glycerol, 4mM DTT and 2 mM ATP; 100µl/well). As described above, cells were sonicated and centrifuged, and total protein content in the supernatants was quantified using the Bio-Rad method. The protein concentration in the samples was equalized with Lysis buffer and the proteins were then denaturated with 2× concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue). The samples were used to evaluate proteasome activity or Western Blot analysis.

Western Blot

Protein were separated by SDS-PAGE, in 6%, 7.5% or 11% polyacrylamide gels, transferred to PVDF membranes in 10mM CAPS buffer with 10% of methanol (overnight, 4 °C, 40 V), and immunoblotted. The blocking of the membranes was performed with 5% non-fat dry milk, prepared in TBS supplemented with 0.1% Tween-20 for 45 min. The membranes were then incubated with the appropriate primary antibodies (overnight at 4°C), washed, and exposed to alkaline phosphatase-conjugated secondary antibodies (1h at room temperature). Alkaline phosphatase activity was visualized by enhanced chemifluorescence (ECF) on the Storm 860 Gel and Blot Imaging System (GE Healthcare) and quantified using the ImageQuant software (GE Healthcare). The following primary antibodies were used: anti-ubiquitin (mono- and

polyubiquitinated conjugates; 1:1000), anti-GFP (1:1000), anti-spectrin (1:1000). Anti-β-tubulin was used as loading control (1:300000) in all experiments and the results expressed after normalization

Stripping and reprobing of the membranes

In order to reprobe membranes with additional primary antibodies, namely those used against β-tubulin, used as experimental loading control, ECF was removed by washing the membranes with TBS-T for 30-40 min. After this washing step, the membranes were washed for 5 min with 0.2 M NaOH and washed again, abundantly with water. Membranes were again blocked for 1 h, with 5% non-fat milk in TBS-T, and incubated with the anti-β-tubulin antibody (1:300000). Secondary antibodies were incubated for 1 h at room temperature.

Proteasomal peptidase activity assay

The chemotrypsin-like activity of the proteasome was assayed by monitoring the production of 7-amino-4-methylcoumarin (AMC) from the fluorogenic substrate Suc-LLVY-AMC (25 µM; Peptide Institute, Inc, Osaka, Japan). Hippocampal cell extracts (17 µg of protein) were incubated with Suc-LLVY-AMC in 50 mM Tris-HCl (pH 8.0) and 0.5 mM EDTA, in a final volume of 100 µl. The release of fluorescent AMC was measured using a SPECTRAmax Gemini EM microplate reader (Molecular Devices), at an excitation and emission wavelengths of 360 nm and 460 nm, respectively, for 60 min at 37°C, at 5 min intervals. All the experiments were performed in the presence of 2 mM ATP. Specific activity was determined by subtracting the activity measured in the presence of 10 µM MG132, a proteasome inhibitor.

Lactate dehydrogenase (LDH) assay

After the OGD insult, the cells were further incubated in culture conditioned medium for the period of time mentioned in figure captions. The LDH leakage to the extracellular medium was evaluated by a colorimetric assay, using the CyoTox 96 Non-Radioactive assay kit (Promega), according to the manufacturer´s instruction. Briefly, the extracellular medium was removed and diluted with an equal volume of H_2O to a final volume of 100 µl, and 50 µl of substrate mix was added to the diluted sample, at room temperature, protected from the light. Incubation with the substrate was performed for 30 min and the reaction was stopped with 50 µl of stop solution. The activity of LDH was measured using a SPECTRAmax Gemini EM (Molecular Devices) microplate reader, at an excitation wavelength of 490 nm. The percentage of LDH released to the medium was determined as the ratio between LDH activity in the extracellular medium and total LDH activity (100% cell death) obtained by cell lysis.

Nuclear morphology staining

Hippocampal neurons were cultured for 7 days on poly-D-lysine-coated glass coverslips, at a density of 80×10^3 cells/cm^2. After the appropriate stimulus (see figure caption), cells were fixed for 20 min, at room temperature, in 4% sucrose/ 4% paraformaldehyde dissolved in PBS, washed once with ice-cold PBS and the nuclei were then stained with Hoechst 33342 (1µg/ml), for 10 min, protected from the light, at room temperature. After this, cells were washed once with ice-cold PBS and the coverslips were mounted with a fluorescent mounting medium (DAKO). Images were captured using a Zeiss Axiovert 200 fluorescent microscope coupled to an Axiocam

camera. Three independent coverslips were prepared for each experimental condition, and at least 200 cells were counted in each case.

Statistical analysis

Graphs and statistical analysis were performed using Graph Pad Prism 5. Results are presented as mean±S.E.M. of the indicated number of experiments and statistical analysis were assessed by Students t-test or one-way variance analysis (ANOVA) followed by the Dunnett's and Bonferroni´s post test as mentioned in figure captions.

CHAPTER 4 - RESULTS

Excitotoxic glutamate stimulation downregulates the proteasome activity and induces hippocampal neuronal death

Excitatory neurotransmission in mammalian brain is mainly driven by the excitatory neurotransmitter glutamate (Santos et al., 2009). This type of neurotransmission is thought to underlie many aspects related to learning and memory formation (Santos et al., 2009). However, overactivation of glutamate receptors can induce cell death (Choi et al., 1987; Almeida et al., 2005), and is associated with many pathological conditions, including stroke (Mattson et al., 2003). Also, *in vivo* models of stroke, such as transient forebrain ischemia, have shown a decrease in proteasome activity with a concomitant increase in protein aggregates (Ge et al., 2007). However, the mechanisms involved in the downregulation of the proteasome activity in brain ischemia have not been elucidated.

In this study we evaluated the effect of excitotoxic stimulation with glutamate on the proteasome chymotrypsin-like activity, using the fluorogenic substrate Suc-LLVY-AMC. Hippocampal neurons (7 DIV) were stimulated with 125 µM glutamate for 20 min, and further incubated in cultured conditioned medium for different periods of time. Excitotoxic stimulation of hippocampal neurons induced a transient downregulation of the chymotrypsin-like activity of the proteasome, and the maximal effect was observed 4-5 h after the insult (Figure 15A), when the activity of the proteasome was decreased to about 50% of the control (P<0.001). For longer incubation periods after the toxic insult the inhibition of the proteasome activity was not as significant.

In additional experiments we measured the effect of stimulation with different concentrations of glutamate on the viability of cultured hippocampal neurons.

Hippocampal neurons were incubated with 10, 20, 50 and 125 µM glutamate and cell death was evaluated after 4 h of incubation in culture conditioned medium, by fluorescence microscopy and using Hoechst 33342. We observed a dose-dependent effect of glutamate, and the maximal effects obtained for 50 and 125 µM glutamate, which increased cell death to 54.4% (P<0.01) and 57.2% (P<0.01), respectively. Taken together, these results indicate that excitotoxic glutamate stimulation downregulates the chymotrypsin-like activity of the proteasome under conditions that induce hippocampal neuronal death.
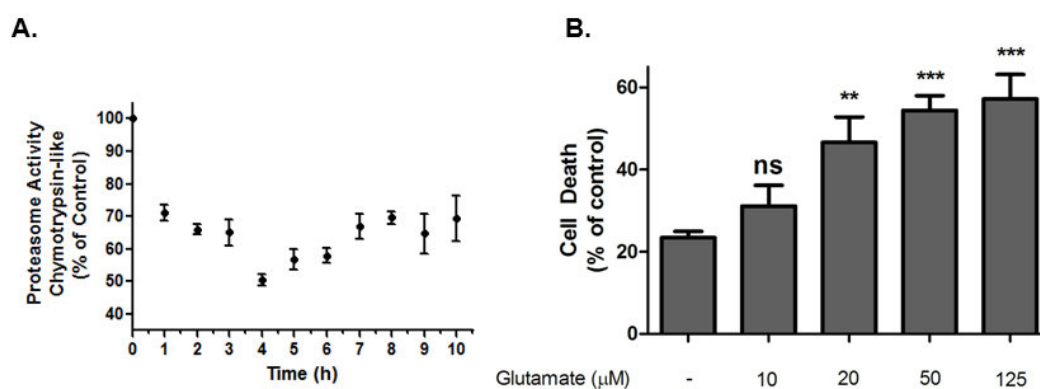

Figure 15- Effect of excitotoxic glutamate stimulation on proteasome activity. A) Hippocampal neurons (7 DIV) were stimulated with 125 µM of glutamate and further incubated with the conditioned medium for the indicated period of time. The proteasome chymotrypsin-like activity was evaluated by using the fluorogenic substrate Suc-LLVY-AMC (25 µM). The control proteasome activity, measured in unstimulated neurons, was set to 100%. The results are the average ± SEM of 3 to 8 experiments, performed in independent preparations. Statistical analysis was performed by ANOVA, followed by Dunnett's post test, comparing all the conditions with the control. All effects are statistically significant with ***P<0.001. B) Effect of increasing concentrations of glutamate on cell death. Hippocampal neurons (7 DIV) were stimulated for 20 min with 10, 20, 50 or 125 µM and further incubated in culture conditioned medium for 4 h. After fixation, cell death was evaluated by fluorescence microscopy with Hoechst 33342. The results are the average ± SEM of 9 experiments, performed in independent preparations. Statistical analysis was performed by ANOVA, followed by Dunnett's post test, comparing all the conditions with the control. All effects are statistically significant with ***P<0.001, *P<0.05 and **P<0.01, except where indicated as n.s. (P>0.05).

Proteasome activity during glutamate induced excitotoxicity

To validate the results obtained using a fluorogenic substrate (previous section) which showed a downregulation of the proteasome activity under excitotoxic conditions, we used a GFP-based reporter strategy to monitor the rate of proteasome activity (Dantuma et al., 2000) under the same experimental conditions. Ub^{G76V}-GFP is a fusion protein comprising an ubiquitin fusion degradation (UFD)-targeted green fluorescent protein (GFP) that is constitutively targeted for proteasomal degradation under basal conditions (Dantuma et al., 2000). After transfection, hippocampal neurons (5 DIV) were further incubated for 48 h in cultured conditioned medium, to allow protein expression, before stimulation with 125 µM of glutamate for 20 min. After 4 h or 8 h of incubation in culture conditioned medium, we found a 2.67 ($P<0.05$, third lane) and 3.49 ($P<0.01$, fourth lane) fold increase in the GFP protein levels, respectively (Figure 16A). Similarly, the GFP protein levels increased by about 2.67 fold when the cells were incubated for 4 h with 1 µM of the proteasome inhibitor MG132 (Figure 16A, $P<0.05$, second lane). Taken together, the results obtained with the GFP reporter and using the fluorogenic substrate of the proteasome indicate that excitotoxic stimulation of hippocampal neurons downregulates proteasome activity.

To determine the effect of proteasome inhibition on the viability of cultured hippocampal neurons (7 DIV) the cells were incubated with 1 µM and 10 µM MG132 for 5 h, and cell death was assessed by fluorescence microscopy with Hoechst33342. This incubation period with the proteasome inhibitor was chosen based on the results showing that i) excitotoxic stimulation with glutamate induces a maximal inhibition of the proteasome at 4-5 h after the insult (Figure 15A), and ii) cell death was observed at

4 h after glutamate stimulation (Figure 15B). We found that cell viability was not significantly affected (P>0.05) in the presence of 1 µM MG132 (Figure 16B) and, as expected, under the same conditions there was an accumulation of polyubiquitin conjugates, a common hallmark associated with proteasome inhibition, as shown by Western Blot analysis (Figure 16C, compare the first lane with the second). The accumulation of polyubiqutin conjugates was significantly higher when hippocampal neurons were incubated with 10 µM MG132 for 5 h (Figure 16C, compare second lane with the third).

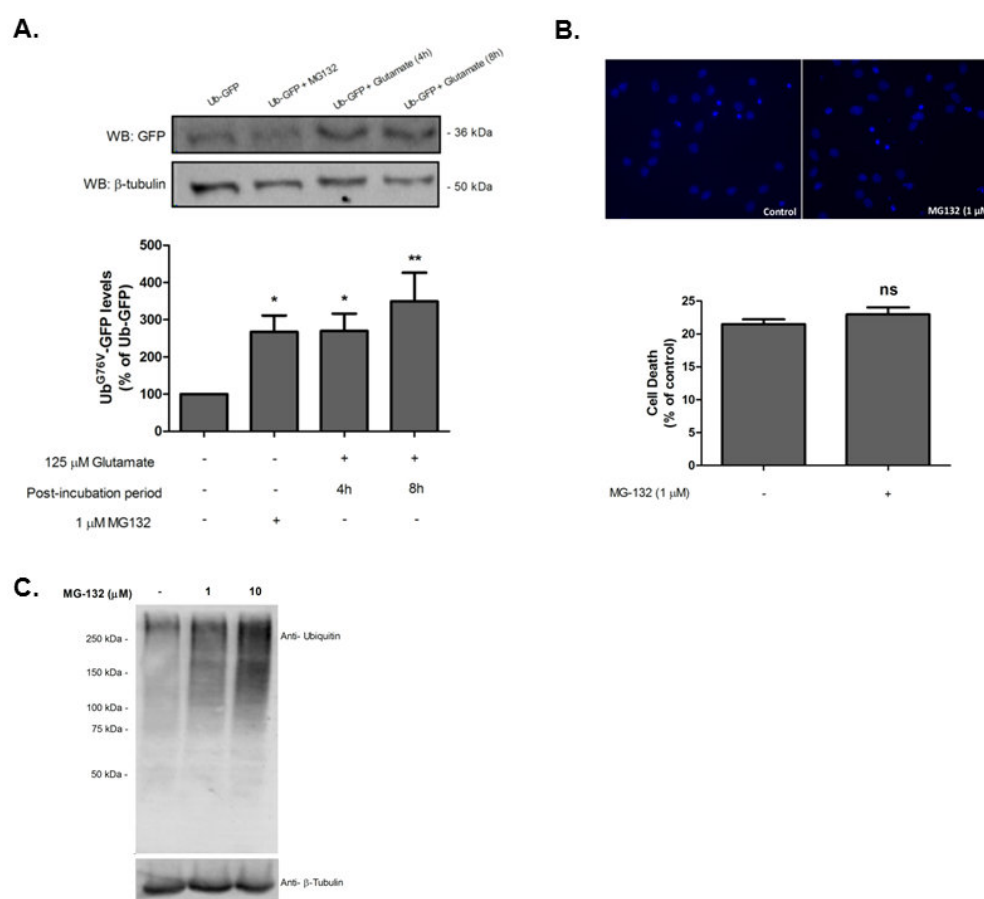**Figure 16- Effect of proteasome inhibition in hippocampal cultured neurons.** A) Effect of excitotoxic stimulation with glutamate on the activity of the proteasome as determined with a fluorescent protein tagged for proteasomal degradation. Ub^{G76V}-GFP transfected hippocampal neurons were incubated with 125 µM glutamate for 20 min and further incubated in culture conditioned medium for 4h or 8h. The

abundance of Ub^{G76V}-GFP was evaluated by Western blot using an anti-GFP antibody. The control Ub^{G76V}-GFP levels, determined in non-stimulated neurons, was set to 100%. The results are the average ± SEM of 3-4 independent experiments performed in different preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's test (*P<0.01, **P<0.01). B) Effect of proteasome inhibition on cell death. Hippocampal neurons (7 DIV) were incubated with 1 μM MG132 during 5 h. After fixation, cell death was evaluated by fluorescence microscopy with Hoechst33342. The results are the average ± SEM of 8 different experiments performed in independent preparations. Statistical analysis was performed by unpaired Students t-test comparing the experimental condition with the control with n.s (P<0.05). C) Effect of proteasome inhibition on the abundance of ubiquitin-conjugated proteins. Hippocampal neurons (7 DIV) were incubated with 1 or 10 μM MG132 during 5 h. The accumulation of ubiquitinated-proteins was determined by Western Blot using an antibody anti-ubiquitin.

Proteasome inhibition induces hippocampal cell death

The ubiquitin-proteasome system (UPS) regulates multiple cellular functions (Hershko et al., 1998; Ehlers et al., 2003; Colledge et al., 2003). Also, mutations in the ubiquitination machinery, in proteasome substrates or even in the proteasome itself are associated with many neurodegenerative diseases (Ciechanover et al., 2003). Similarly, proteasome inhibition with synthetic compounds induces apoptosis in neurons (Qiu et al., 2000) by promoting the release of apoptotic factors such as cytochrome c, Smac and others from the mitochondria (Sun et al., 2004). Since proteasomal degradation of ubiquitinated substrates is the major pathway for ubiquitin recycling through associated deubiquitinating enzymes (Reyes-Turcu et al., 2009), its inhibition induces an accumulation of ubiquitinated proteins decreasing, at the same time, free ubiquitin levels (Patrick et al., 2003; Xu et al., 2009).

The experimental conditions used in the previous section did not allow confirming that that proteasome inhibition induces cell death. This was further

investigated by incubating cultured hippocampal neurons with 0.05 µM, 0.1 µM or 1 µM β-lactone, a selective and irreversible proteasome inhibitor (Lee et al., 1998), for 6 h. At these concentrations, the proteasome chymotrypsin-like activity was decreased to 63.6 % (P<0.01), 28.9 % (P<0.001) and 18.9 % (P<0.001) of the control, respectively (Figure 17A). Along with proteasome inhibition, the number of polyubiquitinated-conjugated proteins was increased, in a dose-dependent manner, as seen in the Western Blot analysis (Figure 17B, C), confirming the inhibition of the proteasome under the experimental conditions used.

To further address the effect of proteasome inhibition on cell viability, cultured hippocampal neurons were incubated with 0.05 µM and 1 µM β-lactone for different periods of time. We found that proteasome inhibition with β-lactone can indeed induce hippocampal cell death. When applied at a concentration of 0.05 µM or 1 µM β-lactone for 12 h the proteasome inhibitor induced 22.5% (P<0.05) and 26.9% (P<0.01) neuronal death, respectively (Figure 17D). However, no difference (P>0.05) was observed using the same concentrations for 8 h incubation period (Figure 17D). These results suggest that the lack of effect of MG132 on cell survival shown in Fig. 16B is probably due to the short incubation period used.

We can therefore conclude that proteasome inhibition with pharmacological compounds induce hippocampal cell death, with a concomitant increase in polyubiquitin-conjugates. Furthermore, the results show that 8 h incubation period with β-lactone is not enough to induce a typical apoptotic-like cell death morphology.
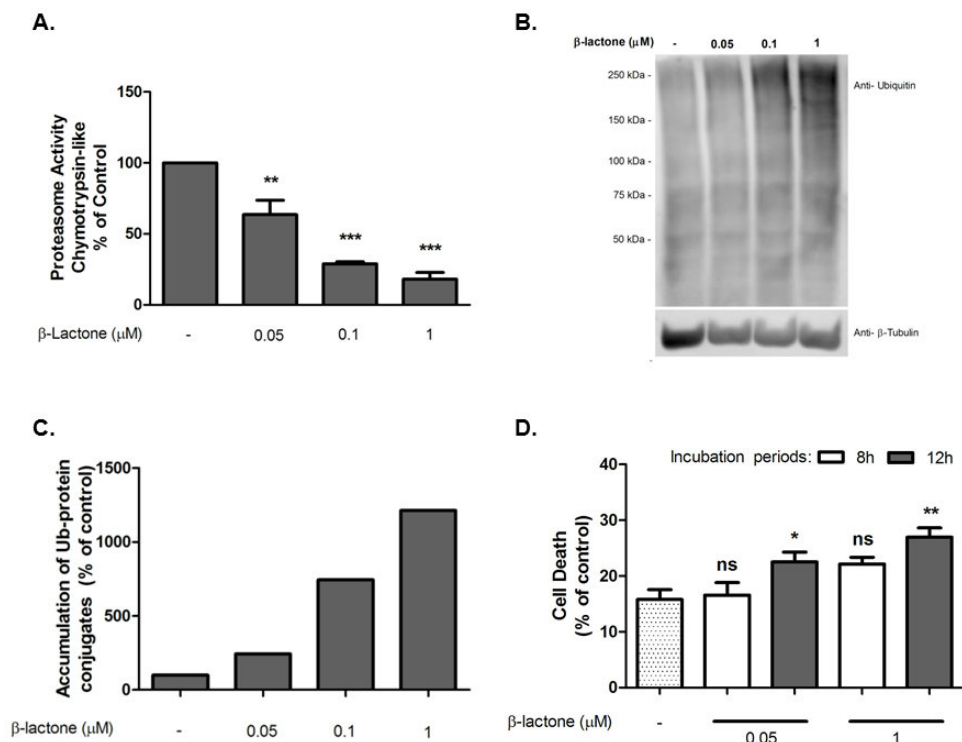
Figure 17- Proteasome inhibition induces hippocampal cell death. A-C) Effect of proteasome inhibition on the abundance of ubiquitin-conjugated proteins. Hippocampal neurons (7 DIV) were incubated with 0.05, 0.1 or 1 μM β-lactone during 6 h. A) The chymotrypsin-like activity was measured with the fluorogenic substrate 25 μM Suc-LLVY-AMC. The results are the average ± SEM of 3 different experiments performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's test (**P<0.01, ***P<0.001). B) The accumulation of ubiquitinated-proteins was determined by Western Blot, using an antibody anti-ubiquitin (A) and the quantification of the results obtained for the 75 kDa-250 kDa proteins is shown in C. The control condition, corresponding to neurons not exposed to the proteasome inhibitor, was set to 100%. D) Effect of proteasome inhibition with β-lactone on cell death. Hippocampal neurons (7 DIV) were incubated for 8h or 12h with 0.05 or 1.0 μM β-lactone. After fixation, cell death was evaluated by fluorescence microscopy with Hoechst33342.The results are the average ± SEM of 3 different experiments performed in independent preparations Statistical analysis was performed by one-way ANOVA, followed by Dunnett's test (ns, P>0.04; *P<0.05; **P<0.01).

Oxygen and Glucose Deprivation (OGD) induces cell death in cultured cortical neurons

Oxygen and glucose deprivation is a well-known *in vitro* model of transient global ischemia (Goldberg et al., 1993). After depriving mixed cortical cultures both from oxygen and glucose for 50 min, overstimulation of glutamate receptors and consequent Ca^{2+} influx was described to induce ~50% of cell death (Goldberg et al., 1993; Grabb et al., 2002). Under excitotoxic conditions the influx of Ca^{2+} is responsible for activating several intracellular proteases, including calpains and caspases (Takano et al., 2005). Calpain and caspase activation leads to the cleavage of several intracellular proteins, including α-spectrin, a 280 kDa cytoskeleton protein. While calpain activation is responsible for generating 150 kDa and 145 kDa spectrin fragments, activation of caspase-3 generate the same 150 kDa and an additional 120 kDa fragment (Wang et al., 2000). Thus, evaluation of these truncation species is a good tool to assess calpain/caspase activation in neurons subjected to OGD.

We established a culture of cortical neurons in our laboratory as a model to study the mechanisms of neuronal death in *in vitro* ischemia. First, cell density was optimized ($94.7x10^3$ cells/cm^2) and then the duration of OGD insult as well as the recovery times after the lesion. In the studies performed with 10 DIV cortical neurons, OGD times ranging from 40 to 135 min and recovery times between 3 to 24 h did not induce neuronal death (data not shown). Since neuronal loss promoted by glutamate increases in older cultures (Choi et al., 1987), we wondered whether OGD would have a stronger impact in the viability of neurons maintained in culture for a longer period. In fact, exposure of 14-15 DIV cortical neurons to 2 h of OGD followed by a 12 h

incubation period in culture conditioned medium increased LDH release by 13.8 % (P<0.01) relative to the control (Figure 18A). Furthermore, at 30 min, 1 h or 2 h after OGD there was an increase in SBDP, which was correlated with a decrease in the expression of the full-length protein (Figure 18B, upper panel). Quantitative analysis of the results shows an increase in the SBDP by 37.1 % (P>0.05), 46.1 % (P<0.05) and 42.5 % (P<0.05) when compared to control, respectively (Figure 18B, lower panel).

We can therefore conclude from these data that 2 h of OGD induced cortical neuron death, as determined 12 h after the insult. However, at 30 min after OGD it was possible to observe α-spectrin cleavage into 145 and 150 kDa fragments, suggesting that at least calpains are activated in this model. However, statistical significance was only observed at 1 h and 2 h after oxygen and glucose deprivation.
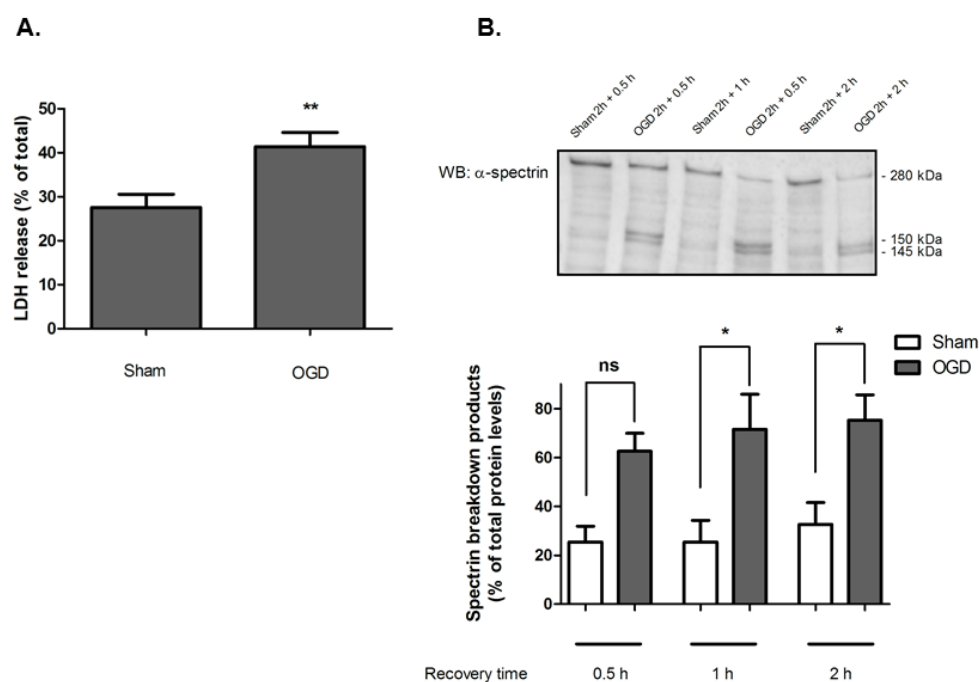
Figure 18- OGD induces cell death in cultured cortical neurons. A) Effect of OGD in the viability of cultured cortical neurons. Cortical neurons (14-15 DIV) were subject to OGD for 2 h and allowed to recover for 12 h in cultured conditioned medium. Cell death was evaluated by the LDH assay and the results were normalized to the full-kill (100% cell death). The results are the average ± SEM of 7 experiments performed in independent preparations. Statistical analysis was performed by unpaired

Students *t*-test comparing the experimental condition with the control (**P<0.01). B) Effect of OGD on α-spectrin cleavage. Cortical neurons (14 DIV) were subjected to OGD for 2 h and allowed to recover for 30 min, 1h or 2h. Full-length α-spectrin and breakdown products were analyzed by Western Blotting (upper panel) and the percentage of SBDP was quantified by normalizing to total α-spectrin (280, 150 and 145 kDa bands) in each condition. The results are the average ± SEM of 3 different experiments performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni's test (ns, P>0.05;*P<0.05).

CHAPTER 5 - DISCUSSION

In this work we found that brief excitotoxic application of glutamate induces a time-dependent decrease in the proteasome chymotrypsin-like activity (Figure 15A) and this effect was correlated with increased cell death (Figure 15B). Although maximal inhibition was seen at 4 h after the insult, the activity did not fully return to basal levels. We further investigated the effect of the excitotoxic stimulation with glutamate on the activity of the proteasome using a GFP based probe that is constitutively degraded by the proteasome (Dantuma et al., 2000; Djakovic et al., 2009). In agreement with the results obtained with the fluorogenic substrate of the proteasome, excitotoxic stimulation of hippocampal neurons transfected with Ub^{G76V}-GFP (Dantuma et al., 2000) increased the accumulation of the protein in the cells, showing a decrease of the proteolytic activity of the proteasome (Figure 16A). In a previous study this approach also showed that prion proteins act as an endogenous proteasome inhibitor thus increasing the GFP signal (Deriziotis et al., 2008).

In a recent study from our laboratory using native gel electrophoresis it was shown that excitotoxic treatment with glutamate increases the 20S proteasome activity while the 26S proteasome activity was downregulated, suggesting that 26S proteasomes are dismantled rather than being inhibited by an unknown mechanism (unpublished data). Moreover, the Ub^{G76V}-GFP protein is constitutively directed for proteasomal degradation in a manner that does not require further ubiquitination, showing that its accumulation is promoted by 26 proteasome impairment rather than by a deficit in the ubiquitination machinery. These results are also correlated with the observed disassembly of the 26S proteasome upon transient forebrain ischemia (Asai et al., 2002).

We also showed that incubation of hippocampal neurons with the proteasome inhibitor β-lactone induces an accumulation of ubiquitinated proteins in a concentration dependent manner (Figure 17C), while cell death was only observed for longer exposure periods (Figure 17D). These results are in agreement with others where proteasome inhibition was correlated with an apoptotic cell death in cultured cortical neurons (Qiu et al., 2000), and is associated with an increased accumulation of ubiquitinated proteins (Xu et al., 2009). The lack of cell death for shorter incubation periods with the proteasome inhibitor (Figure 17D) is also in agreement with a previous report showing that neuronal death is only observed for longer times of exposure (>12 h) (Ding et al., 2006). Given the delayed effects of proteasome inhibitors on neuronal survival, the glutamate-induced downregulation of the proteasome activity is likely not play a major role in excitotoxic cell death. However, inhibition of the proteasome may play a more important role in the demise process when combined with other alterations in the proteolytic machinery of the cell, such as the increase in calpain activity that is characteristic of the excitotoxic and ischemic injuries.

We established in our laboratory the appropriate experimental conditions to use cultured cortical neurons as a model to study the mechanisms of neuronal death after oxygen and glucose deprivation, an *in vitro* model of ischemia. Our results show that OGD for 2 h induced an increase around 13.8% of cell death relative to the control control in 14-15 DIV cortical neurons (Figure 18A). However, no cell death was observed in 10 DIV cell cultures using the same protocol. During cortical neuronal development the mRNA for the GluN1 subunit increases progressively between days 1 and 21 *in vitro*. The mRNA for GluN2A only increases after 7 DIV while the mRNA for GluN2B increased between days 1 to 7 (Zhong et al., 1994). In contrast, expression of all four AMPAR subunits (GluA1-4), both mRNA and protein, are increased during

in vitro neuronal maturation (Orlandi et al., 2011). The lack of cell death upon deprivation of 10 DIV cortical neurons from oxygen and glucose may be explained by their immature state. Since NMDAR antagonists are neuroprotective against glutamate excitotoxicity (Goldberg et al., 1993), the lack of these receptors at the cell surface can be neuroprotective as well. Additionally, data published from Grabb and co-workers, shows that 50 min of OGD increases cell death by 50% when evaluated 24 h later. However, it is not entirely possible to compare these results with ours, due to differences in the OGD protocol, recovery times, cell culture density and cell culture components.

We also found that α-spectrin is cleaved into 145 and 150 kDa fragments in cerebrocortical neurons exposed to OGD. This 145 kDa fragment is generated by calpain cleavage while the 150 kDa can be generated both by calpain and caspases (Wang et al., 1998; Wang et al., 2000), suggesting that calpains are activated with this OGD protocol. An additionally 120 kDa cleavage product can also be generated by caspase cleavage (Wang et al., 1998), but we did not observe this fragment in our WB analysis (data not shown).

A major hallmark of necrosis is the LDH leakage to the extracellular medium occurring when cell membrane integrity is compromised (Cummings et al., 2012), and calpain activation is also often associated with necrotic processes (Wang et al., 2000). During transient forebrain ischemia protein synthesis is severely impaired, and it has been shown that translation activity is required for apoptosis (Paschen et al., 2003). However, in neuronal primary cultures early apoptotic cells are not cleared by phagocytosis, and often become late apoptotic cells, also known as secondary necrosis, when cell membrane becomes permeabilized (Poon et al., 2010). Furthermore, calpain activation can induce a caspase-independent apoptotic cell death mechanism by

inducing nuclear translocation of AIF which promotes DNA fragmentation and chromatin condensation (Thal et al., 2011). However, it remains to be determined whether neuronal death after exposure of cortical neurons to OGD, under the experimental conditions used in this work, occurs by necrosis or apoptosis, and further experiments are required to elucidate this point.

CHAPTER 6 - CONCLUSION

Previous studies showed a downregulation of the activity of the proteasome in experimental models of transient forebrain ischemia, which mimic stroke to a large extent, including the excitotoxic phenomena, ATP depletion, generation of ROS, Ca^{2+} unbalance and others. However, these studies did not elucidate the role of the excitotoxic injury in the downregulation of the proteasome activity. In this work we showed that excitotoxic stimulation of cultured hippocampal neurons induces a transient downregulation of the proteasome activity, which was correlated with neuronal death.

However, inhibition of the proteasome with chemical inhibitors only showed a delayed effect on neuronal survival, indicating that the alteration of the proteasome after the excitotoxic insult, and the consequent accumulation of ubiquitinated proteins, may not play a major role in the demise process. In fact, whether proteasome dysfunction during brain ischemia is neuroprotective, i.e. if can prevent a further increase in cell death, or detrimental still remains to be elucidated (Meller et al., 2009). The precise mechanism initiated by the excitotoxic cascade that leads ultimately to proteasome disassembling also remains unclear. However, dissecting these mechanisms can be a potential therapeutical target to prevent cell death observed during stroke and other neurodegenerative diseases.

CHAPTER 7 - FUTURE PERSPECTIVES

Recent results from our laboratory showed that excitotoxic stimulation with glutamate promotes: i) 26S proteasome disassembly into its major components, reducing the overall activity; ii) accumulation of ubiquitinated proteins both in the cytoplasm and nuclear compartments; iii) the decrease in chymotrypsin-like activity of the 26S proteasome was slightly prevented by cathepsin-L and caspases inhibitors and, iv) the overall activity of DUBs is compromised, but no effect was observed on the Uch-L1 activity. Given these results, it will be important to address these same questions in our cortical cell culture using the OGD model of in vitro ischemia. In fact, evaluation of proteasome activity and analysis of the accumulation of ubiquitinated proteins upon 2 h of OGD is ongoing work in the laboratory. Additionally, to further validate the method experiments should be conducted using NMDA and AMPARs antagonists to understand if cell death induced by OGD is dependent on the activation of glutamate receptors.

Currently we are trying to identify which ubiquitinated proteins are accumulated in cultured cortical neurons upon proteasome disassembly. For this purpose, we are using tandem-repeated ubiquitin binding entities (TUBEs) (Hjerpe et al., 2009), which are based on UBA domains with high affinity for tetra-ubiquitin (Hjerpe et al., 2009). This technique allows isolating proteins with K48 and to a less extent K63 polyUb chains attached, and the identification of these proteins by mass-spectrometry will unravel novel therapeutic targets to prevent neuronal loss upon an ischemic episode.

During the apoptotic process caspases are activated, and the controlled cleavage of several cytosolic proteins promotes DNA fragmentation, chromatin condensation and cell shrinkage (Wang et al., 2000). Additionally, it has been shown that several

components of the UPS can undergo selective degradation by caspases. Rpt5, Rpn2, Rpn10 (Adrain et al., 2004), Rpn2 (Sun et al., 2004), α2, α6 and Rpt1 (Jang et al., 2007) are the best characterized caspase substrates, and their cleavage contributes to proteasome impairment in cells committed to die. Purifying proteasomes from cerebrocortical neurons transduced with a Rpt6-HA tagged subunit, which is successfully incorporated in wild-type proteasomes (Djakovic et al., 2012), will allow investigating whether any of the subunits is cleaved under these conditions. There are several candidate proteins responsible for maintaining the 26S proteasome and, therefore, their degradation by caspases and/or by calpains may destabilize and disassemble the proteasome. Ecm29 is a protein responsible to tether the CP to the RP (Leggett et al., 2002), the Rpn12 subunit is responsible to join the lid and the base subcomplexes of the RP (Tomko et al., 2011), the Rpn6 subunit interacts with the α2 and the Rpt6 subunits, acting as a clamp that holds together the CP and RP (Pathare et al., 2012), and Hsm3 is a chaperon responsible to induce a proper scaffolding of the RP (Barrault et al., 2012). All these proteins can be potential candidates involved in proteasome disassembly after OGD and in brain ischemia. Strikingly, several components of the RP can be themselves targeted for proteasomal degradation, suggesting another route for degradation in which proteasome activity can be subject of autoregulation, and this pathway should also be considered (Tai et al., 2010). Moreover, heat-shock protein 70 has been shown to protect RP from degradation upon 26S disassembly mediated by oxidative stress suggesting that overexpression of this protein during recovery periods may be beneficial by preventing proteasome impairment (Grune et al., 2011).

One important question remains to be answered in this field. It is not known whether cell death is the cause or effect of proteasome impairment. Usp14 is a RP

associated DUB present substoichiometrically when compared to other resident 26S proteasome subunits (Tai et al., 2010), and its inhibition accelerates proteolysis in cells thereby ameliorating oxidative stress induced toxicity (Lee et al., 2010). The use of this compound can be used to counteract proteasome downregulation during ischemia by promoting an increase in activity in the remaining assembled proteasomes (Lee et al., 2010).

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