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About the cover: The cover shows the symbol for the concept yin and yang. Yin yang represents complementary opposites that interact within a greater whole, as part of a dynamic system. VGLUT1 and VGLUT2 play the same function of glutamate uptake into synaptic vesicles, but they have some complementarity within the glutamatergic synapses. They show a largely complementary distribution in the CNS, which may correlate with different probabilities of glutamate release, and are expressed differently during development. In this thesis, we found that VGLUT2 is downregulated after excitotoxic and ischemic insults, in contrast with VGLUT1.

Clivagem de VGLUT1 e VGLUT2 em condições excitotóxicas e de isquémia cerebral

VGLUT1 and VGLUT2 cleavage under excitotoxic conditions and in cerebral ischemia

Andrea Catarina Amaro de Campos Lobo

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Abbreviations

AD	Alzheimer's disease
AIF	Apoptosis inducing factor
Akt/PKB	Protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease activating factor-1
ATP	Adenosine 5′-triphosphate
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BNPI	Brain-specific Na^+-dependent Pi cotransporter
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
CA	*Cornu Ammonis*
CAD	Caspase-activated deoxyribonuclease
Cl^-	Chloride ion
CaMK	Calcium/Calmodulin-dependent protein kinase
CaN	Calcineurin
cDNA	Complementary DNA
CINC	Cytokine-induced neutrophil chemoattractant
CLAP	Chymostatin, leupeptin, antipain and pepstatin
CNS	Central nervous system
Contra	Contralateral
Ctr	Control
DA	Dopamine
DIABLO	Direct IAP binding protein with low pI
DIV	Days in vitro

DNA	Deoxyribonucleic acid
DNPI	Differentiation-associated Na^+-dependent Pi cotransporter
DTT	Dithiothreitol
ECF	Enhanced chemifluorescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EPSC	Excitatory postsynaptic current
ER	Endoplasmic Reticulum
GAD	Glutamic acid descarboxylase
GFP	Green fluorescent protein
Glu	Glutamate
GRIP	Glutamate receptor-interacting protein
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
HBSS	Hank's balanced salt solution
HD	Huntington's disease
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HI	Hypoxia-ischemia
Hsp70	Heat-shock protein 70
HtrA2	High temperature requirement protein
IAP	Inhibitor of apoptosis
ICAM	Intercellular adhesion molecule
IL	Interleukin
Ipsi	Ipsilateral
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	IP_3 receptor
KA	Kainate
KCC	K^+-Cl^- co-transporter
LDCV	Large dense core vesicle

LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
MCP	Monocyte chemoattractant protein
mEPSC	Miniature excitatory postsynaptic current
mRNA	Messenger RNA
NAD^+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCX	Na^+/Ca^{2+} exchanger
NKCC1	Na^+-K^+-Cl^- co-transporter
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
O_2^-	Superoxide
OGD	Oxygen and glucose deprivation
OH	Hydroxyl
PBS	Phosphate buffered saline
PC-12	Pheochromocytoma cell line
PCR	Polymerase chain reaction
PD	Parkinson’s disease
PEST	Sequence rich in the amino acids: proline, glutamate, serine and threonine
Pi	Inorganic phosphate
PID	Peri-infarct depolarization
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PKM	Protein kinase M

PMSF	Phenylmethylsulfonyl fluoride
PSD	Post-synaptic density
PTD	Protein transduction domain
PTZ	Pentylenetetrazol
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RYR	Ryanodine receptor
SAP97	Synapse associated protein 97
SBDPs	Spectrin breakdown products
SDS-PAGE	SDS-poliacrylamide gel
SERCA	Sarcoplasmic/ER Ca^{2+} ATPase
SLC	Solute carrier
SLMV	Synaptic-like microvesicle
Smac	Second mitochondrial activator of caspases
SSV	Small synaptic vesicle
Striat	Striatum
SV	Synaptic vesicle
TAT	Trans-activator of transcription
TGF	Transforming growth factor
TGN	*trans* Golgi network
TNF	Tumor necrosis factor
TNFR	TNF receptor
tPA	Tissue plasminogen activator
TRP	Transient receptor potential
UPS	Ubiquitin proteasome system
VAchT	Vesicular acetylcholine transporter
VGCC	Voltage-gated Ca^{2+} channels
VGLUT 1	Vesicular glutamate transporter type 1

VGLUT 2	Vesicular glutamate transporter type 2
VGLUT 3	Vesicular glutamate transporter type 3
VMAT1	Vesicular monoamine transporter type 1
VMAT2	Vesicular monoamine transporter type 2

Resumo

Os acidentes vasculares cerebrais (AVC) constituem uma doença complexa, causada sobretudo pelo bloqueio de uma artéria responsável pela irrigação do cérebro (isquémia), provocando graves danos neurológicos. Os AVC são uma das principais causas de morte e de incapacidade ou invalidez a longo prazo na população adulta em países desenvolvidos, incluindo Portugal. Os AVC isquémicos iniciam-se com a oclusão de vasos sanguíneos cerebrais, frequentemente as artérias cerebral média ou anterior, e desencadeiam uma cascata de eventos nomeadamente uma falha na bionergética, excitotoxicidade, stress oxidativo, despolarização da membrana plasmática, e inflamação, que causam morte celular.

A excitotoxicidade é um dos eventos iniciais após um AVC isquémico, e consiste na estimulação excessiva de receptores de glutamato devido a uma acumulação excessiva de glutamato no espaço extracelular. Os mecanismos excitotóxicos podem provocar morte celular de forma aguda (necrose) ou iniciar eventos moleculares que levam a morte celular apoptótica retardada. A excitotoxicidade desempenha também um papel importante noutras doenças neurológicas, como a epilepsia e doenças neurodegenerativas, e é considerada um dos principais alvos terapêuticos para tratar AVC.

A investigação sobre a isquémia cerebral tem-se focado sobretudo nos efeitos pós-sinápticos ao nível das sinapses glutamatérgicas. Considerando que a libertação de glutamato por exocitose depende da acumulação deste neurotransmissor em vesículas sinápticas mediada por transportadores vesiculares de glutamato (VGLUTs), e que alterações nos níveis de VGLUTs afectam directamente a libertação de glutamato, neste trabalho investigaram-se possíveis variações nos níveis de VGLUTs após insultos excitotóxicos ou isquémicos.

Os resultados obtidos mostram a clivagem do VGLUT2 por calpaínas em neurónios de hipocampo em cultura após estímulo excitotóxico com glutamato, e após privação de oxigénio e glucose. A clivagem do VGLUT2 foi também observada após injecção de cainato no hipocampo, e depois de oclusão transitória da artéria cerebral média (MCAO) em murganhos. Por outro lado, o VGLUT1 só foi clivado após estímulo excitotóxico de glutamato, ainda que esse efeito tenha sido menos significativo que o observado para o VGLUT2. Além disso, verificou-se um aumento dos níveis de VGLUT1 após injecção de cainato no hipocampo de murganhos. A incubação de vesículas sinápticas isoladas do córtex cerebral de ratos e de uma proteína de fusão de GST com o C-terminal do VGLUT2 com calpaína recombinante também causou a clivagem do VGLUT2. Na clivagem de GST-VGLUT2 C-terminal observou-se a formação de produtos de clivagem. Ensaios de

immublotting usando anticorpos específicos contra diferentes regiões do VGLUT2, assim como a sequenciação do terminal amínico das formas truncadas da proteína de fusão GST-VGLUT2, permitiram identificar dois locais de clivagem pela calpaína no VGLUT2, nos aminoácidos Asn534 e Lys542. A utilização de um péptido designado TAT-VGLUT2, contendo a sequência de aminácidos da região do terminal carboxílico do VGLUT2, reduziu especificamente a clivagem do VGLUT2 em neurónios de hipocampo sujeitos a estimulação excitotóxica de glutamato, sem afectar a capacidade da calpaína na clivagem de outros substratos.

O C-terminal do VGLUT2 também possui uma sequência de consenso (DELD) para clivagem pela caspase-3, mas o transportador não foi clivado por esta protease em neurónios de hipocampo sujeitos a estimulação excitotóxica de glutamato, nem em neurónios sujeitos a indução de apoptose com estaurosporina ou remoção dos factores tróficos. No entanto, foi observada a clivagem e formação de um produto de clivagem quando a proteína de fusão GST-VGLUT2 C-terminal foi incubada com caspase-3 recombinante, e a formação deste produto não ocorreu após mutação dos resíduos de asparatato para alanina na sequência de consenso.

Experiências de imunocitoquímica usando neurónios de hipocampo transfectados com proteínas de fusão da GFP com VGLUT2, ou com as formas truncadas do transportador nos aminoácidos 534 e 542, mostraram que a clivagem do VGLUT2 por calpaína reduz a sua localização sináptica. Os resultados obtidos neste trabalho permitem concluir que o VGLUT2, ao contrário do VGLUT1, é clivado por calpaínas em condições excitotóxicas ou isquémicas, alterando a sua localização intracelular. Esta alteração na distribuição do VGLUT2 pode afectar a transmissão sináptica glutamatérgica e contribuir para uma redução da morte celular em situações de isquémia ou excitotoxicidade. O facto de o VGLUT2 ser sobretudo expresso nas fases iniciais do desenvolvimento permite sugerir que a sua clivagem por calpaína pode ter um papel importante em acidentes vasculares cerebrais no recém-nascido.

Abstract

Stroke is a complex disease mainly caused by a blockage of an artery responsible for blood supply to the brain (ischemia), causing severe neurological impairment. It constitutes a leading cause of death and long term adult disability in the developed countries, including Portugal. The blood vessel occlusion in the ischemic brain injury, commonly the middle and anterior cerebral arteries, is followed by a cascade of events namely bionergectic failure, excitotoxicity, oxidative stress, peri-infarct depolarizations, and inflammation, ultimately leading to cell death.

Excitotoxicity is one of the first events in the ischemic stroke, and consists in the overstimulation of glutamate receptors caused by an extracellular accumulation of glutamate. Excitotoxic mechanisms can cause acute cell death (necrosis) or initiate events that ultimately lead to delayed apoptotic-like cell death. Excitotoxicity is also involved in other neurological disorders, such as epilepsy and neurodegenerative diseases, and it is indeed considered an important target for stroke therapy.

So far, research in cerebral ischemia has been focusing on the postsynaptic effects in glutamatergic synapses. However, considering that the exocytotic release of glutamate depends on the uptake of the neurotransmitter into synaptic vesicles mediated by vesicular glutamate transporters (VGLUTs), and that changes in VGLUTs levels will directly affect the vesicle loading with glutamate and neurotransmitter release, we investigated putative changes in VGLUT levels after excitotoxic or ischemic insults.

We found that VGLUT2 was cleaved by calpains following glutamate excitotoxic stimulation or oxygen and glucose deprivation in cultured hippocampal neurons. VGLUT2 cleavage was also observed after intrahippocampal injection of kainate and following transient middle cerebral artery occlusion (MCAO) in mice. In contrast, VGLUT1 was only cleaved after stimulation of hippocampal neurons with glutamate, but to a lower extent than VGLUT2, and was upregulated after intrahippocampal injection of kainate. Incubation of isolated cerebrocortical synaptic vesicles with recombinant calpain also lead to a downregulation of VGLUT2, and a fusion protein of GST with VGLUT2 C-terminal region was cleaved upon incubation *in vitro* with recombinant calpain. Immunoblot analysis using antibodies that target different regions of VGLUT2, and N-terminal sequencing of the cleavage products resulting from incubation of recombinant calpain with the GST-VGLUT2 C-terminal fusion protein, showed that calpain cleaves VGLUT2 at amino acids Asn534 and Lys542. Accordingly, incubation of cultured hippocampal neurons with a TAT-VGLUT2 peptide, containing the C-terminal region of VGLUT2, inhibited the downregulation

of the hippocampal neurons subjected to excitotoxic conditions, without affecting calpain activity towards other substrates.
Although VGLUT2 contains a consensus DELD sequence that is typically targeted by caspase-3, the transporter was not cleaved by this protease in hippocampal neurons following glutamate stimulation, or after induction of apoptosis with staurosporine or by trophic factor withdrawal. However, incubation of the GST-VGLUT2 C-terminal fusion protein with recombinant caspase-3 was able to produce a cleavage product, that was not found upon mutation of the aspartic acid residues in the consensus sequence to alanine residues.
Immunocytochemistry of hippocampal neurons expressing GFP fusion proteins with the full length VGLUT2, or VGLUT2 truncated at Asn534 or at Lys 542, showed that the truncated forms that result from calpain cleavage of VGLUT2 have reduced the transporter synaptic localization. Taken together our results show that VGLUT2, unlike VGLUT1, is cleaved by calpain under excitotoxic or ischemic conditions, which is likely to affect glutamatergic neurotransmission and may contribute to a reduced cell death during excitotoxic and ischemic events. The fact that VGLUT2 is mainly expressed early in development suggests that calpain cleavage may have a role in neonatal brain injury.

Chapter 1

Introduction

1.1. Cerebral Ischemia

Stroke is a complex and devastating disease, constituting the second leading cause of death worldwide. The disease is the leading cause of acquired disability in adults, and is a considerable social burden due to excessive costs of long hospitalizations, and rehabilitation (Brouns and De Deyn, 2009; Lloyd-Jones et al., 2009; Moskowitz et al., 2010). In Portugal, stroke is also one of the main causes of death, and statistics indicates that the country has the highest stroke mortality in Western Europe (Correia et al., 2004). Stroke can be subdivided into ischemic and hemorrhagic (Doyle et al., 2008). Ischemic strokes are more frequent and constitute 87% of all cases. The ischemic strokes can be caused by a thrombosis, an embolism or a general hypo-perfusion, all of which result in a constraint of blood flow to the brain (Dirnagl et al., 1999; Doyle et al., 2008; Lloyd-Jones et al., 2009). The remaining 13% are hemorrhagic strokes or caused by a cardiac arrest (Lloyd-Jones et al., 2009; Moskowitz et al., 2010). Symptoms of stroke include impaired vision and speech, dizziness, muscular weakness or numbness and severe headache (Candelario-Jalil, 2009; Moskowitz et al., 2010). The incidence of stroke is also predicted to increase with an aging population (Donnan et al., 2008; Moskowitz et al., 2010).

Because of its high metabolic activity, together with large concentrations of glutamate (Choi, 1992), the brain is particularly vulnerable to ischemic insults. These can occur as a consequence of a thrombosis or an embolic occlusion of a cerebral blood vessel, more frequently the middle and anterior cerebral arteries (Candelario-Jalil, 2009; Durukan and Tatlisumak, 2009; Moskowitz et al., 2010).

The ischemic core is the irreversibly damaged tissue characterized by <20% of normal blood flow levels, reduced ATP levels and irreversible energetic failure (Lo, 2008a). Cells in the core are killed rapidly by proteolysis, lypolysis, bioenergetic failure and collapse of ion homeostasis (Doyle et al., 2008; Brouns and De Deyn, 2009). In the peripheral areas of stroke, between the normal brain and the damaged core, lies an area called ischemic penumbra or peri-infarct zone (Astrup et al., 1981). In this region, blood flow deficits are less severe, hardly sufficient to support basal ATP levels and normal ionic gradients (Moskowitz et al., 2010). Therefore, in the penumbra region the tissue is functionally impaired but potentially salvageable, and can be rescued by enhancing blood flow or interfering with the ischemic cascade (Lo, 2008a, b; Brouns and De Deyn, 2009). Along time, the infarct core expands into the ischemic penumbra, so, accurate detection of this tissue at risk can help to identify patients who might benefit from the treatments that restore blood flow at an early time point. Currently, recombinant tissue plasminogen activator

(rtPA) is the only approved drug that has shown significant benefits in acute stroke patients, by promoting clot lysis and reperfusion (NINDS, 1995; Paciaroni et al., 2009). However, the use of tPA to treat stroke patients remains low (Gropen et al., 2006), due to the short window of opportunity to administrate this treatment and risk of hemorrhagic complications (Brouns and De Deyn, 2009).

Within the ischemic penumbra, multiple mechanisms involved in cell death have been identified, such as bioenergetic failure, excitotoxicity, oxidative and nitrative stress, peri-infarct depolarizations, inflammation and apoptosis (Fig. 1.1) (Doyle et al., 2008; Candelario-Jalil, 2009; Moskowitz et al., 2010).

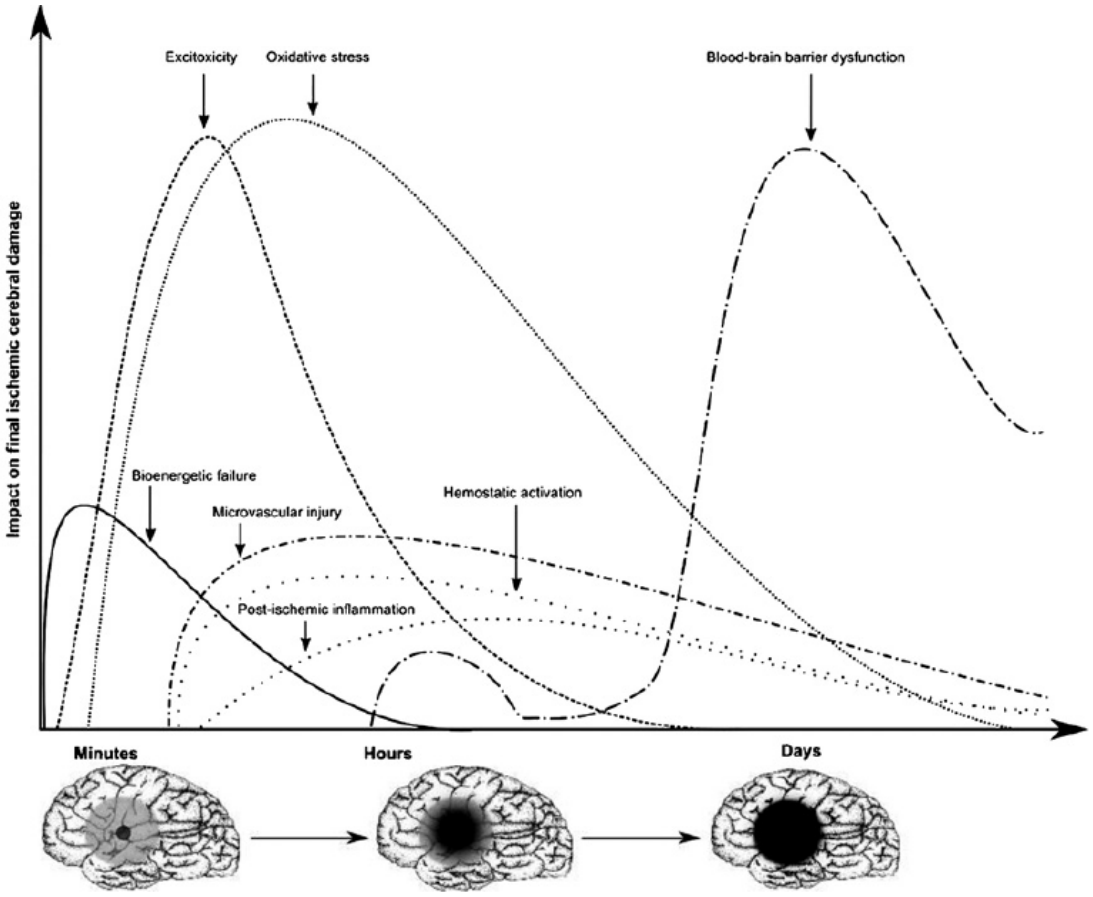

Figure 1.1. Damaging mechanisms underlying acute focal cerebral ischemic stroke. The x-axis represents the evolution of cascades over time, and the y-axis refers to the expected impact of each event on the final ischemic damage. In absence of early

reperfusion, cells in the ischemic penumbra (light grey) die due to ongoing injury, resulting in an expansion of the ischemic core (black) (Brouns and De Deyn, 2009).

1.1.1. Excitotoxicity

Brain tissue has a high consumption of oxygen and glucose, and depends almost exclusively on oxidative phosphorylation for energy production (Dirnagl et al., 1999; Verweij et al., 2007). The impairment of cerebral blood flow reduces the delivery of substrates, particularly oxygen and glucose, and ATP production (Martin et al., 1994). This leads to the dysfunction of energy-dependent ion transport pumps, and to the consequent depolarization of neurons and glia (Katsura et al., 1994; Martin et al., 1994). The Na^+/K^+ ATPase at the plasma membrane of neurons consumes nearly 70% of the energy supplied to the brain (Edvinsson and Krause, 2002). This ion pump maintains high K^+ and low Na^+ intracellular concentrations, that are essential for the propagation of action potentials. After ischemia, the inhibition of ATP synthesis by mitochondria leads to a rapid ATP consumption, which causes a neuronal membrane depolarization with the release of K^+ and Na^+ entry into cells (Caplan, 2009). Energy failure also impedes the plasma membrane Ca^{2+} ATPase to maintain the very low calcium concentrations usually found within the cells (Doyle et al., 2008). This, together with the activation of voltage-dependent calcium channels, leads to the release of neurotransmitters, especially glutamate, which plays a critical role in the ischemic damage (Fig. 1.2) (Nicholls and Attwell, 1990; Brouns and De Deyn, 2009). A large concentration gradient of glutamate is maintained across the plasma membrane by sodium-dependent glutamate transporters located at the plasma membrane, which maintain a high cytosolic glutamate concentration (approximately 10 mM) when compared to the synaptic concentration (in the micromolar range) (Hsu, 1998). Membrane depolarization during ischemia also induces a reversal of glutamate uptake carriers and enables glutamate to exit the cells along its concentration gradient, further increasing its accumulation in the extracellular space (Nicholls and Attwell, 1990; Rossi et al., 2000).

The extracellular accumulation of glutamate leads to a sustained stimulation of synaptic and extra-synaptic AMPA, Kainate and NMDA–type glutamate receptors, which will result in neuronal dysfunction and death, a process called excitotoxicity (Choi, 1988; Sims and Zaidan, 1995; Kroemer et al., 2009). In particular, the stimulation of AMPA and NMDA receptors causes an influx of Na^+, and NMDA receptors are also characterized by a high Ca^{2+} permeability and conductance properties (Fig. 1.2) (Dong et al., 2009). Activation of group I mGluR receptors, which includes mGluR1 and mGluR5, increases the intracellular inositol trisphospate (IP_3), thereby activating protein kinase C and releasing Ca^{2+} from neuronal stores

(Nakanishi, 1994; Pin and Acher, 2002; Friedman, 2006). Therefore, overactivation of NMDA receptors and metabotropic glutamate receptors (mGluR) contribute to a $[Ca^{2+}]_i$ overload. AMPA receptors are not normally calcium permeable due to the GluA2 subunit, but the downregulation of this subunit after ischemia increases the calcium permeability of these receptors, and thus allow AMPA receptors to contribute to delayed cell death (Liu et al., 2006; Peng et al., 2006). Furthermore, there are evidences suggesting that the cation-dependent Cl^- transporter type 1 (NKCC1) is involved in the initial steps of ischemic neuronal damage, by facilitating excessive Na^+ and Cl^- entry into cells (Staub et al., 1994; Dong et al., 2009).
Moreover, water passively enters the cells following the ion influx, since the influx of Na^+ and Cl^- is larger than the efflux of K^+, and this results in cytotoxic edema (Furukawa et al., 1997; Beck et al., 2003). The edema affects the perfusion of regions adjacent to the core, increasing intracranial pressure, and causing vascular compression and herniation (Dirnagl et al., 1999; Siesjo, 2008).

1.1.2. Calcium Dysregulation

The universal second messenger Ca^{2+} is a neuronal signaling molecule and a critical player in ischemia-induced neuronal death. The extracellular calcium concentration is approximately 1.2 mM, and the intracellular concentration is maintained in the range of 50 to 300 nM (about four thousand times lower than the extracellular concentration). Most cellular processes regulated by Ca^{2+} have a K_m value around 0.1-1 μM, but during ischemia the intracellular Ca^{2+} levels rise to 50-100 μM (Edvinsson and Krause, 2002).
Calcium ion entry into the cells occurs through several routes, and many of them are deregulated after excitotoxicity or brain ischemia (Szydlowska and Tymianski, 2010). The main determinant of Ca^{2+} toxicity is the route by which these ions enter the cells. In particular, the sustained influx of Ca^{2+} through glutamate receptor channels represents a common pathway of neuronal cell death (Choi, 1987; Friedman, 2006). Excessive levels of glutamate in the CNS can result in elevated intracellular Ca^{2+} levels, which in turn cause a rise in the Ca^{2+} concentration in mitochondria and in the endoplasmic reticulum (ER) (Fig. 1.2).
Depletion of Ca^{2+} ions from the ER has been considered an initial signal for ER dysfunction and damage in ischemic neurons (Paschen and Doutheil, 1999; Pisani et al., 2000). Deregulation of Ca^{2+} homeostasis in the ER involves an accumulation of Ca^{2+} in ER stores, and consequent release following ischemia (Chen et al., 2008b).

In the mitochondria, Ca^{2+} accumulates through a proton electrochemical gradient generated by the electron transport chain, creating a mitochondrial potential. Ca^{2+} influx decreases mitochondrial electrochemical gradient, thereby reducing ATP synthesis (Pepe, 2000). In response to elevated $[Ca^{2+}]_i$, cells direct ATP spending to drive plasma membrane pumps to expel Ca^{2+}. Together, the accumulation of intramitochondrial Ca^{2+}, reduced ATP synthesis and increased ATP usage were suggested to be a cause of cell death (Schinder et al., 1996; Racay et al., 2009).
Moreover, several ion channels and pumps, including Na^{+}/Ca^{2+} exchanger (NCX) (Bano et al., 2007; Jeffs et al., 2007), acid-sensiting ion channels (Xiong et al., 2004), hemmichannels (Contreras et al., 2004) and Transient Receptor Potential (TRP) channels (Aarts and Tymianski, 2005; Sun et al., 2009), account for the irreversible increase of intracellular Ca^{2+} after ischemia..
When cytoplasmic calcium concentrations hit non-physiological levels, several mechanisms are activated to remove it. However, in ischemia or excitotoxicity, such mechanisms fail or are not sufficient. The elevated intracellular Ca^{2+} activates proteases, lipases, and endonucleases (Ankarcrona et al., 1995). One of the the most important proteases activated after ischemia and $[Ca^{2+}]_i$ overload are calpains (Higuchi et al., 2005). Calpains cleave proteins involved in Ca^{2+} homeostasis, such as NCX, L-type Ca^{2+} channels, IP_3 receptors and NMDA and AMPA receptor subunits (Hell et al., 1996; Araujo et al., 2007; Bevers and Neumar, 2008; Vosler et al., 2008). Calpains also cleave cytosolic enzymes such as Ca^{2+}/calmodulin-dependent protein kinase (CaMK) and the protein phosphatase calcineurin (CaN) (Vosler et al., 2008). Lipases are also activated by calcium, creating free radical species that ultimately lead to lipid peroxidation and membrane damage in the ischemic brain (Farooqui and Horrocks, 1998).

Zn^{2+} is also a neuronal signaling molecule and a critical player in ischemic cell death (Choi and Koh, 1998; Bitanihirwe and Cunningham, 2009). It is present at high concentration in the presynaptic terminals of a subset of glutamatergic neurons and is particularly abundant at presynaptic vesicles, being released from nerve terminals in response to synaptic activity (Assaf and Chung, 1984; Frederickson, 1989; Bitanihirwe and Cunningham, 2009). Like glutamate, Zn^{2+} is neurotoxic both *in vitro* (Weiss et al., 1993) and *in vivo* (Yanamoto et al., 2000).

1.1.3. Oxidative and Nitrative stress

Oxidative and nitrative stress are processes that contribute to ischemic injury. They occur when the production of free radicals prevails over the endogenous antioxidant

defenses. Several evidences have shown that reactive oxygen and nitrogen molecules are important mediators of tissue injury following ischemic stroke (Fig. 1.2) (Chan, 2001; Kelly et al., 2008). The redox environment modulates signal transduction cascades between prodeath and prosurvival pathways (Crack and Taylor, 2005), and the reactive species may also act as executioners of cell death (Chan, 2001). The brain is especially vulnerable to radical-mediated injury due to its reduced antioxidant defenses (Coyle and Puttfarcken, 1993; Adibhatla and Hatcher, 2010).

Brain ischemia generates superoxide (O_2^-), produced by mitochondria, which is a major source of reactive oxygen species (ROS) produced during electron transport and oxidative phosphorylation processes. O_2^- is the primary radical from which hydrogen peroxide (H_2O_2) is formed, and H_2O_2 is the source of hydroxyl radical (OH) (Beckman and Koppenol, 1996). Recently, it was shown that NADPH oxidase produces the majority of O_2^- *in vivo* and *in vitro* during NMDA receptor activation and ischemia (Brennan et al., 2009; Girouard et al., 2009). The excessive accumulation of ROS causes the destruction of cellular molecules and participates in signaling mechanisms that result in apoptotic cell death (Halliwell, 1994; Sugawara and Chan, 2003).

Nitic oxide (NO) and related oxidation products are also key players in excitotoxicity. Ischemia activates nitric oxide synthase (NOS) and increases the generation of NO, which combines to O_2^- to produce peroxynitrite, a highly toxic oxidant (Beckman and Koppenol, 1996; Beckman et al., 1996; Iadecola, 1997). Reactive nitrogen species have important cellular effects, such as inhibition of mitochondrial enzymes, DNA damage, and activation of Ca^{2+} permeable channels (Aarts and Tymianski, 2005; Pacher et al., 2007). As a second mechanism, NO modifies protein groups by covalently attaching to cysteine residues, forming S-nitrosothiol derivatives, and this will affect cell survival by altering the function of critical regulatory proteins such as caspases (Na et al., 2008).

1.1.4. Peri-infarct Depolarizations

As described above, in brain ischemia neurons depolarize due to the reduction of energy supply. Peri-infarct depolarizations (PIDs) are characterized by cellular sustained depolarization, increased glutamate release and loss of membrane ionic gradients (Somjen, 2001; Doyle et al., 2008; Gniel and Martin, 2010). PIDs propagate through the penumbra and are triggered by the release of K^+ and glutamate from the ischemic core (Fig. 1.2) (Somjen, 2001). Repeated depolarization in the penumbra may mediate tissue damage by allowing Ca^{2+} to

accumulate within neurons, and is therefore associated with increased ischemic injury (Hossmann, 1996; Doyle et al., 2008). It was demonstrated that PIDs occur in animal stroke models where spreading depression correlates with infarct maturation and core expansion (Gill et al., 1992; Strong et al., 2000).
Recently, the existence of PIDs in the injured human brain was observed, and this suggests that inhibition of spreading depression could be an important therapeutic strategy to contain the evolution of the ischemic injury (Fabricius et al., 2006).

1.1.5. Inflammation

Inflammation contributes to stroke brain injury, participating in the delayed response to ischemia. The inflammatory response is a complex process that involves many different cell types, inflammatory mediators and extracellular receptors, and occurs over many days (Kleinig and Vink, 2009). Cerebral blood vessels are the first to be exposed to the ischemic insult, and their reaction to injury is important for the inflammatory response. Initially, cytokines such as tumor necrosis factor α (TNFα) and interleukin (IL)-1β, are produced by endothelial cells and microglia (Rothwell and Hopkins, 1995; Gong et al., 1998; Huang et al., 2006), and induce expression of adhesion molecules, including intercellular adhesion molecule (ICAM) -1 and -2, and P- and E-selectin (Haring et al., 1996; Lindsberg et al., 1996; Zhang et al., 1998). Adhesion molecules promote neutrophil dislocation (mediated by selectins), activation and adherence to the endothelium (mediated by integrins and immunoglobulins), and these cells cross the vascular wall and enter the cerebral parenchyma, a process that is promoted by the blood brain barrier (BBB) disruption. The recruitment of neutrophils can obstruct the microcirculation and therefore prevent the restoration of cerebral blood flow. This causes additional tissue damage and is known as the ischemic no-reflow phenomenon (Yamasaki et al., 1997; Huang et al., 2006). After their penetration into the brain, neutrophils also cause tissue damage through the release oxygen-free radicals and proteolytic enzymes (Chou et al., 2004; Huang et al., 2006). Neutrophils are followed by monocytes and lymphocytes (Fig.1.2). Lymphocytes are usually excluded from the CNS, but they are found in the brain within 24h after ischemia (Schroeter et al., 1994). The primary immune cells in the brain are the microglia, which transform into macrophages upon activation (Fig. 1.2), and accumulate at the border zone together with blood-born macrophages. They clear dead cells and cellular remains, and produce inflammatory mediators and toxic molecules (Schilling et al., 2003). Chemokines, such as cytokine-induced neutrophil chemoattractant (CINC) and monocyte chemoattractant protein-1 (MCP-1), are also produced in the injured brain and guide

the migration of inflammatory cells towards the injured tissue (Yamasaki et al., 1995; Ivacko et al., 1997; Huang et al., 2006). Recently, T cell subsets have also been implicated as modulators of secondary infarct progression (Liesz et al., 2009).
These findings suggest that controlling the postischemic inflammation may block secondary events that increase brain injury. However, inflammation also promotes critical events required for tissue repair (Amantea et al., 2009).

1.1.6. Apoptosis

Brain cells affected by excessive glutamate receptor activation, $[Ca^{2+}]_i$ overload, ROS formation and mitochondrial and DNA damage, die by necrosis or apoptosis (Fig. 1.2). The decision depends on the magnitude and type of stimulus, and the type of cell (Leist and Nicotera, 1998). Necrosis is the predominant mechanism following acute stroke, whereas mild injury preferentially induces cell death via an apoptotic-like mechanism. Because the ischemic penumbra sustains moderate injury and preserves ATP, delayed apoptosis predominates in this region (Bonfoco et al., 1995; Yuan, 2009).
Mitochondria play an essential role in apoptosis and necrosis (Schinzel et al., 2005), serving as a source of pro and antiapoptotic proteins and cytochrome c. Release of cytochrome c from the mitochondria plays a crucial role in mediating apoptosis after ischemia, and is caused by ionic imbalance and mitochondrial swelling, or by the formation of a pore in the mitochondrial membrane. The complex interaction of the Bcl-2 family of proteins promotes (*e.g.* Bax, Bad, Bid) or prevents (*e.g.* Bcl-2, Bcl-XL) pore formation (Kroemer and Reed, 2000; Adams and Cory, 2001; Antonsson et al., 2001). After the opening of the mitochondrial pores, two groups of proapoptotic proteins are released from the intermembrane space into the cytosol (Saelens et al., 2004). The first group includes cytochrome c, Second-mitochondria-derived activator of caspases (Smac)/DIABLO and the serine protease HtrA2/Omi, and once released these proteins activate the caspase-dependent mitochondrial pathway (Du et al., 2000; Garrido et al., 2006; Nakka et al., 2008). Cytochrome c activates the cytosolic protein Apaf-1 and procaspase-9, and forms the “apoptosome” in the presence of ATP (Love, 2003; Hill et al., 2004). This leads to caspase-9 activation, which in turn will activate caspase-3, followed by caspase-2,-6, and -10 activation downstream (Slee et al., 1999; Sugawara et al., 2004). Both caspase-3 and caspase-9 have been shown to play a key role in neuronal death after ischemia (Rami et al., 2003; Cao et al., 2004a). Caspase-11 is also a critical initiator, activating of caspase-1 and -3 after ischemia (Kang et al., 2000).

Caspase-3 is considered the most important of the executioner caspases. It cleaves homeostatic, cytoskeletal, repair, metabolic and cell signaling proteins. Among its substrates is PARP, which leads to DNA injury, and the inhibitor protein ICAD, thereby causing DNA fragmentation by stimulating the caspase-activated deoxyribonuclease (CAD) (Endres et al., 1997; Sakahira et al., 1998; Didenko et al., 2002; Goto et al., 2002). Caspase activation can be modulated by protein inhibitors of apoptosis (IAP), which prevent their activation and inhibit their activity (Deveraux and Reed, 1999; Miller, 1999; Tanaka et al., 2004). The Smac and HtrA2/Omi are released upon apoptotic stimuli and bind IAPs, thus promoting activation of caspase-3 (Chai et al., 2000; Du et al., 2000; van Loo et al., 2002; Siegelin et al., 2005).
Caspase-3 can also be activated by caspase-8, which is activated by members of the death receptor family, such as the Fas receptor and the tumor necrosis factor receptor (TNFR) (Loh et al., 2006). Furthermore, downstream caspases are also activated as in the mitochondrial pathway (Li et al., 1998; Hengartner, 2000). Caspase-8 also truncates Bid, activating this protein and initiating the mitochondrial pathway of apoptosis (Cao et al., 2004b).

The second group of proapoptotic proteins is composed of apoptosis-inducing factor (AIF), endonuclease G and CAD (Elmore, 2007). AIF-induced cell death is caspase-independent and constitutes an alternative pathway of cell death after the cellular energy depletion that prevents caspase activation. In fact, ATP depletion acts as a stimulus for AIF release (Daugas et al., 2000; Cho and Toledo-Pereyra, 2008). AIF is released from mitochondria in response to activation of the nuclear protein PARP, that generates the PAR polymer, its toxic product (Yu et al., 2006). Recent studies indicate that AIF is truncated by calpain, a process essential for its translocation to the nucleus, where it promotes DNA fragmentation and chromatin condensation (Cao et al., 2003; Cao et al., 2007). This caspase-independent neuronal apoptosis has been reported after ischemic stroke (Culmsee et al., 2005; Gao et al., 2010), and seems to be particularly relevant in adult brain injury, since AIF is more expressed in the adult brain (Cao et al., 2003). In contrast, the expression of caspases and the caspase dependence of ischemic neuronal death markedly decline with brain maturation (Yakovlev et al., 2001). Endonuclease G nuclear translocation has also been associated with apoptosis after ischemic stroke (Li et al., 2001; Lee et al., 2005).

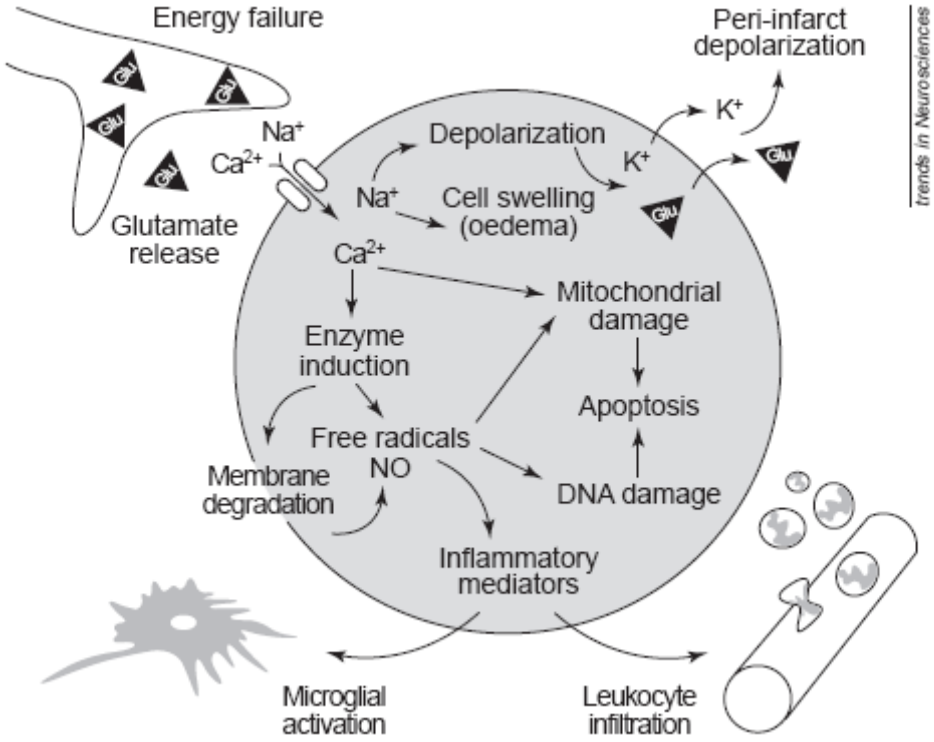

Figure 1.2. Simplified overview of pathophysiological mechanisms in the focally ischemic brain. Energy failure leads to depolarization of neurons. Activation of specific glutamate receptors increases intracellular Ca^{2+}, Na^+ and Cl^- levels, while K^+ is released into the extracellular space. Diffusion of glutamate and K^+ in the extracellular space can propagate a series of spreading waves of depolarization (peri-infarct depolarizations). Water shifts to the intracellular space via osmotic gradients and cells swell (edema). Intracellular Ca^{2+} overactivates numerous enzymes. Free radicals are generated, which damage membranes, mitochondria and DNA, triggering apoptotic cell death. Free radicals also induce the formation of inflammatory mediators, which activate microglia and lead to the invasion of blood-born inflammatory cells via upregulation of endothelial adhesion molecules (Dirnagl et al., 1999).

1.1.7. Neonatal/Perinatal Brain Injury

Injury in the brain during early development is a significant cause of mortality and long-term incapacity. Perinatal brain injury occurs immediately before or after delivery, while neonatal injury occurs from the perinatal period until 4 weeks of age. Causes of early brain injury include stroke, birth trauma, metabolic and genetic

disorders, *status epilepticus*, and asphyxia events resulting in hypoxia-ischemia (HI), the last being the predominant cause. (Volpe, 2001; Gonzalez and Ferriero, 2008). Hypoxic-ischemic brain damage in neonates is a major risk factor of a variety of human neurological disorders, especially motor and learning disabilities, epilepsy and seizures. Survivors of such injury can experience significant and lifelong cognitive, sensory and motor disorders, for which there is no established therapy (Dilenge et al., 2001; Johnston, 2001; Johnston et al., 2001; Ferriero, 2004; Hossain, 2008).

HI damages different regions of the immature brain at different ages, particularly when it occurs during stages of critical cellular or tissue differentiation, which have a serious impact on brain maturation. HI often leads to oligodendrocyte/white matter injury in premature infants, while term infants usually develop neuronal cortical/ subcortical lesions (Volpe, 2001; Gressens and Luton, 2004). The neonatal and adult brain do not respond similarly to injury, and show distinct susceptibility to oxidative stress and excitotoxicity, in addition to differences in gene regulation during ischemia (McQuillen and Ferriero, 2004).

Neuronal death during the neonatal period seems to occur in two phases: primary from cellular hypoxia and energy depletion, and secondary by reperfusion and increased excitotoxicity, free radical and nitric oxide formation, followed by secondary energy failure and delayed cell death (Balduini et al., 2004; Perlman, 2006). The developing brain is characterized by a high level of plasticity and enhanced excitability, associated to activity-dependent synaptogenesis and learning. Several factors related to synaptic transmission and neuronal excitability suffer maturational changes during early development, such as the expression and molecular composition of neurotransmitter receptors and transporters, mechanisms of ion homeostasis and oxidative stress pathways. These factors can add to the increased vulnerability of the immature brain compared to the adult brain (Jensen, 2002; Sanchez and Jensen, 2003).

Differences in ionotropic glutamate receptor expression are one of the factors thought to account for the vulnerability of the developing brain to HI (Deng et al., 2004). NMDA receptors are present on developing oligodendrocytes and play a significant role in white matter injury (Karadottir et al., 2005; Salter and Fern, 2005). The properties of NMDA receptor contribute to their enhanced activity during early postnatal development. For instance, NMDAR-mediated synaptic currents appear to be more slowly decaying during early postnatal development in the forebrain, leading to a much longer depolarization and glutamate induced neuroplasticity, probably due to a ratio GluN2B to GluN2A expression higher in the early postnatal brain compared to the adulthood (Sanchez and Jensen, 2003; Walz et al., 2010).

The maturational regulation of AMPARs composition and function also improve glutamate-mediated plasticity in early postnatal life. The ratio of GluA2 expression and other AMPARs subunits is significantly reduced in immature neocortex and hippocampus compared to the adult (Sanchez et al., 2001). It is known that AMPARs lacking GluA2 exhibit a higher Ca^{2+} permeability (Jonas et al., 1994; Washburn et al., 1997), and an increased Ca^{2+} influx through AMPARs in immature neurons may trigger mechanisms for plasticity, although also provide potential mechanism for excitotoxicity.
Oxidative stress is also an important component of injury in the neonatal brain (Ferriero, 2001). The neonatal brain is particularly sensitive to damage, due to a high rate of oxygen consumption and low concentrations of antioxidants. In the immature rat brain, the endogenous antioxidant defense mechanisms are less active when compared to the mature brain (Khan and Black, 2003; Halliwell, 2006). In humans, mature oligodendrocytes carry increased antioxidant enzymes compared to the oligodendrocyte precursors that exist in the immature brain, and this may somehow explain the susceptibility of premature infants to white matter injury (Baud et al., 2004; Haynes et al., 2005).
Apoptosis is crucial for normal brain development, but is also an important component of injury following HI and stroke (Northington et al., 2005). Activation of apoptotic pathways leads to activation of caspase-3, which is maximally expressed in the neonatal period (Hu et al., 2000). The proapoptotic protein Bax is present in high concentrations in the striatum of rats during the first two postnatal weeks (Lok and Martin, 2002). PARP-1 is also more abundant in the immature brain, and blockade or deletion of PARP-1 protects against excitotoxicity and ischemic injury (Hagberg et al., 2004).

However, there are also strong cellular evidences that neonatal brains possess several strategies that contribute to prevent ischemic damage, such as elevated melatonin concentration, resistance to energetic depletion, and altered NMDAR composition, similar to some adaptations found in animals that experience insults to the nervous system in their natural habitat (Singer, 1999; Tan et al., 2005).
The neurohormone melatonin, besides its role in biological timing, is a potent antioxidant with the ability to scavenge free radicals and to upregulate antioxidant enzymes. Elevated melatonin concentration is an adaptation found in animals that survive ischemia-reperfusion injury. Thus, in a variety of hibernating animals, like hamsters and snakes, melatonin concentration increases quickly during arousal from torpid states (Vanecek et al., 1984; Mendonca et al., 1995; Tan et al., 2005).

Also, diving marine mammals possess high basal melatonin concentrations during the time of the year when diving frequently occurs (Aarseth and Stokkan, 2003).
Hibernating animals undergo physiological events similar to ischemia-reperfusion. During hibernation, cerebral blood flow is reduced, in some species by >90%, and return to the non-hibernating state is an energetically challenging event that induces transient hypoxia (Tan et al., 2005; Dave et al., 2006). For example, the arctic ground squirrel brain is surprisingly resistant to ischemia-reperfusion injury, and they can survive NMDA-induced cell death when compared to laboratory rats (Ross et al., 2006). They apply diverse neuroprotective mechanisms, like increased expression of antioxidant enzymes, changes in NMDARs dynamics and ion channels blockade (Drew et al., 1999; Zhao et al., 2006).
Another example of an adaptation that provides neuroprotection is found in aquatic turtles which are able to spend long periods of time submerged under frozen water, by exhibiting striking resistance to anoxia. To achieve that, they use various mechanisms, such as decreased glutamate:GABA ratio, neurotransmitter dynamics change and ion channels blockade (Nilsson and Lutz, 1991; Lutz and Milton, 2004; Storey, 2007).

1.1.8. Experimental models of ischemia

A number of experimental models are currently used to study brain ischemia. In this work we used an *in vivo* model of focal cerebral ischemia, and an *in vitro* model of global ischemia.

Oxygen and glucose deprivation (OGD) is an established *in vitro* model of transient global ischemia (Goldberg and Choi, 1993; Ying et al., 1997; Liu et al., 2006; Hertz, 2008). *In vitro* models require longer periods of OGD to induce cell death, and ATP levels do not fall as much as in *in vivo* models. The absence of blood vessels and blood flow simplifies interpretation of the results. We used this model to induce transient global ischemia in primary cultures of hippocampal neurons of embryonic rats. At 14 DIV, the culture medium was exchanged with a deoxygenated, glucose-free salt solution to induce OGD (Liu et al., 2006) for different periods of time.

Focal ischemia is the animal model closer to stroke in humans (Longa et al., 1989), and is conducted experimentally by occlusion of one major cerebral blood vessel such as the middle cerebral artery (MCA). MCA occlusion (MCAO) reduces the cerebral blood flow in both striatum and cortex, but the degree and distribution of the effect depends on the duration of insult, the site of occlusion in the MCA and the

amount of collateral blood flow into the MCA territory (Traystman, 2003). Two surgical approaches are used to give access to the cerebral vasculature to induce focal ischemia: i) the first group requires opening of the skull to allow direct access to the cerebral arteries; ii) to avoid opening the skull, a second group of methods uses intra-arterial access to occlude cerebral arteries The most commonly used of these is thread occlusion of the MCA (Howells et al., 2010). This method was first described by Koizumi et al. (Koizumi et al., 1986) and modified by Longa et al. (Longa et al., 1989). It involves introducing an occluding thread into the extracranial internal carotid artery and advancing until the tip occludes the origin of the MCA. This method is most frequently applied in rats and mice (Howells et al., 2010). The great advantage of these techniques is that the thread can either be left in place for permanent occlusion or withdrawn at any time to permit controlled reperfusion, and is characterized by the presence of a significant ischemic penumbra. In this work, we used the method described in Nygren and Wieloch (2005), in which transient focal ischemia was induced using the intraluminal filament placement technique.

We also used intrahippocampal injection of kainic acid (KA) as a model to induce excitotoxicity. KA is an agonist for two subtypes of ionotropic glutamate receptors (AMPA and kainate receptors), and administration of KA has been shown to increase production of ROS, mitochondrial dysfunction, and apoptotic-like cell death in neurons in many regions of the brain, particularly in the hippocampal subregions CA1, CA3 and dentate gyrus. Therefore, KA-injection is often used as a model to study neurodegeneration (Tomioka et al., 2002; Wang et al., 2005).

1.2. Calpains

The calpains constitute a family of cellular cysteine proteases that are activated by Ca^{2+} at neutral pH (Huang and Wang, 2001; Croall and Ersfeld, 2007), and are expressed in the cytosol, including in the synaptic terminal of neurons (Doshi and Lynch, 2009). The calpain family of proteases was identified by the discovery of what is now known as μ-calpain in 1964 in rat brain (Guroff, 1964; Huston and Krebs, 1968). The cDNA for this protein was isolated in the mid-1980s (Ohno et al., 1984) and, based on sequence homology, there are now 15 identified calpain family members on the human genome. For most calpains just the mRNA was identified, and they are classified as ubiquitous or tissue specific based on their expression patterns (Huang and Wang, 2001; Goll et al., 2003). Only the catalytic subunit

isoforms 1,2,3,5 and 10 and two regulatory subunit isoforms were identified in the brain, but the most abundant and well-characterized are μ-calpain and m-calpain (also called calpain 1 and 2, respectively). Both exist as heterodimers, each with a different 80 kDa catalytic subunit encoded by the CAPN1 gene in chromosome 11 or the CAPN2 gene in chromosome 1, respectively, combined with a common 30 kDa regulatory subunit (Calpain 4).

Calpain 1 and 2 catalytic subunits share nearly 50% sequence homology, and analysis of their amino acid sequence predicts four functional domains (I, II, III and IV) (Ohno et al., 1984; Goll et al., 2003; Bevers and Neumar, 2008; Doshi and Lynch, 2009). Domain I, the N-terminal region, contains the site where autolytic cleavage occurs, and is important for regulating the activity and dissociation of the two subunits. Domain II contains the cysteine and histidine residues involved in calpain catalytic activity and interacts with calpain substrates (Carafoli and Molinari, 1998; Ono et al., 1998; Nakagawa et al., 2001). Domain III has an unknown function, but is probably implicated in Ca^{2+} binding and in other electrostatic interactions (Strobl et al., 2000; Tompa et al., 2001). Domain IV, the C-terminal end of the catalytic subunit, has five EF-hand Ca^{2+}-binding motifs (Maki et al., 2002). The most relevant differences between calpain 1 and 2 are that calpain 1 has a less drastic separation of the catalytic residues, and its Ca^{2+}-binding region in domain IV is more flexible. These facts might explain the principal biochemical distinction between μ- and m-calpain, which is the concentration of calcium needed for *in vitro* activation. While μ-calpain requires 3 to 50 μM Ca^{2+} for half-maximal activity, m-calpain demands higher levels, 0.4 to 0.8 mM Ca^{2+} (Goll et al., 2003). However, they have similar physiological functions and pathological actions (Liu et al., 2008). The regulatory subunit contains two domains. Domain V, the N-terminal region, is a glycine-rich, hydrophobic domain, that is thought to function as a membrane anchor. Domain VI, the C-terminal end, is a Ca^{2+} binding region, like domain IV of the catalytic subunit (Hosfield et al., 1999; Strobl et al., 2000). The Ca^{2+} binding domains of the catalytic and regulatory subunits associate to form heterodimeric calpain. Calpains are usually activated by an elevation of intracellular Ca^{2+} (Wu and Lynch, 2006).

Calpain activity is directly regulated by calpastatin, a specific endogenous inhibitor of μ- and m-calpain in mammalian cells. It interacts with domain II of the catalytic subunit (the substrate binding region), thereby preventing substrate binding in a competitive manner. Calpastatin only binds to calpain at intracellular Ca^{2+} concentrations above basal levels (Murachi, 1989; Hood et al., 2004). Both initial activation and subsequent calpain activity are inhibited by calpastatin, and each calpastatin molecule is able to inhibit four calpain molecules, due to its repeated

domain structure (Maki et al., 1987). Calpastatin activity is regulated by phosphorylation through cyclic adenosine monophosphate-dependent protein kinase, thus increasing its ability to inhibit m-calpain and decreasing inhibition of μ-calpain (Salamino et al., 1994).

Calpains act in two manners: under physiological conditions they undergo controlled activation, involving only a few calpain molecules, whereas during sustained Ca^{2+} overload observed in pathological conditions they become overactivated, with all available calpain molecules involved. Calpains 1 and 2 are selective concerning substrate, since only about 5% of cellular proteins are degraded by these proteases even under severe conditions (Liu et al., 2008). Calpains substrate specificity impedes complete classification. These proteases cleave at preferred sequences in association with favorite tertiary structures, but without known unequivocal rules. According to the PEST hypothesis, *i.e.* the presence of region(s) rich in Pro, Glu/Asp, and Ser/Thr flanked by Arg/Lys residues is related with the short lifetime of proteins (Rogers et al., 1986; Rechsteiner and Rogers, 1996), calpains cleave their substrates near a PEST sequence. This negatively charged region is thought to bind Ca^{2+} and deliver peptide bonds for calpain activity (Tompa et al., 2004). However, whether the presence of PEST sequences supply a signal for calpain cleavage is not a clear issue, since mutations of these sequences did not abolish calpain cleavage of some substrates (Molinari et al., 1995; Carillo et al., 1996), and there are some calpain substrates that lack PEST sequences (Carillo et al., 1996). It was also observed that calpain substrates often possess a calmodulin binding motif (Wang et al., 1989; Tompa et al., 2004).

1.2.1. Physiological functions of calpain in CNS

Calpains intervene in several intracellular signaling pathways mediated by Ca^{2+} by proteolysis, thereby altering the function or facilitating further degradation of target proteins (Tompa et al., 2004; Lynch and Gleichman, 2007). Calpain participate in many neuronal processes like excitability, neurotransmitter release, synaptic plasticity, vesicular trafficking, and signal transduction, by cleaving membrane receptors, cytoskeletal proteins, postsynaptic density proteins, and intracellular mediators important for synaptic function (Lynch and Baudry, 1984; Tomimatsu et al., 2002; Wu and Lynch, 2006; Bevers and Neumar, 2008; Doshi and Lynch, 2009).

Long term potentiation (LTP) is a form of synaptic plasticity thought to underlie memory formation (Lynch et al., 2007). This process involves synaptic remodeling, that may require calpain-mediated proteolysis of spectrin, which is known to regulate proteins expressed at cell surface and cellular morphology (Lynch and Baudry,

1987; Glantz et al., 2007; Zadran et al., 2010a). It was shown that calpain mediated spectrin cleavage is implicated in dendritic spine changes associated to LTP induction (Vanderklish et al., 2000). LTP also requires rapid insertion of AMPARs in the postsynaptic membrane, and these receptors are stabilized in the postsynaptic density (PSD) through interaction with anchoring proteins (Shi et al., 1999; Kessels and Malinow, 2009). Calpain cleaves the glutamate receptor interacting protein (GRIP), disrupting its interaction with GluA2, and causing rapid insertion of AMPARs into postsynaptic membranes (Lu et al., 2001; Zadran et al., 2010a). Furthermore, calpain 2 cleaves PSD95, which induces changes in the organization of the PSD, modifying the anchoring of NMDARs and enhancing the efficacy of synaptic activity (Vinade et al., 2001). Reduction of calpain 1 activity by 50% also shown to greatly reduce the incidence and magnitude of LTPs (Vanderklish et al., 1996).

Calpain activation in dopaminergic neurons in response to Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels (VGCCs) cleaves protein kinase C (PKC), releasing the constitutively active protein kinase M (PKM) fragment, which regulates maintenance of burst firing, a process that enhances synaptic dopamine release (Tobler et al., 2005; Liu et al., 2007). PKM is also implicated in addiction and motivation and is associated with learning and memory, suggesting that calpains could contribute to these processes independently of LTP mechanisms (Osten et al., 1996; Steketee et al., 1998).

Under physiological conditions, activation of calpain is temporary, since the rise in intracellular Ca^{2+} is transient, returning rapidly to basal levels. Once the resting conditions are established, calpain molecules return to their inactive state.

Recent studies have revealed that m-calpain can also be activated independently of calcium. Both brain-derived neurotrophic factor (BDNF) and epidermal growth factor (EGF) were able to activate m-calpain via MAPK-dependent phosphorylation in cultured cortical and hippocampal neurons, thereby providing a novel mechanism by which this calpain isoform plays a crucial role in synaptic plasticity (Zadran et al., 2010b).

1.2.2. Calpains in CNS pathological conditions

Hyperactivation of calpains in response to sustained increase in $[Ca^{2+}]_i$ is related with severe cellular damage caused by physical trauma or ischemic insults, and was implicated in various neurodegenerative disorders, including Alzheimer's disease (AD) (Liang et al., 2010), Parkinson's disease (PD) (Esteves et al., 2010) and Huntington's disease (HD) (Reijonen et al., 2010).

Calpain substrates following ischemic injury exist in several cellular components, including synapse, plasma membrane, ER, mitochondria and nucleus, and throughout various functional categories, especially neurotransmitter receptors, postsynaptic structural proteins, calcium regulatory proteins and signaling proteins (Bevers and Neumar, 2008).

A critical component of the postischemic injury is membrane depolarization as a consequence of ATP reduction, which results in the opening of voltage-gated Na^+ and Ca^{2+} channels and the release of glutamate. The excessive glutamate release causes an overactivation of glutamate receptors, further contributing to alterations in ion homeostasis. Calpain itself may promote the sustained calcium overload by cleaving and altering the function of several proteins involved in $[Ca^{2+}]_i$ homeostasis (Neumar, 2000). Some examples are L-type calcium channels (Hell et al., 1996), the NCX (Bano et al., 2005) and the plasma membrane Ca^{2+} ATPase (Pottorf et al., 2006). Cleavage of these proteins reduces the cell ability to maintain a low $[Ca^{2+}]_i$, contributing to cytosolic calcium overload.

Calpain activity also alters calcium regulation through cleavage of glutamate receptors (Fig. 1.3). NMDARs subunits GluN2A, -2B and -2C undergo C-terminal proteolysis by calpain *in vitro* (Guttmann et al., 2001; Wu et al., 2005), but only the GluN2B was shown to be cleaved in neurons (Simpkins et al., 2003; Dong et al., 2006). The truncated receptors remain at the cell surface, and are predicted to be active, although the lack of the C-terminal region probably affects their interaction with intracellular signaling molecules. The C-terminus of the mGluR1 receptor is cleaved by calpain after NMDA exposure, but retains its ability to signal for the intracellular release of Ca^{2+} from intracellular stores, but is unable to activate the neuroprotective PI3K-Akt signaling pathway (Xu et al., 2007). Calpain also cleaves AMPARs subunits GluA1, -2 and -3, removing their C-terminal region (Bi et al., 1998; Bi et al., 2000). This cleavage leads to a loss of total GluA1-3 subunits, and their dissociation from PSDs, thereby reducing AMPARs-mediated currents and enhancing the receptors turnover (Lu et al., 2000; Jourdi et al., 2005). NMDA and AMPA receptor subunits cleavage by calpain can be modulated by the interaction with other proteins. When PSD-95 binds to GluN2 subunits protects them from calpain cleavage (Dong et al., 2004), and PSD-95 cleavage by calpains alters the anchoring of NMDA receptors (Vinade et al., 2001). Furthermore, calpain cleaves proteins associated with the stabilization of AMPARs, such as SAP97 and GRIP, which affects the receptors turnover (Jourdi et al., 2005).

Structural changes occurring at synapses following ischemic insults involve rapid changes in dendritic spine density (Faddis et al., 1997), and calpains appear to be involved in this process by cleaving cytoskeletal proteins such as spectrin (Siman et

al., 2004) and MAP2 (Fig. 1.3) (Buddle et al., 2003). Spectrin proteolysis is a hallmark of postischemic brain injury, and the appearance of spectrin breakdown products in the cerebrospinal fluid can be used as a biomarker of ischemia (Siman et al., 2004).

Calpain also alters signaling molecules in the ischemic brain. It cleaves the calmodulin-dependent phosphatase calcineurin, producing a constitutively active form (Wu et al., 2004; Shioda et al., 2006). It also cleaves and produces an active form of CaMK IIα, and degrades nNOS (Hajimohammadreza et al., 1997). The presynaptic growth-associated protein 43, involved in the regulation of neurotransmitter release, is also a calpain substrate (Sato and Kawashima, 2001; Zakharov et al., 2005).

The ER has also an important role in ischemic injury (Section 1.1.2). Calpains are associated with the membrane of the ER, and cleave several proteins in this organelle (Fig. 1.3) (Hood et al., 2004). The ryanodine receptor (RYR) and IP_3 receptor are Ca^{2+} channels found in the ER membrane. The RYR channel is activated by a rise in cytosolic Ca^{2+}, and its cleavage by calpain 2 increases the mean channel open time with no change in the channel conductance, causing a permanent ER Ca^{2+} drain (Rardon et al., 1990; Zahradnikova et al., 2010). The IP_3R channel opening is stimulated by IP_3 binding, and Ca^{2+} release through this channels is also stimulated by increased intracellular Ca^{2+} (Ding et al., 2010). Calpain proteolysis of IP_3R decreases IP_3 binding (Dahl et al., 2000). The sarcoplasmic/ER Ca^{2+} ATPase (SERCA) is responsible for energy-dependent ER Ca^{2+} sequestration and is also cleaved by calpain after ischemia. This cleavage apparently causes an uncoupling of SERCA ATPase activity and Ca^{2+} uptake (Parsons et al., 1999; French et al., 2006).

The mitochondrial calcium overload plays an important role in mitochondrial dysfunction after ischemic brain injury (Section 1.1.6). In this condition, calpain may contribute to mitochondrial dysfunction through cleavage of several proteins, namely members of the Bcl2 family of proteins (Fig. 1.3). Calpain cleaves Bax, producing a fragment that leads to cytochrome c release (Choi et al., 2001); Bid, activating the protein (Chen et al., 2001; Takano et al., 2005); and the antiapoptotic Bcl-xL, which is converted into a proapoptotic protein (Nakagawa and Yuan, 2000). Calpain also plays a significant role in apoptotic cell death through direct and indirect interactions with members of the caspase family of proteases. Apaf-1 and caspase-9 cleavage by calpain was correlated with a reduced activity of caspase-3 (Chua et al., 2000; Reimertz et al., 2001). In contrast, calpain cleavage converts procaspase-7 into its active form (Ruiz-Vela et al., 1999). Contradictory results have been reported regarding the functional consequence of caspase-3 cleavage by calpain, which has

been considered inhibitory (McGinnis et al., 1999) or activator (Blomgren et al., 2001). Calpain has recently been shown to cleave AIF, inducing its release from the mitochondria and translocation to the nucleus (Polster et al., 2005; Cao et al., 2007). Although calpain appears to downregulate certain forms of caspase-mediated cell death, it may also promote caspase-independent cell death through the AIF-mediated mechanism.

Calpain activation may contribute as well to necrotic cell death through the release of hydrolytic enzymes usually stored in the lysosomes, especially cathepsins, in a process described as the “calpain-cathepsin cascade”. This consists of calpain-mediated disruption of lysosomal membranes, for instance by cleaving Hsp70.1, followed by diffusion of cathepsin B all over the cytoplasm and nucleus (Fig. 1.3) (Yamashima, 2004; Yamashima and Oikawa, 2009).

Finally, calpain cleaves nuclear proteins and causes the translocation of cytosolic proteins to the nucleus after proteolysis (Fig. 1.3). Within the nucleus, it cleaves CaMKIV, reducing the phosphorylation of its targets (Tremper-Wells and Vallano, 2005), and PARP-1, normally a DNA repair enzyme that becomes overactivated after toxic insults, causing ATP consumption (Pellicciari et al., 2004). β-catenin and glycogen synthase kinase 3 are cytosolic calpain substrates that migrate to the nucleus after cleavage by calpain, and the β-catenin cleavage induces gene transcription (Abe and Takeichi, 2007; Goni-Oliver et al., 2007). AIF, as previously referred, translocates to the nucleus following calpain cleavage, where it promotes DNA fragmentation and chromatin condensation (Cao et al., 2003; Cao et al., 2007).

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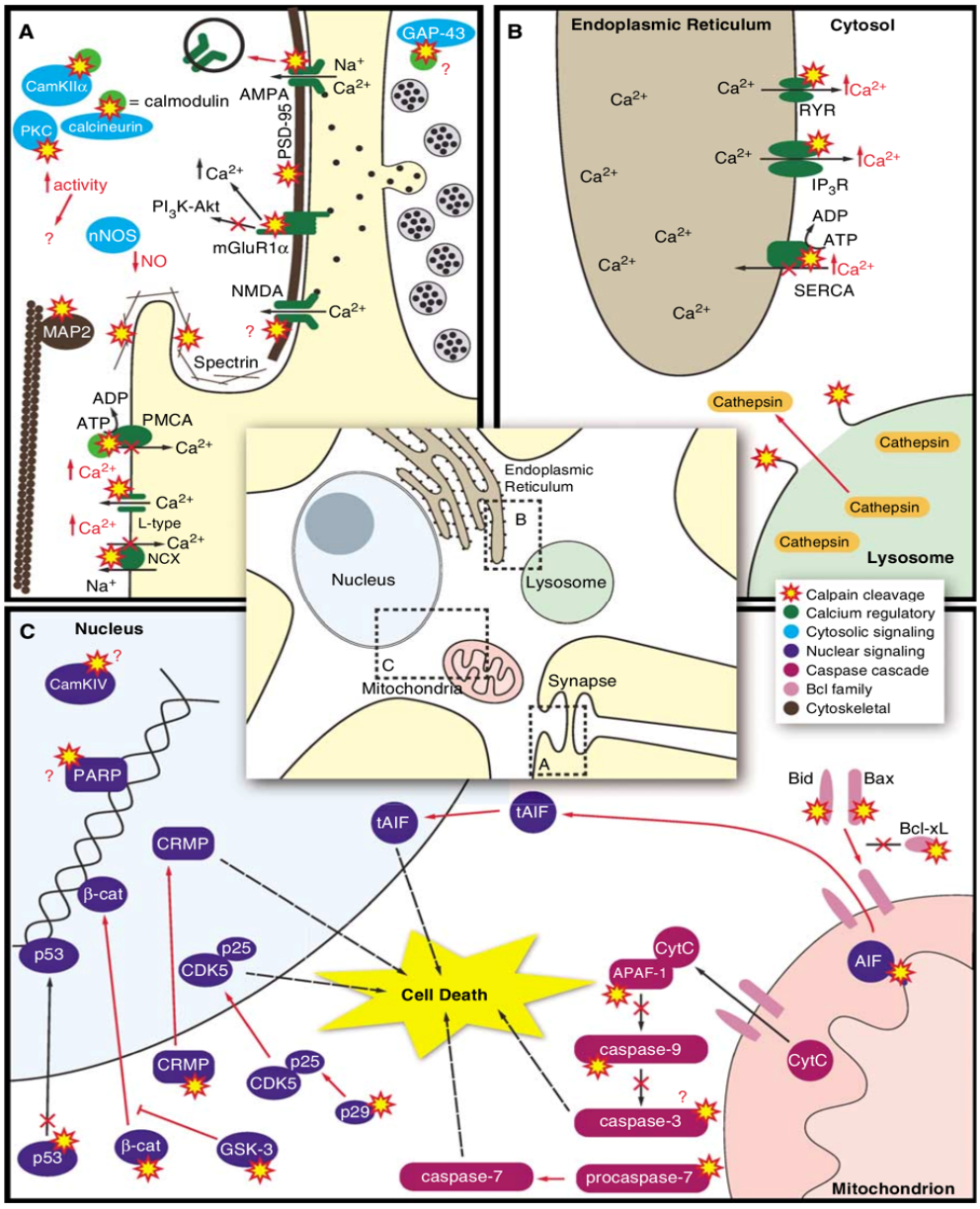


Figure 1.3. Subcellular localization of calpain substrates in postischemic neurons. Postischemic calpain activity occurs in a wide range of subcellular regions, namely the synapse and plasma membrane (A), endoplasmic reticulum and lysosomes (B), and mitochondria and nucleus (C). The substrate profile within each location suggests multiple distinct mechanisms by which calpain activity can contribute to postischemic neurodegeneration (Bevers and Neumar, 2008).

1.3. Caspases

Caspases comprise an ubiquitous family of aspartate-specific cysteine proteases, with enzymatic activity ruled by a predominant specificity for protein substrates containing Asp and by the use of a Cys chain for catalyzing peptide cleavage (Alnemri et al., 1996; Fuentes-Prior and Salvesen, 2004). Caspases are synthesized as inactive polypeptides composed of one large (α) and one small (β) subunit joined by a small spacer and a variable N-terminal prodomain. During activation, the catalytic domain is cleaved and the spacer and the prodomain are removed, and the large and small subunits interact closely with each other. In its active form, caspases are heterodimers of catalytic domains, with two active sites per molecule (Chan and Mattson, 1999; Pop and Salvesen, 2009). Caspases were classically divided into "apoptotic" and "inflammatory", although most apoptotic candidates (caspase-2, -3, -6, -7, -8,-9, and -10) have at least one non-apoptotic role (Bredesen, 2008). Within the apoptotic subgroup, the terms "initiator" or "apical" *versus* "effector" or "downstream" caspases distinguish the caspases that initiate the cascade (caspase-8, -9, and -10) from those that are activated by the initiators to execute apoptosis (caspase-3, -6, and -7) (Chan and Mattson, 1999; Pop and Salvesen, 2009). Upon activation of plasma membrane death receptor (*e.g.* Fas receptor) or following selective mitochondrial cytochrome c release, the apical caspases-8 and -9 are activated, respectively, and in turn activate the effector caspases-3 and -7 (Riedl and Shi, 2004; Kumar, 2007).

Caspase-3 is considered the most important of the effector caspases, and has been shown to play an important role in neuronal death after ischemia (Chen et al., 1998; Rami et al., 2003; Cao et al., 2004a) (Section 1.1.6). The most-important specificity determinant for calpain-3 cleavage is the Asp in P1 and P4 positions, being DExD the preferred cleavage motif. This motif appears in many proteins that are cleaved during cell death (Nicholson, 1999; Wang, 2000; Pop and Salvesen, 2009). Caspase-3 tends to produce "limited fragments" of its substrates, leaving them as fingerprints of its activity. Among its substrates are cytoskeletal proteins, enzymes involved in signal transduction, and DNA-repairing proteins (Nicholson, 1999; Wang, 2000)

Because proteolysis is irreversible, activation of caspases is tightly regulated by mechanisms that include caspase inhibiton and degradation. Caspase activation can be modulated by IAPs (Section 1.1.6), or by calpain direct and indirect interaction with members of the caspase family of proteases (Section 1.2.2), to quote some examples.

1.4. Glutamatergic neurotransmission in physiological and pathological conditions

Glutamate is an ubiquitous anionic amino acid that exists in all cell types, but in the brain it acts as a signaling molecule, being stored and released from the glutamatergic neurons subpopulation. Glutamate was discovered in 1908, but only in 1984 it was considered the main excitatory neurotransmitter in the CNS, used by around half of the neurons in the brain (Fonnum, 1984; Liguz-Lecznar and Skangiel-Kramska, 2007).

Glutamate is derived from glutamine through enzymatic conversion involving the phosphate-activated glutaminase (PAG) (Albrecht et al., 2007), and is stored in small synaptic vesicles at nerve terminals. Following membrane depolarization and Ca^{2+} entry into cells, synaptic vesicles fuse with the plasma membrane and release the glutamate by exocytosis into the synaptic cleft. After being released, glutamate is transported back to the neuron or into the glial cells by the action of excitatory amino acid transporters (EAATs). In astrocytes, glutamate is converted to glutamine by glutamine synthase, and glutamine is transferred back to neurons, probably through the sequential action of amino acid system N and A transporters (Albrecht et al., 2007; Liguz-Lecznar and Skangiel-Kramska, 2007; Lee and Pow, 2010).

1.4.1. Vesicular Glutamate Transporters (VGLUTs)

Vesicular glutamate transporters (VGLUTs) are responsible for the vesicular storage of glutamate, and therefore play an important role in glutamatergic signalling Glutamate uptake into synaptic vesicles is driven by a proton-dependent electrochemical gradient ($\Delta\Psi$) that exists across the vesicle membrane and is created by the vacuolar-type H^+-ATPase (Liu and Edwards, 1997; Bellocchio et al., 2000; Forgac, 2000; Fremeau et al., 2004a). This allows H^+ entry into synaptic vesicles, making the vesicular lumen more acidic and generating a pH gradient (ΔpH). The main driving force for the activity of VGLUTs is the difference in membrane potential instead of the pH gradient (Fig. 1.4) (Tabb et al., 1992; Moriyama and Yamamoto, 2004). The presence of Cl^- contributes to the acidification of synaptic vesicles, presumably by dissipating the luminal positive charge and enabling the H^+ pump to generate a larger ΔpH (Forgac, 2000). Cl^- also exerts an allosteric effect on vesicular glutamate transport, with synaptic vesicles exhibiting a maximal glutamate uptake at 2-10 mM Cl^- concentration (Hartinger and Jahn, 1993; Wolosker et al., 1996). VGLUTs exhibit high substrate specificity for L-glutamate, but a relatively low affinity (Km= 1-3 mM) (Shigeri et al., 2004; Takamori, 2006).

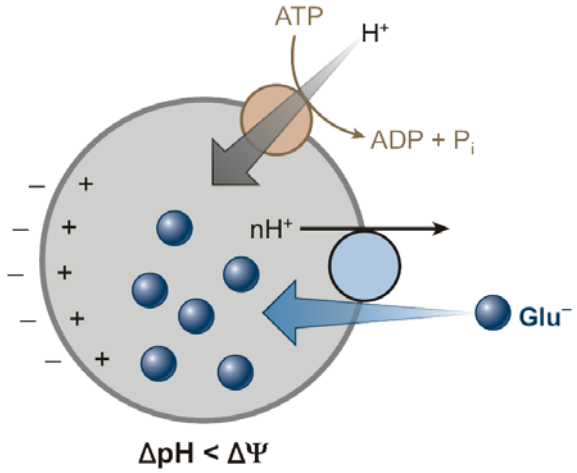


Figure 1.4. Mechanism of glutamate transport into the synaptic vesicles. VGLUTs use proton electrochemical gradient generated by vacuolar-type H^+- ATPase to carry glutamate into the synaptic vesicles. VGLUTs transport the negatively charged glutamate and rely more on $\Delta\Psi$ than ΔpH (Chaudhry et al., 2008).

The first VGLUT protein was cloned in 1994 and originally termed BNPI (brain-specific Na^+-dependent inorganic phosphate cotransporter), since it is capable of Na^+-dependent uptake of inorganic phosphate (Pi) (Ni et al., 1994). However, the protein was found to be specifically expressed and localized in the synaptic vesicles of glutamatergic neurons, suggesting its presynaptic role at glutamatergic synapses (Bellocchio et al., 1998; Takamori et al., 2000). Additional studies demonstrated that BNPI functions as a vesicular glutamate transporter and is sufficient to provide a glutamatergic phenotype in neurons (Bellocchio et al., 2000; Takamori et al., 2000). Therefore, the transporter was later renamed VGLUT1. The results showing that about half of the glutamatergic neurons lack VGLUT1 suggest the presence of other VGLUT proteins. A cDNA homologous to VGLUT1 was cloned in 2000, and initially called DNPI (differentiation-associated Na^+-dependent Pi transporter) (Aihara et al., 2000). DNPI also exhibited VGLUT activity similar to BNPI, and was named VGLUT2 (Bai et al., 2001; Fremeau et al., 2001; Hayashi et al., 2001; Herzog et al., 2001; Takamori et al., 2001; Varoqui et al., 2002). Another VGLUT (VGLUT3) was identified, with a high homology to VGLUT1 and VGLUT2 amino acid sequence (Fremeau et al., 2002; Gras et al., 2002; Schafer et al., 2002; Takamori et al., 2002). The three isoforms show almost 90% homology in the membrane crossing domains, whereas the N- and C-terminal regions have little homology and account to

functional differences (Takamori, 2006). According to their primary amino acid sequences, VGLUTs are part of the SLC17 type I phosphate transporter family, and seem to contain 12 transmembrane domains with both amino and carboxyl terminals facing the cytoplasm (Fig. 1.5) (Moriyama and Yamamoto, 2004; Reimer and Edwards, 2004; Jung et al., 2006).

VGLUTs transport L-glutamate in synaptic vesicles, while in the plasma membrane they co-transport Na^+ and Pi in the reverse direction (Bellocchio et al., 1998; Aihara et al., 2000; Bellocchio et al., 2000; Hayashi et al., 2001; Takamori et al., 2001). The purification and functional reconstitution of VGLUT2 has shown that this protein can catalyze Na^+-dependent phosphate transport in addition to glutamate/H^+ exchange (Juge et al., 2006). Moreover, it was shown that a fraction of the VGLUTs is indeed present on the presynaptic plasma membrane (Hagiwara et al., 2005), and since glutamate is synthesized by PAG at presynaptic terminals, phosphate influx through VGLUTs might stimulate PAG activity, by accumulating more glutamate close to synaptic vesicles (Bellocchio et al., 1998). Therefore, if mechanisms exist to regulate expression and modulate the activity of plasma membrane VGLUTs, these transporters will also regulate the process of synaptic refilling with glutamate.

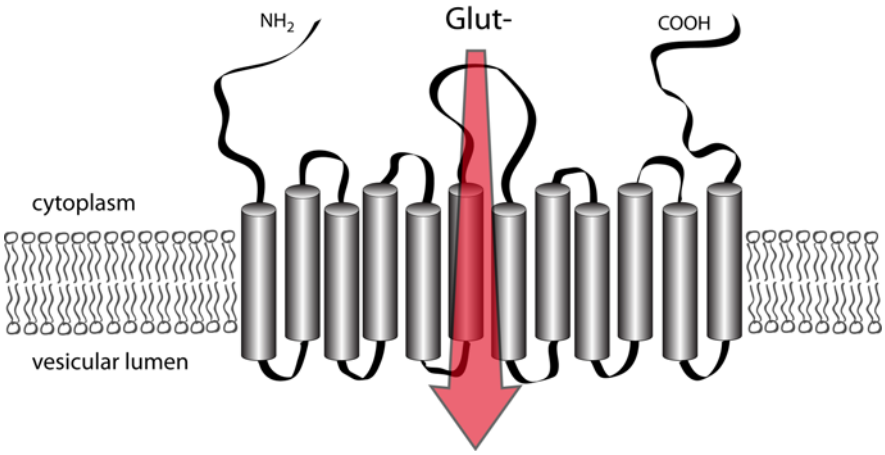


Figure 1.5. Predicted topology of VGLUTs (Moriyama and Yamamoto, 2004; Jung et al., 2006).

VGLUT1 and VGLUT2 are expressed exclusively in glutamatergic neurons, and are markers for these neurons in the CNS. These isoforms show a generally complementary expression in the CNS, with limited intersection. Cerebral cortex, hippocampus and amygdala express predominantly mRNA for VGLUT1, while

VGLUT2 mRNA is mainly localized in the diencephalic zone (*e.g.* thalamus, hypothalamus) and brain stem region (Fremeau et al., 2001; Herzog et al., 2001; Kaneko and Fujiyama, 2002). In the cerebellum, VGLUT1 mRNA is localized in granule cells (parallel fibers), while VGLUT2 mRNA localizes in the climbing fibers (Fremeau et al., 2001; Hisano et al., 2002). At the protein level, VGLUT1 immunoreactivity is distributed throughout the cerebral cortex, but for VGLUT2 only localizes in layer IV and VI (Fremeau et al., 2001; Varoqui et al., 2002). In the hippocampus, the dentate gyrus essentially stains for VGLUT1, but the granule and pyramidal cell layers also label for VGLUT2 (Bellocchio et al., 1998; Fremeau et al., 2001). In the cerebellum, protein expression for VGLUT1 and VGLUT2 is in accordance to the mRNA expression (Fremeau et al., 2001). The division of central excitatory neurons into expressing VGLUT1 or VGLUT2 suggests that these cells might differ in some aspects of the packaging and regulated release of glutamate. Although no functional differences were found between VGLUT1 and VGLUT2, the expression of the two isoforms appears to correlate with differences in the probability of glutamate release. It was proposed that VGLUT1 is present at synapses with low release probability, known to exhibit long-term potentiation (LTP), while VGLUT2 is expressed in synapses that exhibit high release probability, associated with long-term depression (LTD) (Fremeau et al., 2001; Varoqui et al., 2002; Liguz-Lecznar and Skangiel-Kramska, 2007). In fact, a recent study reported the reduction in the magnitude of LTP in the hippocampal CA1 region of heterozygous VGLUT1-deficient mice where the VGLUT1 expression is reduced to about 60%, whereas heterozygous VGLUT2-deficient mice were practically unchanged in their propensity to generate LTP (Balschun et al., 2010).

VGLUT1 and VGLUT2 also undergo transcriptional regulation during development. In rodents, VGLUT1 transcription increases weeks after birth, while that of VGLUT2 declines (Boulland et al., 2004). The highest level of VGLUT2 detected early in development is in accordance to the high release probability shown for this isoform, promoting survival and maturation of the synapses (Liguz-Lecznar and Skangiel-Kramska, 2007). A developmental switch from VGLUT2 to VGLUT1 occurs in the hippocampus, cortex and cerebellum, and associates with the physiological maturation of synapses within particular structures (Miyazaki et al., 2003; Nakamura et al., 2005). This phenomenon is connected to the co-expression of the two isoforms in the same nerve terminals, which can be seen transiently in early postnatal life or even permanently in adulthood in the regions where the developmental switch occurs, and is thought to be involved in high neuronal plasticity (Nakamura et al., 2005; Herzog et al., 2006). In these neurons, VGLUTs can be segregated to different synapse populations (segregation model) (Fremeau

et al., 2004b) or colocalized in the same synapse. According to the last hypothesis, VGLUTs can be distributed in distinct synaptic vesicles (commingling model) or colocalized in the same vesicle (colocalization model) (Wojcik et al., 2004; Herzog et al., 2006).
VGLUT3 is expressed in a limited number of synapses in multiple brain regions, such as the striatum, hippocampus, cerebral cortex, hypothalamus and raphe nuclei (Fremeau et al., 2002; Gras et al., 2002; Schafer et al., 2002; Herzog et al., 2004). It was also found in hippocampal and cortical GABAergic neurons (Herzog et al., 2004), cholinergic neurons in the striatum and serotonergic neurons in the raphe nuclei (Gras et al., 2002; Schafer et al., 2002). Furthermore, VGLUT3 is found postsynaptically at dendrites and cell bodies (Fremeau et al., 2002; Fremeau et al., 2004a), suggesting that this isoform might be involved in somatodendritic release of glutamate (Herzog et al., 2004). VGLUT3 is also expressed in a subset of astrocytes throughout the brain (Fremeau et al., 2002). Differently from VGLUT1 and 2 isoforms, VGLUT3 does not colocalize with other VGLUT isoforms (Boulland et al., 2004).

Quantal size, the postsynaptic response to the fusion of a single synaptic vesicle, is a fundamental parameter controlling the strength of synaptic transmission. Variability in excitatory quantal size is remarkably attributable to presynaptic changes in glutamate release (Liu et al., 1999; Hanse and Gustafsson, 2001; Franks et al., 2003), since glutamate exocytosis of a single synaptic vesicle is usually insufficient to saturate glutamate receptors (Liu et al., 1999; McAllister and Stevens, 2000; Ishikawa et al., 2002; Erickson et al., 2006; Edwards, 2007). Several studies addressed the putative role of VGLUTs expression in quantal neurotransmitter release at excitatory synapses. One study showed that glutamatergic neurotransmission was drastically reduced in neurons from VGLUT1-deficient mice, in which the remaining activity correlated with the expression of VGLUT2. This reduction was reverted with VGLUT1 overexpression, showing that VGLUT1 expression levels determined the amount of glutamate loaded into vesicles and released, thereby regulating synaptic transmission (Wojcik et al., 2004). A different study showed that VGLUT1 overexpression at hippocampal excitatory synapses results in enhanced evoked and miniature responses, and these changes were correlated to an increase in the amount of glutamate release per vesicle (Wilson et al., 2005). Deletion of the gene encoding VGLUT2 in mice caused perinatal lethality and a 95% reduction in evoked glutamatergic responses in cultured thalamic neurons. Heterozygous knockout mice, where VGLUT2 expression is reduced to 50%, showed behavioral phenotypes consistent with a deficit in thalamic processing,

such as acquisition of neuropathic pain. There was also a reduction in mEPSCs amplitude in heterozygous and VGLUT2 knockout mice thalamic neurons, demonstrating that VGLUT2 expression controls the amount of glutamate molecules loaded into a single vesicle (Moechars et al., 2006). Increasing the expression of *Drosophila* VGLUT (DVGLUT) leads to an increase in quantal size, accompanied by an increase in synaptic vesicle volume, demonstrating that DVGLUT expression determines the size and glutamate content of synaptic vesicles (Daniels et al., 2004). Together, these findings suggest that the expression of vesicular transporters is used endogenously to directly regulate the extent of glutamate release, providing a presynaptic mechanism for controlling the quantal efficacy of excitatory transmission. Interestingly, VGLUT1 knockout mice survived 2 weeks after birth, while the VGLUT2-deficient mice died immediately after birth, reinforcing the importance of VGLUT2 in early development., probably implicated in life-supporting mechanisms immediately *ex utero* (Wallen-Mackenzie et al., 2010).

Since changes in VGLUT protein levels may affect glutamate release, studies have been performed to characterize the expression of these transporters in different neurological disorders. VGLUT1 and VGLUT2 expression was shown to be altered in Parkinson's disease, and both transporters were found to be upregulated in the Parkinsonian putamen, whereas VGLUT1 was downregulated in the prefrontal and temporal cortex of PD patients (Kashani et al., 2007). VGLUT1 expression in the cerebral cortex and hippocampus was increased after chronic administration of antidepressant drugs (Moutsimilli et al., 2005; Tordera et al., 2005). In addition, in schizophrenic patients, VGLUT1 expression was diminished in the hippocampus and the dorsolateral prefrontal cortex, while VGLUT2 expression was enhanced in the thalamus (Smith et al., 2001; Eastwood and Harrison, 2005). Several evidences have also implicated VGLUTs in epilepsy. VGLUT1 expression was elevated in patients with temporal lobe epilepsy, contributing to a rise in extracellular glutamate levels and hyperexcitability (van der Hel et al., 2009), and mice lacking VGLUT3 exhibited enhanced cortical excitability and primary, generalized epilepsy, accompanied by little or no change in ongoing motor behavior (Seal et al., 2008). VGLUT2 heterozygous mice also show an increased susceptibility in pentylenetetrazol (PTZ) induced clonic seizures, a model for generalized epilepsy (Schallier et al., 2009). Finally, several studies have implicated changes in VGLUTs levels in several models of brain ischemia. VGLUT2 levels were shown to be reduced after transient global ischemia in the CA1 layer of the gerbil hippocampus (Kim et al., 2006), and in the cortex and caudate-putamen of rats subjected to transient MCAO (Sanchez-Mendoza et al., 2010). In contrast, VGLUT1 was found to

be upregulated in hippocampus following focal hypoxic ischemia (Kim et al., 2005), and in the cortex and caudate-putamen of rats subjected to transient MCAO (Sanchez-Mendoza et al., 2010).

1.4.2. Glutamate receptors

Once released from the presynaptic nerve terminal, glutamate binds to specific receptors in the postsynaptic membrane to conduct excitatory transmission. Pre-synaptic glutamate receptors act in the modulation of glutamate release. The effects of glutamate are mediated by activation of ionotropic or metabotropic receptors, which differ in their molecular, biochemical, physiological and pharmacological properties (Kew and Kemp, 2005). The ionotropic glutamate receptors have been classified into three distinct subgroups, α.amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA), N-methyl-D-aspartate (NMDA) and Kainate (KA) receptors (Dingledine et al., 1999; Mayer, 2004). AMPA and kainate receptors are responsible for most of the fast excitatory transmission in the vertebrate CNS. They are voltage-independent ion channels and permeable to Na^+ and K^+, leading to a net depolarizing influx of cations upon activation by glutamate (Swanson et al., 1997). AMPA receptors are composed of four possible subunits, GluA1-4, which associate in different stoichiometries to form receptors with distinct properties (Greger et al., 2007). NMDA receptors are ligand-gated ion channels that exhibit strong voltage dependence owing to the blocking of the receptor channels at negative membrane potentials by extracellular magnesium. As a result, these receptors contribute little to the postsynaptic response during low-frequency synaptic activity. However, when the cell is depolarized, Mg^{2+} dissociates from its binding site within the NMDAR channel, allowing Ca^{2+} as well as Na^+, to enter the dendritic spine (Cull-Candy et al., 2001). Functional NMDA receptors are heterotrimeric complexes containing both GluN1 and GluN2 subunits (Prybylowski and Wenthold, 2004). Metabotropic glutamate receptors are coupled to G proteins (which in turn stimulate second messenger signalling pathways), and, as such, they mediate slower synaptic responses, occurring over seconds and minutes, rather than milliseconds as occurs for ionotropic glutamate receptors. There are three groups of mGluR, distinguished based on sequence homology, signal transduction mechanisms and agonist selectivity (Pin and Acher, 2002; Kim et al., 2008; Niswender and Conn, 2010).

LTP and LTD are forms of synaptic plasticity thought to underlie learning and memory processes (Braunewell and Manahan-Vaughan, 2001; Lynch, 2004). LTP refers to a persistent increase in efficacy of synaptic transmission, following a short

period of presynaptic high frequency stimulation with the depolarization of the postsynaptic membrane. On the other hand, LTD is characterized by a reduction in the synaptic strength, following low-frequency synaptic stimulation. NMDA receptors are the most important known trigger for long-term modification of synaptic strength, and their activation and the resulting Ca^{2+} influx through the receptor channel are required for LTP and LTD in the hippocampus. The rise in intracellular Ca^{2+} concentration is a signal to activate several signaling pathways: LTP is partially mediated by CaMKII activation (Okamoto et al., 2009), while LTD requires calcineurin activation (Kourrich et al., 2008).

Excitotoxicity caused by overactivation of glutamate receptors plays a key role in the neuronal death characteristic of several neuronal disorders, namely ischemia, epilepsy and neurodegenerative disorders (Szydlowska and Tymianski, 2010), and was described in Section 1.1.1.

1.5. Trafficking of vesicular neurotransmitter transporters

Since synaptic transmission requires the efficient vesicular packaging of neurotransmitters, mediated by vesicular neurotransmitter transporters, the targeting of these proteins to synaptic vesicles (SVs) is tightly regulated (Liu and Edwards, 1997; Colgan et al., 2007). In addition to small synaptic vesicles (SSVs), neurotransmitter transporters can also traffic to large dense core vesicles (LDCVs). SSVs and LDCVs differ in several important ways, including their subcellular sites of release (Fei et al., 2008). Vesicular monoamine transporter 2 (VMAT2) localizes to both SSVs and LDCVs in neurons, and changes in VMAT2 trafficking may affect the relative distribution of the transporter to the two types of synaptic vesicles and the subcellular site of monoamine release (Nirenberg et al., 1996, 1997). Besides monoamines, other neurotransmitters are also released from LDCVs. VGLUT3 also localizes to dendrites (Fremeau et al., 2002), and glutamate release from dendrites modulates signaling in cortical pyramidal cells (Harkany et al., 2004). GABA release from dendrites mediates retrograde signaling in the cortex (Zilberter et al., 1999). These observation suggest that both VGLUT and VGAT also localize to other types of secretory vesicles besides SSVs, that are only located at nerve terminals, and the final location of the transporters may regulate the subcellular sites of glutamate and GABA release (Fei et al., 2008).

SSVs proteins like vesicular neurotransmitter transporters are thought to first traffic to the plasma membrane during SSVs biogenesis (Prado and Prado, 2002). Vesicular proteins probably exit the *trans* Golgi network (TGN) through constitutive secretory vesicles (Regnier-Vigouroux et al., 1991), but some SSV proteins may exit the TGN on more specialized organelles (Ahmari et al., 2000). Vesicles exiting the TGN do not contain all the proteins found in mature SSVs, and more trafficking steps are necessary for these vesicles to fully maturate (Nakata et al., 1998; Hannah et al., 1999). Studies on the glutamatergic and the cholinergic systems suggest that mature synaptic vesicles are formed at presynaptic terminals after constitutive recycling or from endosomal intermediates (Nakata et al., 1998; Ahmari et al., 2000; Santos et al., 2009). Synaptic vesicle proteins in constitutive secretory vesicles are thought to be delivered to the plasma membrane, and go through several steps of exo and endocytosis that sort and cluster proteins (Regnier-Vigouroux et al., 1991; Santos et al., 2001; Barbosa et al., 2002). Endocytosis is also required for SSVs recycling, and both fast and slow endocytic pathways have been identified (Koenig and Ikeda, 1996; Jahn and Rizzoli, 2007). Trafficking via fast versus slow endocytic pathways determine whether a SSV goes to a ready releasable or to a reserve pool of vesicles (Richards et al., 2000; Kidokoro et al., 2004; Evans and Cousin, 2007).

LDCVs are generated by a different pathway, and do not require endocytosis for their biogenesis (Kelly, 1993). LDCVs proteins, as they exit the TGN, are sorted to the regulated secretory pathway. LDCVs are generated in an immature form, and require the removal of some proteins to maturate (Tooze et al., 1991).

Vesicular neurotransmitter transporters contain several motifs involved in the trafficking of membrane proteins (Bonifacino and Traub, 2003). Thus, VMAT2 contains a dileucine motif (IL) encoded in the C-terminal region, which is required for its endocytosis in PC12 cells and in hippocampal neurons and for sorting to synaptic-like microvesicles (SLMVs) in PC12 cells (Tan et al., 1998; Li et al., 2005; Yao and Hersh, 2007). Additional signals are necessary for VMAT2 localization to LDCVs, including glutamate residues (EE) upstream of the dileucine motif, that are conserved in VMAT1 and *Drosophila* VMAT (DVMAT) (Krantz et al., 2000; Greer et al., 2005). The hydrophobic residues (IL) in the dileucine motif may help sorting VMAT2 to LDCVs, and it is thought that the hydrophobic and the acidic residues (EE) in the dileucine motif function as a single unit (Bonifacino and Traub, 2003). Another acidic cluster present at the C-terminal domain of VMAT2 is needed for the transporter sorting to LDCVs in PC12 cells, and possibly has a similar function in neurons (Waites et al., 2001). VMAT2 also possess other signals important for

trafficking, such as glycosylation, since it was shown that a decrease in glycosylation associates with a reduced VMAT2 localization to SVs *in vivo* (Cruz-Muros et al., 2008). A recent study has also implicated N-linked glycosyl groups of the luminal loop in VMAT2 sorting to LDCVs in PC12 cells (Yao and Hersh, 2007).

The vesicular acetylcholine transporter (VAchT) contains a dileucine motif (LL), which is required for the transporter endocytosis in non-neuronal cells and in the cholinergic cell line SN56, and regulates the transporter sorting to SLMVs in PC12 cells (Tan et al., 1998; Santos et al., 2001; Ferreira et al., 2005; Colgan et al., 2007). The VAchT also has an atypical tyrosine-based motif (YNYY) in the C-terminal trafficking domain, that was shown to be involved in its endocytosis, suggesting that this transporter may contain two distinct endocytic motifs (Kim and Hersh, 2004). Both dileucine and tyrosine motifs are sufficient and necessary for sorting VAChT to SLMVs (Kim and Hersh, 2004; Ferreira et al., 2005).

VGLUT1 contains a dileucine-like motif (FV) that seems to play a role in the transporter endocytosis and recycling to SVs in hippocampal neurons (Voglmaier et al., 2006). VGLUT1 also holds two polyproline domains that functions together with the dileucine-like motif (De Gois et al., 2006; Vinatier et al., 2006; Voglmaier et al., 2006). At one of these polyproline motifs (PPRPPPP) VGLUT1 interacts with endophilins 1 and 3, proteins that have been implicated in endocytosis and in the remodeling of lipid membranes (Guichet et al., 2002; Verstreken et al., 2002). Deletion of the polyproline motif in VGLUT1 only decreases the transporter endocytosis in prolonged periods of exocytosis and SV recycling (Voglmaier et al., 2006). It was proposed that VGLUT2 and VGLUT3 recycle less efficiently due to the absence of the polyproline motif. In fact, VGLUT2 expressing synapses depress more rapidly and recover more slowly in response to high frequency stimulation than VGLUT1 expressing synapses (Fremeau et al., 2004b). Recently, it was reported that deletion of DVGLUT C-terminal domain had a small but consistent effect in reducing internalization and SV localization of the transporter (Grygoruk et al., 2010). It was also shown that expression and possible localization of VGLUT1 to SVs follows a circadian pattern (Yelamanchili et al., 2006).

1.6. Objectives

Excitotoxicity caused by a raise in glutamate release and overactivation of glutamate receptors is an important trigger of cell death in brain ischemia. The resulting $[Ca^{2+}]_i$ overload activates several proteases, namely caspases and calpains, which will cleave several substrates, changing its subcellular localization and/or its function.

In this study we investigated presynaptic changes in glutamatergic synapses under excitotoxic or ischemic conditions, focusing on the vesicular glutamate transporters type 1 and 2, the two VGLUT isoforms that confer neurons its glutamatergic phenotype (Fremeau et al., 2001; Fremeau et al., 2004a). So far, studies have mainly addressed the mechanisms underlying postsynaptic changes in glutamatergic synapses under excitotoxic conditions and brain ischemia. In this work we investigated presynaptic changes in the glutamatergic synapses following excitotoxic stimulation and brain ischemia. In particular, we focused on the vesicular glutamate transporters, since their abundance regulate directly the accumulation of glutamate into synaptic vesicles and the exocytotic release of the neurotransmitter (Daniels et al., 2004; Wojcik et al., 2004; Wilson et al., 2005; Moechars et al., 2006; Edwards, 2007), providing a concise presynaptic mechanism for controlling the quantal efficacy of excitatory transmission. To address this question, cultured hippocampal neurons were subjected to excitotoxic stimulation with glutamate or to oxygen and glucose deprivation conditions. These conditions resemble, to some extent, the toxic effects of glutamate that occur during stroke in humans. Because *in vitro* models are remote from the complexity of the *in vivo* condition, we also determined the changes in VGLUT protein levels in an *in vivo* model of excitotoxicity, consisting in the intrahippocampal injection of kainate, and following transient focal cerebral ischemia (MCAO). VGLUT protein levels were assessed by western blot using specific antibodies. The results showed a significant downregulation of VGLUT1 and VGLUT2 in cultured hippocampal neurons subjected to excitotoxic conditions, but in *in vitro* and *in vivo* brain ischemia VGLUT2 was selectively downregulated.

The C-terminal region of VGLUT2 and VGLUT1 possess PEST sequence(s) which are directed towards the cytoplasm in both transporters. This type of sequence is thought to constitute a signal for rapid intracellular proteolysis (Rogers et al., 1986; Rechsteiner and Rogers, 1996) and has been associated to protein cleavage by calpains (Molinari et al., 1995; Shumway et al., 1999). Furthermore, the VGLUT2 C-terminal region also contains a DELD consensus sequence for putative caspase-3 cleavage. Given the high vulnerability of VGLUT2 under excitotoxic conditions, and

considering the role played by calpains and caspase-3 in cell death under the same conditions, we tested for the role of both proteases in glutamate-induced downregulation using specific chemical inhibitors. Calpain inhibition significantly prevented VGLUT2 downregulation under excitotoxic conditions, and the direct effect of calpain in the cleavage of the transporter was further investigated in *in vitro* experiments, by incubating synaptic vesicles from the cerebral cortex with a recombinant form of the protease. Additional studies were performed in which a fusion protein of the C-terminal region of VGLUT2 with GST was incubated with recombinant calpain I and the cleavage products were analyzed by N-terminal Edman sequencing. A fusion protein of the protein transduction domain (PTD), derived from the TAT protein of the HIV virus, with the VGLUT2 C-terminal region, targeted by calpain, was also used to assess VGLUT2 cleavage in hippocampal neurons subjected to excitotoxic conditions. The fusion protein containing the VGLUT2 C-terminal region and GST was also used to determine the caspase-3 cleavage site, in experiments where the protein was incubated with the recombinant protease. Whether caspase-3 contributes to the cleavage of VGLUT2 was further investigated under two experimental conditions known to induce caspase-3 mediated apoptotic neuronal death: trophic factor deprivation and stimulation with staurosporin.

Given the VGLUT2 cleavage by calpain and the formation of a truncated form observed using a N-terminal antibody for VGLUT2, we reasoned that the truncation could affect the intracellular trafficking and subcellular localization of the transporter. This was investigated in cultured hippocampal neurons transfected with GFP fusion proteins with the full length and the cleaved forms, and the synaptic distribution of the transporter was evaluated by colocalization with the synaptic marker synapsin.

Chapter 2

Material & Methods

2.1. DNA constructs

To clone the VGLUT2 C-terminal in pGEX6P2, the cDNA encoding the C-terminal of rat (*Rattus norvegicus*) VGLUT2 was obtained by RT-PCR from rat cortex RNA. The first strand cDNA was produced using the first strand cDNA Synthesis Kit for RT-PCR (AMV) procedure (Roche) and the resulting cDNA was amplified by PCR using primers forward 5' CGGAATTCTATTTGCCTCAGGAGAGAAG 3' and reverse 5' CCGCTCGAGTTATGAATAATCATCTCGGT 3'. This product was cloned into the pGEM vector (Promega), and then subcloned into pGEX6P2 using EcoRI and XhoI restriction sites, in frame with glutathione S-transferase (GST) encoded in the plasmid. The pGEX-VGLUT2 C-terminal sequence was confirmed by DNA sequencing reactions. The plasmids encoding for the fusion proteins of GST-VGLUT2 C-terminal mutants, D522A and D525A, were produced by site-directed mutagenesis, using PCR.

The rat full length VGLUT2 in pCMV6b was a kind gift from J. Takeda (Department of Diabetes and Endocrinology, Division of Molecule and Structure, Gifu University, Japan). The plasmid encoding VGLUT2 rat protein fused with green fluorescent protein (GFP) was generated by molecular cloning. The cDNA of rat VGLUT2 was amplified by PCR using the primers forward 5' CGGAATTCTATGGAGTCGGTAAAACAAAG 3' and reverse 5' CGGGATCCTTATGAATAATCATCTCGG 3', and subcloned into the pGEM vector (Promega) through EcoRI and BamHI restriction sites. VGLUT2 was then subcloned into pEGFPC1 (BD Biosciences Clontech) in frame with GFP. The pEGFPC1-VGLUT2 sequence was verified by DNA sequencing reactions. Immunocytochemistry experiments using antibodies anti-VGLUT2 and anti-GFP showed that the GFP-VGLUT2 fusion protein has the same subcellular localization as VGLUT2 endogenous protein in cultured hippocampal neurons (Figure 3.21). The truncated pGFPC1-VGLUT2 plasmids were generated by insertion of stop codons after amino acids 533 or 541 by directed mutagenesis, using PCR.

The rat full length VGLUT1 in pcDNA3.1(+) at HindIII/XhoI site was a kind gift of S. Takamori (Center for Brain Integration Research, Tokyo Medical and Dental University, Tokyo, Japan). The plasmid encoding the GST-VGLUT1 C-terminal fusion protein was produced by performing PCR using the primers forward 5' CCGGAATTCTCGGGAGAGAAACAGCCGTGG 3' and reverse 5' CCGCTCGAGTCAGTAGTCCCGGACAGGGGG 3' to amplify VGLUT1 C-terminal. The PCR product was cloned into the pGEX4T1 vector (Pharmacia) with EcoRI and XhoI. The resulting plasmid pGEX4T1-VGLUT1 C-terminal sequence was confirmed by DNA sequencing reactions.

To produce the fusion protein TAT-VGLUT2 C-terminal, the cDNA of VGLUT2 C-terminal region was amplified by PCR using pGEX-6P-VGLUT2 C-terminal as template, with the primer forward 5' CCGGATATCTTTGCCTCAGGAGAGAAGCAACC 3' and primer reverse 5' CCGGAATTCTTATGAATAATCATCTCGGTCCTTA 3'. The PCR product was cloned into plasmid ET 30 (+) using EcoRV and EcoRI. The pET 30 (a+) fused with Bcl-xL, encoding peptide sequences in the order His-6 tag, Thrombin cleavage site, PTD domain, HA tag and Bcl-xL, was a kind gift from G. Cao (Department of Neurology and Pittsburgh Institute of Neurodegenerative Disorders, Pittsburgh,USA). This plasmid was digested with the enzymes referred above to remove Bcl-xL cDNA, and the VGLUT2 C-terminal cDNA was inserted.

2.2. Hippocampal Cultures

High density cultures of rat hippocampal neurons were prepared from E18-E19 Wistar rat embryos as previously described (Almeida et al., 2005; Caldeira et al., 2007). Briefly, after dissection, the hippocampi were treated with trypsin (0.06%, 15 min, 37ºC; GIBCO Invitrogen) in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS: 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red). The hippocampi were washed with HBSS containing 10% fetal bovine serum, to stop trypsin activity, and then washed once in HBSS to remove serum and avoid glia growth. Finally, the hippocampi were transferred to Neurobasal medium (GIBCO Invitrogen), supplemented with B27 (1:50 dilution; GIBCO Invitrogen), glutamate (25 µM), glutamine (0.5 mM) and gentamicin (0.12 mg/mL), the cells were dissociated mechanically, and platted on poly-D-lysine coated 6 microwell plates (MW6) at a density of $9x10^4$ cells/cm^2. The cells were kept at 37°C in a humidified incubator with 5% CO_2/95% air, for 7 to 14 days in vitro (DIV).

Low density hippocampal cultures were prepared as previously described (Goslin et al., 1998). Briefly, hippocampi were dissected from E18 rat embryos, dissociated using trypsin (0.25%) and triturated. Neurons were plated at a final density of 1-5 x 10^4 cells/dish (60 mm culture dishes) on poly-D-lysine-coated in neuronal plating medium (MEM supplemented with 10% horse serum, 0.6% glucose and 1 mM pyruvic acid). After 2-4 h, coverslips were flipped over an astroglial feeder layer in Neurobasal medium supplemented with B27 supplement (1:50 dilution), 25 µM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin. The neurons grew face

down over the feeder layer but were kept separate from the glia by wax dots on the neuronal side of the coverslips. To prevent the overgrowth of glia, neuron cultures were treated with 5 μM cytosine arabinoside after 3 DIV. Cultures were maintained in a humidified incubator with 5% CO_2/95% air, at 37 °C, for up to 2 weeks, feeding the cells once per week by replacing one-third of the medium.

2.3. Excitotoxic stimulation with glutamate

Hippocampal neurons (7 DIV) were exposed to 125 μM glutamate for 20min in Neurobasal medium and further incubated in culture conditioned medium for the indicated periods of time. Pre-incubations of 2h were used when cells were treated with the calpain inhibitors MDL2817 (Calbiochem) and ALLN (Calbiochem) (50 μM), or with the pan-caspase inhibitor Z-VAD-FMK (Biomol) (50 μM). Under control conditions hippocampal neurons were not exposed to glutamate.

2.4. Induction of apoptosis

Hippocampal neurons were exposed to 30nM (Prehn et al., 1997; Krohn et al., 1998) or 300nM (Krohn et al., 1999; Lankiewicz et al., 2000) staurosporine, for 24h and 10min, respectively, and further incubated in culture conditioned medium for 14 h.

Trophic factor withdrawal was also used to induce apoptosis. Hippocampal neurons were washed four times with Locke's buffer (154 mM NaCl, 5.6mM KCl, 2.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5 mM glucose and 5 mM HEPES, pH 7.2) and further incubated in Locke's buffer for 48h, as previously described (Chan et al., 1999).

2.5. Oxygen-Glucose Deprivation (OGD)

Hippocampal neurons (14 DIV) were incubated in a glucose-free saline buffer (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 26 mM $NaHCO_3$), under an anaerobic atmosphere (10% H_2, 85% N_2, 5% CO_2) (Forma Anaerobic System, Thermo Fisher Scientific) at 37°C for the indicated time periods. After the OGD challenge, cultures were incubated in culture conditioned medium and returned to the humidified 95% air/5% CO_2 incubator for 8h. Control neurons

(Sham) were washed and incubated in the saline buffer described above, supplemented with 10 mM glucose, and kept in the humidified 95% air/5% CO_2 incubator at 37°C for the indicated time periods. When appropriate the calpain inhibitor MDL28170 (50 μM) was added 2h before and during OGD.

2.6. Preparation of hippocampal culture extracts

Hippocampal cultures were washed twice with ice-cold PBS, and once more with PBS buffer supplemented with 1 mM dithiothreitol (DTT) and a cocktail of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride (PMSF), CLAP (1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin; Sigma). The cells were then lysed with RIPA (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS at a final pH 7.5) supplemented with 50 mM sodium fluoride (NaF), 1.5 mM sodium ortovanadate (Na_3VO_4) and the cocktail of protease inhibitors. After centrifugation at 16,100 xg for 10 min at 4ºC, protein in the supernatants was quantified using the Bicinchoninic acid (BCA) assay kit (Pierce), and the samples were denaturated with 2× concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM Na_3VO_4, and 0.01% bromophenol blue). Extracts used for VGLUT1 and VGLUT2 analysis were not subjected to a boiling step to avoid VGLUT aggregation.

2.7. Western blot

Protein samples were separated by SDS-PAGE, in 12% polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MA), and immunoblotted, as previously described (Caldeira et al., 2007). Blots were incubated with primary antibodies overnight at 4°C, washed, and exposed to alkaline phosphatase-conjugated secondary antibodies (1:20000 dilution for anti-rabbit IgG and 1:10000 dilution for mouse IgG) for 1h at room temperature. Alkaline phosphatase activity was visualized by enhanced chemifluorescence (ECF) on the Storm 860 Gel and Blot imaging system, and quantified using the ImageQuant program (GE Healthcare). The following primary antibodies were used: anti-VGLUT1 (1:5000, Synaptic Systems, Germany), anti-VGLUT2 (1:1000, Ab:510-582aa [Synaptic Systems, Germany]; 1:750, Ab:N-terminal [USBiological]), anti-GST (1:2000, GE Healthcare), anti-spectrin (1:1000, Chemicon), anti-active caspase-3 (1:1000, Cell Signaling Technology) and anti-GFP (1:2000, Abcam, UK). The anti-

Synaptophysin (1:10000, Synaptic Systems, Germany or Sigma) antibody was used as loading control.

2.8. Neuron transfection with calcium phosphate

Constructs were recombinantly expressed in primary cultures of hippocampal neurons using a calcium phosphate co-precipitation method as previously described, with minor modifications (Jiang et al., 2004; Kaech and Banker, 2006). Briefly, 4 µg or 10 µg of plasmid DNA for 60 mm coverslips or MW6, respectively, were diluted in Tris-EDTA buffer (TE) pH 7.3 and mixed with an HEPES-Calcium chloride solution pH 7.2 (2.5M $CaCl_2$ in 10mM HEPES). This DNA/TE/Calcium mix was added to an equivalent volume of 2xHEPES buffered saline solution (270 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11 mM Dextrose, 42 mM HEPES), pH 7.2. The precipitates were allowed to develop for 30min, and vortexed every 5 min, to ensure that precipitates had similar small sizes. Meanwhile, hippocampal neurons were incubated with cultured conditioned medium supplemented with 2 mM of Kynurenic acid. The precipitates were added dropwise to each coverslip or well of the MW6, and incubated at 37°C, 5% CO_2, for 3h. The cells were then washed with culture medium containing 2 mM Kynurenic acid, slightly acidified with HCl (~5mM), and returned to the 37°C/5% CO_2 incubator for 15 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 48-72h to allow protein expression.

2.9. Immunocytochemistry

Hippocampal neurons were fixed in 4% sucrose/paraphormaldehyde (in PBS) and permeabilized with 0.3% Triton X-100 in PBS. The neurons were then incubated with 10% BSA in PBS for 30 min at 37°C, to block nonspecific staining, and incubated with primary antibodies diluted in 3% BSA in PBS, overnight at 4°C. The cells were then washed 5 times with PBS for 5 min each, and incubated with the appropriate secondary antibodies, for 1h at 37°C. The coverslips were mounted in a fluorescent mounting medium (DAKO, Denmark). Imaging was performed on a Zeiss Axiovert 200 fluorescent microscope, using a 63× oil objective. The primary antibodies used were anti-VGLUT2 (1:200 Ab: 510-582; Synaptic Systems, Germany), anti-HA (1:200, Abcam), anti-GFP (1:200, MBL; 1:200, Abcam) and anti-Synapsin (1:200, Millipore). The secondary antibodies used were Alexa Fluor 488

and 594, and Texas Red (Invitrogen). Images were quantified using image analysis software (ImageJ).

2.10. Intra-hippocampal injection of kainate

Intra-hippocampal injection of kainate was performed as previously described (Tomioka et al., 2002; Takano et al., 2005), with minor modifications. Briefly, wild type adult male mice (C56BL6) were deeply anesthetized with avertin (2,2,2 Tribromoethanol, 2-methyl-2-butanol), placed in a stereotaxic apparatus, and given a unilateral injection of 1 nmol of kainate (in 0.3 µl of PBS) into the hippocampal CA1 region, using a 10µl motorized syringe (Hamilton), after drilling a small hole with a surgical drill. The coordinates of the injection were anterior-posterior: 2.3 mm, medial-lateral: 1.5 mm, and dorsal-ventral: 1.7 mm from the bregma. Two min after the needle insertion, kainate was injected at a constant flow rate of 0.05 µl/ min. The needle remained in place for an additional 2 min to prevent reflux of fluid. The body temperature of mice was monitored and maintained at 37°C during surgery and 30 min after the injection, using an homeothermic heating blanket (Harvard Apparatus) with feedback regulation. The death rate in this experiment was less than 5%.

4, 8 or 12h after injection mice were sacrificed and a 2mm section around the hippocampus was taken with the help of a 1 mm coronal mouse matrice. The slices were immediately frozen with dry ice and the contralateral and the damaged ipsilateral areas of the hippocampal slices were taken using a Harris Unicore 2mm tip (Pelco International). Samples were then homogenized in RIPA supplemented with 50 mM NaF, 1.5 mM Na_3VO_4 and the cocktail of protease inhibitors (PMSF, DTT and CLAP) and processed for western blot.

2.11. Middle Cerebral Artery Occlusion (MCAO)

Focal cerebral ischemia was induced by the transient occlusion of the right middle cerebral artery (MCA), using the intraluminal filament placement technique as described previously (Nygren and Wieloch, 2005). Briefly, adult male mice were anesthetized by inhalation of 2.5 % isoflurane (*IsobaVet*, *Schering-Plough Animal Health*, England) in O_2: N_2O (30:70). Anesthesia was subsequently reduced to 1.5 - 1.8 % isoflurane and sustained throughout the occlusion period. Body temperature was kept at approximately 37 °C throughout the surgery period. In order to monitor regional cerebral blood flow (rCBF), an optical fiber probe (*Probe 318-I*, *Perimed*,

Sweden) was fixed to the skull at 2 mm posterior and 4 mm lateral to the bregma and connected to a laser Doppler flow meter (*Periflux System 5000*, *Perimed*, Sweden). A filament composed of 6-0 polydioxanone suture (PSD II, Ethicon) with a silicone tip (diameter of 225 to 275 μm) was inserted into the External Carotid Artery and advanced into the Common Carotid Artery. The filament was retracted, moved into the Internal Carotid Artery and advanced until the origin of the MCA, given by the sudden drop in rCBF (approximately 70% of baseline). After 45 min, the filament was withdrawn and reperfusion observed. The animals were placed in a heating box at 37°C for the first 2 h post-surgery and thereafter transferred into a heating box at 35°C, to avoid post-surgical hypothermia. Thirty minutes after the onset of reperfusion, 0.5 mL of 5% glucose were administered subcutaneously. Temperature and sensorimotor deficits were assessed at one and two hours, and in the morning after the surgery.

The Ethics Committee for Animal Research at Lund University approved animal housing conditions, handling and surgical procedures. Nine to 11 weeks old C57BL/6J male mice (weight - 21.5 g to 27.9 g; *Taconic*, Denmark) were housed under diurnal conditions with free access to water and food before and after surgery.

Mice were sacrificed 24 h after the occlusion and 1 mm sections were done with the help of a 1 mm coronal mouse matrice. The slices were immediately frozen with dry ice and the damaged ipsilateral and contralateral areas of the slices were taken using a Harris Unicore 2 mm tip (Pelco International). Samples were then homogenized and processed for western blot.

2.12. Expression of recombinant proteins

To produce the GST-VGLUT2 C-terminal fusion protein (pGEX4T1-VGLUT2 C-terminal), the plasmid was transformed into *Escherichia coli* (*E. coli*) BL21 and protein expression was induced by treating the *E. coli* culture in the exponential phase of growth (A_{600nm}= 0.8-2) with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) for 30 min at 30ºC. The protein was extracted and purified from the bacterial pellet through affinity chromatography on glutathione Sepharose 4B (GE Healthcare), according to manufacturer's recommendations.

To express and purify the TAT-VGLUT2 C-terminal fusion protein, the plasmid (pET-PTD-HA-VGLUT2 C-terminal) was transformed as described above, and protein expression was induced with 0.5 mM IPTG for 4 h at 37ºC. The protein was extracted from the bacterial pellet and purified using an affinity column containing

nickel (Bio-Scale Mini Profinity IMAC Cartridge 5 mL- Bio-Rad) by FPLC (AKTA Prime - GE Healthcare), according to the column manufacturer's recommendations. The purified proteins were analyzed in SDS-PAGE gels and stained with Coomassie Blue.

2.13. In vitro assay with recombinant calpain

Incubation of GST fusion proteins with calpain:

GST-VGLUT1 C-terminal and GST-VGLUT2 C-terminal fusion proteins (100 µg) were diluted in 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 2mM DTT and 2.5mM of $CaCl_2$ (or 1 mM EDTA), and incubated with or without 2.5 U/ml of recombinant calpain I (Human erythrocytes, Calbiochem), for 15 min at 37°C. Incubation was stopped using concentrated denaturating buffer and samples were boiled for 5 min at 95°C. The peptides were then separated by SDS-PAGE Tricine gels, in order to allow the separation of low molecular weight proteins (Schagger and von Jagow, 1987). The gels were stained with Commassie blue colloidal solution (Candiano et al., 2004). The same experimental procedure was used to transfer the proteins to a PVDF membrane (Millipore, MA), and the bands indicated were cut and analyzed by N-terminal Edman sequenciation, at the Protein Sequencing facility in University of Leeds, UK (Dr Jeff N Keen).

Incubation of synaptic vesicles with calpain:

Cerebrocortical synaptic vesicles were isolated from the rat brain as previously described (Synaptic Systems - Protocol for preparation of P2 and LP2 fractions). The vesicles (5 µg) were diluted in 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 2 mM DTT and 2.5mM of $CaCl_2$ (or 1mM EDTA), and incubated with or without 2.5 U/ml of recombinant calpain I (Human erythrocytes, Calbiochem), for different periods of time at 37°C. Incubation was stopped with concentrated denaturation buffer and samples were not boiled to prevent VGLUT1 and VGLUT2 aggregation. When appropriate, calpain inhibitors MDL28170 (50 µM) or ALLN (50 µM) were added during the assay. Samples were analyzed by western blot using anti-VGLUT1 or anti-VGLUT2 antibodies.

2.14. In-vitro assay with recombinant caspase-3

GST-VGLUT2 C-terminal fusion protein (5 µg) was diluted in 20 mM PIPES, 100 mM KCl, 1 mM EDTA, 10% Sucrose, 10 mM DTT and (or without) 0.1% CHAPS, and incubated with 2 U/ml of recombinant caspase-3 for 20 h at 37°C. Incubation was stopped using concentrated denaturation buffer and samples were boiled for 5 min at 95°C. Control reactions were performed in the absence of the enzyme. When appropriate, the caspase-3 inhibitor Z-DEVD-FMK (30 mM) was added during the assay. The samples were analyzed by western blot using an anti-GST antibody.

2.15. Statistical Analysis

The immunoreactivity obtained in each experimental condition was calculated as a percentage of the control. Data are presented as mean ± SEM of at least three different experiments, performed in independent preparations. Statistical analysis of the results was performed using one-way ANOVA analysis followed by either Dunnett's or Bonferroni's multiple comparison test: n.s. non significant, ***$p<0.001$, **$p<0.01$,*$p<0.05$.

Chapter 3

Results

3.1 VGLUT1 and VGLUT2 are cleaved after excitotoxic stimulation with glutamate

In order to assess the effect of excitotoxic stimulation on the protein levels of VGLUT1 and VGLUT2, cultured hippocampal neurons (7 DIV) were stimulated with 125 µM of glutamate for 20 min, and were further incubated in culture conditioned medium for different periods of time. VGLUT protein levels were assessed by western blot using antibodies against the C-terminal region of both transporters. Stimulation of cultured hippocampal neurons under these conditions causes 40-50% of cell death (Almeida et al., 2005). The results show a time-dependent downregulation of both transporters, although the effects on VGLUT2 were observed at an earlier time point after the glutamate stimulation and were more robust than those observed for VGLUT1 (Fig. 3.1). Thus, VGLUT2 protein levels decreased by about 70% 12h after the insult, whereas VGLUT1 protein levels dropped by 40% at 14h after excitotoxic stimulation. In contrast, no effect on the synaptophysin protein levels was observed, showing that not all vesicular proteins are deregulated by the excitotoxic stimulation.

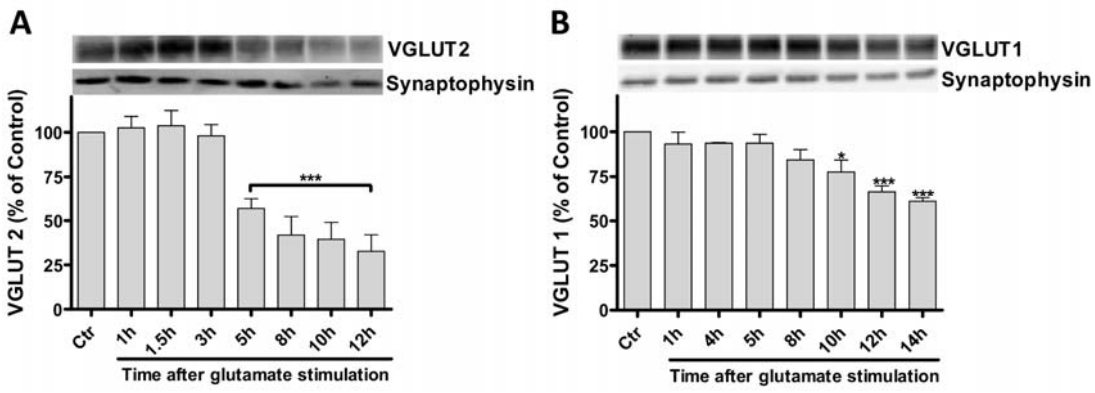


Figure 3.1. Glutamate-induced cleavage of VGLUT1 and VGLUT2 in cultured hippocampal neurons. The cells (7 DIV) were subjected to excitotoxic stimulation with glutamate (125 µM for 20 min) and the extracts were prepared after incubation in culture conditioned medium for the indicated periods of time. Samples were analyzed by western blot with an antibody against the C-terminal region of VGLUT2 (A) or VGLUT1 (B). The immunoreactivity with an antibody against synaptophysin was used as a loading control. The results are the average ± SEM of 4 to 5 independent experiments performed in distinct preparations. Control (Ctr) VGLUT levels, not exposed to glutamate, were set to 100%. Statistical analysis was performed using One Way ANOVA, followed by the Dunnett's

multiple comparison test performed for each condition as compared to the control condition (*p<0.05, ***p<0.001).

The VGLUT2 cleavage induced by glutamate was also assessed using an antibody against its N-terminal region (Fig. 3.2-A). These experiments showed that the downregulation of the full length VGLUT2 protein levels is associated with the time-dependent formation of a stable cleavage product (tVGLUT2) with approximately 55 kDa. This cleavage product was also observed in cultured hippocampal neurons transfected with a GFP-VGLUT2 fusion protein and subjected to excitotoxic stimulation with glutamate, and further incubated with culture conditioned medium for 8h; in this case western blot was performed with an anti-GFP antibody (Fig. 3.2-B).

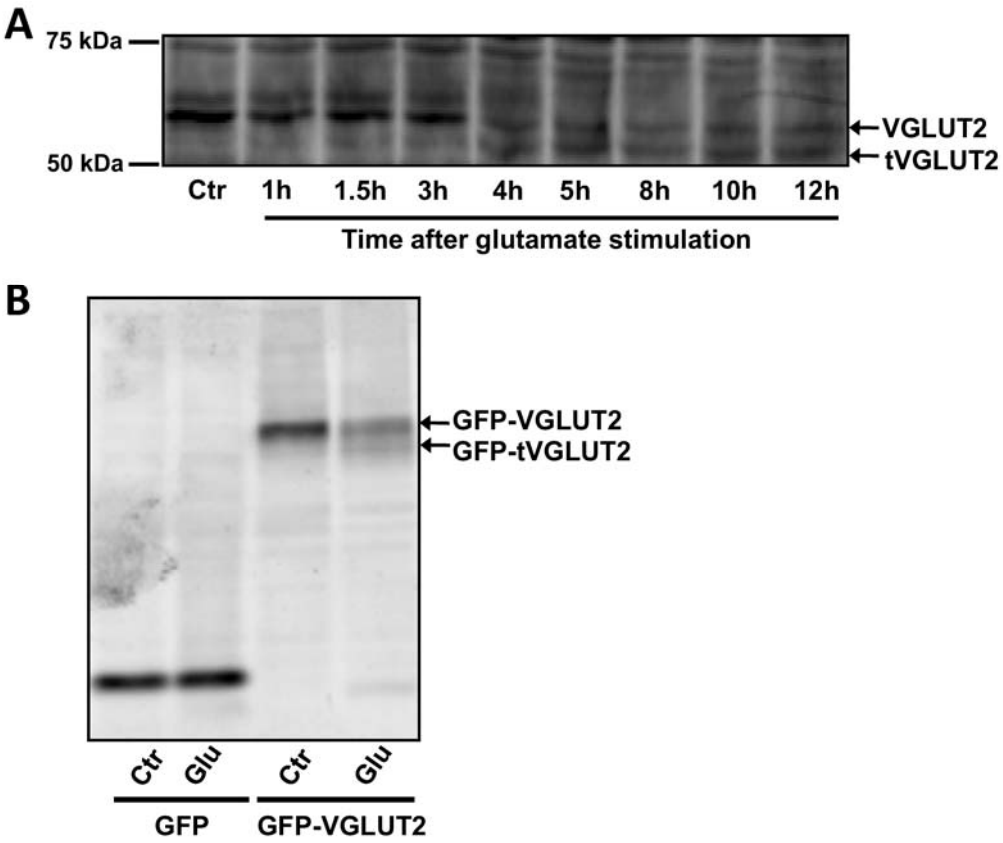


Figure 3.2. Glutamate-induced cleavage of VGLUT2 in cultured hippocampal neurons. The cells (7 DIV) were subjected to excitotoxic stimulation with glutamate (125 μM for 20 min) and the extracts were prepared after incubation in culture conditioned medium for the

indicated periods of time. Samples were analyzed by western blot with an antibody against VGLUT2 N-terminal region (A). Cultured hippocampal neurons were transfected at 7 DIV with GFP or GFP-VGLUT2, and 48h later were stimulated with glutamate for 20 min followed by 8h incubation in culture conditioned medium. Cell extracts were analyzed by western blot using an antibody against GFP (B).

3.2. VGLUT2 is selectively downregulated in excitotoxic conditions *in vitro* and *in vivo*

Transient incubation of cultured neurons under oxygen and glucose deprivation is often used as an *in vitro* model of transient global ischemia (Hertz, 2008). Transient global ischemia induces delayed neuronal death, especially in the hippocampal CA1 region (Liu et al., 2006).

To test the effect of OGD on VGLUT1 and VGLUT2 protein levels, cultured hippocampal neurons (14 DIV) were subjected to oxygen and glucose deprivation for 1, 1.5 or 2h, and protein levels were assessed 8h after the insult, by western blot using antibodies against the C-terminal region of both transporters. These OGD conditions cause 30% (at 1.5h) to 40% (at 2h) of cell death (Fernandes, 2009).

The results show a selective time dependent downregulation of VGLUT2 (40% decrease after 2h OGD) but, in contrast, OGD did not affect VGLUT1 levels, as determined by western blot (Fig. 3.3). Moreover, no effect was observed on the synaptophysin protein levels.

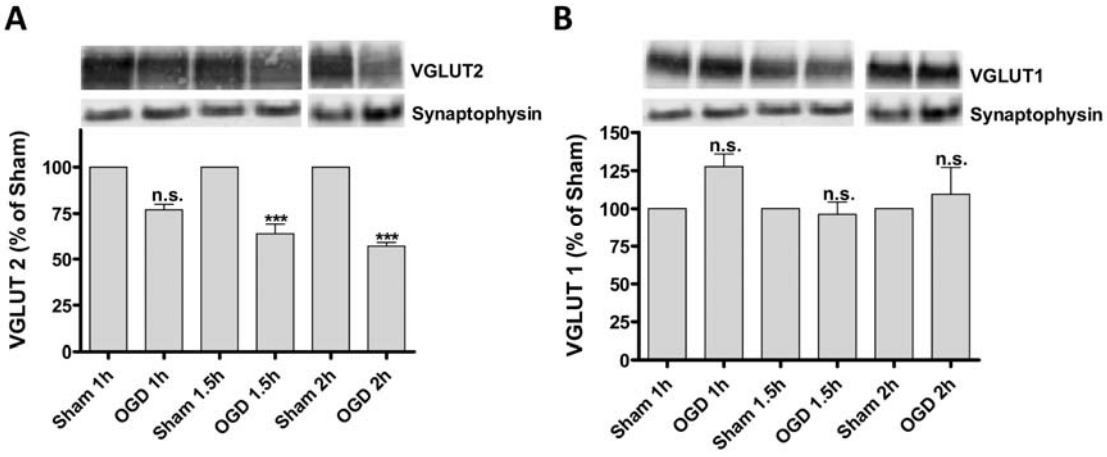


Figure 3.3. VGLUT2 is cleaved after transient OGD. Cultured hippocampal neurons (14 DIV) were subjected to oxygen glucose deprivation (OGD) for 1 to 2 h and further incubated

in culture conditioned medium for 8h. VGLUT2 (A), VGLUT1 (B) and synaptophysin protein levels were assessed by western blot. The results are the average of ±SEM of 3 to 7 independent experiments performed in different preparations. Sham VGLUT levels were set to 100%. Statistical analysis was performed using One Way ANOVA followed by Bonferroni's multiple comparison test. n.s.- non significant, ***p<0.001 as compared with the Sham condition.

To determine if VGLUT1 and VGLUT2 cleavage also occurs under excitotoxic conditions *in vivo*, adult mice were subjected to intra-hippocampal injection of kainate (Tomioka et al., 2002; Takano et al., 2005), and the protein levels of both transporters was determined by western blot at different time points after the insult, using antibodies against the C-terminal region of both transporters. Kainate injection decreased VGLUT2 protein levels in the ipsilateral hemisphere (as compared to the contralateral hemisphere) by about 20% when determined 12h after the insult, but no differences were observed for the other time points tested. Surprisingly, an increase of about 40% in VGLUT1 protein levels was observed in the ipsilateral hemisphere 8h after kainate injection, compared to the contralateral hemisphere levels, with no differences observed at 4 or 12h (Fig. 3.4). Furthermore, the excitotoxic insult with kainate did not affect synaptophysin protein levels.

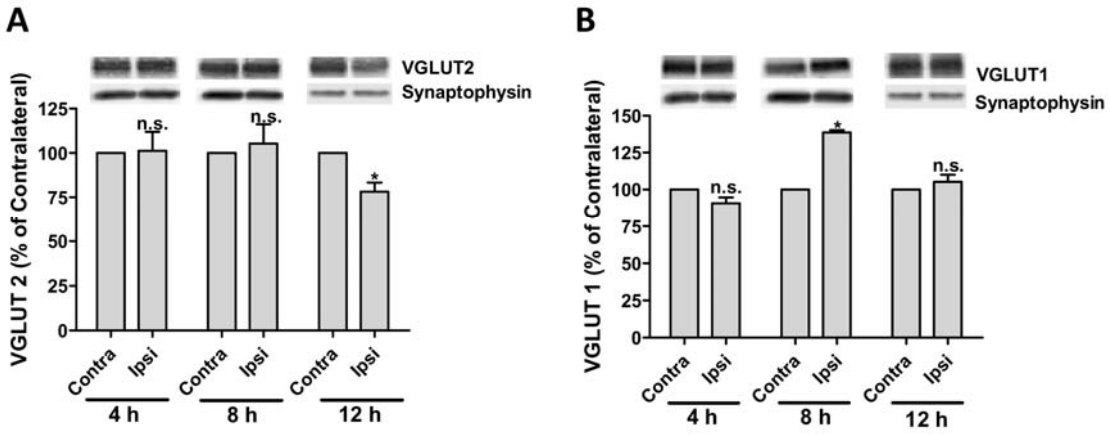


Figure 3.4. VGLUT2 is cleaved under excitotoxic conditions *in vivo*. Adult mice (C56BL6) were injected with kainate (1 nmol in 0.3µl phosphate buffer) in the right hippocampus and 4, 8 or 12h later the ipsilateral and the contralateral hippocampi were collected from the coronal sliced brain. Hippocampal extracts were analyzed by western blot using antibodies against VGLUT2 (A), VGLUT1 (B) and synaptophysin, and the results are the average of ± SEM of 3 to 5 independent experiments, performed in different animals.

Contralateral VGLUT levels were set to 100%. Statistical analysis was performed using One Way ANOVA followed by Bonferroni's multiple comparison test. n.s.- not significant, *p<0.05 as compared with the contralateral hemisphere.

A decrease in VGLUT2 protein levels was also observed in the brain of mice subjected to middle cerebral artery occlusion (MCAO), a model of transient focal ischemia (Traystman, 2003). Adult mice were subjected to a 45 min occlusion of the left middle cerebral artery, and extracts were prepared from the ipsilateral and contralateral brain hemispheres (cerebral cortex and striatum) 24h after injury. VGLUT2 cleavage was observed in the striatum (Fig. 3.5), but not in the cerebral cortex, as assessed by western blot using the antibody against VGLUT2 C-terminal region. Synaptophysin protein levels were used as loading control.

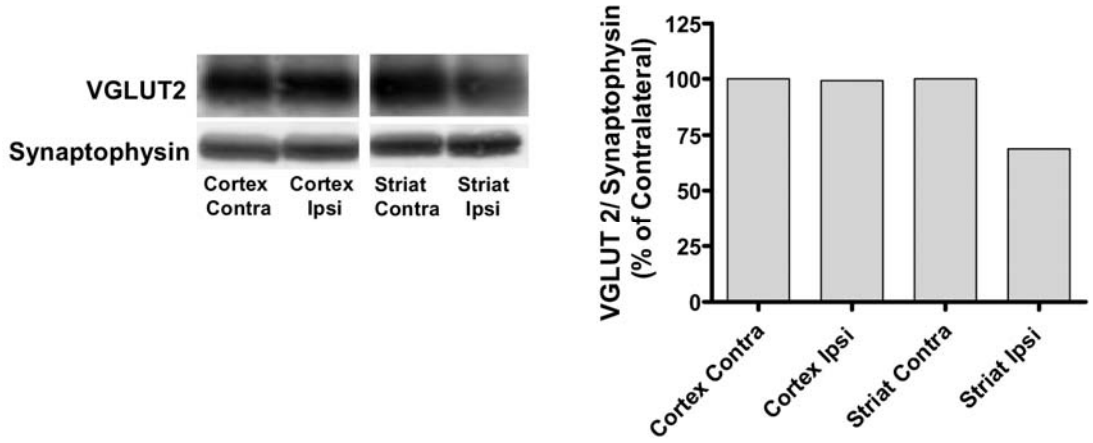


Figure 3.5. VGLUT2 is cleaved under ischemic conditions *in vivo*. Adult mice (C56Bl6) were subjected to a transient 45min occlusion of the left middle cerebral artery (MCAO). VGLUT2 and synaptophysin levels were determined by western blot in the cerebral cortex and striatum (Striat) of the Ipsilateral (Ipsi) and Contralateral (Contra) brain hemispheres, 24 h after the lesion. The ratio between VGLUT2 levels and the loading control synaptophysin is plotted in the graphic. The ratio in the contralateral hemisphere was set to 100%. The results represent one of two independent experiments performed in different animals.

3.3. Calpain cleaves VGLUT2 under *in vitro* excitotoxic conditions

Since calpains play a key role in neuronal death following excitotoxicity and ischemia (Bevers and Neumar, 2008), we investigated if these proteases are involved in VGLUT2 cleavage after excitotoxic stimulation with glutamate. Cultured hippocampal neurons (7 DIV) were pre-incubated with the chemical inhibitors of calpains ALLN or MDL28170 for 2h, and subjected to the glutamate insult (125 µM for 20 min) in the presence of the inhibitors. VGLUT2 protein levels were analyzed by western blot using the antibody raised against the C-terminal of the transporter, 5h after glutamate stimulation. Both calpain inhibitors used, ALLN and MDL28170, prevented VGLUT2 cleavage induced by glutamate (Fig. 3.6).

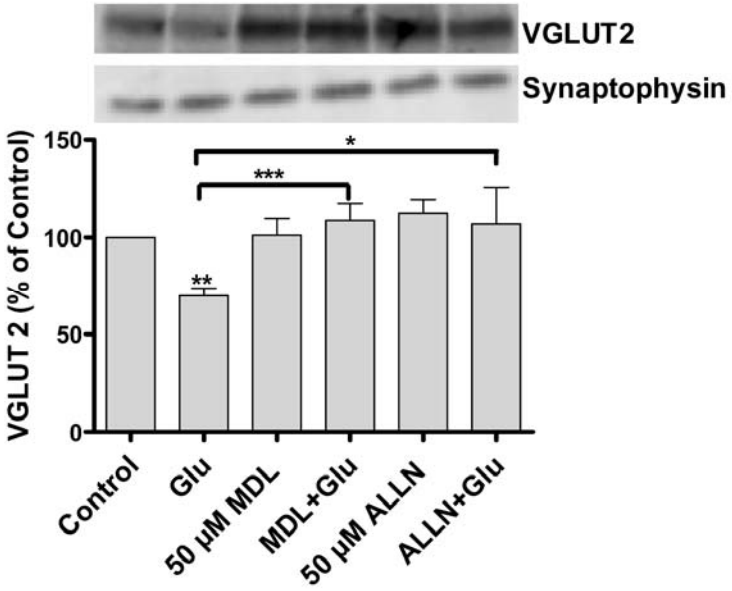


Figure 3.6. VGLUT2 is cleaved by calpains after excitotoxic stimulation with glutamate. Cultured hippocampal neurons (7 DIV) were stimulated with 125 µM glutamate for 20min, and further incubated in culture conditioned medium for 5h. Where indicated the cells were pre-incubated with MDL28170 or ALLN and the inhibitors were also present throughout the experiment. VGLUT2 and synaptophysin protein levels were determined by western blot. The results are the average ± SEM of 3 to 9 independent experiments performed in distinct preparations. Control VGLUT2 levels were set to 100%. Statistical analysis was performed using One Way ANOVA, followed by the Bonferroni's multiple comparison test (n.s.- non significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$).

To determine if calpains are also involved in VGLUT2 downregulation induced by OGD insult, cultured hippocampal neurons (14 DIV) were pre-incubated with MDL 28170 for 2h, and then subjected to the OGD insult for 1.5h in the presence of the inhibitor. VGLUT2 protein levels were assessed by western blot 8h after OGD, . using the antibody raised against the C-terminal region of the transporter The results show that incubation with MDL28170 abrogated VGLUT2 downregulation caused by OGD (Fig. 3.7).
Taken together, the results indicate that VGLUT2 is cleaved by calpains after excitotoxic or ischemic *in vitro* insults.

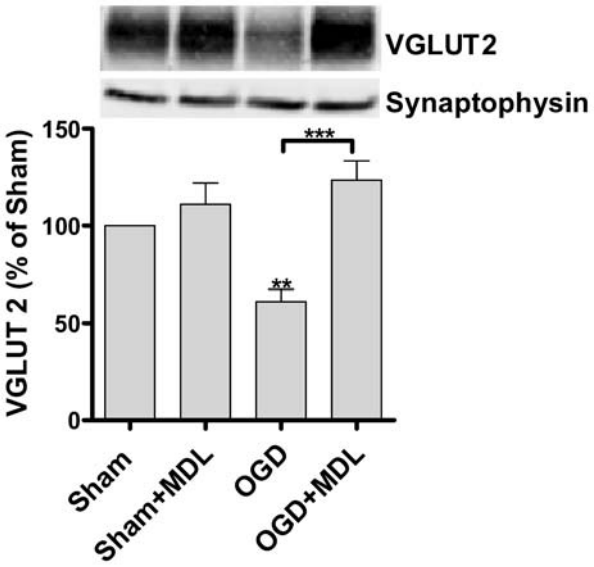


Figure 3.7. VGLUT2 is cleaved by calpains after OGD. Cultured hippocampal neurons (14 DIV) were subjected to oxygen glucose deprivation (OGD) for 1.5h and further incubated in conditioned culture medium for 8h. Where indicated the cells were pre-incubated with MDL28170 and the inhibitor was also present throughout the experiment. VGLUT2 and synaptophysin protein levels were determined by western blot. The results are the average ± SEM of 6 independent experiments performed in distinct preparations. Sham VGLUT2 levels were set to 100%. Statistical analysis was performed using One Way ANOVA, followed by the Bonferroni's multiple comparison test (n.s.- non significant, **$p<0.01$, ***$p<0.001$).

In order to confirm the cleavage of VGLUT2 by calpains, isolated cerebrocortical synaptic vesicles were incubated with recombinant calpain I in a Ca^{2+}-containing medium, and VGLUT2 protein levels were determined by western blot, using the

antibody against the transporter C-terminus, at different incubation periods (5,10, 15 and 60 min). These *in vitro* experiments showed a downregulation of VGLUT2 protein levels by calpain I, and the effect was inhibited by MDL28170 and ALLN (Fig. 3.8-A). Also, no decrease in VGLUT2 protein levels was observed in experiments where synaptic vesicles were incubated with calpain in a Ca^{2+}-free medium to prevent the activity of the protease. Taken together, these evidences show that VGLUT2 is a calpain substrate. Although a decrease in VGLUT1 protein levels was also observed when isolated synaptic vesicles were incubated with calpain (Fig. 3.8-B), the effect was not as significant as that observed for VGLUT2. As expected, no effects were observed on the synaptophysin protein levels.

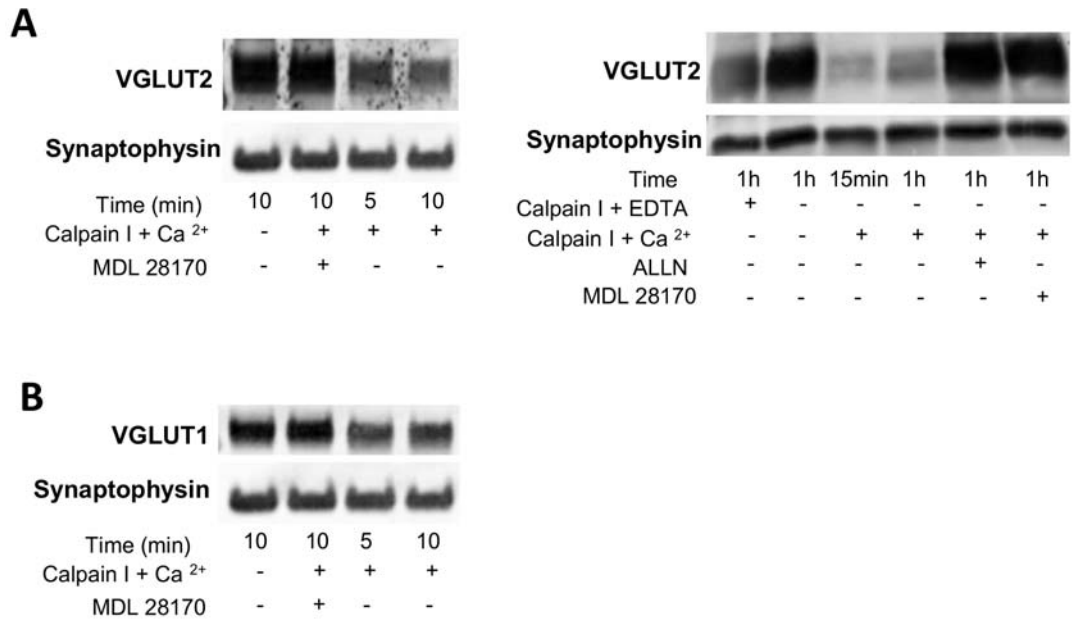


Figure 3.8. VGLUT2, but not VGLUT1, is cleaved by recombinant calpain I. Cerebrocortical synaptic vesicles (5µg) were incubated with recombinant calpain I (2.5U/ml) for the indicated periods of time at 37ºC, in the presence (2.5mM Ca^{2+}) or in the absence (1mM EDTA) of calcium. Where indicated the effect of the calpain inhibitors MDL 28170 (50 µM) or ALLN (50 µM) was tested. Extracts were analyzed by western blot using antibodies against VGLUT2 (A)and VGLUT1 (B) C-terminal regions, or synaptophysin.

3.4. VGLUT2 is cleaved by calpain in two different sites in the C-terminal region

According to the predicted topology, VGLUT2 has 12 transmembrane domains (Transmembrane helices as determined with the protein prediction online software – TMHMM; Center of Biological Sequence Analysis, Denmark) and both the N- and C-terminal regions are directed towards the cytoplasm (Jung et al., 2006). The presence of a putative PEST sequence (emboss epestfind at http://emboss.sourceforge.net/, amino acids 502 to 515, score: +15.77) in the C-terminal region of VGLUT2 suggests that this region might be targeted by calpain (Fig. 3.9-A). These amino acid sequences, enriched in proline, glutamate, serine and threonine, are typically targeted by calpains, which may then cleave neighbor sequences (Rogers et al., 1986; Rechsteiner and Rogers, 1996; Tompa et al., 2004). As an initial approach to investigate the VGLUT2 amino acid sequences targeted by calpains, western blot experiments were performed using antibodies that bind specific epitopes on VGLUT2, in the N- (18 amino acids sequence, more specific information about the epitope is not available) and C-terminal (amino acids 510-582) regions of the transporter. Full-length VGLUT2 reacted with both the antibodies, whereas the cleavage product was only detected by the antibody raised against VGLUT2 N-terminal, suggesting that the cleavage site(s) is located within VGLUT2 C-terminal region, probably between amino acids 510 and 582 (Fig. 3.9-B).

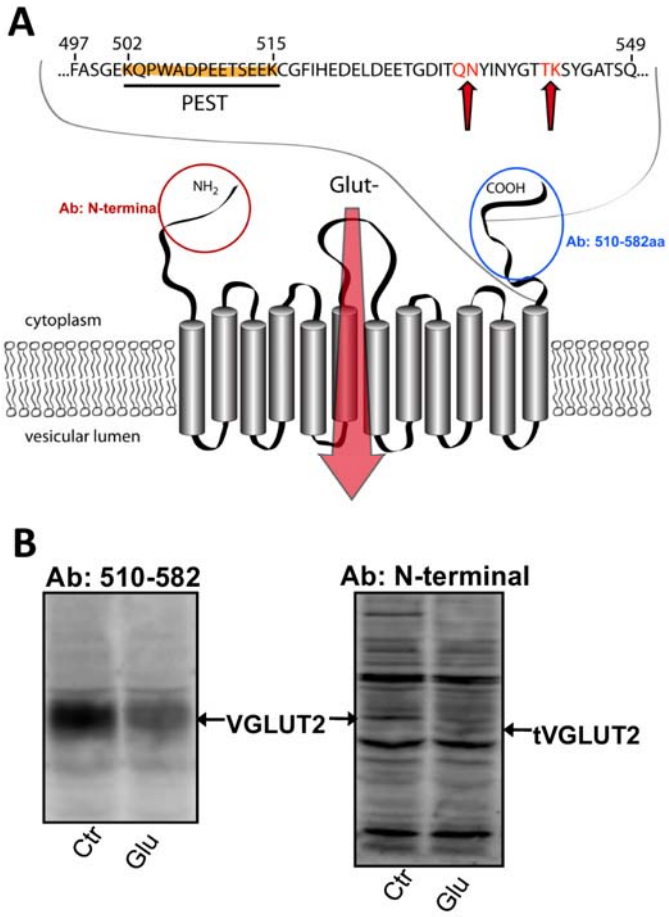


Figure 3.9. Excitotoxic stimulation with glutamate gives rise to a VGLUT2 cleavage product that binds to an antibody raised against the N-terminal region of the transporter. (A) Predicted topology of VGLUT2 highlighting the epitopes targeted by the antibodies used, the predicted calpain cleavage sites identified (Asn534 and Lys542) and the PEST sequence. (B) The antibody against the N-terminus of VGLUT2 binds a sequence of 18 amino acids within this region, and the antibody against the C-terminus region recognizes amino acids 510 to 582. Thus, the C-terminus antibody binds only the full length form of VGLUT2, whereas the N-terminus antibody can also interact with the truncated form (*e.g.* Figure 3.2-A).

To further investigate the putative cleavage site(s) in the C-terminal region of VGLUT2, we used the algorithm of Tompa et al. (2004), which allows predicting the amino acid sequences targeted by calpain. The results in Fig. 3.10-A show that the

highest scores are found close to the putative PEST sequence, and between amino acids 528 and 553. Since it was proposed that calpains cleave their substrates in rather disordered segments of the proteins, we used a bioinformatic tool (metaPrDOS) to predict the disorder tendency along the VGLUT2 amino acids sequence (Ishida and Kinoshita, 2008). The results show that VGLUT2 is more disordered at the N- and C-terminal (particularly between amino acids 506 to 582) regions (Fig. 3.10-B).

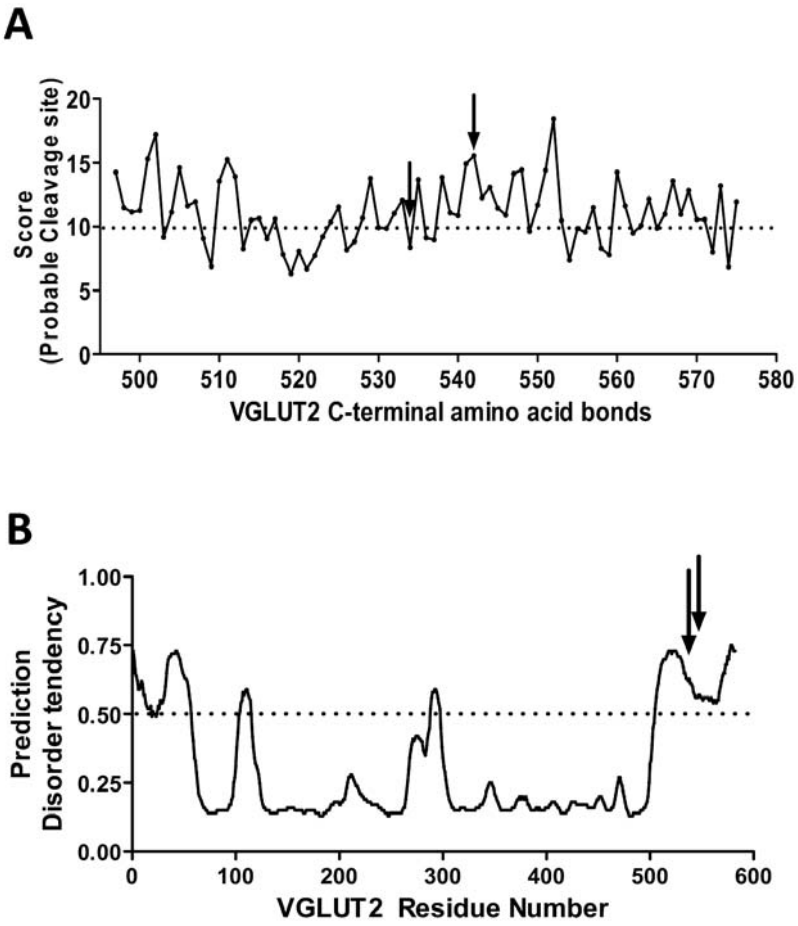


Figure 3.10. Prediction of VGLUT2 calpain cleavage sites using bioinformatic tools. Analysis of the probability of cleavage by calpain in the C-terminal region of VGLUT2 based on the model for prediction of calpain cleavage sites published by Tompa et al. (2004). (B) Prediction of the disorder tendency in VGLUT2 based on a bioinformatic analysis of the

amino acid sequence of the protein (Ishida and Kinoshita, 2008). Arrows point to the predicted calpain cleavage sites identified in the present work (Asn534 and Lys542).

The probable location of the cleavage site(s) at VGLUT2 C-terminal region is further supported by the results showing cleavage by calpain I of a GST fusion protein with the C-terminal region of VGLUT2 in a Ca^{2+}-containing medium, as assessed by western blot using an anti-GST antibody (Fig. 3.11-A). Cleavage of the fusion protein gives rise to a truncated product with an apparent molecular weight of 32 kDa (oval shape in Fig. 3.11-A) and, as expected from the Ca^{2+}-dependency of calpain activity, no effect was observed when the experiments were conducted in Ca^{2+}-free medium.
The calpain cleavage sites were determined by incubating the fusion protein of GST with VGLUT2 C-terminal region with recombinant calpain I. The peptides were separated by SDS-PAGE and stained with Coomassie Blue (Fig. 3.11-B), or alternatively transferred to a PVDF membrane before staining. The band indicated in Fig. 3.11-B was processed to be sequenced by N-terminal Edman sequencing, and two peptides were identified, one comprising amino acids 534 to 582, the other which correspondes to the amino acids 542 to 582 of the C-terminus of VGLUT2. These results indicate that VGLUT2 is cleaved by calpain I at amino acids Asn534 and Lys542 (Fig. 3.11-B). These cleavage sites are indicated by the arrows at Fig. 3.9-A, and Fig. 3.10-A and –B. It was not possible to identify the other bands formed upon calpain incubation, raising the possibility that other cleavage sites exist in the C-terminal region of VGLUT2, upstream the cleavage sites identified in this work.

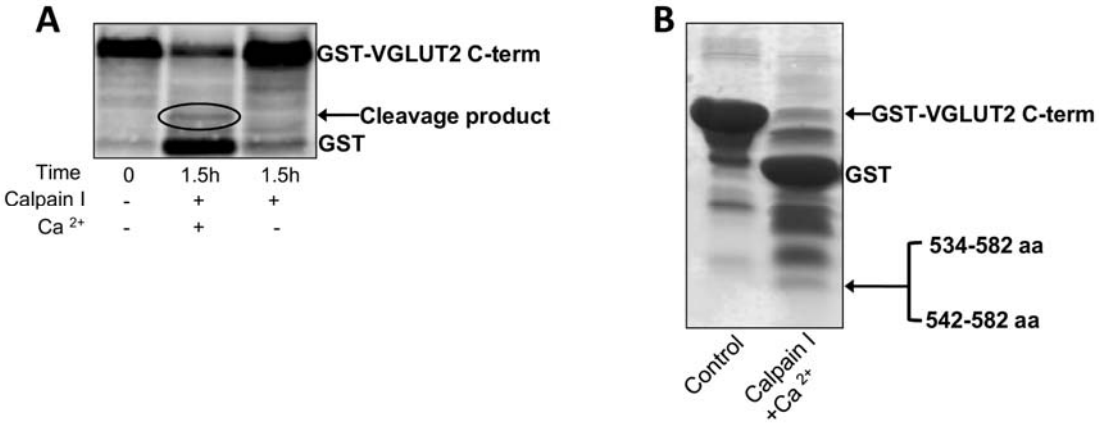


Figure 3.11. Identification of the putative calpain cleavage sites of VGLUT2. (A) GST-VGLUT C-terminal (amino acids 497 to 582) fusion protein was incubated with recombinant calpain I (2.5 U/ml) for 15 min at 37ºC in the presence or absence of Ca^{2+} (2.5mM). Extracts

were analyzed by western blot using an antibody against GST. (B) The GST-VGLUT2 C-terminal fusion protein (100µg) was incubated with recombinant calpain I (2.5U/ml) in the presence of Ca^{2+} (2.5mM), for 15 min at 37ºC. The peptides were separated by SDS-PAGE and stained immediately (or after transfer to a PVDF membrane) with Commassie Blue. The PVDF membrane stained with Coomassie Blue with the indicated fragment (arrow) was processed for N-terminal Edman sequencing.

3.5. VGLUT1 is not cleaved by calpain

The predicted topology of VGLUT1 is similar to that of VGLUT2, with 12 transmembrane domains and N- and C-terminal regions facing the cytoplasm (Transmembrane helices as determined with the protein prediction online software – TMHMM; Center of Biological Sequence Analysis, Denmark). This prediction, together with the presence of two putative PEST sequences (emboss epestfind at http://emboss.sourceforge.net/, amino acids 494 to 507, score: +10.35, and amino acids 513 to 545, score: +13.25) in the C-terminal region of VGLUT1, prompted us to investigate whether VGLUT1 is a target for calpain cleavage (Rogers et al., 1986; Rechsteiner and Rogers, 1996) (Fig. 3.12). The VGLUT1 antibody was raised against an epitope within the last transmembrane domain and the C-terminal region (amino acids 456 to 560), and binds to the full-length form of the transporter, suggesting that VGLUT1 cleavage under excitotoxic conditions (Fig. 3.1-B) may occur between amino acids 456 and 560.

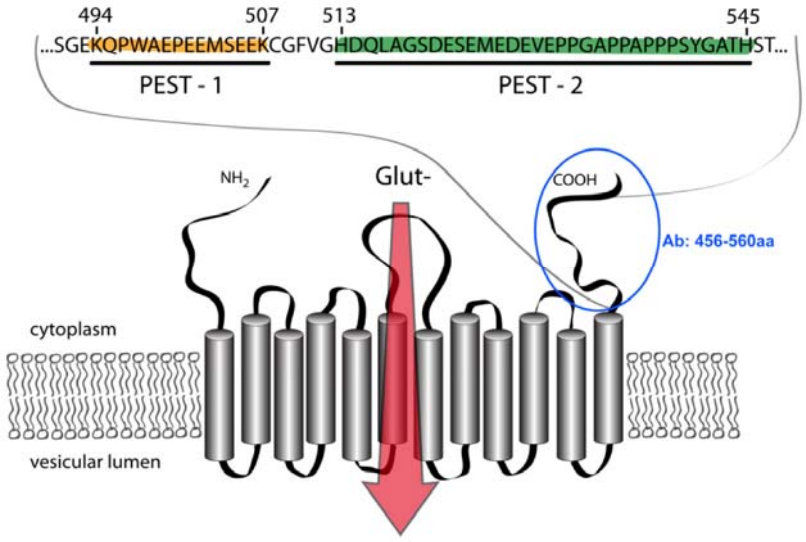


Figure 3.12. Identification of the putative PEST sequences and location of the antibody epitope on VGLUT1 C-terminal region. Predicted topology of VGLUT1 showing the antibody binding site and the two PEST sequences located in VGLUT1 C-terminal region. The antibody was raised against a peptide between amino acids 456 and 560.

To further evaluate the putative cleavage site(s) in the C-terminal region of VGLUT1, we used the algorithm of Tompa et al. (2004), which allows predicting amino acid sequences targeted by calpain. The results in Fig. 3.13-A show that the highest scores are found near the second putative PEST sequence. The bioinformatic tool (metaPrDOS) was also used to predict the disorder tendency along VGLUT1 sequence, and the results show that the protein is more disordered in that N- and C-terminal (particularly amino acids 497 to 560) regions (Fig. 3.13-B).

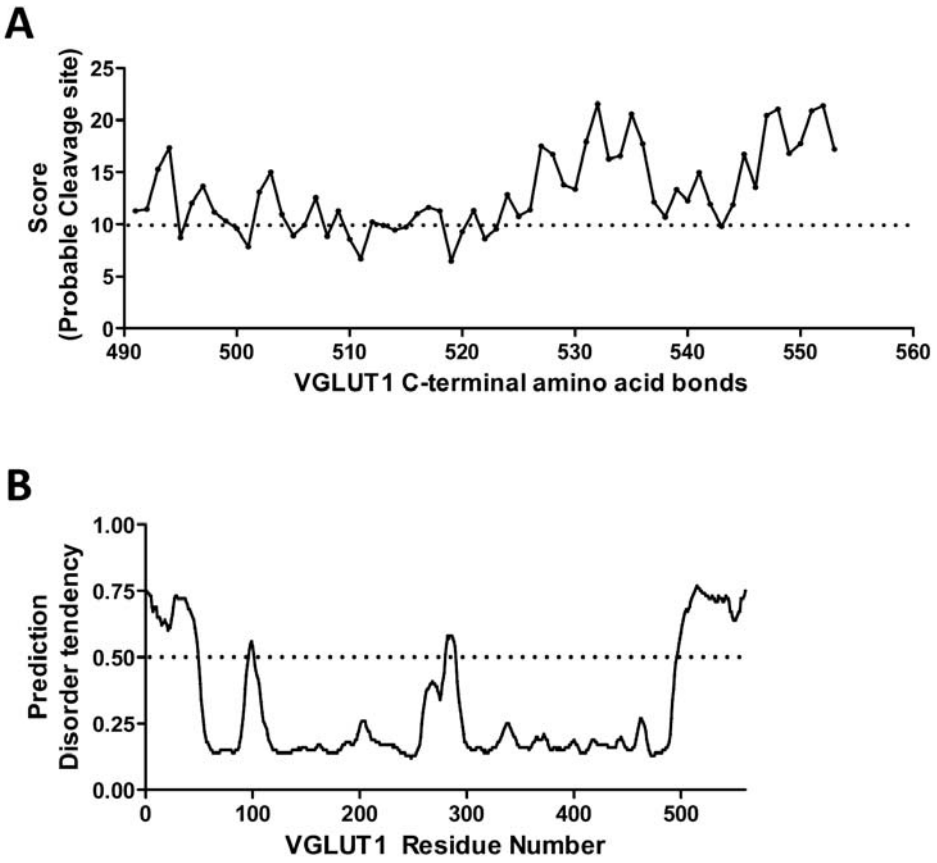


Figure 3.13. Prediction of VGLUT1 calpain cleavage sites using bioinformatic tools. Analysis of the probability of cleavage by calpain in the C-terminal region of VGLUT2 based on the model for prediction of calpain cleavage sites published by Tompa et al. (2004). (B) Prediction of the disorder tendency in VGLUT1 based on a bioinformatic analysis of the amino acid sequence of the protein (Ishida and Kinoshita, 2008).

In order to determine whether calpain cleaves VGLUT1 C-terminal region, a fusion protein of GST with a peptide corresponding to this part of the transporter was incubated with recombinant calpain I and stained with Coomassie Blue. The results show no cleavage of the fusion protein by calpain, indicating that the C-terminal region of VGLUT1 is not a calpain substrate (Fig. 3.14).

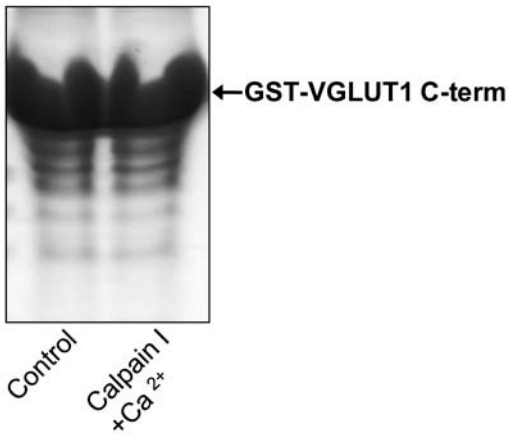


Figure 3.14. GST-VGLUT1 is not cleaved by calpain I. GST-VGLUT1 C-terminal (amino acids 491 to 560) fusion protein (100µg) was incubated with recombinant calpain I (2.5U/ml) in the presence of Ca^{2+} (2.5mM), for 15 min at 37ºC. The peptides were separated by SDS-PAGE and stained immediately with Coomassie Blue.

3.6. Characterization of VGLUT2 cleavage by caspase-3

The analysis of the C-terminal region of VGLUT2 shows that it contains a consensus DELD sequence (amino acids 522 to 525) (Fig. 3.15), that is typically targeted by caspase-3 (Thornberry et al., 1997; Chang and Yang, 2000). This sequence is located close to the putative PEST sequence and the identified calpain cleavage sites (Ans534 and Lys542).

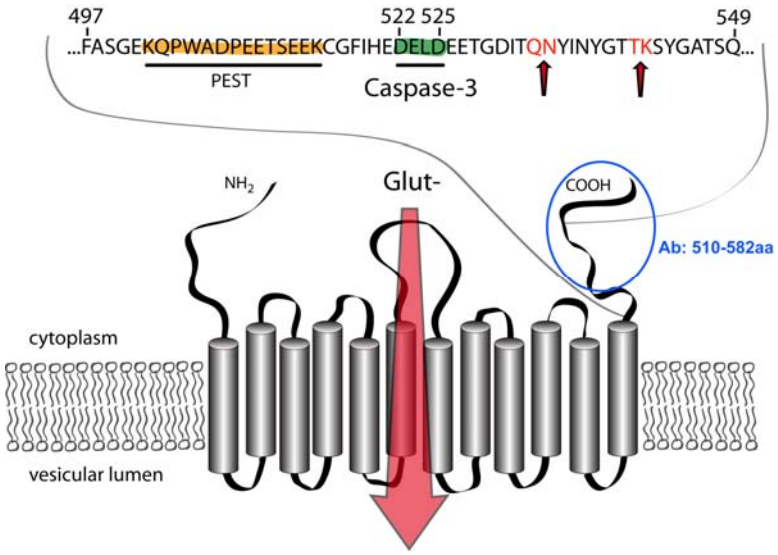


Figure 3.15. Identification of the consensus DELD sequence targeted for caspase-3 on VGLUT2 C-terminal region. Predicted topology of VGLUT2 highlighting the DELD consensus site. The epitope targeted by the C-terminal antibody used, the calpain cleavage sites identified (Asn534 and Lys542) and the PEST sequence are also indicated.

To investigate if VGLUT2 is cleaved by caspase-3, *in vitro* experiments were performed using a fusion protein of GST with VGLUT2 C-terminus and recombinant caspase-3. Fusion protein levels were determined by western blot, with an antibody against GST, after 20h of incubation. The results show that cleavage by caspase-3 gives rise to a product with approximately 32 kDa, and this effect was sensitive to the caspase-3 inhibitor Z-DEVD-FMK (Fig. 3.16-A; oval shape). Mutation of the two aspartate residues at positions 522 and 525 to alanines prevented GST-VGLUT2 protein cleavage, showing that the DELD consensus sequence is targeted by caspase-3 (Fig. 3.16-B). The effect of the detergent CHAPS in caspase-3 activity was also tested, since it was shown that this caspase loses about 40% activity upon CHAPS removal from the buffer used to perform the *in vitro* assay (Stennicke and Salvesen, 1997). However, we observed that presence or absence of CHAPS did not affect the cleavage of the fusion protein GST-VGLUT2 C-terminus by caspase-3.

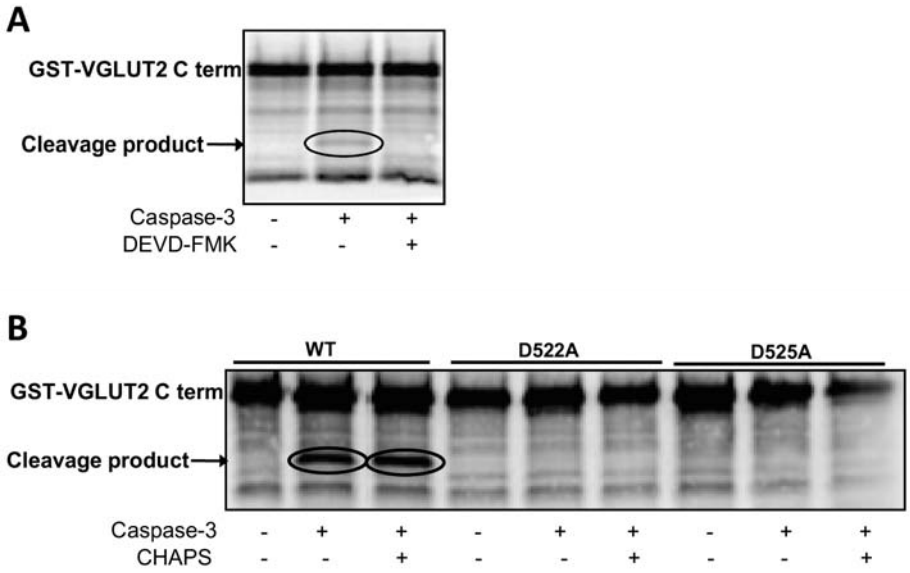


Figure 3.16. Characterization of GST-VGLUT2 C-terminus cleavage by caspase-3. (A) GST-VGLUT2 C-terminal fusion protein was incubated with recombinant caspase-3 (2 U/ml) for 20h at 37ºC. Where indicated, Z-DEVD-FMK (30 μM) was used to inhibit caspase-3 activity. (B) The consensus site for cleavage by caspase-3 (DELD) located in VGLUT2 C-terminal region (*e.g.* Figure 3.15) was mutated separately in the two aspartic acid residues to alanine, and the recombinant proteins were incubated with caspase-3 in the presence or absence of the detergent CHAPS, as indicated. Immunoblots were performed using an anti-GST antibody.

The role of caspases in VGLUT2 downregulation under excitotoxic conditions was addressed using the pan-caspase inhibitor Z-VAD-FMK (Fig. 3.17). Cultured hippocampal neurons (7 DIV) were pre-incubated with the inhibitor for 2h, and subjected to excitotoxic stimulation with glutamate (125 μM for 20 min) in the presence of the inhibitor. VGLUT2 protein levels were assessed 5h after the glutamate insult by western blot with the antibody raised against the C-terminal region of the transporter. The results show a slight (about 15%), but not significant, effect on the cleavage of VGLUT2, indicating that caspases do not play a significant role in glutamate-evoked VGLUT2 downregulation.

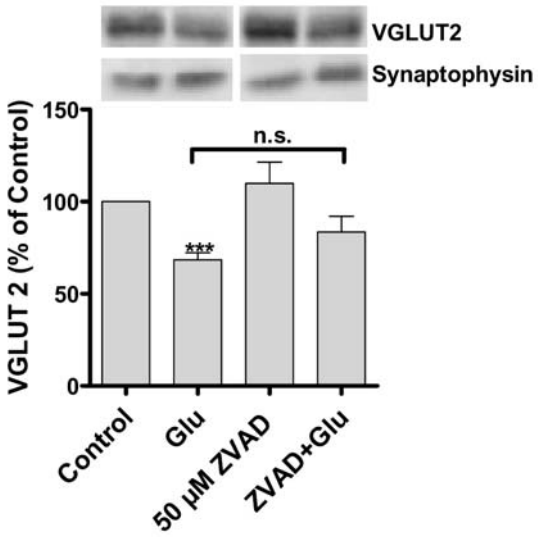


Figure 3.17. VGLUT2 cleavage under excitotoxic conditions is not mediated by caspases. Cultured hippocampal neurons (7 DIV) were subjected to excitotoxic stimulation with 125 µM glutamate for 20min, and further incubated in culture conditioned medium for 5h. In the experiments where the effect of Z-VAD-FMK was tested, the cells were pre-incubated with the inhibitor for 2h and throughout the experiment. The results are the average ± SEM of 4 independent experiments performed in distinct preparations. Control VGLUT2 levels were set to 100%. Statistical analysis was performed using One Way ANOVA, followed by the Bonferroni's multiple comparison test (n.s.- non significant, ***p<0.001).

The putative effect of caspase-3 on the cleavage of VGLUT2 was further investigated using two stimuli that typically induce apoptotic-like cell death through activation of this caspase, staurosporin [STS; (Bertrand et al., 1994; Koh et al., 1995; Young et al., 2010)] and trophic factor deprivation (Chan et al., 1999; Glazner et al., 2000). Cultures hippocampal neurons (7 DIV) were exposed to 30 nM STS for 24h, a concentration that induces 50% of cell death in cultured hippocampal neurons (Prehn et al., 1997; Krohn et al., 1998), or to 300 nM STS for 10min (Krohn et al., 1999; Lankiewicz et al., 2000), and further incubated in culture conditioned medium for 14h. Although STS activated caspase-3, as determined by western blot using an antibody against the cleaved (at Asp185; active) form of the enzyme, no downregulation of VGLUT2 protein levels was observed (Fig. 3.18-A).

When deprived from trophic support, cultured hippocampal neurons undergo apoptosis (Chan et al., 1999; Glazner et al., 2000), resulting in the death of around

40% of the neurons during the 48h period (Guo et al., 2008). The results show that under these conditions there was no VGLUT2 downregulation, despite the activation of caspase-3 (Fig. 3.18-B). Taken together, these results indicate that the VGLUT2 cleavage by caspase-3 observed *in vitro* is not relevant under the experimental conditions tested.

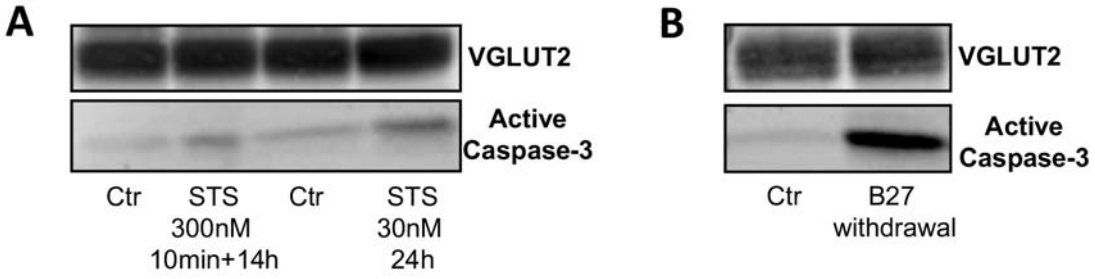


Figure 3.18. VGLUT2 is not cleaved under conditions that induce caspase-3 activation. Cultured hippocampal neurons (7 DIV) were treated with staurosporine for 10min (300 nM) or 24h (30 nM), and further incubated in culture conditioned medium for 14h (A), or incubated in culture medium lacking B27 for 48h (B). Extracts were immunoblotted using antibodies against VGLUT2 and the active form of caspase-3. The results are representative of three independent experiments performed in different preparations.

3.7. TAT-VGLUT2 peptide blocks glutamate-induced cleavage of VGLUT2

To further analyze the role of VGLUT2 cleavage by calpains, we used a fusion protein of the C-terminal region of VGLUT2 with the protein transduction domain (PTD) derived from the human immunodeficiency virus TAT protein, that we designated TAT-VGLUT2 peptide (Fig. 3.19-A). This strategy was previously used to transfer peptides across cell membranes (Cao et al., 2002; Wadia et al., 2004). The fusion protein was purified to near homogeneity, as determined by Coomassie Blue staining, and western blot analysis showed that the peptide could be detected by the antibody against VGLUT2 C-terminal region (Fig. 3.19-B).

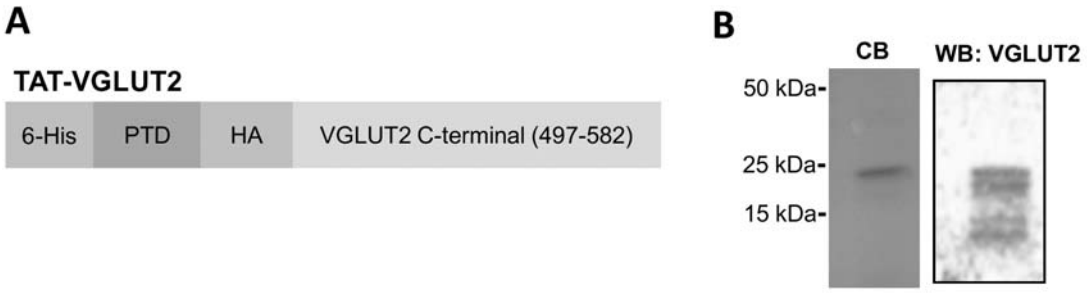


Figure 3.19. TAT-VGLUT2 C-terminal protein construct and its purification. (A) Schematic representation of the TAT-VGLUT2 C-terminal peptide. The peptide contains a His-6 tag, a PTD domain, a HA tag and the VGLUT2 C-terminal region. (B) Test for TAT-VGLUT2 protein expression. Coomassie Blue (CB) staining shows that the protein was purified to near homogeneity (left) and western blot shows that the protein could be detected using a VGLUT2 antibody against its C-terminal region (right).

The peptide was highly efficient in transducing cultured hippocampal neurons (7 DIV), as determined by immunocytochemistry after 20 min of exposure to the peptide, using antibodies directed towards the VGLUT2 C-terminal (red) and the HA tag (green) of the peptide (Fig. 3.20-A). However, there were some cells labeled only with the anti-HA antibody, possibly due to transduction of a peptide lacking the VGLUT2 C-terminal sequence.

We reasoned that this peptide would compete with endogenous VGLUT2 for calpain and therefore protect the transporter from cleavage. Cultured hippocampal neurons (7 DIV) were incubated with 1 μM of TAT-VGLUT2 for 1.5h followed by excitotoxic stimulation with glutamate (125 μM for 20 min) in the presence of the peptide. VGLUT2 protein levels were assessed 8h after the glutamate insult by western blot with the antibody raised against the C-terminal region of the transporter. The results in Fig. 3.20-B show that the peptide significantly reduced (by about 30%) VGLUT2 cleavage induced by glutamate, but was without effect on spectrin cleavage, as determined with an antibody that binds specifically to the product resulting from the cleavage of spectrin by calpains (Bahr, 2000; Caba et al., 2002; Munirathinam et al., 2002). The latter results show that the TAT-VGLUT2 did not act by inhibiting total calpain activity. Also, no effects on synaptophysin protein levels were observed (Fig. 3.20-B).

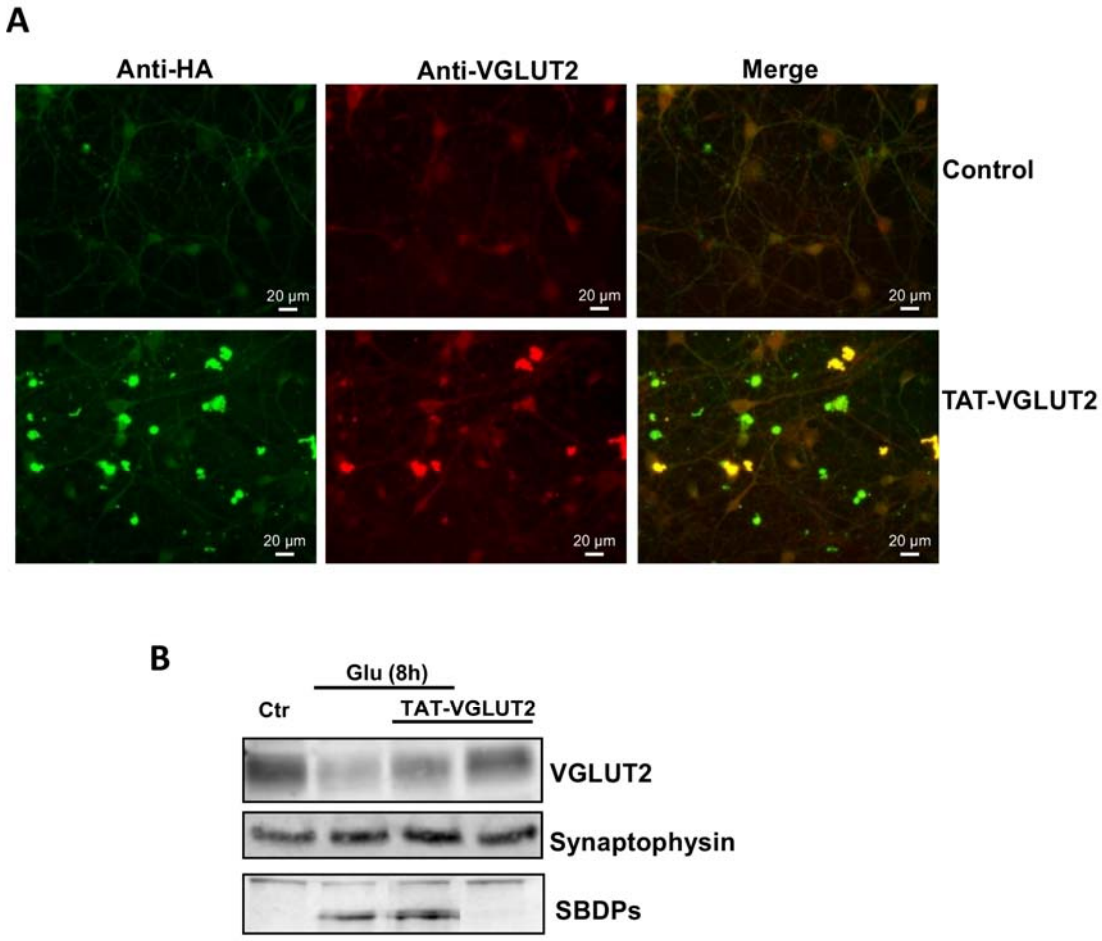


Figure 3.20. TAT-VGLUT2 C-terminal protein transduces into neurons and suppresses VGLUT2 cleavage under excitotoxic conditions. (A) Representative immunofluorescence images showing the transduction of TAT-VGLUT2 into hippocampal neurons. The cells were incubated with the peptide for 20 min and after fixation the preparation was incubated with antibodies against HA (green) and VGLUT2 (red). (B) Effect of TAT-VGLUT2 transduction into cultured hippocampal neurons on glutamate-evoked VGLUT2 downregulation. The cells (7 DIV) were treated with the peptide (1 µM) for 1.5h, subjected to excitotoxic stimulation with glutamate (125 µM, 20min), and further incubated in culture conditioned medium for 8h. Extracts were analyzed using antibodies against VGLUT2, synaptophysin and spectrin breakdown products (SBDPs). The results are representative of two independent experiments performed in different preparations.

3.8. VGLUT2 cleavage changes the distribution of the protein along the axons

Since the N- and C-terminal regions of VGLUT2 are turned to the cytoplasm, both regions may play a role in the trafficking of the transporter through interaction with other cytoplasmic proteins. In order to understand how VGLUT2 cleavage affects the subcellular localization of the protein, we compared the distribution of the GFP fusion protein with full-length VGLUT2 or the truncated forms (Δ534-582 or Δ542-582, corresponding to the proteins generated by calpain cleavage at the identified sites), generated by insertion of stop codons after amino acids 533 or 541 by directed mutagenesis (Fig. 3.21-A). Control immunocytochemistry experiments in cultured hippocampal neurons demonstrated that the GFP-VGLUT2 (green) colocalized with total VGLUT2 (endogenous + fusion protein; red) along neurites in cultured hippocampal neurons (Fig. 3.21-B), showing that the fusion of GFP to the N-terminal of VGLUT2 does not affect its intracellular traffic.

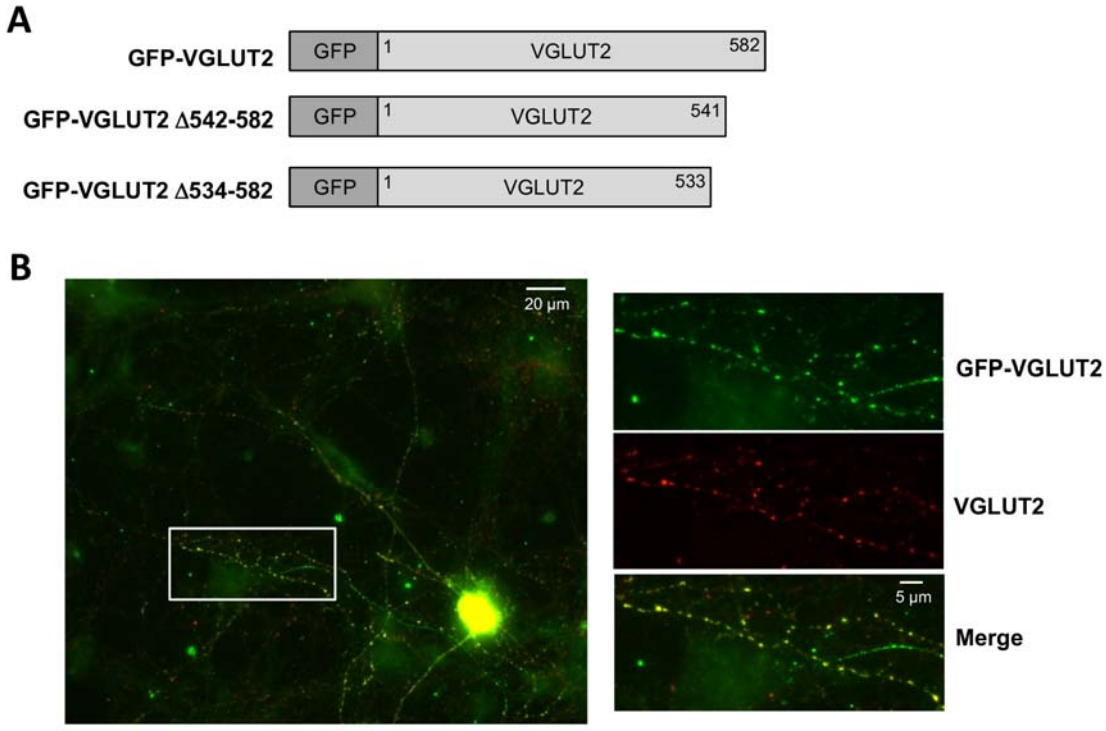
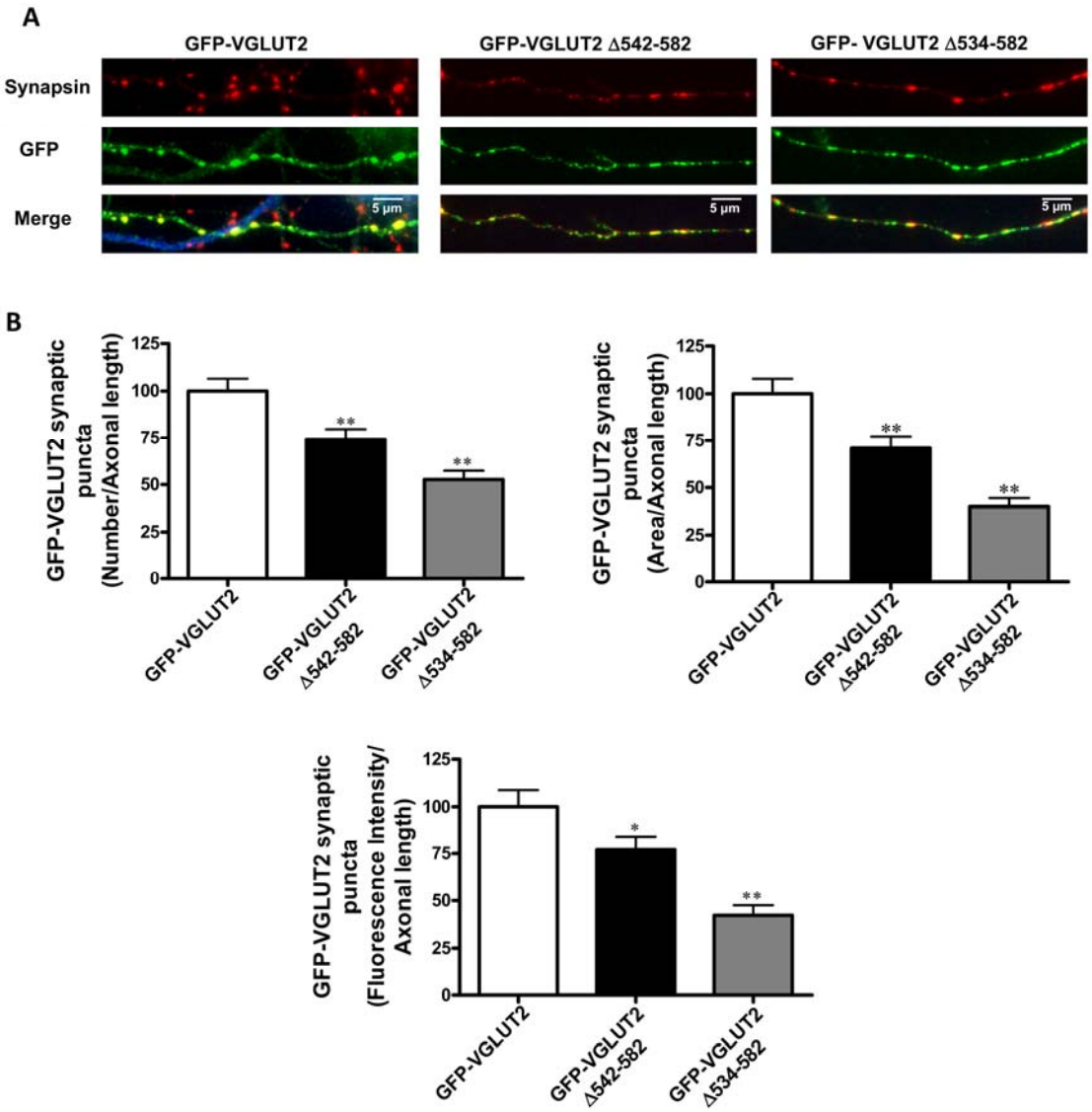


Figure 3.21. GFP fusion with VGLUT2 does not affect intracellular trafficking of the protein. (A) Schematic representation of the GFP-VGLUT2 and truncated constructs. (B)

Cultured hippocampal neurons (7 DIV) were transfected with GFP-VGLUT2 and immunocytochemistry was performed using antibodies for GFP (green) and VGLUT2 (red). Transfected neurons are shown in the left, and enlarged images of their neurites in the right.

The GFP-VGLUT2 fusion protein showed a punctate distribution along neurites, colocalizing with synapsin, a presynaptic marker. The colocalization with synapsin was significantly reduced in the GFP fusion proteins with truncated forms of VGLUT2 (Δ534-582 or Δ542-582), as assessed by the density, average area and fluorescence intensity of the GFP-VGLUT2 puncta that colocalize with the presynaptic marker (Fig. 3.22-A,B). Analysis of total GFP-VGLUT2 expression showed no significant difference in the number of puncta observed in the neurites of cells transfected with the full-length transporter and the truncated forms. However, there was a reduction in the average area of the puncta containing clustered truncated transporters. Also, the total fluorescence intensity along neurites was significantly lower when the shorter form of the transporter (Δ534-582) was expressed in hippocampal neurons (Fig. 3.22-C). Taken together, the results show that VGLUT2 cleavage alters the subcellular distribution of the transporter, significantly reducing its synaptic localization, which may affect glutamatergic synaptic transmission.


A
GFP-VGLUT2
GFP-VGLUT2 Δ542-582
GFP- VGLUT2 Δ534-582
Synapsin
GFP
Merge
5 µm
B
GFP-VGLUT2 synaptic puncta (Number/Axonal length)
GFP-VGLUT2 synaptic puncta (Area/Axonal length)
GFP-VGLUT2 synaptic puncta (Fluorescence Intensity/ Axonal length)
125
100
75
50
25
0
**
*
GFP-VGLUT2
GFP-VGLUT2 Δ542-582
GFP-VGLUT2 Δ534-582

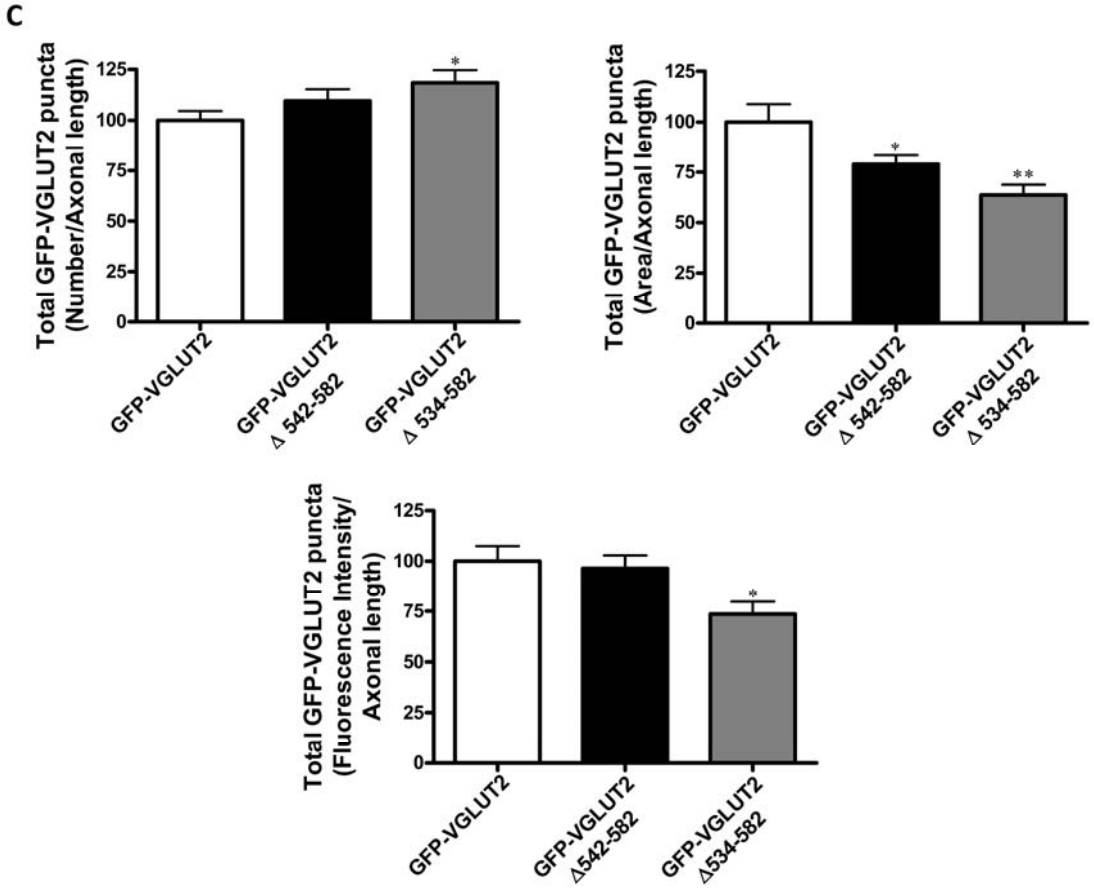


Figure 3.22. VGLUT2 calpain cleavage reduces its synaptic localization. (A) Cultured hippocampal neurons (7 DIV) were transfected with GFP-VGLUT2, or with the mutated forms Δ534-582 or Δ542-582, and immunocytochemistry was performed using antibodies for GFP (green) and Synapsin (red). Synaptic GFP-VGLUT2 is defined as the GFP-VGLUT2 that colocalizes with synapsin. Transfected neurons were analyzed for the GFP-VGLUT2 synaptic (B) and total (C) cluster number, area and fluorescence intensity. The results are the average ± SEM of 3 independent experiments performed in distinct preparations ($n \geq 54$). GFP-VGLUT2 full-length levels were set to 100%. Statistical analysis was performed using One Way ANOVA, followed by the Dunnet's multiple comparison test (*$p<0.05$, **$p<0.01$) as compared with neurons transfected with the GFP-VGLUT2 construct. The data presented in this figure were obtained by Tatiana Catarino.

Chapter 4

Discussion

The deregulation of glutamatergic synaptic transmission with overactivation of glutamate receptors contributes to neuronal death in many disorders of the nervous system, including brain ischemia.

In this work, we show that the vesicular glutamate transporter type 2 is cleaved under excitotoxic conditions *in vitro* and *in vivo*, and in a model of transient focal ischemia *in vivo*. VGLUT1 was also downregulated under excitotoxic conditions *in vitro* but not in transient focal brain ischemia. The cleavage of VGLUTs under excitotoxic conditions, and in particular the changes in the subcellular distribution of VGLUT2, are likely to contribute to the deregulation of the glutamatergic synapses in the ischemic brain.

Several evidences show that VGLUT2 cleavage is mediated by calpains: i) chemical calpain inhibitors prevent VGLUT2 cleavage in cultured hippocampal neurons subjected to an excitotoxic stimulation with glutamate or challenged with oxygen-glucose deprivation; ii) incubation of synaptic vesicles and GST-VGLUT2 C-terminal region fusion protein with recombinant calpain induces VGLUT2 cleavage. These findings are in agreement with the key role played by calpains in neuronal death following excitotoxic or ischemic insults (Bevers and Neumar, 2008). Calpains are also activated after MCAO in the cerebral cortex and in the striatum, contributing to the observed neuronal death (Takagaki et al., 1997), and may mediate the VGLUT2 cleavage observed in the striatum in mice subjected to this model of ischemia. Calpain activation was also observed in the CA1 region of the hippocampus following transient brain global ischemia, contributing to neuronal death (Yamashima et al., 2007), and has been shown to contribute to neurodegeneration after intrahippocampal injection of kainate (Higuchi et al., 2005), and so calpain may also be responsible for VGLUT2 cleavage in these model of excitotoxicity *in vivo*.

In contrast to the involvement of calpains in neurodegeneration, a few reports suggest that calpains also promote survival, by contributing to the activation of the Akt survival pathway (Tan et al., 2006), protecting against TNFα-induced apoptosis (Lu et al., 2002) or cleaving and inactivating caspases (Chua et al., 2000). A recent study also found that calpain-induced downregulation of some GluN2 subunits in rat

striatal neurons promotes tolerance to excitotoxicity mediated by NMDAR (Kambe et al., 2010).

Our observations of downregulation of VGLUT2 after excitotoxic stimulation or ischemic insults are in agreement with the results of previous works showing that VGLUT2 protein levels are reduced after transient global ischemia in the CA1 layer of the gerbil hippocampus (Kim et al., 2006) and in the cortex and caudate-putamen of rats subjected to transient MCAO (Sanchez-Mendoza et al., 2010). In the latter study, MCAO was shown to upregulate VGLUT1 protein levels (Sanchez-Mendoza et al., 2010), in agreement with the effect observed in this work for VGLUT1 after excitotoxic stimulation in the hippocampus *in vivo*. VGLUT1 levels were also shown to be elevated in hippocampus following focal hypoxic ischemia (Kim et al., 2005), and VGLUT1 upregulation was correlated with an increase in glutamatergic transmission, contributing to a rise in extracellular glutamate levels and hyperexcitability in patients with temporal lobe epilepsy (van der Hel et al., 2009).

Western blot experiments using antibodies against VGLUT2 N- and C-terminal regions suggested that the transporter is cleaved at the C-terminal region. According to the predicted topology, this region is directed towards the cytoplasm, and incubation of a GST fusion protein with the C-terminal region of VGLUT2 with calpain allowed the identification of two cleavage sites at amino acid residues Asn534 and Lys542. The presence of more than one cleavage site was also found in other calpain substrates (Haacke et al., 2007; Meary et al., 2007; Grumelli et al., 2008). The C-terminal region of VGLUT2 contains a putative PEST sequence, which is thought to constitute a signal for rapid intracellular proteolysis (Rogers et al., 1986; Rechsteiner and Rogers, 1996) and has been associated with protein cleavage by calpains (Molinari et al., 1995; Shumway et al., 1999). However, whether the presence of PEST sequences provides a signal for calpain cleavage is not a clear issue, since mutations of these sequences did not abolish calpain cleavage (Molinari et al., 1995; Carillo et al., 1996), and there are some calpain substrates that lack PEST sequences (Carillo et al., 1996). The results are also in

agreement with several studies which predict that calpain cleavage should occur in rather disordered regions of the proteins (Fontana et al., 1997; Tompa et al., 2004), and the VGLUT2 C-terminal region fits this criterion.

Incubation of hippocampal neurons with the TAT-VGLUT2 C-terminal peptide specifically prevented VGLUT2 cleavage under excitotoxic conditions, without affecting calpain activity, as assessed by spectrin cleavage, a hallmark of calpain activity (Neumar et al., 2001; Siman et al., 2004). The peptide was taken up by a significant number of neurons as previously described for other TAT peptides (Cao et al., 2002; Wadia et al., 2004), and was able to compete with endogenous VGLUT2 for calpain cleavage. The use of a TAT fusion peptide also reduced calpain-mediated cleavage of metabotropic glutamate receptor 1 (mGluR1), and provided neuroprotection from excitotoxic insults *in vitro* and *in vivo* (Xu et al., 2007). Similarly, neuroprotective effects of the TAT-Bcl xL peptides (Cao et al., 2002; Ju et al., 2008) and the TAT-GluN2B peptide (Cui et al., 2007) have been reported. We have not tested the effect of TAT-VGLUT2 peptide on excitotoxic neuronal death but inhibition of VGLUT2 cleavage may increase excitotoxic cell death, since the transporter mediates glutamate uptake into synaptic vesicles in excitatory synapses (Hayashi et al., 2001). In particular, the preservation of VGLUT2 may increase the exocytotic release of glutamate in cerebral ischemia while ATP is still available, increasing cell death. If this is the case, VGLUT cleavage in brain ischemia may constitute an endogenous neuroprotective mechanism.

We also addressed the putative role of caspases in VGLUT2 downregulation, since these proteases are implicated in ischemic neuronal cell death (Yamashima, 2000), and VGLUT2 contains a consensus DELD sequence that is a target for caspase-3 cleavage.(Thornberry et al., 1997; Chang and Yang, 2000). *In vitro* assays using the fusion protein GST-VGLUT2 C-terminus incubated with recombinant caspase-3 showed cleavage of VGLUT2, as seen by the appearance of a cleavage product. However, the pan-caspase inhibitor Z-VAD-FMK did not affect the glutamate-evoked downregulation of VGLUT2, ruling out a contribution of this group of proteases to the

downregulation of the transporter. Since excitotoxic conditions induce a minor activation of caspase-3 (Almeida et al., 2005; Higuchi et al., 2005), we tested for the role of this protease in the cleavage of VGLUT2 under conditions that induce caspase-3-mediated apoptotic neuronal death. Incubation of hippocampal neurons with staurosporine (Bertrand et al., 1994; Koh et al., 1995; Young et al., 2010) or trophic factor withdrawal (Chan et al., 1999; Glazner et al., 2000) did not downregulate VGLUT2 protein levels, although caspase-3 activation was observed. The results indicate that caspase-3 cleavage of VGLUT2 observed *in vitro* may not have a relevant role *in vivo*, at least in the models tested in the present work.

The cleaved forms of VGLUT2, lacking the amino acid sequences 542-582 or 534-582, showed a reduced synaptic localization when compared to the full-length transporter in transfected cultured hippocampal neurons. The number of synaptic clusters formed by the truncated transporters was lower when compared with the full-length protein, and the area and intensity of each cluster was also decreased. At least for the truncated form ∆542-582 the total fluorescence intensity along neurites was similar to that observed for the full-length transporter, indicating that the loss of synaptic clustering is not due to a downregulation of the number of transporters along the axons. This change in the distribution of the protein along neurites may be related with the fact that VGLUT2 C-terminal region has several amino acid sequences that have been reported to be involved in the synaptic delivery and internalization of vesicular transporters (Fig. 4.1), namely a dileucine-like (FV) motif, with the presence of upstream glutamate residues, and a tyrosine-based motif. The dileucine-like motif was found to be involved in the endocytosis and recycling of VGLUT1 to synaptic vesicles (SVs) in cultured neurons (De Gois et al., 2006; Vinatier et al., 2006; Voglmaier et al., 2006). This motif is also found in the VMAT2, being required for the endocytosis of the transporter in PC12 cells and hippocampal neurons (Li et al., 2005), and to the sorting to synaptic-like microvesicles (SLMVs) in PC12 cells (Tan et al., 1998). The presence of glutamate residues (EE) upstream of the dileucine motif in VMAT2 may be required for sorting into the regulatory

secretory pathway (Li et al., 2005). A dileucine motif is also present in the VAChT (Colgan et al., 2007), and together with an additional novel tyrosine-based motif (YNYY) allows internalization and localization to SLMVs (Varoqui and Erickson, 1998; Kim and Hersh, 2004; Ferreira et al., 2005). Recently, it was reported that deletion of *Drosophila* VGLUT (DVGLUT) C-terminal domain had a small but consistent effect in reducing internalization and the SV localization of the transporter (Grygoruk et al., 2010). VGLUT2 also has three putative glycosylation sites at amino acid residues Asn100, Asn101 and Asn470 (UniProtKB Database), located at two intravesicular domains. Intravesicular glysosylation sites were also found in the VMAT2, where they play a role in the localization of the transporter at SVs *in vivo* (Cruz-Muros et al., 2008). Whether these putative glycosylation sites play a similar role in the regulation of the distribution of VGLUT2 remains to be determined. When VGLUT2 is cleaved by calpain, almost all of these motifs are lost, and only two acidic clusters (EE) are kept in the cleaved form. The loss of the majority of the putative trafficking motifs may affect the subcellular distribution of the transporter, and contribute to the fact that the truncated forms of VGLUT2 are found in non-synaptic sites at a higher extent than the full length form.

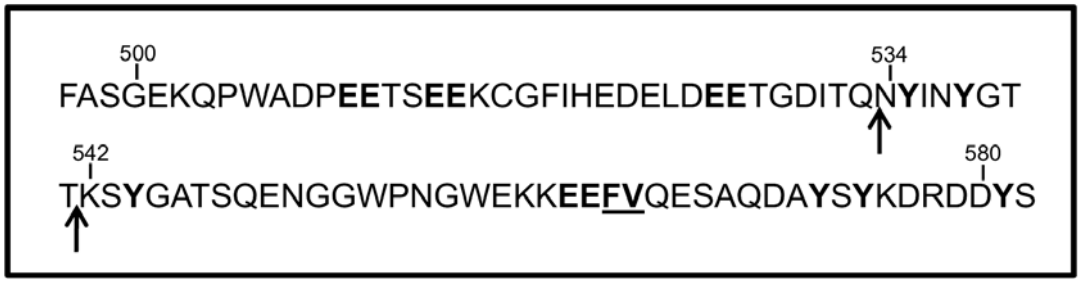


Figure 4.1. Putative trafficking signals in VGLUT2 C-terminal region. Representation of VGLUT2 C-terminal region showing in bold putative trafficking signals based on the characterization reported for other vesicular transporters. The arrows indicate the two calpain cleavage sites identified in the present work.

VGLUT1 was also downregulated in hippocampal neurons subjected to excitotoxic stimulation, but although bioinformatic analysis of the transporter amino acid sequence showed two putative PEST sequences, the protein was not cleaved by calpains *in vitro*. Other proteases may contribute to VGLUT1 cleavage under excitotoxic conditions, including lysosomal hydrolytic aspartyl (cathepsin D) and cystein (cathepsins B, H, L) proteases. Cathepsin D mediates execution of neuronal death induced by transient forebrain ischemia and excitotoxicity (Adamec et al., 2000), while cathepsins B and L were shown to contribute to hippocampal neuronal death after excitotoxicity and global ischemia (Yamashima, 2000; Wang et al., 2006). The spreading of hydrolytic enzymes into the cytoplasm upon lysosomal membrane injury or rupture was observed after brain ischemic injury (Yamashima et al., 1998; Yamashima, 2000, 2004; Qin et al., 2008). Lysosomal membrane injury may occur through calpain activation following an ischemic insult, according to the "calpain-cathepsin hypothesis", or may be induced by free-radicals (Yamashima, 2000). Moreover, it was recently observed that cathepsins may cleave other proteins following glutamate excitotoxicity in cultured cortical neurons, such as the glutamic acid decarboxilase (GAD) 65/67 (Monnerie and Le Roux, 2008). VGLUT1 may also be cleaved by the ubiquitin-proteasome system (UPS). The UPS was shown to play a role in the cleavage of GAD 65/67 isoforms in cultured hippocampal neurons following excitotoxicity stimulation with glutamate (Baptista et al., 2010). Other proteins cleaved by the UPS after brain ischemia include A-kinase anchor protein (AKAP) 121 (Carlucci et al., 2008) and PKC-gamma (Matsumoto et al., 2004).

Taken together, our results show that ischemic or excitotoxic insults cause a VGLUT2 cleavage by calpains, reducing its synaptic targeting, in contrast with VGLUT1 which was only downregulated in hippocampal neurons subjected to excitotoxic stimulation. Given the role of VGLUTs in glutamate release, a critical step to the excitotoxic damage that characterizes the ischemic brain, the changes in the synaptic distribution of VGLUT2 after cleavage may play an important neuroprotective role in this pathology. The reduction of VGLUT2 levels may

contribute to an inhibition of glutamate release after an excitotoxic event. Since VGLUT2 and VGLUT1 expression during development is not constant, with VGLUT1 levels increasing gradually after birth, and VGLUT2 showing a higher expression early in development (Boulland et al., 2004), the specific decrease of VGLUT2 levels after ischemia might be particularly important in perinatal or neonatal brain injury. Several studies indicate that the neonatal mammalian brain is much more resistant to ischemic injury than conspecific adult brains (Duffy et al., 1975; Singer, 1999; Chen et al., 2008a). Strong cellular evidences confirm that the neonatal brain possesses several strategies that contribute to prevent ischemic injury (Weil et al., 2008). An example is the elevated melatonin concentration (a potent anti-oxidant) found in the brain of newborns during delivery (Tan et al., 2005), which may provide neuroprotection from brain ischemia. Similar adaptations provide neuroprotection during arousal from hibernation (Tan et al., 2005) or diving mammals (Aarseth and Stokkan, 2003), and this may be an adaptation that aids animals to survive ischemia in their natural habitat. Resistance to energetic depletion and altered NMDA receptor composition, distribution and attenuated receptor currents have also been described in response to hypoxia in neonatal mammalian brains (Duffy et al., 1975; Singer, 1999).

Chapter 5

Conclusions

Stroke is a complex disease characterized by a significant neurological impairment, being a leading cause of death and acquired disability in developed countries, including Portugal. Excitotoxicity is one of the first events after ischemic stroke, and constitutes an important trigger of cell death. Although excitotoxicity is considered an important target for therapeutic intervention, until now the extensive research in the field has not provided a drug that can be used in stroke patients. The only drug approved for stroke therapy is tPA, which promotes clot lysis and allows reperfusion of the injured brain area. The study of molecular changes in neurons following excitotoxic injury may contribute to the development of novel therapeutic strategies. In the present work, we investigated potential changes in the vesicular glutamate transporters 1 and 2, which directly regulate the extent of glutamate release, thus providing a presynaptic mechanism for controlling the efficacy of excitatory transmission.

We showed that VGLUT2 is cleaved under excitotoxic conditions *in vitro* and *in vivo*, and after transient focal cerebral ischemia. In contrast, VGLUT1 is less or not cleaved, and is even upregulated after excitotoxicity *in vivo*. VGLUT2 cleavage was mediated by calpains after excitotoxic stimulation with glutamate and upon OGD, giving rise to a cleavage product observed with an antibody against the N-terminal region of VGLUT2. Calpains are also activated after MCAO in the cerebral cortex and striatum, contributing to neuronal death (Takagaki et al., 1997), and may mediate VGLUT2 cleavage observed in the striatum after MCAO. Furthermore, calpains are activated and contribute to neurodegeneration after intrahippocampal injection of kainate (Higuchi et al., 2005), and may contribute to VGLUT2 cleavage in this model of excitotoxicity *in vivo*. Calpain cleavage of VGLUT2 was also observed when synaptic vesicles and a GST-VGLUT2 C-terminal fusion protein were incubated with recombinant calpain I.

Two calpain cleavage sites in the C-terminal region of VGLUT2 were identified, at amino acid residues Asn534 and Lys542, close to a putative PEST sequence, which has been associated to protein cleavage by calpains (Rechsteiner and Rogers, 1996; Shumway et al., 1999). Accordingly, incubation of hippocampal neurons with a TAT-VGLUT2 C-terminus peptide that competed with endogenous VGLUT2 for calpain cleavage, without inhibiting calpain activity, prevented the downregulation of VGLUT2 in hippocampal neurons subjected to excitotoxic conditions. Inhibition of VGLUT2 cleavage by the TAT-VGLUT2 peptide may increase excitotoxic neuronal death since the transporter will mediate glutamate uptake into synaptic vesicles and increase exocytotic neurotransmitter release, thereby contributing to glutamate induced excitotoxicity. Although VGLUT1 also possesses two putative PEST

sequences, we found no evidences suggesting that this transporter may be cleaved by calpains.

The C-terminal region of VGLUT2 possesses a DELD consensus sequence that typically targets substrate proteins for cleavage by caspase-3. However, although we observed that recombinant caspase-3 cleaved a fusion protein containing the C-terminal region of VGLUT2, the transporter was not cleaved by caspase-3 in cultured hippocampal neurons after excitotoxic stimulation with glutamate, or following induction of apoptosis with staurosporine or by withdrawal of trophic factors. These results indicate that caspase-3 is not likely to play a relevant role in VGLUT2 cleavage *in vivo*.

Using GFP fusion proteins with the full-length VGLUT2, or with the cleaved forms of the transporter at Asn534 and Lys542, we showed that VGLUT2 truncation by calpain reduces its synaptic localization. This may decrease the release of glutamate by exocytosis, thus reducing its toxic effects. Although the sequences contributing to the synaptic delivery of VGLUT2 have not been identified, the transporter contains several sequences that have been shown to play a similar role in other vesicular neurotransmitter transporters. Since some of these sequences are deleted when the transporter is cleaved by calpains, it will be interesting to investigate how they contribute to synaptic targeting and the VGLUT2 binding proteins involved.

Since VGLUT2 and VGLUT1 expression during development is not constant, with VGLUT1 levels increasing gradually after birth, and VGLUT2 showing a higher expression early in development, the specific decrease of VGLUT2 levels after ischemia might be a protective mechanism in perinatal or neonatal brain injury. This fits the hypothesis proposed in several studies which sustain that the neonatal mammalian brains are more resistant to ischemic brain injuries than conspecific adult brains (Duffy et al., 1975; Singer, 1999; Chen et al., 2008a). In future studies it would be interesting to investigate if VGLUT2 cleavage decreases excitotoxic or ischemic cell death, by specifically inhibiting the cleavage of the transporter by calpain using the TAT-VGLUT2 C-terminal peptide, which does not affect other calpain substrates (Xu et al., 2007), and evaluating cell death after excitotoxic and ischemic insults.

The effect of calpains on VGLUT2 cleavage described in this work adds to a list of proteins targeted by the protease under excitotoxic and ischemic conditions. Calpains were shown to be involved in neurodegeneration, but also to play some neuroprotective effects (Chua et al., 2000; Lu et al., 2002; Tan et al., 2006; Kambe

et al., 2010). VGLUT2 cleavage may provide neuroprotection in brain ischemia, which may be important early in development

Chapter 6

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