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ABBREVIATIONS

A	Alanine
ADAR2	Adenosine deaminases acting on RNA-2
ALS	Amyotrophic lateral sclerosis
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AMPAR	AMPA receptor
AP-1	Activator protein-1
ATPase	Adenosine triphosphatase
Ca^{2+}	Calcium ion
CLAP	Chymostatin, Leupeptin, Antipain and Pepstatin cocktail
CNS	Central Nervous System
CTZ	Cyclothiazide
D	Glutamate
DIV	Days *in vitro*
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
ECF	Enhanced Chemifluorescence
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
G	Glycine
G418	Geneticin
Glu	Glutamate
Gly	Glycine
GRIP	Glutamate receptor interacting protein
GTP	Guanosine triphosphate
H^+	Hydrogen ion
HBSS	Hank's balanced salt solution
HEK293	Human embryonic kidney 293
HEKGluR4	HEK293 cells constitutively expressing $GluR4_{flip}$ subunits
IgG	Immunoglobulin
iGluR	Ionotropic glutamate receptor
IP	Immunoprecipitation
IP3	Inositol trisphosphate
JIP	JNK interacting protein
JNK	c-Jun N-terminal kinase
K^+	Potassium ion
KA	Kainate
LTD	Long-term depression
LTP	Long-term potentiation

MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
Mg^{2+}	Magnesium ion
mGluR	Metabotropic glutamate receptor
MK-801	5-methyl-10,11-dihydro-5H-dibenzo-cyclohepten-5,10-imine maleate
MLK	Mixed-lineage kinase
mPTP	Mitochondrial permeability transition pore
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
Na^{+}	Sodium ion
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo-quinoxaline-2,3-dione
NMDA	*N*-methyl-D-aspartate
NMDAR	NMDA Receptor
nNOS	Neuronal NO synthase
NO	Nitric oxyde
NSF	N-ethylmaleimide sensitive fusion protein
OGD	Oxygen-glucose deprivation
PARP	Poly-ADP-ribose polymerase
PBS	Phosphate Buffer Saline
PICK1	Protein interacting with C-kinase-1
PKC	Protein kinase C
POSH	Plenty of SH3 domains
Pro	Proline
Q	Glutamine
R	Arginine
REST	Repressor element-1 silencing transcription factor
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
SAPK	Stress-activated protein kinase
TBS	Tris-buffered saline
TBS-T	TBS with 0.1% Tween20
TCA cycle	Tricarboxylic acid cycle
Thr	Threonine
Tyr	Tyrosine
VGCC	Voltage-gated Ca^{2+} channels
Zn^{2+}	Zinc ion

ABSTRACT

Ca^{2+}-permeable α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors have been reported to be crucial for glutamate-induced neuronal death in some acute and chronic neurodegenerative diseases. Indeed, the selective death of CA1 hippocampal neurons after transient global ischemia is related to molecular changes in the composition of AMPARs, which render them Ca^{2+}-permeable. However, the excitotoxic mechanisms associated with Ca^{2+}-permeable AMPARs are not completely understood and therefore further studies are required to identify the downstream signaling pathways activated under ischemic stimulation. A thoroughly knowledge of such pathways can be of outstanding relevance to the development of therapeutic strategies that target protein interactions mediated by ischemic insults, while sparing the physiological activity of AMPA receptors. Recently, the AMPA receptor subunit GluR4 was shown to be a possible physiological JNK substrate (Thomas et al., 2008). Our previous results in HEK-GluR4 cells, which constitutively express GluR4-containing Ca^{2+}-permeable AMPARs, showed that excitotoxicity increased the GluR4 phosphorylation on T855 (Vieira, M., unpublished data), the residue described as specifically phoshorylated by JNK. Thus, to investigate the role of JNK in the phosphorylation of GluR4-T855, we stimulated HEK-GluR4 cells in the presence of the JNK pathway inhibitors CEP11004 and SP600125, which caused a decrease in the GluR4-T855 phosphorylation levels, therefore indicating the involvement of a kinase of the JNK pathway in this event. To evaluate the role of GluR4-T855 phosphorylation to the excitotoxic response mediated by those receptors, HEK293-A cells were transduced with wild type GluR4 or with the GluR4 mutants GluR4-T855A and GluR4-T855D and then submitted to excitotoxic stimulation. The evaluation of cell viability did not

indicate a function of phosphorylated GluR4-T855 on GluR4-containing Ca^{2+}-permeable AMPAR-mediated excitotoxicity.

On the other hand, to pursue our aim of identifying the molecular mechanisms coupling Ca^{2+}-permeable AMPARs to excitotoxic pathways, we challenged cultures of hippocampal neurons with OGD, an *in vitro* model of brain ischemia that induces synaptic targeting of Ca^{2+}-permeable AMPARs (Liu et al., 2006). We evaluated the effect of 1-3 hours OGD stimuli in the hippocampal neuron viability. Mature (14-15 DIV) hippocampal cultures displayed a decrease in the metabolic activity, assessed by the MTT test, which correlated with the time-length of the OGD stimuli. In order to characterize the contribution of the different ionotropic glutamate receptors to the reduction in neuronal viability, we used the selective NMDA receptor antagonist MK-801 and the AMPA/KA receptor antagonist NBQX. In hippocampal neuron cultures with 8 DIV, analysis of the nuclear morphology indicates that both MK801 and NBQX prevented the OGD-induced cell death, pointing for a contribution of NMDA and AMPA/KA receptors to the excitotoxic response. However, in mature cultures with 14-15 DIV, only NBQX prevented changes in the nuclear morphology, showing that in these cultures the AMPA/KA receptors are the main ionotropic glutamate receptors responsible for the OGD-induced excitotoxicity. We also observed a decrease in the levels of GluR2 in hippocampal slices submitted to mild OGD, by performing biotinylation of plasma membrane-associated proteins, suggesting that OGD induces changes in surface membrane AMPAR trafficking similar to those described for cultured hippocampal neurons. Taken together, these results suggest that developmental changes in the composition of ionotropic glutamate receptors, or in the machinery associated to the glutamatergic neurotransmission, regulates the OGD-induced excitotoxic response of hippocampal neurons. Moreover, our results support the OGD

challenge in hippocampal neurons as an ischemic model suitable for the *in vitro* study of excitotoxic mechanisms coupled to GluR2-lacking Ca^{2+}-permeable AMPARs. Additionally, our results support the recent observation that GluR4-T855 is a new substrate of JNK. However, this phosphorylation event does not influence the GluR4-containing Ca^{2+}-permeable AMPAR-mediated excitotoxicity.

Keywords: AMPAR, Ca^{2+}-permeable AMPARs, Excitotoxicity, GluR4, OGD, GluR2

RESUMO

Os receptores AMPA permeáveis a Ca^{2+} têm revelado ter um papel crucial na morte de neurónios induzida pela libertação excessiva de glutamato em algumas doenças neurodegenerativas graves. De facto, estes receptores têm sido relacionados com a morte selectiva dos neurónios da zona CA1 do hipocampo após isquémia global, durante a qual ocorrem alterações moleculares na composição destes receptores, tornando-os permeáveis a Ca^{2+}. No entanto, os mecanismos moleculares que relacionam estes receptores com a activação de vias de sinalização excitotóxicas não são ainda totalmente conhecidos, pelo que são necessários mais estudos com vista à identificação das vias de sinalização activadas em situação de isquémia. A compreensão dessas vias pode ser de elevada importância para o desenvolvimento de estratégias terapêuticas dirigidas à disrupção selectiva de interacções proteicas mediadas por insultos isquémicos, sem, no entanto, interferir com a actividade fisiológica destes receptores. Recentemente, mostrou-se que a subunidade GluR4 dos receptores AMPA pode constituir um substrato da cinase JNK em condições fisiológicas (Thomas e tal., 2008). Resultados prévios obtidos em células HEK293-GluR4 (expressando constitutivamente receptores AMPA permeáveis a Ca^{2+} contendo GluR4) mostraram que a excitotoxicidade aumenta a fosforilação do resíduo T855 do GluR4 (Vieira, M., resultados não publicados), resíduo especificamente fosforilado pela JNK. Assim, para confirmar a fosforilação do resíduo T855 pela JNK em condições de excitotoxicidade, células HEK-GluR4 foram submetidas a estimulação excitotóxica na presença de inibidores farmacológicos da JNK, CEP11004 e SP600125. Verificou-se a diminuição nos níveis de fosforilação do mesmo resíduo, o que revela a participação de uma cinase da via de sinalização da JNK neste processo. Para compreender a contribuição da fosforilação do GluR4 para a morte celular mediada por estes receptores, células

HEK293-A foram transfectadas com GluR4 normal ou com mutantes desta subunidade, GluR4-T855A e GluR4-T855D, sendo posteriormente submetidas a estimulação excitotóxica. A viabilidade celular observada nestas células não confirma uma função da fosforilação do GluR4 para o mecanismo de morte celular desencadeado após a sobreactivação de receptores AMPA permeáveis a Ca^{2+}.

Por outro lado, no sentido de procurar identificar os mecanismos moleculares que relacionam os receptores AMPA permeáveis a Ca^{2+} com vias de excitotoxicidade, culturas primárias de neurónios do hipocampo foram submetidas a OGD, um modelo *in vitro* para a isquémia cerebral que induz o endereçamento destes receptores para a sinapse (Liu et al., 2006). A viabilidade das culturas de hipocampo foi avaliada após 1h-3h de estímulo de OGD. As culturas maturas (14-15 DIV) submetidas a OGD apresentaram uma redução na actividade metabólica, avaliada pelo teste do MTT, correlacionada com a duração do tempo do estímulo de OGD. Além disso, para caracterizar a contribuição dos diferentes receptores ionotrópicos do glutamato para a redução da viabilidade celular, usaram-se os antagonistas MK-801 e NBQX, selectivos para receptores NMDA e AMPA/KA, respectivamente. Nas culturas de neurónios do hipocampo com 8 DIV, a análise da morfologia nuclear indicou que ambos os antagonistas impediram a morte celular induzida por OGD, apontando uma contribuição dos receptores NMDA e AMPA/KA para a resposta excitotóxica. Contudo, em culturas maturas com 14-15 DIV, apenas o antagonista NBQX evitou as alterações na morfologia nuclear dos neurónios, demonstrando que, nestas culturas, os receptores AMPA/KA poderão ser o tipo predominante de receptores ionotrópicos do glutamato para a excitoxicidade induzida por OGD. Por fim, observou-se, através da biotinilação de proteínas associadas à membrana plasmática, que em fatias de hipocampo submetidas a um estímulo breve de OGD, os níveis da subunidade GluR2 à superfície

das células são reduzidos, o que sugere um efeito do estímulo de OGD no endereçamento destes receptores para a membrana plasmática dos neurónios do hipocampo, semelhante ao observado já em culturas do hipocampo. De uma forma geral, os resultados obtidos sugerem que modificações na composição de receptores ionotrópicos ao longo do desenvolvimento dos neurónios, ou na maquinaria associada aos processos de neurotransmissão do glutamato, influenciam a resposta excitotóxica mediada por OGD em neurónios do hipocampo. Além disso, estes resultados apoiam o estímulo de OGD como modelo *in vitro* para a isquémia apropriado para estudos de sinalização de excitotoxicidade mediada por receptores AMPA permeáveis a Ca^{2+}.

Chapter 1 Introduction

1.1. Glutamate transmission

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system since it was found that diverse agents able to block glutamate actions also prevent physiologic synaptic excitation. Glutamate is also the most predominant excitatory neurotransmitter in the mammalian central nervous system (CNS). Besides its role in neurotransmission, it participates in protein and fatty acid synthesis and interfaces closely with carbohydrate metabolism, acting as a precursor of intermediates in Krebs cycle, so that glucose is the physiologic precursor of most glutamate in the brain (Hara & Snyder, 2007).

At chemical synapses, glutamate, which is stored in vesicles, is released from the pre-synaptic cell in a process triggered by nerve impulses. In the post-synaptic cell, glutamate receptors bind glutamate and are activated. Because of its role in synaptic plasticity, it is believed that glutamate is involved in cognitive functions like learning and memory in the brain (Catarzi et al., 2007). The synaptic action of this neurotransmitter is terminated when glutamate transporters, present in both neuronal and glial membranes, rapidly remove it into nerve terminals and/or astrocytic glia. This latter mechanism is the most predominant in the reuptake of glutamate (Hara & Snyder, 2007).

1.2. Glutamate ionotropic receptors

Glutamate has the ability to activate two major classes of receptors, the metabotropic glutamate receptors (mGluRs) and the ionotropic glutamate receptors

(iGluRs). The mGluRs mediate slow synaptic responses, due to their coupling to intracellular G proteins. To date, the heterogeneous mGluRs family consists of at least eight subtypes (1-8) that have been classified into three groups (groups I–III) based on sequence similarity, pharmacology and transduction mechanism (Catarzi et al., 2007).

The iGluRs are heterotetrameric cation channels, comprising three functionally distinct subtypes: α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainate (KA) and *N*-methyl-D-aspartate (NMDA) receptors. These receptors are mainly concentrated at postsynaptic sites, where they contribute to a variety of different functions (Cull-Candy et al., 2006). Glutamate ionotropic receptors pass electric current in response to glutamate binding, and the three classes were initially distinguished by the different affinity of the various glutamate analogues on receptor activation and their different physiological properties. Later, the cloning of glutamate receptors in the early nineties (Hollmann & Heinemann, 1994) came in support of the initial classification of the ionotropic glutamate receptors in three subtypes.

NMDA receptors are activated by the glutamate analogue NMDA (*N*-methyl-D-aspartate), and possess a recognition site for glycine, a co-agonist, which is required for the receptor activation. When activated, NMDA receptors open a channel that conducts Na^+ and Ca^{2+}. However, under basal conditions, the channel is blocked by Mg^{2+}. This blockade is removed by cellular depolarization (Figure 1). Continuously strong stimulation increases NMDA receptor activation and plays an important role in long-term potentiation (LTP).

AMPA receptors, another class of ionotropic glutamate receptors, respond selectively to glutamate derivatives such as AMPA (*α*-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and kainate, opening a channel that allows Na^+ and, less frequently, Ca^{2+} influx, causing cell membrane to depolarize. Both NMDA and AMPA

receptors are localized in the same cells. Thus, when Na^+ ions flows through AMPA receptors, triggering cell depolarization, the blockade of NMDA receptors by Mg^{2+} is relieved and the receptors activated, leading to Ca^{2+} entry through those receptors.

Finally, kainate receptors, a third class of ionotropic glutamate receptors, bind with high affinity to kainate, opening a channel that allows for Na^+, and in some cases Ca^{2+}, influx. Increasing evidence links kainate receptors to several critical neuronal cell processes, such as synaptic plasticity and regulation of neurotransmitter release, synaptogenesis and synaptic maturation. However, these receptors may also be implicated in pathophysiological conditions such as excitotoxicity (Pinheiro & Mulle, 2006).

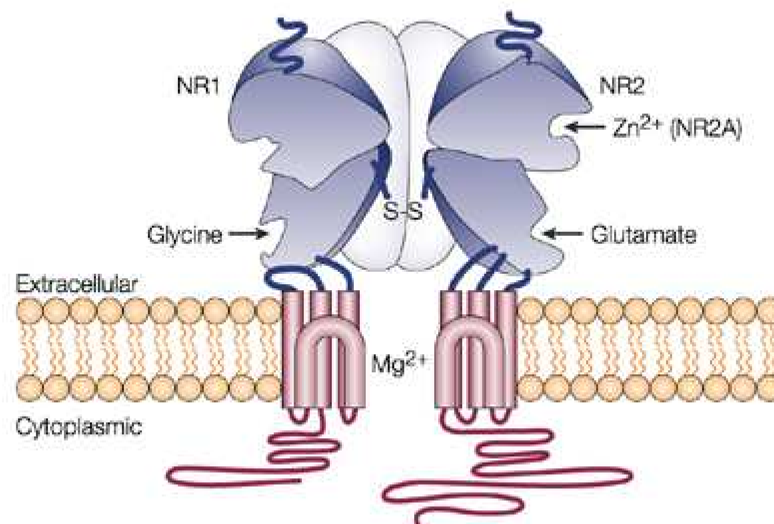


Figure 1. Model of the NMDA receptor. The channel pore is blocked by Mg^{2+} in a voltage-dependent manner. Depolarization of the neuron relieves this blockade and glutamate and glycine binding cause the channel to open, allowing Ca^{2+} influx (adapted from Witt et al., 2004).

1.3. Excitotoxicity

Many neurotransmitters likely participate in signaling events that influence neurotoxicity, but glutamate appears to be the principal one (Hara & Snyder, 2007). In fact, by the same time glutamate was considered the most prevalent excitatory neurotransmitter in the central nervous system (CNS), there were evidences that glutamate could also be a potent neurotoxin. It was shown that injections of glutamate could induce acute neuronal necrosis in several regions of the developing brain in newborn mice and destroy inner neural layers of the retina when injected systemically into immature mice that did not possess a fully developed blood-brain barrier (Olney, 1969). This damaging process was believed to be mediated by glutamate receptors since application of glutamate receptor antagonists attenuates synaptic transmission, acting as neuroprotective agents (Simon et al., 1984).

In brain injury leading to cell depolarization, glutamate transporters can work in reverse, causing an excess of glutamate to accumulate outside the cells. This process induces glutamate receptors overactivation, allowing for intracellular ionic changes, in particular regarding the unbalanced influx of Ca^{2+}, Na^{+} and even Zn^{2+} into the cell. Ca^{2+}, for instance, activates enzymes and signal transduction pathways responsible for triggering neuronal damage and even cell death, as established by many studies of neurotoxicity in cultured neurons. Especially NMDA receptors are given a key role in mediating at least certain aspects of glutamate neurotoxicity, owing to their high Ca^{2+} permeability. This pathological process, whereby excitatory amino acids produce neurodegeneration, is referred to as excitotoxicity (Sattler & Tymianski, 2001).

Excitotoxicity due to glutamate receptor overactivation is associated with many diseases, like spinal cord injury, cerebral ischemia (stroke), epileptic seizures, and neurodegenerative diseases of the central nervous system such as multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease and Huntington's disease.

1.3.1. Calcium and neurotoxicity

To target the neurotoxic glutamate effects, therapeutic approaches based on the inhibition of glutamate release or on the blockade of its receptors were developed. However, those putative treatments revealed to be ineffective, since glutamate receptor blockade interferes with normal brain function and may trigger many neurological side effects (Sattler & Tymianski, 2001). Therefore, research has rather focused on the identification of intracellular signaling and regulatory pathways triggered by overactivation of glutamate receptors.

Calcium, one of the most important intracellular messenger molecules, can mediate a variety of cellular responses. Ca^{2+} influx occurs by at least three types of channels: voltage-sensitive Ca^{2+} channels, store-operated channels and receptor-operated channels, like the NMDA receptor (Hara & Snyder, 2007). Within the cell there are several components that store Ca^{2+}, such as the endoplasmic reticulum (ER), the mitochondria and the many Ca^{2+}-binding proteins. The largest store of intracellular Ca^{2+} is the ER, in which Ca^{2+} is retained by a Ca^{2+}-ATPase pump. Neurotransmitter or hormone receptor activation of phospholipase C (PLC), and subsequent production of inositol trisphosphate (IP3), releases Ca^{2+} from this reservoir upon IP3 binding to its receptors in the ER. Mitochondria accumulate Ca^{2+} via a membrane uniporter and

release it by reversal of this uniporter, by a Na^{+}-H^{+} dependent exchanger or by a mitochondrial permeability transition pore (mPTP). The mPTP channel has been extensively related to cell death through release of Ca^{2+} and other substances, despite its possible role in the regulation of intracellular Ca^{2+} concentration. Free cytosolic Ca^{2+} concentrations are maintained at approximately 100 nM, mainly by the ER pump and by extrusion through the plasma membrane Ca^{2+}-ATPase and the Na^{2+}-Ca^{2+} exchanger (Hara & Snyder, 2007).

As mentioned before, Ca^{2+} concentration can increase with NMDA receptor activation. Immoderate influx of extracellular Ca^{2+}, along with any Ca^{2+} release from intracellular reservoirs, causes free Ca^{2+} concentrations to rise. Elevated levels of cytosolic Ca^{2+}, exceeding the capacity of intracellular Ca^{2+}-regulatory mechanisms, lead to cell systems derangement and activation of cell death pathways. Ca^{2+} influx can also occur via voltage-sensitive Ca^{2+} channels (VSCCs), which are sensitive to the membrane potential. However, despite the excitotoxicity-mediated cell membrane depolarization and activation of such channels, the contribution of VSCCs to neuronal damage remains to be clarified (Sattler & Tymianski, 2001).

Although it is generally accepted that Ca^{2+} overloading is a responsible factor for neuronal cell death, there have been difficulties in correlating intracellular Ca^{2+} concentration and excitotoxicity. Several studies suggest a linear correlation between total Ca^{2+} loading and neurodegeneration, but this relationship might be regulated by many factors, such as Ca^{2+} gradient, route of Ca^{2+} influx and Ca^{2+} homeostatic mechanisms (Tymianski, 1996). Therefore, not only the changes in total Ca^{2+} concentration, but specially the route of Ca^{2+} entry and its associated biochemical signaling pathways, may impart specificity to the signaling mechanisms activated in physiological and pathological processes. In fact, recent studies of Ca^{2+} signal

transduction pathways revealed that Ca^{2+} differently affects processes central to the development and plasticity of neuronal cells, such as cell survival, synaptic strength and cell death, depending on its route of entry into a neuron (Ghosh & Greenberg, 1995, Sattler & Tymianski, 2001). Thus, researchers have been focusing as to how the signaling mechanisms activated by Ca^{2+} entry through different glutamate receptors would influence neuronal cell death during an excitotoxic insult.

At the present, some of the molecular mechanisms by which Ca^{2+} entry through NMDA receptors might trigger excitotoxicity have been described (Sattler & Tymianski, 2001). However, despite the strong belief that NMDA receptors have the major role in excitotoxicity-mediated cell death, due to their high Ca^{2+} permeability, there are evidences that AMPA receptors can also be of considerable importance in this process.

1.4. AMPA receptors

1.4.1. Structure of AMPA receptors

AMPA receptors constitute the class of ionotropic glutamate receptors that mediates the fast depolarization in glutamate-mediated neurotransmission, playing a key role in synaptic plasticity in the vertebrate central nervous system. Changes in synaptic strength, such as those occurring on long-term potentiation (LTP) and long-term depression (LTD), which are thought to be involved in learning and memory formation, are associated with changes in AMPA receptor distribution and phosphorylation. Furthermore, deregulation of AMPA receptors activity is related to many pathologic brain injuries (Santos et al., 2008).

AMPA receptors are homo- or heteromeric assemblies of four subunits, GluR1-4 (A-D), which are encoded by separate genes. AMPA mRNA can be detected at very early stages of development, but the expression of the subunits is developmentally regulated (Palmer et al., 2005), and can also be found in glial cells (Gallo & Russell, 1995). Also, GluR1, 2 and 3 mRNAs are largely expressed throughout the CNS, while GluR4 is expressed in a restricted spatial and temporal fashion (Santos et al., 2008).

AMPA receptors subunits have four hydrophobic membrane domains with an extracellular N-terminal domain and an intracellular C-terminal tail, which is different among the subunits (Figure 2). For instance, GluR1, GluR4 and an alternative splicing form of GluR2, GluR2L, have longer cytoplasmic tails, whereas GluR2, GluR3 and an alternative form of GluR4, GluR4c, present a short cytoplasmic domain (Santos et al, 2008). Interactions with specific proteins through the C-terminal tail of each subunit have an important role in controlling the trafficking and stabilization of AMPA receptors at the synapses, including after neuronal insults. As such, AMPA receptors composed of GluR2 and 3 constitutively replace existing synaptic receptors, but, in contrast, those constituted by GluR1 and 2L or GluR2 and 4 are targeted to synapses during plasticity (Shi et al., 2001).

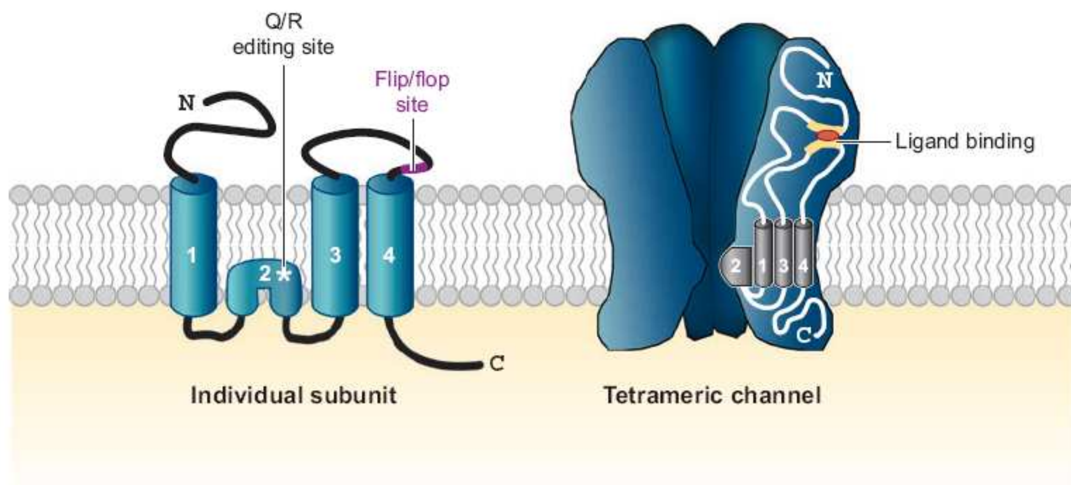


Figure 2. Structure of the AMPA receptor subunits and the tetrameric channel. The individual subunits are formed of four transmembrane domains. The channel comprises four subunits, generally two dimers which, in turn, are usually two different subunits (GluR1/2 or GluR2/3, for example) (from Shepherd & Huganir, 2007).

The presence of GluR2 in heteromeric AMPA receptor assemblies determines the permeability to cation ions, like Ca^{2+} or Zn^{2+}, due to an arginine (R) residue in the second membrane-domain. The presence of GluR2 also influences channel kinetics, conductance, receptor assembly, trafficking from the endoplasmic reticulum and targeting to and from synapses (Liu & Zukin, 2007).

1.4.2. Diversity of AMPA receptor subunits

A wide variety of functionally distinct AMPA receptors are present in neuronal tissues due to alternatively spliced sequences of the respective mRNA, phosphorylation and RNA editing of the different subunits, as well as receptor composition. However,

we will briefly describe the RNA splicing and editing processes, since these are more related with Ca^{2+}-permeable AMPARs, the subtype of glutamate receptors focused in this work.

a) RNA splicing

Alternative RNA splicing increases the molecular diversity of AMPA receptor subunits. All the AMPA receptor subunits are expressed in two distinct isoforms, *flip* and *flop*, which are generated by alternative splicing of a region near the fourth transmembrane domain. Despite the forms only differ in a few amino acids, the resulting receptors show different functional properties, such as desensitization and ER export kinetics (Tanaka et al., 2000, Santos et al., 2008). For instance, homomeric AMPA receptor channels formed by $GluR4_{flop}$ subunits desensitize four times faster than $GluR4_{flip}$ channels, a difference also found between $GluR3_{flop}$ and $GluR3_{flip}$ channels (Mosbacher et al., 1994).

b) GluR2 RNA editing

RNA editing, a process that consists in the enzymatic modification of the DNA encoded information, is an important mechanism of receptor regulation. When the GluR2 mRNA undergoes RNA editing, a glutamine (Q) codon is substituted by an arginine (R) codon in the region that codifies the second membrane-associated segment, which forms the pore of the receptor. Unedited subunits, containing a neutral glutamine residue, consist of Ca^{2+}-permeable AMPA receptors, whereas the presence of the positively charged arginine residue in the channel pore blocks the entrance of Ca^{2+} and

other divalent cations (Sommer et al., 1991, Higuchi et al., 1993, Tanaka et al., 2000, Cull-Candy et al., 2006). AMPA receptors that lack this subunit exhibit high Ca^{2+}-permeability. As such, the GluR2 subunit is a key component of AMPA receptors function.

The majority of AMPA receptors in the CNS contain the GluR2(R) subunit and are, therefore, impermeable to Ca^{2+}. In neonatal and adult rat brain, nearly 100% of GluR2 mRNA is edited at the Q/R site, rendering GluR2-containing AMPA receptors Ca^{2+}-impermeable, whereas the unedited form is detectable only in the embryonic brain, in a small percentage (1%) of total GluR2 mRNA (Liu & Zukin, 2007). However, a significant amount of Ca^{2+}-permeable AMPA receptors is found in neurons and glial cells of various brain regions. The dynamic regulation of these receptors is crucial in synaptic plasticity, neuronal development and neurological disease (Cull-Candy et al., 2006; Liu & Zukin, 2007).

In the AMPAR family, desensitization kinetics are controlled by editing of GluR2, GluR3 and GluR4 at a site preceding the fourth transmembrane region, a process that is 80-90% complete in the adult rat brain (Tanaka et al., 2000).

1.4.3. Regulation of Ca^{2+} permeability through AMPA receptors

Under physiologic conditions, the majority of AMPA receptors in the CNS is Ca^{2+}-impermeable, reflecting the presence of edited GluR2 subunits. The levels of Ca^{2+} -permeable AMPA channels are regulated in response to physiological activation, through mechanisms that might involve particular protein-protein interactions with GluR2 and that regulate the insertion of GluR2-lacking and GluR2-containing receptors in the membrane (Cull-Candy et al., 2006; Santos et al., 2008). It has been found that

two proteins, both containing GluR2-interacting PDZ domains, protein interacting with C-kinase-1 (PICK1) and N-ethylmaleimide sensitive fusion protein (NSF), are required for the activity-dependent trafficking of the receptors. Both proteins participate in a dynamic process of regulation and control of the GluR2-content at the cell surface, thus indirectly controlling Ca^{2+} permeability of synaptic receptors.

Ca^{2+} entrance through Ca^{2+}-permeable AMPA receptors is involved in synaptic plasticity, synaptogenesis and formation of neuronal circuitry (Tanaka et al., 2000), but it can also be implicated in glutamate-induced neuronal toxicity. The subunit composition and Ca^{2+} permeability of AMPA receptors can be changed after neuronal insults such as seizures (Friedman et al., 1994, Friedman, 1998, Grooms et al., 2000, Sommer et al., 2001,), transient global ischemia (Pellegrini-Giampietro et al., 1992, Gorter et al., 1997, Opitz et al., 2000, Noh et al., 2005, Liu et al., 2006), by the administration of certain substances, such as corticosteroids (Nair et al., 1998), and also in chronic neuronal diseases, such as amyotrophic lateral sclerosis (Kawahara et al., 2004, Kwak et al., 2005, Liu & Zukin, 2007). These changes are due to downregulation of GluR2 expression, a deficiency in the RNA editing process and changes in receptor trafficking. In these cells, acute loss of GluR2 renders them more vulnerable to neuronal insults.

1.4.3.1. Global ischemia primes neuronal death mediated by Ca^{2+}-permeable AMPARs

Cerebral global ischemia is the third leading cause of death in the Western world. In many cases, it triggers delayed neurological and cognitive deficits. Transient global or forebrain ischemia, observed in patients during cardiorespiratory arrest, cardiac

surgery or in animals models, can induce selective and delayed neuronal death of hippocampal CA1 neurons, which will present signs of apoptosis only a few days after the insult (Petito et al., 1987, Böttinger et al., 1998). In general, impairment of cerebral blood flow restricts the delivery of substrates and the maintenance of ionic gradients. Consequently, there is loss of membrane potential and depolarization of neurons. As a result, dendritic as well as presynaptic voltage-dependent Ca^{2+} channels are activated and glutamate is released into the extracellular space. Since ionic concentrations are impaired, there is a transient rise in glutamate release, due to the deficient, or reversal of, uptake during the ischemic event. Early after reperfusion, despite the normal morphology of the cells and the normal levels of intracellular Ca^{2+}, it occurs a rise in intracellular free Zn^{2+} in CA1 neurons, which is followed by a delayed rise in Ca^{2+} intracellular concentration through ionotropic glutamate receptors. These receptors thus mediate the critical excitotoxic events that result in neuronal death (Tanaka et al., 2000, Kwak & Weiss, 2006, Liu & Zukin, 2007).

Under physiologic conditions, hippocampal neurons abundantly express GluR2-containing AMPA receptors, which are Ca^{2+}-impermeable. As mentioned before, ischemic insults trigger downregulation of GluR2 expression, as well as a decrease on the protein abundance in the CA1 neurons, inducing a long-lasting switch in AMPA receptors properties due to an exchange of GluR2-containing for GluR2-lacking receptors. Moreover, since these cells do not express high levels of Ca^{2+}-binding proteins or extrusion pumps, that would compensate for the rise in Ca^{2+} concentration, acute loss of GluR2 enhances vulnerability to glutamate-induced pathogenecity (Liu & Zukin, 2007). As such, and given that NMDA receptors antagonists seem to be less effective than AMPA receptors antagonists in preventing ischemia, this class of glutamate receptors is increasingly believed to be an important mediator of ischemia-

induced neuronal death (Buchan et al., 1991, Pellegrini-Giampietro et al., 1992, Li et al., 1993, Gorter et al., 1997, Opitz et al., 2000, Noh et al., 2005, Liu et al., 2006). In fact, promoting the expression of AMPA receptors impermeable to Ca^{2+} ions, enhances protection of CA1 neurons after transient global ischemia whereas overexpression of the unedited form of GluR2, GluR2(Q), causes the death of hippocampal CA3 neurons and dentate gyrus cells, which otherwise would be resistant to ischemia-triggered damage (Liu et al., 2004). Moreover, the use of selective GluR2-lacking receptor blockers confers neuroprotection in models of global ischemia (Noh et al, 2005). Altogether, these results pointed out that AMPA receptors permeable to Ca^{2+} (GluR2-lacking) may play a significant role in ischemia-induced neurotoxicity. Moreover, there are emerging evidences that show that not only GluR2 expression is affected, but also receptor trafficking and GluR2 mRNA editing are deregulated in response to ischemic insults (Gorter et al., 2005, Liu et al., 2006, Dixon et al., 2009). For instance, in hippocampal CA1 synapses, GluR2-containing AMPA receptors are anchored at the synapse by glutamate receptor interacting protein (GRIP), but, following Ca^{2+} entry, such as after an ischemic insult, PICK1 competes with GRIP for binding to GluR2, leading to the disruption of GluR2 binding to GRIP. Association of GluR2-containing AMPA receptors with PICK1 promotes receptor internalization, whereas GluR2-lacking AMPA receptors are inserted in the synapse. Thus, ischemic insults might be responsible for the replacing of GluR2-containing AMPA receptors by GluR2-lacking AMPA receptors at insulted CA1 synapses. Therefore, the removal of GluR2-containing receptors allows for the insertion of GluR2-lacking receptors in synaptic sites, which may contribute to the selective vulnerability of hippocampal neurons (Liu et al., 2006, Liu & Zukin, 2007, Dixon et al., 2009) (Figure 2).

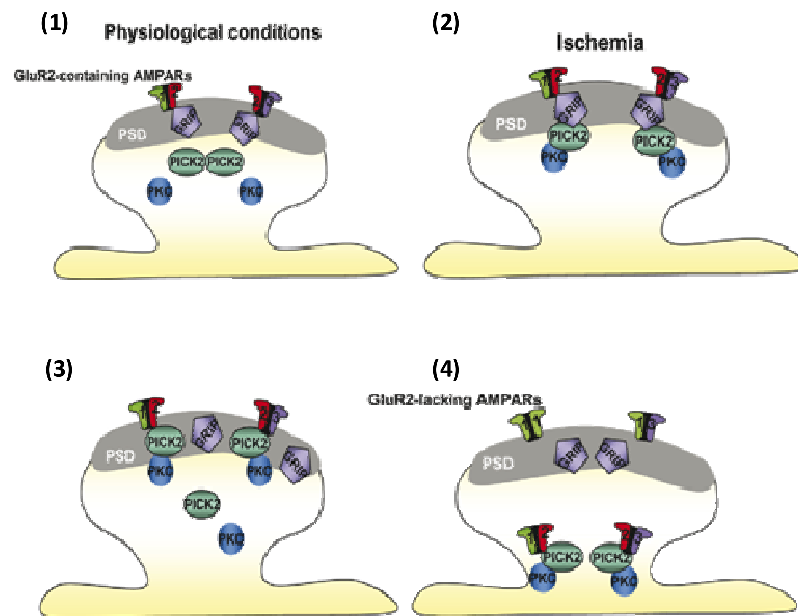


Figure 3. Ischemia-induced switch in AMPA receptor subtypes at CA1 synapses. (1) In the basal state, GluR2-containing AMPA receptors are stabilized at the postsynaptic membrane by association with GRIP. **(2)** Ischemic insults activate PKC and promote binding of PKC to PICK1. The PICK1–PKC complex binds to the GRIP–GluR2 complex. **(3)** PICK1 competes with GRIP for binding to GluR2, leading to disruption of GluR2 binding to GRIP. **(4)** Association of GluR2-containing AMPA receptors with PICK1 promotes receptor internalization, while synaptic incorporation of GluR2-lacking AMPA receptors occurs, thereby replacing GluR2-containing AMPA receptors at insulted CA1 synapses (adapted from Liu & Zukin, 2007).

Regarding an increase in Ca^{2+}-permeable AMPA receptors due to a decrease in GluR2 mRNA editing, it was shown that ischemia causes a cell-specific reduction in the expression of the enzyme normally responsible for GluR2 Q/R site editing in the CA1 hippocampal neurons. Thus, the GluR2 subunits that forms the receptors are

predominantly unedited, which in addition to a reduced GluR2 expression, is responsible for the upregulation of Ca^{2+}-permeable AMPARs (Peng et al., 2006).

1.4.3.2. The role of Zn^{2+}

Besides contributing to an increase in the intracellular Ca^{2+} concentration, another way in which Ca^{2+}-permeable AMPA receptors might mediate cell damage is by allowing a delayed Zn^{2+} influx. Zn^{2+} ions are present in presynaptic nerve terminals in the mammalian central nervous system, serving as endogenous signaling mediators, and are released simultaneously with glutamate at certain synapses. Zn^{2+} accumulates in hippocampal neurons during ischemia, and the addition of Zn^{2+} chelators has proven to be neuroprotective (Koh et al., 1996, Calderone et al., 2004). Since AMPA channels are highly permeable to these ions, they must be the principal way of entry of Zn^{2+} (Jia et al., 2002, Yin et al., 2002).

Zinc can induce injury through various mechanisms, such as mitochondrial disruption, production of ROS (Sensi et al., 1999, Sensi et al., 2000, Jiang et al., 2001) and PARP (poly (ADP-ribose) polymerase) activation, a protein involved in many cellular processes such as DNA repair and programmed cell death (Kim & Koh, 2002). Curiously, Zn^{2+} is more potent at inducing cell damage than Ca^{2+}, acting by disruption of mitochondrial activity, which is believed to be related to degeneration after Zn^{2+} accumulation (Kwak & Weiss, 2006).

1.4.3.3. Ca^{2+}-permeable AMPARs and other diseases

Besides the emerging role in ischemia, these receptors have also been described to be implicated in other CNS diseases, such as ALS (motoneurons express Ca^{2+}-permeable AMPARs, which renders these cells particularly vulnerable to AMPAR-mediated excitotoxicity), white matter injury (Ca^{2+}-permeable AMPARs expressed by oligodendrocyte precursors might play a role in hypoxic–ischemic cerebral white matter injury), human glioblastoma (malignant brain tumor cells also express these receptors; replacement of these receptors with Ca^{2+}-impermeable ones has shown to cause migration suppression and death of glioblastoma cells) and epilepsy (in various seizure models, changes in GluR2 subunit expression, and thus changes in the abundance of Ca^{2+}-permeable AMPARs, have been identified (Sanchez et al., 2001, Zhang et al., 2004; Sanchez et al., 2005, Cull-Candy et al., 2006).

Ca^{2+}-permeable AMPARs are more widespread than originally thought. They are expressed in both neurons and glia, and are activated during synaptic transmission, participating in mechanisms of learning and memory. Even though Ca^{2+} influx through these receptors is modest when compared to that through NMDARs, the location and kinetics of this influx enables them to serve distinct cellular functions. As such, and since selective Ca^{2+}-permeable AMPA receptors antagonists are limited and not available for human trials, other approaches, such as modulation of GluR2 trafficking or manipulation of RNA editing, seem to be better therapeutic alternatives approaches against ischemia-triggered damage (Cull-Candy et al., 2006).

Other approaches may also come from the understanding of the pathways involved in triggering cell death upon activation of these receptors. Although there can be many cell death pathways known to be activated during different types of stimuli,

one of emerging interest in excitotoxic insults is the mitogen-activated protein kinases (MAPKs) pathways, namely the JNK pathway, which is sensitive to stress conditions.

1.5. MAP kinases signaling

Several environmental stimuli influence and control various aspects of complex celular processes, to which cells need to respond properly, in order to survive. These stimuli include both physical stimulation, such as mechanic stress, heat, radiation, pH, osmolarity, and chemical stimulation with, for instance, cytokines, hormones, growth factors, as well as other environmental stresses. These physical and chemical signals direct many aspects of cell function, including migration, proliferation, differentiation, survival and death (Davis, 2000, Cui et al., 2007). MAPKs are evolutionary conserved enzymes, found in fungi, plants and mammals, which are capable of organizing a response to chemical and physical stimuli, thus controlling cell adaptation.

Many signal transduction pathways cooperate and participate in a high interactive network towards the integration of the information in the cell, involving second messengers production and protein kinases activation, which relies on the interaction of the transducers with the proper targets. One of the major signaling pathways responsible for transducing the external stimuli into a cellular response includes the mitogen-activated protein kinases (MAPK) pathway, which are involved in many aspects of cellular regulation (Davis, 2000, Pearson et al., 2001, Cui et al., 2007).

MAPK pathways comprise a sequence of at least three kinases. These are MAPKK kinases (MAPKKKs or MEKKs), MAPK kinases (MAPKKs or MEKs) and MAP kinases (MAPKs). These kinases phosphorylate and activate the downstream

kinases sequentially (Figure 4). Another feature of the MAPKs cascade is that they are activated by dual phosphorylation, in serine/threonine or threonine/tyrosine residues.

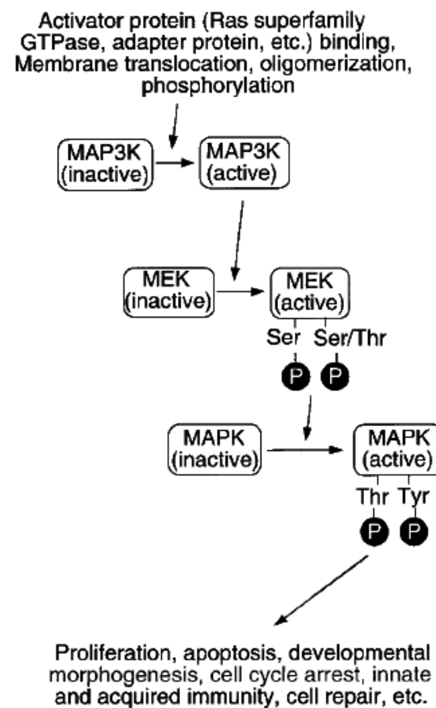


Figure 4. The MAPK signaling module. Different stimuli activate the MAPKKK, which activates MAPKK by phosphorylation. These, in turn, activate MAPK by phosphorylation, which then triggers the appropriate responses (from Kyriakis & Avruch, 2001).

MAPKKKs can be regulated through many ways, such as receptor activation, membrane recruitment of adaptor molecules, activation of small GTP-binding proteins and phosphorylation (Kyriakis & Avruch, 2001, Cui et al., 2007). It may also require further upstream kinases as well as scaffold proteins. Scaffold proteins might have the capacity to bind other proteins simultaneously, thereby organizing and facilitating specific interactions between the components. Thus, different stimuli can activate specific MAPK signaling components, that scaffolding proteins help to bring together.

In mammals, three major groups of MAPKs have been identified: the extracellular signal-regulated kinases (ERKs), the p38 and the c-Jun N-terminal kinases (JNKs). Each group is activated by a protein kinase cascade, similar to that of Figure 5, but each one responds to specific stimuli and has specific substrates. The ERK and p38 groups are related to enzymes initially found in the yeast *S. cerevisae* and have the dual phosphorylation motifs Thr-Glu-Tyr and Thr-Gly-Tyr, respectively. As for the JNKs, they represent a group originally identified in mammals, and contain the dual phosphorylation motif Thr-Pro-Tyr (Davis et al., 2000). Each MAPKK can be activated by more than one MAPKKK, which increases the complexity of MAPK signaling. For instance, ERK1/2 requires activation of the MAPKKK c-Raf in order to be activated by growth factors, but pro-inflammatory stimuli activate ERK1/2 via another MAPKKKs (Chang & Karin, 2001). Despite the fact that two different stimuli can activate the same MAPK, given that there are many different extracellular signals, the first elements on the kinase cascade, MAPKKKs, show high divergence in structure and gene number compared with MAPKKs and MAPKs. As such, each MAPKKK is responsible for a specific response to a specific stimuli. This is even more complex with JNK and p38 cascades, which are activated by many stimuli acting on diverse MAPKKKs, whose physiological functions and specificities remain to be clarified (Chang & Karin, 2001).

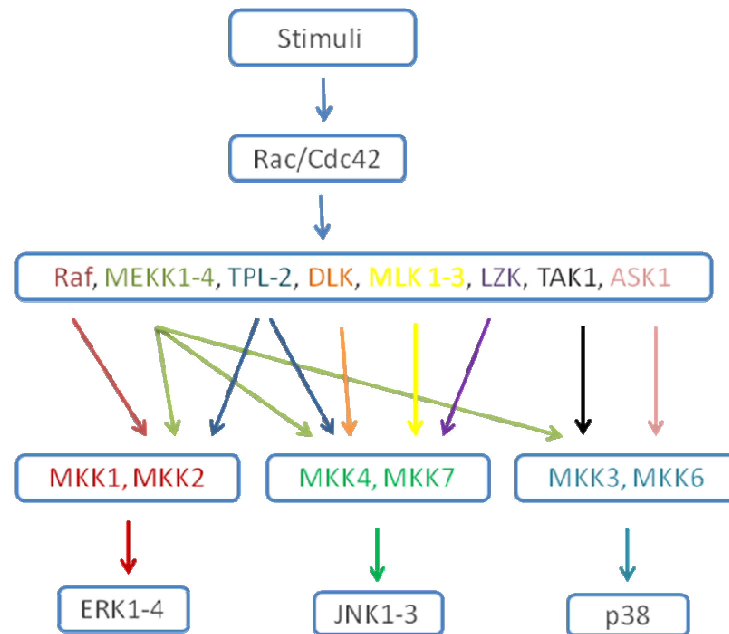


Figure 5. Components of the MAPK signaling pathways (adapted from Cui et al., 2007).

1.5.1. The JNK pathway

The c-Jun N-terminal kinases (JNKs) are also known as stress-activated protein kinases (SAPKs), since they can be activated by many different environmental stresses, as well as by cytokines and growth factors. Therefore, the MAPK JNK is a multifunctional protein kinase involved in many physiologic, as well as pathological, cell processes. In fact, it is known that JNK can mediate embryonic morphogenesis and regulate cell proliferation (Davis et al, 2001). However, JNK can also be involved in apoptotic neuronal cell death induced by growth factor deficiency, DNA damage, oxidative stress, tumor necrosis factor and excitotoxic stress (Cui et al., 2007).

There are three *JNK* genes identified in mammals: *Jnk1*, *Jnk2* and *Jnk3*. Whereas JNK1 and JNK2 are expressed throughout the organism, JNK3 is only highly expressed in the CNS neurons, existing in lower levels in cardiac smooth muscle and

testis. There at least ten isoforms identified in the human brain as a result of alternative splicing of the three genes, which localize in different chromosomes (Gupta et al., 1996). As such, different JNK isoforms can play specific roles within the cell. Furthermore, it is proposed that there is a great complementation between the three genes, and that the expression of the splice variants vary with the tissue. Altogether, this limits experiments with *Jnk* knockouts, since the results will only be credited if all JNK isoforms are lacking (Davis, 2000).

Despite the fact that JNK can be activated by many different MAPKKKs, the JNK cascade involved in cell death has been extensively studied and thus some participants are known. The cascade mainly comprises Rac1/Cdc42, mixed-lineage (MLKs), MKK4/7 and JNKs (Davis, 2000, Cui et al., 2007).

Other components of the JNK pathway, such as the scaffold proteins, are also involved in JNK activation. Namely, the proteins POSH (plenty of SH3) and JIP (JNK interacting protein) have been identified as scaffolding proteins for the JNK apoptotic cascade elements (Dickens et al., 1997, Xu et al., 2003, Kukekov et al., 2006). For instance, disruption of the *Jip1* gene in mice prevents JNK activation upon exposure to excitotoxic and anoxic stresses, which suggests that JIP1 is a critical scaffold protein for the JNK signaling pathway induced by excitotoxicity (Whitmarsh et al., 2001).

It is generally accepted that scaffold proteins confer a great advantage to the efficient and specific activation of a signaling cascade in a restricted region of the cell by a particular stimulus. The binding of multiple components of the cascade increases their proximity and the efficient interaction among them. In fact, since MAPKKs and JNK are activated by dual phosphorylation, the close proximity due to the scaffold protein increases their activation (Davis, 2000, Kukevov et al., 2006).

1.5.2. JNK substrates

JNK can phosphorylate a variety of substrates, including transcription factors and non-nuclear proteins. It has been well established that JNK can bind the NH_2-terminal domain of c-Jun, a component of the AP-1 (activator protein-1) transcription factor family, and therefore triggers nuclear events elicited by extracellular stimuli, which includes the regulation of stress-responsive genes expression. AP-1 activation is thought to be mediated, at least in part, by the phosphorylation of c-Jun, which is the most potent activator of transcription within the AP-1 family. JNK phosphorylates c-Jun on Ser-63 and Ser-73, which activates its transcriptional activity (Pulverer et al., 1991, Smeal et al., 1991, Davis, 2000, Chang & Karin, 2001). JNK appears to be essential for AP-1 activation caused by stress and some cytokines, but not in response to other stimuli (Davis, 2000).

JNK can also phosphorylate other transcription factors, including JunB, JunD and ATF (Gupta et al., 1996) and the sites of phosphorylation are located in the activation domain of the transcription factor. These transcription factors are also components of the AP-1 transcription factor complexes. Other transcription factors, such as ELK1, NFAT (Chow et al., 1997) and p53 (Buschmann et al., 2001) can also be phosphorylated by JNK.

In addition to the regulation of cell death by transcriptional mechanisms, JNK can also activate transcriptional-independent cell death pathways. Several studies have shown that JNK can phosphorylate both pro- and anti-apoptotic proteins, thus regulating their activity. For instance, JNK can phosphorylate Bcl-2 (Maundrell et al., 1997) and Bcl-X_L (Kharbanda et al, 2000), both anti-apoptotic proteins, in order to decrease their

protective effect, and pro-apoptotic proteins, such as Bim (Lei et al., 2003), enhancing their pro-apoptotic activity (Cui et al., 2007).

Despite the establishment of JNK as an apoptosis mediator, it is not clear whether apoptosis is the only functional response of JNK activity since the major part of the stimuli that activates JNK does not trigger cell death. This is due to blockade of the JNK signaling pathway by activated cell survival cascades, such that JNK activity depends on the overall activation and integration of other signaling pathways. The apoptotic response might also be due to the duration of JNK activation, since only continued, but not transient, activation leads to an apoptotic cell death. As such, further studies are required to understand the role of JNK in signaling cell death and survival (Davis, 2000, Chang et al., 2001).

In fact, JNK might have important physiological roles, owing to its high expression and constitutive activity in neurons. Accordingly, JNKs have been implicated in the regulation of synaptic plasticity (Chen et al., 2005; Zhu et al., 2005), though there is still little knowledge on the neuronal JNK substrates that control these mechanisms. Recently, Thomas and collaborators (2008) observed that the AMPAR subunits GluR2L and GluR4 are JNK substrates. This study shows that, in cortical neurons, JNK constitutively phosphorylates GluR4 at Thr-855 and GluR2L at Thr-912. Moreover, it was shown that stimuli which increase the glutamatergic signaling decrease the phosphorylation of the GluR4 and GluR2L subunits, while the block of neuronal activity with tetrodotoxin (TTX) increases JNK-mediated phosphorylation of both subunits. Accordingly to this work, GluR2L phosphorylation by JNK contributes to the regulation of the synaptic trafficking of the receptors containing this subunit. However, the physiological implication of GluR4 phosphorylation by JNK remains to be clarified.

1.6. Ischemia-mediated cell death: Oxygen-glucose deprivation

As mentioned before, some ischemic insults lead to excitotoxicity by directing GluR2-lacking AMPA receptors to synaptic sites, by downregulating GluR2 expression or by causing an impairment in the editing process of GluR2 subunit, rendering AMPA receptors permeable to Ca^{2+} influx (Liu et al., 2006; Peng et al., 2006, Liu & Zukin, 2007). In fact, it was shown that global ischemia-induced Ca^{2+}-permeable AMPA receptors expression primes cell death selectively on the vulnerable area of CA1 hippocampal neurons (Gorter et al., 1997, Ying et al., 1997, Liu et al., 2004).

Despite the fact that the relation between Ca^{2+}-permeable AMPA receptors and neurotoxicity still remains to be clarified, previous work has shown that excitotoxic cell death via Ca^{2+}-permeable AMPA receptors requires the activation of the AP-1 transcription factor, whose activity can be regulated by JNKs (Santos et al., 2006). The JNK pathway is activated upon cellular stress stimuli, such as excitotoxic stimuli and cerebral ischemia (Borsello et al., 2003, Kuan et al., 2003). Indeed, disruption of the *Jnk3*, but not *Jnk1* or *Jnk2* genes, not only reduced the phosphorylation levels of c-Jun, but also greatly protected hippocampal neurons against injury after excitotoxic and ischemia-hypoxia injuries (Yang et al., 1997, Kuan et al., 2003).

Oxygen-glucose deprivation (OGD) is an established *in vitro* model for transient global ischemia. Neurons subjected to this type of insult are lead towards cell death. It has been shown that OGD can induce neuronal damage by upregulating Ca^{2+}-permeable AMPA receptors (Liu et al., 2006, Dixon et al., 2009). The entire process through which neurons are killed remains to be elucidated, however, some components have already been identified. For instance, using a peptide capable of inhibiting the interaction between JNK and its targets, it was observed that JNK inhibition confers a strong

protection to neurons subjected to OGD (Hirt et al., 2004). Thus, the JNK signaling pathway is involved in the process that mediates cell death upon ischemic insults, and might be a good candidate for a link between Ca^{2+}-permeable AMPA receptors and neuronal degeneration of the hippocampal neurons. Furthermore, it has been observed that components of the JNK signaling pathway can participate in the death mechanism following an OGD challenge. For instance, JIP1 is present in the same subcellular compartment as activated JNK after exposure to OGD, suggesting that JIP1 may exert a scaffolding function for the activated JNK signaling cascade (Whitmarsh et al., 2001) and JNK3, since targeted deletion of *Jnk3* not only reduces the stress-induced JNK activity, but also protects mice from brain injury after the OGD challenge (Kuan et al., 2003).

1.7. Objectives

The major goal of our work is to pinpoint excitotoxic signaling pathways associated to Ca^{2+}-permeable AMPA receptors, namely the contribution of the JNK pathway to cell death. Initially, the role of AMPA receptors in excitotoxicity was surpassed by the study of NMDA receptor activation and its effect on neuronal damage, but several evidences favor Ca^{2+}-permeable AMPARs over NMDA receptors regarding their contribution to selective neurodegeneration. For instance, the increase in the number of Ca^{2+}-permeable AMPA receptors during neuronal insults, such as *in vitro* ischemia, leads cells towards death. The change in the subtype of AMPARs at the cell surface increases divalent cation permeability, thereby promoting degeneration of the insulted neurons. The influx of Ca^{2+} and Zn^{2+}, both of which have considerable impact on mitochondria, further increases the vulnerability to neuronal insults and may relate

these ions to the cell death mechanism taking place following ischemia (Kwak & Weiss, 2006). Although we have shown the involvement of the AP-1 transcription factor in the excitotoxic cell death mediated by Ca^{2+}-permeable AMPARs (Santos et al., 2006), the links between glutamate receptors and the activation of JNK, a kinase that might regulate the activity of AP-1, are not fully understood. The molecular components of the JNK signaling complexes involved in excitotoxic cell death are not known and it is not clear how the JNK cascade is organized from the upstream activator under excitotoxic conditions. Thus further research is required to identify the molecular interactions between Ca^{2+}-permeable AMPA receptor subunits and proteins of the JNK excitotoxic signaling pathways. In order to isolate the contribution of Ca^{2+}-permeable AMPAR in excitotoxicity-mediated cell death, we used human embryonic kidney 293 (HEK293) cells that constitutively express the $GluR4_{flip}$ subunit (HEK-GluR4), which oligomerizes and forms Ca^{2+}-permeable AMPARs (Iizuka et al., 2000), and primary cultures of hippocampal neurons which, upon an OGD challenge, increase the synaptic number of Ca^{2+}-permeable AMPARs (Liu et al., 2006).

In previous work we observed showed that, in HEK-GluR4 cells, excitotoxic stimulation increased the levels of phospho-GluR4, at Thr855, the residue that is selectively phosphorylated by JNK (Vieira, M., unpublished data). Thus, we investigated whether GluR4-T855 phosphorylation was carried out by a kinase of the JNK pathway and, importantly, the role of phospho-GluR4-T855 to the excitotoxic response mediated by GluR4-containing Ca^{2+}-permeable AMPARs.

On the other hand, we aimed to characterize the contribution of different ionotropic glutamate receptors to primary cultures of hippocampal neurons submitted to OGD, an established *in vitro* model for transient global ischemia, to understand the role of excitotoxicity in this ischemic insult-mediated cell death. Moreover, we investigated

a change in the surface membrane content of GluR2-containing AMPARs, which is inversely related to the presence of Ca^{2+}-permeable AMPARs.

The study presented in this report is part of an ongoing work whose goal is to identify the link between Ca^{2+}-permeable AMPARs and excitotoxic death pathways. The results are meant to provide a contribution to the development of therapeutic strategies aiming at the selective disruption of the excitotoxic neuronal death mediated by Ca^{2+}-permeable AMPA receptors, while sparing the physiological activity of the receptors.

Chapter 2 Materials & Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), the protease inhibitors chymostatin, leupeptin, antipain and pepstatin (CLAP) (stock solution 1mg/ml), L-Glutamine, peniciline-streptomicine, glutamic acid, poly-D-lysine, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Fetal bovine serum (FBS), trypsin, geneticin (G418), MEM-Non essential amino acids Neurobasal medium and B27 supplement were purchased from GIBCO, as part of Invitrogen Life Technologies (Carlsbad, California, USA). Cyclothiazide (CTZ), NBQX and MK-801 were obtained from Tocris Cookson Ltd (Bristol, UK). The BCA assay kit, biotin and neutravidin beads were purchased from Pierce, as part of Thermo Fisher Scientific (Rockford, Illinois, USA). Lipofectamine™ 2000 and OptiMEM were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Protein A sepharose CL-4B and Enhanced ChemiFluorescence (ECF) were obtained from GE Healthcare (Uppsala, Sweden). Normal rabbit IgGs were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). QuikChange® II XL Site-Directed Mutagenesis was from Kit Stratagene (La Jolla, CA, USA). CEP11004 was a kind gift from Cephalon, Inc. (West Chester, PA, USA). SP600125 was purchased from BIOMOL International LP (Plymouth Meeting, PA, USA. The DNA stain Hoescht 33342 was from Molecular Probes Europe (Leiden, Netherlands). All other reagents of high degree of purity were from Sigma (Saint Louis, Missouri, USA) or from Merck (Darmstadt, Germany).

Stock solutions of CTZ, CLAP and SP00125 were made in dimethyl sulfoxide (DMSO). All the other chemicals were kept in aqueous stocks.

2.1.1. Plasmid constructions

The different constructs used in this work were a kind gift of several individuals.

The pcDNA1-Flag construct, consisting of the Flag epitope, used as an empty vector was an offer from Dr. J. Ham, University College London (London, UK). The pcDNA3.1-Flag-GluR4 construct was a gift from Dr. K. Kainänen, University of Helsinki, Finland.

2.1.2. Antibodies

The antibodies for phospho-SAPK/JNK (T183/Y185) were purchased from Cell Signalling. The antibody for pan-JNK (MAB1387) was obtained from R&D Systems (Minneapolis, Minnesota, USA). The antibodies against phospho-GluR4 (T855), GluR4 and GluR4 IgGs for immunoprecipitation assays were a generous gift from R. Huganir (Howard Hughes Medical Institute, Baltimore, Maryland, USA). The antibody against GluR1 was obtained from Upstate Cell Signalling (Lake Placid, NY, USA). The antibody against GluR2/3 was purchased from CHEMICON International Inc (Temecula, CA, USA). The antibody against transferrin receptor was purchased from Zymed Laboratories Inc. (South San Francisco, CA, USA). The antibody against β-tubulin was from Sigma-Aldrich (Saint Louis, Missouri, USA), whereas the secondary anti-rabbit and anti-mouse antibodies conjugated with alkaline phosphatase were purchased from GE Healthcare (Uppsala, Sweden).

2.2. HEK293 cells and hippocampal neurons

2.2.1. Site-directed mutagenesis

To evaluate the influence of GluR4-T855 phosphorylation on the excitotoxic response mediated by GluR4-containing Ca^{+}-permeable AMPARs, HEK293-A cells were transfected with plasmids containing the full-length rat GluR4 cDNA or, alternatively, the GluR4 mutant forms GluR4-T855A, a non-phophorylatable mutant, and GluR4-T855D, a phopho-mimetic mutant. Point mutations were introduced on the full-length GluR4 cloned into pcDNA3.1 using QuikChange® II XL Site-Directed Mutagenesis Kit. The primers 5'-GGA GAA AAC GGC CGT GTG CTG GCC CCT GAC TGC CCC AAG GCC- 3' and 5'- GGC CTT GGG GCA GTC AGG GGC CAG CAC ACG GCC GTT TTC TCC-3' were used to mutate Thr855 to an alanine residue (GluR4-T855A). Likewise, the primers 5'-GGA GAA AAC GGC CGT GTG CTG GAC CCT GAC TGC CCC AAG GCC-3' and 5'-GGC CTT GGG GCA GTC AGG GTC CAG CAC ACG GCC GTT TTC TCC-3' were used to mutate Thr855 to an aspartate residue (GluR4-T855D).

2.2.2. HEK293 cell culture and transfection

HEK293 cells, constitutively expressing the $GluR4_{flip}$ subunit of AMPA receptors (HEK-GluR4), were cultured in DMEM containing 10% FBS and geneticin (G418) at a density of 0.5×10^{5} cells/cm^{2} in 100 mm plates previously coated with poly-D-lysine (0.1mg/ml). The GluR4 clone, obtained from HEK293 cells transfected with a vector containing human GluR4 cDNA, was selected with G418 (Iizuka et al., 2000). The cultures were allowed to grow at 37ºC in a 5% CO_2/95% air humidified chamber

for 40h, after which they were submitted to excitotoxic stimulation, in the presence of drugs. This cell line was a kind gift from Dr. Iizuka and Dr. Barsoumian, Nippon Boehringer Ingelheim, Kawanishi, Japan.

HEK293-A cells were cultured in DMEM added with MEM Non-essential Amino Acids, L-Glutamine, 1% peniciline-streptomicine and 10% FBS at a density of 0.3×10^5 cells/cm^2 in 48-well plates previously coated with poly-D-lysine (0.1 mg/ml), at 37ºC in a 5% CO_2/95% air humidified chamber. Transfection with wild-type GluR4 or with mutants was carried out one day after plating, using Lipofectamine™ 2000 Reagent, OptiMEM supplemented with 10% FBS and 0.15 µg DNA/cm^2 overnight at 37ºC. After transfection, the medium containing the DNA-Lipofectamine complexes was replaced by fresh antibiotic-containing culture medium. Forty hours later cells were submitted to excitotoxic stimulation.

2.2.3. Hippocampal neuronal cultures

Pimary cultures of rat hippocampal neurons were prepared from the hippocampus of E18–E19 Wistar rat embryos, after 15 minutes treatment with trypsin (0.06%) and deoxyribonuclease I (5.36 mg/ml) in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) (in mM - 5.36 KCl, 0.44 KH_2PO_4, 137 NaCl, 4.16 $NaHCO_3$, 0.34 $Na_2HPO_4{\cdot}2H_2O$, 5 glucose, 1 sodium pyruvate, 10 HEPES, and 0.001% phenol red) at 37ºC. The hippocampi were then washed with HBSS containing 10% fetal bovine serum, in order to stop trypsin activity, and with HBSS before being transferred to culture medium (Neurobasal medium supplemented with B27 supplement (1:50), 25 µM glutamate, 0.5 mM glutamine, and 0.12 mg/ml gentamycin). The cells were mechanically dissociated and plated at a density of 65×10^3 cells/cm^2 in multi-well

plates coated with poly-D-lysine (0.1 mg/ml). At the 7th day *in vitro* (DIV), half of the culture medium was removed and replaced for the double volume of maintenance medium (glutamate-free culture medium). Cells were used at 8 DIV, 14-15 DIV and 21 DIV, as indicated in the legends of the figures.

2.2.4. Preparation of hippocampal slices

For the preparation of hippocampal slices, adult male Wistar rats (8 weeks of age) were used. The animals were decapitated and their brains rapidly removed and put in a plate containing aCSF medium (in mM: 124 NaCl, 3 KCL, 2 $CaCl_2$, 25 $NaHCO_3$, 1.1 NaH_2PO_4, 2 $MgSO_4$, 10 D-glucose, pH 7.4). The hippocampi were dissected in ice-cold aCSF saturated with 95% O2 and 5% CO2. Transverse hippocampal slices 350 µm thick were cut using a McIlwan Tissue Chopper (The Mickle Laboratory Engeneering Co. LTD). Four slices were distributed for each manipulation group and used immediately after preparation.

2.3. Exposure to glutamate

Subconfluent cultures of HEK-GluR4 or HEK-A cells were washed with sodium buffer (132 mM NaCl, 4 mM KCl, 6 mM glucose, 10 mM HEPES, pH 7.4) with 2.5 mM $CaCl_2$and exposed to glutamate (Glu). Cells were stimulated with 100 µM - 1 mM Glu and 100 µM CTZ in sodium buffer for 1h at 37ºC. After stimulation, cells were washed and placed on serum-free DMEM with 2.5 mM $CaCl_2$, and kept at 37ºC in the incubator. Pre-incubations of 5 minutes, 1h and 2h were performed when CTZ,

SP600125 (30 µM) and CEP1104 (1 µM) were used, respectively. Control cells were placed in the sodium buffer without drugs.

2.4. OGD exposure

2.4.1. Oxygen-glucose deprivation of neuronal cultures

To initiate the OGD challenge, cells were transferred to glucose-free saline buffer (in mM: 116 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1 NaH_2PO_4, 1.8 $CaCl_2$, 26 $NaHCO_3$) and introduced in a chamber with anaerobic atmosphere (10% H_2, 85% N_2, 5% CO_2) (Forma Anaerobic System, Thermo Fisher Scientific) at 37ºC for the indicated times. After the OGD challenge, cultures were transferred to the conditioned medium and returned to the humidified 95% air/5% CO_2 incubator. Control neurons were washed and incubated in the saline buffer described above, supplemented with glucose (10 mM), and kept in the in the humidified 95% air/5% CO_2 incubator at 37ºC for the indicated times. After incubation, control neurons were transferred to the conditioned medium and returned to the humidified incubator. In studies performed with the NMDA receptor antagonist MK-801 (10 µM) and the AMPA receptor antagonist NBQX (20-50 µM), a pre-incubation of 15 minutes was done. When indicated, neurons remained in presence of the glutamate receptor antagonist NBQX after the OGD challenge.

2.4.2. Oxygen-glucose deprivation of slices

Immediately after sectioning, four slices were transferred to a 6-well culture plate containing the glucose-free saline buffer used for the OGD challenge, and inserted

in the anaerobic chamber for 45 minutes at 37ºC. Likewise, control slices were transferred to a 6-well plate containing glucose-supplemented saline buffer saturated with 95% air/5% CO_2 and incubated at 37ºC in the humidified incubator with 95% air/5% CO_2 for the same time as OGD-treated slices. After the OGD challenge, both groups of slices were used to perform cell membrane biotinylation assay for analysis of the surface expression of GluR2 subunit.

2.5 Assessment of cell viability

2.5.1. MTT test

Evaluation of cell viability was performed by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), a yellow-colored salt which, when reduced by cellular dehydrogenases in living cells, originates a blue-colored precipitate, the formazan crystals. MTT (0.5 mg/ml) in sodium buffer with 1 mM $CaCl_2$ was added to the cultures 24h after stimulation, and incubated for 3 h at 37ºC. After incubation, the formazan crystals were dissolved in 0.04 M HCl in isopropanol and colorimetrically quantitated (absorbance at 570 nm). Experiments were carried out in triplicate or quadruplicate, and the results were expressed as the percentage of the MTT reduction by control cultures (100%).

2.5.2. Analysis of the nuclear morphology

Rat hippocampal neurons plated in coverslips coated with poly-D-lysine (0.1 mg/ml) were used for analysis of the nuclear morphology by fluorescence microscopy.

Briefly, 16h after the OGD challenge, cells were washed and stained with the fluorescent dye Hoescht 33342 (1 μg/ml) in sodium buffer at 37ºC for 10 minutes. After extensive washing, neurons were fixed in 4% paraformaldehyde in phosphate saline buffer for 15 minutes at room temperature. The coverslips were mounted on glass slides with Dako mounting medium and examined with a Zeiss Axiovert 200 fluorescence microscope (63× objective). The DNA stain Hoescht 33342 fluoresces blue and is cell permeable. Viable cells display a normal nuclear size and a diffuse blue fluorescence, whereas damaged cells display bright blue pyknotic nuclei with condensed or fragmented chromatin (Bonfoco et al., 1997). The experiments were performed in duplicated and approximately 200 cells were counted per coverslip after randomly selection of 6-10 different optical fields. Cell death is expressed as the percentage of dead cells relatively to the total cell number.

2.6. Total extracts preparation

Total cell extracts of HEK-GluR4 cells were prepared 2h after excitotoxic stimulation and used for immunoprecipitation (IP) assays and Western blot (WB) analysis. Total cell extracts of hippocampal neurons were prepared at 7, 15 and 21 DIV and used for WB analysis. Briefly, cells were washed with cold phosphate buffered saline (PBS) (in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4, pH 7.4) and solubilized in ice-cold lysis buffer (in mM: 50 HEPES, 150 NaCl, 2 EGTA, 2 EDTA, 2 Na_3VO_4, 50 NaF, pH 7.4, with 1% Triton X-100 for WB analysis or, alternatively, 0.4% Triton X-100 for IP assays). The lysis buffer was previously supplemented with 1 mM PMSF and 1 μg/ml CLAP, immediately use. The lysates were sonicated (5 x 5sec) and kept on ice for 20 minutes, and then centrifuged at 12000 x g for 20 minutes at 4ºC. The

supernatants were collected and stored at -20ºC. Total protein was quantified using the bicinchoninic acid (BCA) assay kit.

2.7. Immunoprecipitation assay

For the immunoprecipitation (IP) assays, 200-300 μg of total protein were incubated for 1h with 50 μl protein A sepharose beads (CL-4B) at 4°C, and then centrifuged to remove proteins nonspecifically bound to protein A-sepharose beads. The supernatants were incubated overnight with 4 μg of anti-GluR4 polyclonal antibody at 4°C and then protein A-sepharose beads were added and incubated for another 2h at 4ºC. The supernatants were centrifuged at 16100×g for 2 minutes at 4°C, and the pellets were extensively washed with immunoprecipitation buffer (IPB) (in mM: 10 Tris pH 7.0, 2 EDTA, 2 EGTA, 1 PMSF and 1 μg/ml CLAP) enriched with NaCl (twice with IPB containing 200 mM NaCl and twice with IPB containing 300 mM NaCl). The immunoprecipitated proteins were eluted by boiling in 2x sample buffer (0.25 M Tris, pH 6.8, 4% SDS, 200 mM DTT, 20% glycerol, 0.01% bromophenol blue), collected by centrifugation in tube filters (VWR International LLC, West Chester, PA, USA), separated by sodium dodecyl sulphate-polyacrilamide gels (SDS-Page) and analysed by WB. The supernatants treated with normal IgGs from the host species of the primary antibody were used as controls for non-specific binding. Likewise, the inputs (total cell extracts without further treatments) were treated with sample buffer and input samples with 60 μg of total protein were separated by SDS-Page and analysed by WB.

2.8. Biotinylation of cell membranes

After the OGD or the control treatment, slices were transferred to a new 6-well culture plate containing 0.3 mg/ml NHS-SS-Biotin in aCSF saturated with 95% air/5% CO_2 and kept on ice under mild shaking for 45 minutes. Slices were then incubated in aCSF with 1 μm Lysine for 45 minutes, in order to block all reactive NHS-SS-Biotin in excess. Afterwards, slices were solubilized in 250 μl of lysis buffer (1% Triton X-100, 0.1% SDS, 1 mM EDTA, 50 mM NaCl, 20 mM Tris, pH 7.5, supplemented with 1 mM DTT, 0.1 mM PMSF and 1 μg/ml CLAP), sonicated (5 x 5sec) and left on ice for 20 minutes, and then centrifuged at 12000 x g for 20 minutes at 4ºC. The supernatants were recovered and stored at -20ºC. Total protein was quantified using the bicinchoninic acid (BCA) assay kit.

To precipitate biotinylated proteins, 40 μl of Ultra-link immobilized neutravidin plus beads were added to 600-900μg of total protein and incubated at 4ºC for 2h with mild shaking (orbital shaker). The beads were washed three times with lysis buffer by centrifugation at 2500 x g for 2 minutes. Proteins were eluted with a 2x concentrated sample buffer, denaturated at 95ºC for 5 minutes and centrifuged into a tube collector with filter. Biotinylated proteins were then separated by sodium dodecyl sulphate-polyacrilamide gels (SDS-Page) and analysed by WB. Likewise, the inputs (total cell extracts without further treatments) were treated with sample buffer and input samples with 50 μg of total protein were separated by SDS-Page and analysed by WB. The biotinylation protocol was adapted from Thomas-Crusells et al. (2003).

2.9. Western Blot

Total cell extracts denaturated with 2x or 5x sample buffer were resolved by SDS-Page on 8-10% sodium dodecyl sulphate (SDS)-polyacrylamide gels and then electrotransferred at 40V onto a PVDF membrane overnight, at 4ºC. Membranes were blocked for 1h at room temperature in Tris-buffered saline (TBS) (in mM: 20 Tris, 137 NaCl) with 0.1% Tween20 (TBS-T) and 5% non-fat dry milk and incubated with the primary antibodies diluted in TBS-T with 0.5% milk solution (anti-phospho-JNK (1:500), anti-phospho-GluR4 (1:500), anti-GluR1 (1:1000), anti-GluR2/3 (1:200), anti-GluR4 (1:500)) for 1h at room temperature or, alternatively, overnight at 4°C. Membranes were washed with TBS-T (4 x 15 minutes) and incubated for 1h at room temperature with anti-rabbit or anti-mouse secondary antibody (1:20.000), diluted in TBS-T with 0.5% milk. Later, membranes were washed (4 x 15 minutes) with TBS-T, incubated with the ECF reagent for 5 minutes and the protein immunoreactive bands visualized by enhanced chemifluorescence (ECF) on a Storm 860 Gel and Blot Imaging System (Amersham Biosciences, Buckinghamshire, UK). The density of the bands on the membranes was analyzed with the ImageQuant 5.0 software.

For reprobing, membranes were stripped with NaOH 0.2 M for 15-20 minutes at room temperature, to eliminate the antibodies and then blocked with TBS-T with 5% non-fat dry milk and incubated with anti-pan-JNK (1:500), anti-α- tubulin (1:750.000), anti-transferrin receptor (1:500) and anti-GluR4 (1:500), as indicated.

2.10. Statistical analysis

Results are presented as means ± S.E.M. of the number of experiments indicated. Statistical significance was assessed by one-way ANOVA analysis followed by the Dunnett's test (or Bonferroni's test, when necessary). Using the GraphPad Prisms Software, when $p<0.05$, the difference was considered statistically significant.

Chapter 3 Results

3.1. Excitotoxic stimulation studies in HEK293 cell lines

3.1.1. Excitotoxic stimulation of GluR4-containing Ca^{2+}-permeable AMPARs induces JNK-mediated GluR4-T855 phosphorylation

Ca^{2+}-permeable AMPA receptors have a crucial role in the selective neuronal death of CA1 hippocampal neurons in rats submitted to transient global ischemia (Noh et al., 2005, Liu et al., 2006). Despite the progressive knowledge towards the identification of the excitotoxic signaling pathways associated to Ca^{2+}-permeable AMPA receptors, the process of delayed hippocampal neurons demise upon global ischemia is still not completely understood. In HEK-GluR4 cells, excitotoxic stimulation of Ca^{2+}-permeable AMPA receptors activates the AP-1 transcription factor (Santos et al., 2006) and activates JNK, a protein kinase which might regulate the activity of the AP-1 transcription factor (Vieira, M., unpublished results). Therefore, we aimed at pinpointing excitotoxic signaling pathways associated to Ca^{2+}-permeable AMPA receptors, namely the contribution of the JNK pathway to cell death.

Recently, a novel JNK phosphorylation site on the GluR4 subunit of AMPARs was described (Thomas et al, 2008), but the functional meaning of this phosphorylation is not yet clear. We observed an increase in the phosphorylation of the GluR4 subunit of AMPA receptors at T855, the residue selectively phosphorylated by JNK, under excitotoxic conditions (Vieira, M., unpublished results). In order to verify whether the increase in GluR4-T855 phosphorylated levels were due to JNK activity, HEK-GluR4 cells were submitted to excitotoxic stimulation with Glu in the presence of CTZ, for 1h, in the presence or absence of two pharmacological JNK inhibitors, CEP1104 (a MLKs inhibitor and, thus, a JNK pathway inhibitor) and SP600125 (a JNK direct inhibitor).

Total cell extracts, used in subsequent experiments, were obtained 2h post-stimulation, a time-point at which JNK is maximally activated and the GluR4-T855 phospho levels are increased (Vieira, M., unpublished data). We analysed JNK activation by Western Blotting with an anti-phospho JNK antibody. Membranes were then reprobed with an antibody for total JNK and for tubulin (Figure 6A). To evaluate the phosphorylation of GluR4-T855 this subunit of AMPARs was immunoprecipitated with an anti-GluR4 antibody and the levels of phospho-GluR4 assessed by Western Blotting with an antibody which recognizes the GluR4 subunit phosphorylated at T855. Two hundred mg of each extract was incubated with either anti-GluR4 IgGs or normal rabbit IgGs as control for unspecific binding. The membranes were then reprobed with an antibody for total GluR4 (Figure 6B). The results showed that when cells were stimulated with Glu in the presence of CTZ, JNK phosphorylation increased, and this effect was abrogated in the presence of the inhibitors (Figure 6A). Likewise, the phosphorylation levels of GluR4-T855 were increased by the excitotoxic stimulation, whereas under conditions that partially inhibited JNK, the phosphorylation of GluR4-T855 was decreased to levels comparable to the control (Figure 6B). These results are in agreement with previous data showing that overactivation of Ca^{2+}-permeable AMPARs in HEK-GluR4 cells can increase GluR4-T855 phosphorylation (Vieira, M., unpublished data), and suggest that GluR4-T855 is a substrate of the JNK pathway under excitotoxic stimulation. However, the evaluation of GluR4-T855 phophorylation by a kinase of the JNK pathway was performed in a single experiment and therefore needs to be confirmed.

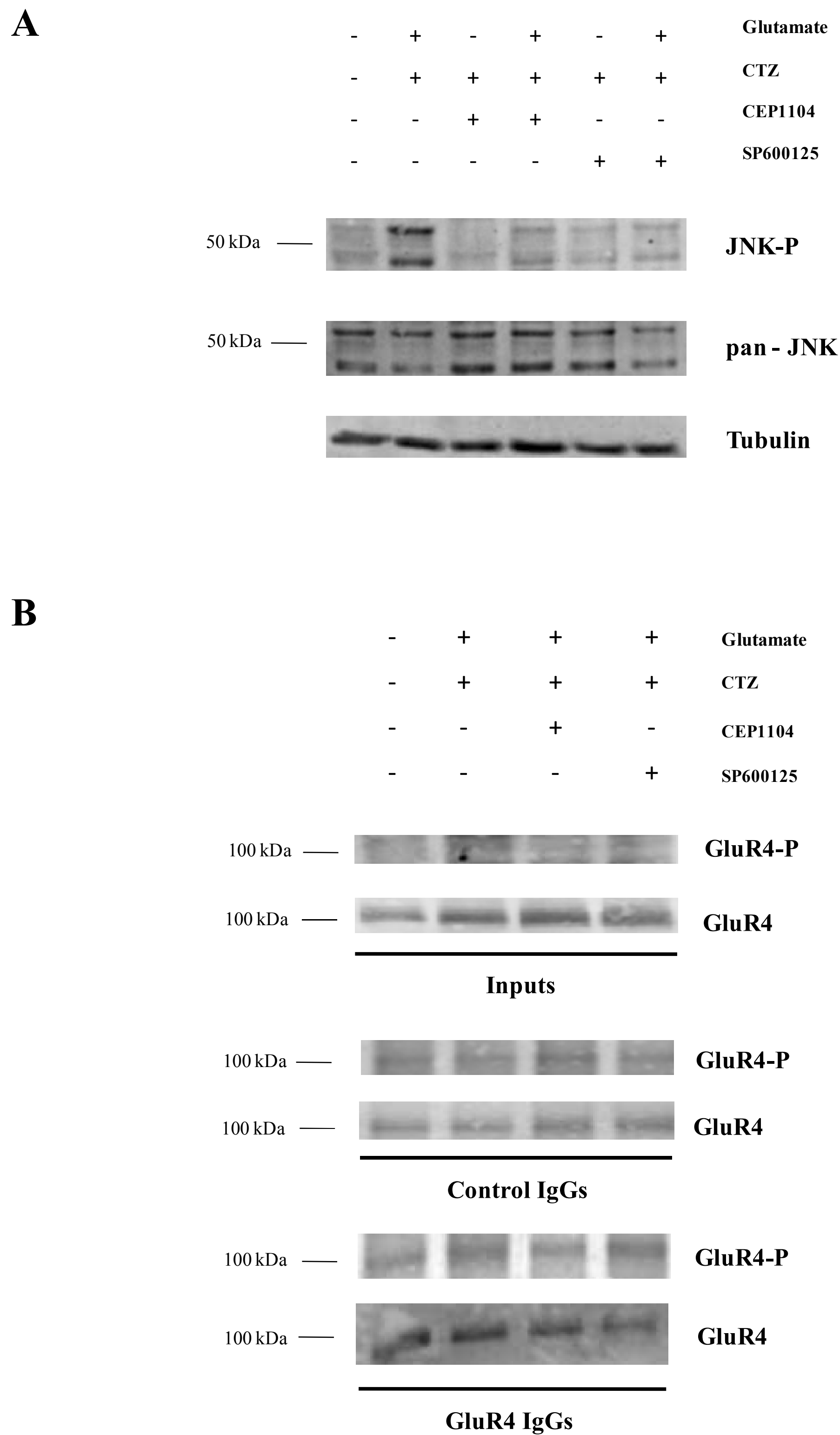


Figure 6. JNK-dependent phosphorylation of GluR4 in HEK-GluR4 cells.

HEK-GluR4 cells were incubated with the MLKs inhibitor CEP1104 (1 µM)

and the JNK direct inhibitor SP600125 (30 μM) for 2h and 1h, respectively, and then stimulated for 1h with 1 mM Glu and 100 μM CTZ (5 minutes pre-incubation with CTZ) in saline buffer (132 mM NaCl, 4 mM KCl, 6 mM glucose, 10 mM HEPES, pH 7.4) containing 2.5 mM Ca^{2+}. Control cells were incubated in saline buffer without drugs. After stimulation, cells were washed and transferred to fresh culture medium and kept in the humidified incubator chamber. Total cell extracts were prepared 2h after stimulation. **(A)** The JNK pharmacological inhibitors blocked the increase in JNK activation induced by the excitotoxic stimulation of Ca^{2+}-permeable AMPARs. Sixty μg of total protein were used in Western blot analysis. Membranes were reprobed with anti-pan-JNK and anti-tubulin antibodies. The figure is representative of three assays performed with extracts of independent experiments. **(B)** The JNK pharmacological inhibitors blocked the increase in phospho-GluR4 (T855) levels induced by the excitotoxic stimulation of Ca^{2+}-permeable AMPARs. Two hundred μg of total protein was used to immunoprecipitate GluR4 using an anti-GluR4 antibody. The immunoprecipitated samples, as well as 60 μg of the total protein from cell extracts (inputs) were used for Western Blot analysis with an anti-phospho-GluR4 (T855) antibody. Membranes were reprobed with an anti-GluR4 antibody.

3.1.2. Phosphorylation of GluR4-T855 does not contribute to excitotoxic cell death triggered by overactivation of GluR4-containing Ca^{2+}-permeable AMPA receptors

In order to address the possible contribution of GluR4-T855 phosphorylation to cell death mediated by GluR4-containing Ca^{2+}-permeable AMPA receptors, we

transduced HEK293-A cells with wild-type GluR4 or with the GluR4 mutants GluR4-T855A, which contains an alanine residue at the phosphorylation site, thus rendering the subunit non-phosphorylable, and GluR4-T855D, that contains an aspartate residue at the phosphorylation site to mimic permanent phosphorylation. Forty hours later, cells were submitted to excitotoxic stimulation with Glu in the presence of CTZ and cell viability was assessed by the MTT test, performed 24h after stimulation. We observed that cells transfected with wild-type GluR4 showed a significant decrease in cell viability following stimulation with Glu in the presence of CTZ, when compared to control cells. However, both the cells containing the GluR4-T855A or the GluR4-T855D mutants were also susceptible to excitotoxic conditions, showing a similar viability to that of cells containing the wild-type GluR4 subunit (Figure 7). In fact, the statistical analysis using the Bonferroni Multiple Comparison test showed no significant differences between the cell viability of HEK293-A cells transduced with the different GluR4 subunits. Therefore, these results suggest that despite the increase in the phosphorylation levels of GluR4-T855 under excitotoxic conditions, this phosphorylation does not contribute to GluR4-containing Ca^{2+}-permeable AMPA receptor-mediated cell death in HEK293-A cells.

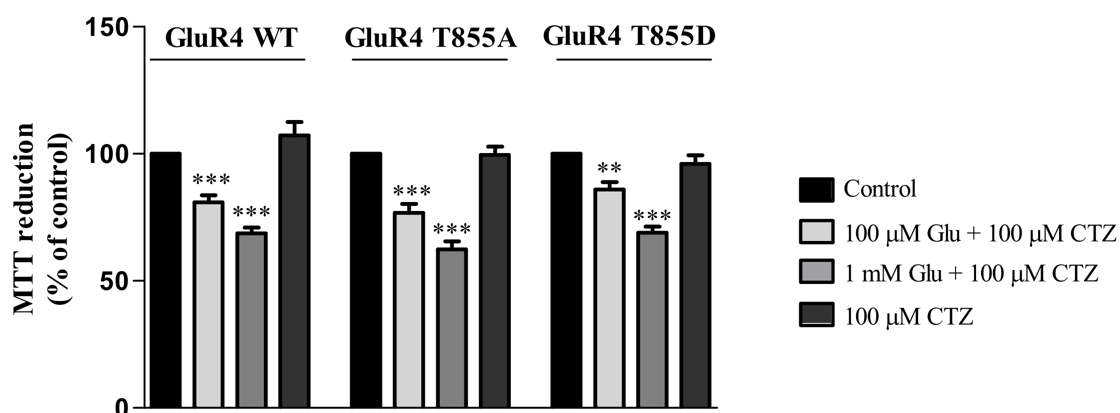


Figure 7. Phosphorylation of the GluR4 subunit at T855 does not contribute to cell death triggered upon overactivation of Ca^{2+}-permeable

GluR4-containing AMPARs. HEK293-A cultures were transduced with GluR4 wild-type, GluR4-T855A, a non-phosphorylatable mutant, or GluR4-T855D, a phosphomimetic mutant, using Lipofectamine 2000. Forty hours after transfection, the cultures were stimulated with toxic concentrations of glutamate and CTZ for 1h, in sodium buffer at 37°C. Cells were pre-incubated for 5 minutes with CTZ. Transfected control cells were incubated in saline buffer without drugs. After stimulation, cells were washed and returned to the incubator in fresh serum- and antibiotic-containing culture medium. Cell viability was determined by the MTT assay 24h after stimulation. Bars represent the mean ± SEM from 6-11 independent experiments (Significantly different from the control, $^{**}p<0.01$, $^{***}p<0.001$).

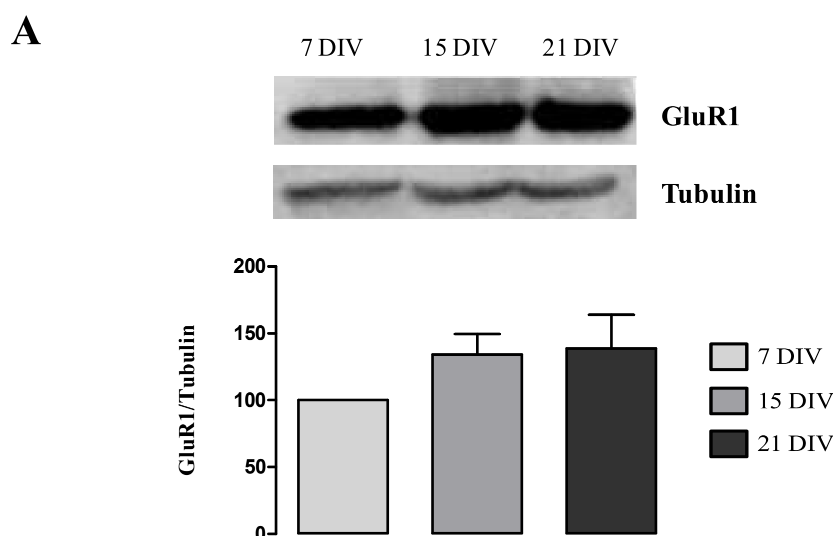
3.2. Excitotoxic stimulation studies in hippocampal neurons

3.2.1. AMPA receptors in primary cultures of hippocampal neurons: abundance of the various subunits at different maturation stages

In order to investigate the excitotoxic mechanisms coupled to Ca^{2+}-permeable AMPA receptors in neuronal cells, we pursued our studies in primary cultures of hippocampal neurons challenged by OGD, an *in vitro* model for cerebral ischemia.

AMPA receptors are homo- or heteromeric assemblies of four subunits, GluR1-4, which can be expressed differently. For instance, whereas GluR1, 2 and 3 mRNAs are largely expressed throughout the CNS, GluR4 is expressed in a restricted spatial and temporal manner (Santos et al., 2008). Therefore, AMPA receptors can have a distinct subunit composition at different maturation stages, which influences the receptor

properties along development. In order to understand how the protein levels of these subunits may vary with the age of the culture, we performed a time-course analysis of the GluR1, GluR2/3 and GluR4 protein patterns in primary hippocampal cultures with 7, 15 and 21 DIV. Total cell extracts obtained at the indicated times were used in immunoblot analysis with anti-GluR1, anti-GluR2/3 and anti-GluR4 antibodies. The total protein loading control was assessed by reprobing the membranes for tubulin. According to our results, GluR1 increased with the age of the culture, with maximal expression levels at 21 DIV (Figure 8A). The contrary occurred with GluR2/3, which had its maximal expression levels at 15 DIV, whereas at 21 DIV their levels abruptly decreased (Figure 8B). As for GluR4, this subunit is less abundant than the others in hippocampal neurons (Santos et al., 2008), which difficults the analysis of its protein level. However, our results suggest that the GluR4 protein level reaches a maximum at 15 DIV and then decreases to lower levels at 21 DIV (Figure 8C). The results show that there is a differential expression of the different subunits during development of the cultures, although not statistically significant. In cultures with 15 DIV, the three subunits are more expressed than at 7 DIV, but in older cultures, only GluR1 remains highly expressed, with GluR2 and GluR4 becoming less abundant. Thus, to pursue our studies we decided to use cultures with 15 DIV.



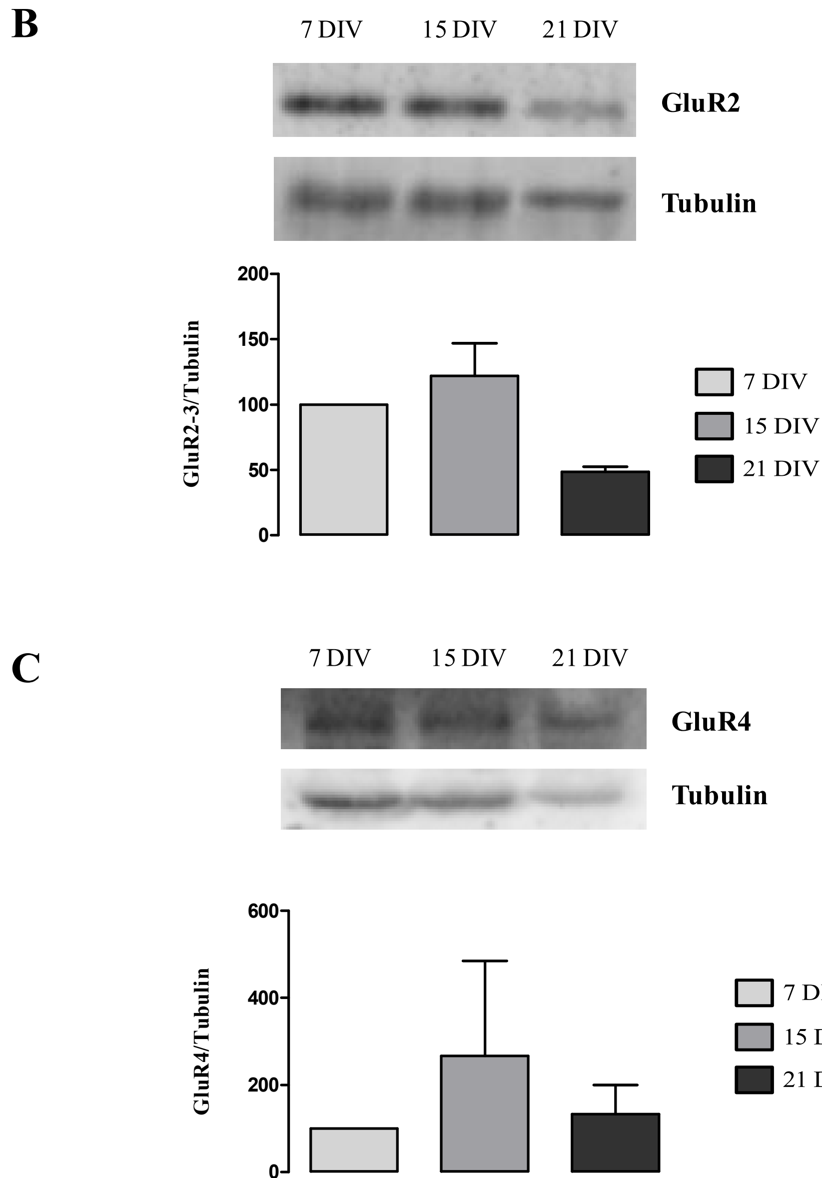


Figure 8. Abundance of the AMPAR subunits in hippocampal neuronal cultures at different maturation stages. Total cell extracts were obtained from hippocampal cultures at 7, 15 and 21 DIV. Sixty µg of total protein was used for immunoblot analysis using anti- GluR1, anti-GluR2/3 and anti-GluR4 antibodies, in order to evaluate the protein level of these subunits. Membranes were reprobed with an anti-tubulin antibody. **(A)** The upper panel shows expression levels of GluR1 at 7, 15 and 21 DIV. The graphic shows the quantification of the ratio of the immunoreactive bands of GluR1 and tubulin. **(B)** The upper panel shows expression levels of GluR2/3 at 7,

15 and 21 DIV. The graphic shows the quantification of the ratio of the immunoreactive bands of GluR2/3 and tubulin. **(C)** The upper panel shows expression levels of GluR4 at 7, 15 and 21 DIV. The graphic shows the quantification of the ratio of the immunoreactive bands of GluR4 and tubulin. The ratio between the different subunits and the levels of tubulin were normalized to the levels of the respective subunits at 7 DIV. Bars represent the mean ± SEM of 3-4 independent experiments.

3.2.2. OGD-induced cell death in mature cultures of hippocampal neurons

Oxygen-glucose deprivation (OGD) is an established *in vitro* model for transient global ischemia. Neurons subjected to this type of insult are lead towards cell death. It has been shown that OGD causes neuronal damage by disturbing AMPA receptors, for instance, but despite the emerging knowledge on this matter, the process through which neurons are killed remains to be elucidated. Our first goal was to establish the period of challenge with OGD and then to characterize the contribution of the ionotropic glutamate receptors to the OGD-induced neuronal death in cultures of hippocampal neurons. As such, hippocampal neurons (14-15 DIV) were exposed to different times of OGD and cell metabolic activity was assessed 24h after the insult by the MTT test.

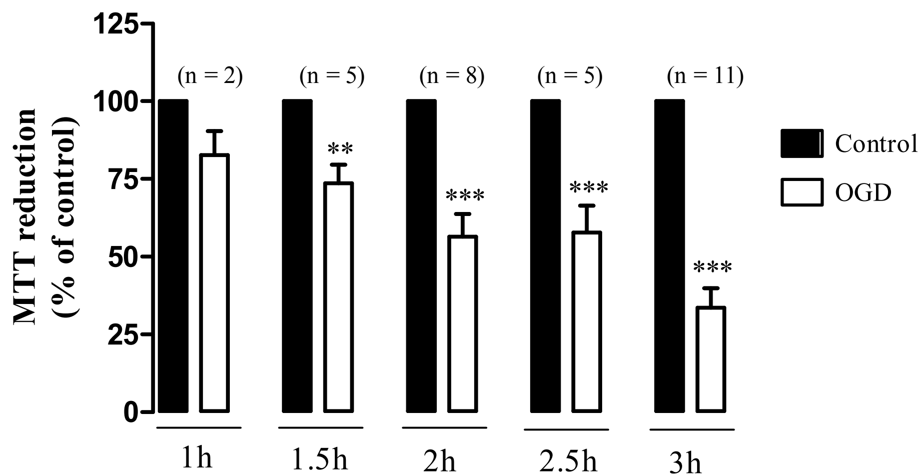
The results show that when OGD was extended to 1.5h or more, neurons presented an increasing level of cell death. Concretely, a 1.5h insult decreased the cell viability by 30%, whereas 2-2.5h of OGD corresponded to a decrease of 40% and a 3h challenge reduced cell viability by almost 60% (Figure 9A).

To assess solely the effect of glucose withdrawal in the cultures, neurons were kept in a glucose-free saline buffer, in the presence of oxygen, for a period

corresponding to the longest time of the OGD challenge (3h). We observed that hippocampal neurons maintained in aerobic conditions, but incubated in saline buffer without glucose, reduced MTT to a similar level than control neurons kept in a glucose-containing saline buffer, suggesting that the absence of glucose for a period of 3h does not reduce the metabolic activity (Figure 9B).

Our results showed that prolonged times of OGD significantly affect cell viability in primary hippocampal neurons, which is in accordance with previous studies (Aarts et al., 2003), but glucose deprivation for up to 3h is not enough to increase neuronal death by itself.

A



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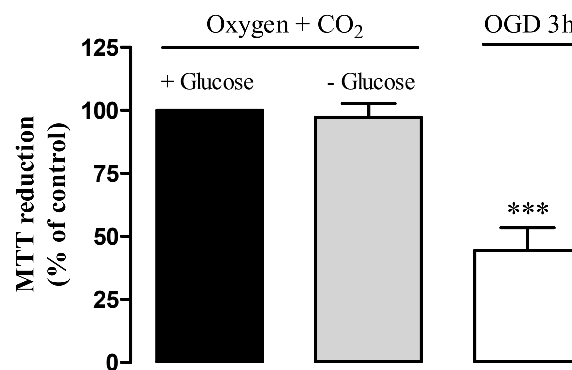
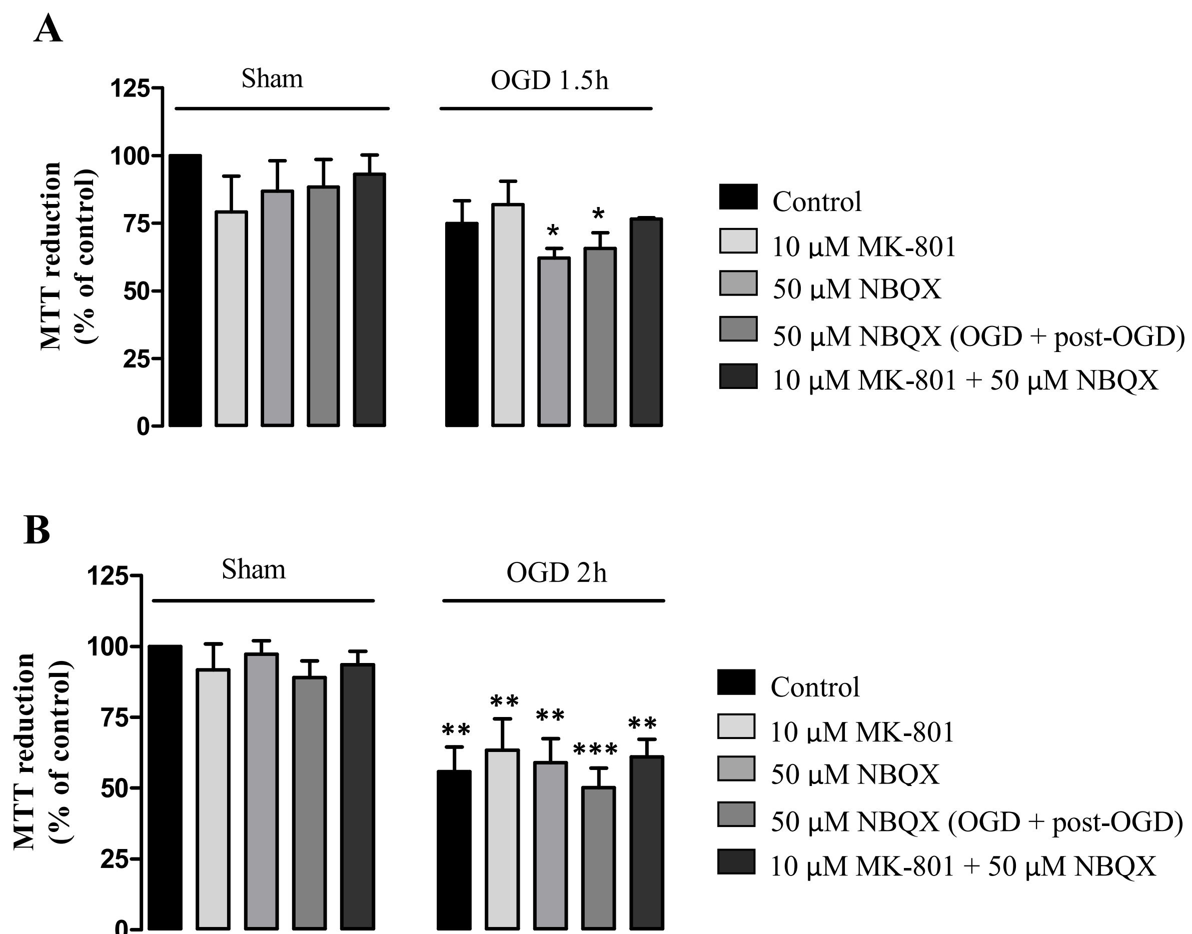


Figure 9. OGD induces hippocampal neuronal death. For the OGD challenge, mature cultures of hippocampal neurons (14-15 DIV) were placed in a glucose-free saline buffer (in mM: 116 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1 NaH_2PO_4, 1.8 $CaCl_2$, 26 $NaHCO_3$) and introduced in a chamber with an anaerobic atmosphere for the indicated times at 37ºC. Then, the cultures were transferred to the conditioned medium and returned to the air/CO_2 incubator. Control neurons were maintained in a saline buffer supplemented with 10 mM glucose in the air/CO_2 incubator for a period of time equivalent to the OGD-challenge. Cell viability was determined by the MTT assay 24h after OGD. **(A)** Time course analysis of OGD-induced hippocampal neuronal death. Cell viability decreases with longer periods of OGD stimulation. Bars represent the mean ± SEM of the indicated number of experiments (Significantly different from the control, $^{**}p<0.01$, $^{***}p<0.001$). **(B)** Effect of glucose deprivation in hippocampal neurons viability. Neurons were deprived of glucose, but not oxygen, for the longest time of the OGD stimulus (3h). Bars represent the mean ± SEM of five independent experiments (Significantly different from the control, $^{***}p< 0.001$).

3.2.3. Effect of the ionotropic glutamate antagonists on the viability of hippocampal neurons challenged with OGD

Excitotoxic neuronal death can be prevented by treating neurons with MK-801 and NBQX, antagonists of NMDA and AMPA/kainate glutamate receptors (Araújo et al., 2003, Ogita et al., 2003). To investigate whether blockade of glutamate receptors would be neuroprotective to OGD-insulted hippocampal neurons, mature hippocampal cultures (14-15 DIV) were subjected to OGD in the absence or in the presence of the

glutamate receptor antagonists MK-801 and NBQX, and neuronal viability was assessed by the MTT test 24h after the insult. We did not observe a neuroprotective effect from the glutamate receptor antagonists, not even when 10 μM MK-801 and 50 μM NBQX were present in combination, for any of the OGD periods evaluated (Figure 10A-C). Moreover, in order to prevent the possible activation of AMPA/kainate receptors that could take place after the OGD stimulation, we additionally included 50 μM NBQX in the conditioned medium during the recovery period. However, this treatment showed also to be ineffective in providing neuroprotection. Interestingly, these results suggest that in hippocampal cultures with 14-15 DIV, OGD-triggered alterations in cell viability, as assessed by the MTT test, cannot be blocked by previous application of ionotropic glutamate receptors antagonists.



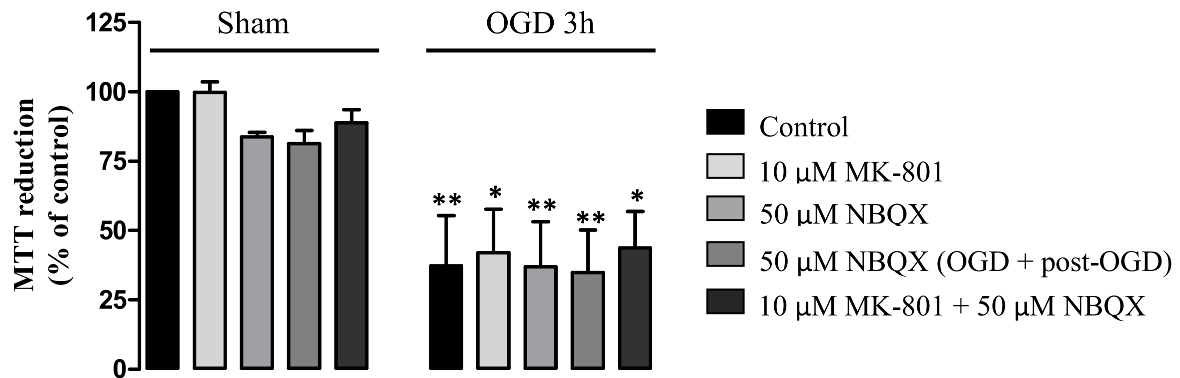


Figure 10. Effect of glutamate receptors antagonists in the MTT reduction by cultured hippocampal neurons subjected to different times of OGD. The NMDAR antagonist MK-801 and the AMPA/KA receptor antagonist NBQX were applied to cultured hippocampal neurons with 14-15 DIV, 20 minutes before the OGD stimulus. After the stimulus, neurons were washed, transferred to the conditioned medium and returned to the air/CO_2 humidified incubator. When indicated, 50 µM NBQX was additionally included in the conditioned medium during the recovery period. In sham experiments, neurons were incubated in the absence (control neurons) or in the presence of the glutamate receptor antagonists in saline buffer supplemented with 10 mM glucose and remained in the air/CO_2 incubator for a period of time equivalent to the OGD-challenge. Cell viability was determined by the MTT assay 24h after the OGD stimulus. The graphics represent the effect of the antagonists in cell viability of cultured hippocampal neurons subjected to 1.5h (A), 2h (B) and 3h (C) OGD challenge. Bars represent the mean ± SEM of 3-4 independent experiments (Significantly different from the sham control, ** p< 0.01, *** p<0.001).

Additionally, cell viability of hippocampal neurons challenged with OGD was investigated by analysis of the nuclear morphology. We compared the possible neuroprotective effect of the glutamate receptor antagonists against the OGD challenge in young (8 DIV) or mature (15 DIV) hippocampal cultures. For that purpose, hippocampal cultures were subjected to a 2.5 h OGD-challenge in the absence or in the presence of MK-801 and NBQX. Sixteen hours after the challenge, cells were washed and stained with the fluorescent dye Hoescht 33342 (1 μg/ml), fixed in 4% paraformaldehyde for 15 minutes at room temperature and then examined with a Zeiss Axiovert 200 fluorescence microscope (63× objective). The results obtained for hippocampal cultures at 15 DIV showed a protective effect only when the antagonist NBQX (20 μM) was present during the OGD insult (Figure 11A), whereas MK-801 (10 μM) was ineffective in inhibiting neuronal death. However, when younger cultures (8 DIV) were challenged in the presence of the antagonists, both MK-801 and NBQX inhibited cell death to values similar to those obtained in sham control cells (Figure 11B). Taken together, these results suggest that the protective effect of the glutamate receptor antagonists is dependent on the age of the culture, since they effectively protected neurons from OGD-induced damage in cultures at 8 DIV, pointing a contribution of both NMDA and AMPA/KA receptors to the excitotoxic response, but not at 15 DIV, in which only NBQX prevented changes in the nuclear morphology. This suggests that, in mature cultures, the AMPA/KA receptors are the main ionotropic glutamate receptors responsible for the OGD-induced excitotoxicity.

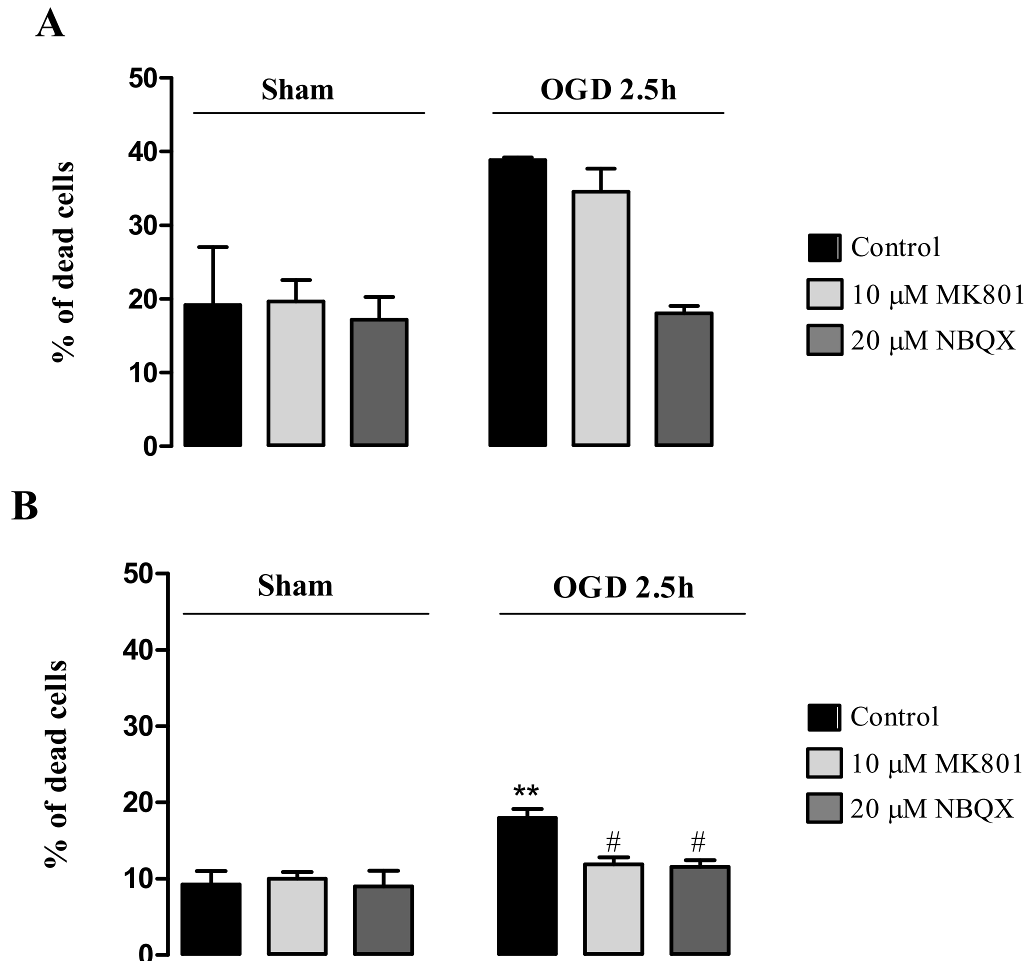


Figure 11. Effect of glutamate receptors antagonists on the nuclear morphology alterations of hippocampal neurons subjected to 2.5h OGD. The NMDAR antagonist MK-801 and the AMPA/KA receptor antagonist NBQX were applied to cultured hippocampal neurons with 14-15 DIV, 20 minutes before the OGD stimulus. After the stimulus, neurons were washed, transferred to the conditioned medium and returned to the air/CO_2 humidified incubator. In sham experiments, neurons were incubated in the absence (control neurons) or in the presence of the glutamate receptor antagonists in saline buffer supplemented with 10 mM glucose and remained in the air/CO_2 incubator for a period of time equivalent to the OGD-challenge. Approximately 16h after the treatment, cells were stained with the nuclear dye Hoescht 33342 (1 µg/ml), fixed with 4% paraformaldehyde for

15 minutes and examined under a fluorescence microscope. Cell death is expressed as the percentage of dead cells relatively to the total cell number. **(A)** Cell death in hippocampal cultures at 15 DIV is not reverted in the presence of NBQX, but not MK801. **(B)** Cell death in hippocampal cultures at 8 DIV is prevented in the presence of the glutamate receptor antagonists, NBQX and MK801. Bars represent the mean ± SEM of three independent experiments (*Significantly different from the sham control, **$p<0.01$, [#]Significantly different from the OGD control, #$p<0.05$).

3.2.4. OGD induces a rapid removal of GluR2 from the surface of hippocampal slices

Recently, Liu and colleagues (2006) showed that OGD primes a decrease in the GluR2 content at the synapse, thus enhancing removal of Ca^{2+}-impermeable AMPA receptors and insertion of Ca^{2+}-permeable AMPARs in the cell membrane of cultured hippocampal neurons. To further confirm whether neuronal insults affect subunit composition of synaptic AMPARs, we subjected 350 μm thick hippocampal slices (8-week Wistar rats) to 45 minutes OGD, a relatively mild OGD stimulus. Immediately after the stimulus, biotinylation of membrane-associated proteins was performed. Nine hundred μg of the total protein were used to precipitate biotinylated proteins with Ultra-link immobilized neutravidin plus beads. Biotinylated proteins and input samples (50 μg of total protein from cell extracts without further treatments) were separated by SDS-PAGE and analysed by WB, using an anti-GluR2/3 antibody.

A preliminary result shows that mild OGD reduces surface GluR2 levels but not total cellular abundance of the protein in hippocampal slices (Figure 12). This finding is consistent with that obtained by Liu et al. (2006) in hippocampal cultures, thus

validating slices as a suitable model for the investigation of OGD-induced alterations in surface AMPAR subunit composition possibly via regulated receptor trafficking. However, additional experiments are required to confirm this result.

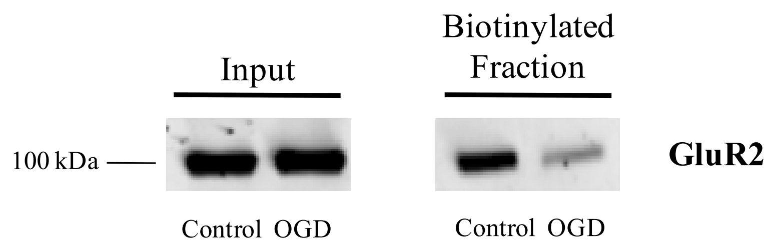


Figure 12. OGD decreases GluR2 surface expression in hippocampal slices. Slices were obtained from adult male Wistar rats and separated in OGD and control groups. For the OGD treatment, slices were transferred to glucose-free saline buffer (in mM: 116 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1 NaH_2PO_4, 1.8 $CaCl_2$, 26 $NaHCO_3$) and inserted in the anaerobic chamber for 45 minutes at 37ºC. Control slices were transferred to glucose-containing saline buffer (10 mM) and incubated at 37ºC in the 95% air/5% CO_2 humidified incubator for the same time as the OGD-treated slices. After the OGD challenge, slices were used to perform biotinylation of cell membrane-associated proteins for analysis of the surface expression of the GluR2 subunit. Mild OGD does not alter total GluR2 protein expression, but reduces the subunit levels at the cell surface.

Chapter 4 Discussion

Ca^{2+}-permeable AMPARs have been increasingly implicated in excitotoxic phenomena associated with neurodegenerative diseases and acute injuries such as ischemic stroke, a leading cause of death and long-term disability in the Western society (American Stroke Association, www.strokeassociation.org; Heart and Stroke Foundation, www.heartandstroke.ca; The Stroke Association, www.stroke.org.uk). Several evidences favor Ca^{2+}-permeable AMPARs over NMDA receptors regarding their contribution to selective neurodegeneration. It has been demonstrated that after transient cerebral global ischemia there is a selective upregulation of Ca^{2+}-permeable AMPA receptors in vulnerable neurons, which primes these cells towards death (Pellegrini-Giampietro et al., 1992; Liu et al., 2006; Dixon et al., 2009). This reinforced our interest in studying the role of Ca^{2+}-permeable AMPARs in cell death triggered by excitotoxic stimuli. A better understanding of the pathophysiology of stroke, in particular the molecular signaling events in neurons has become critical to the development of efficient treatments (Aarts & Tymianski, 2005). Given that previous data from our laboratory showed that overactivation of Ca^{2+}-permeable AMPARs lead to JNK activation and to an increase of the GluR4-T855 phosphorylation in HEK-GluR4 cells (Vieira, M., unpublished observations), we investigated whether this particular phenomenon would be contributing to the excitotoxicity-mediated mechanism of death. Our results confirmed the JNK pathway-dependent phosphorylation of GluR4-T855 under excitotoxic conditions, but not a contribution of this occurrence to the cell death mechanism.

In this work, we also used hippocampal neurons, a neuronal population known to be particularly susceptible to Ca^{2+}-permeable AMPAR-mediated excitotoxicity, to further characterize the mechanism of cell death associated to these receptors. For that purpose, hippocampal neurons were challenged by OGD, an established *in vitro* model

for transient global ischemia. We observed that insulted neurons displayed a decrease in the MTT reduction, suggesting a decrease in the cell metabolic activity and energetic status, which is further lower with longer periods of OGD. Also, the results obtained show that the OGD-induced cell death can be reverted by application of both glutamate receptor antagonists in older, but not younger cultures, suggesting that the cell death mechanisms might vary with neuronal development. Moreover, we observed that, for a mild OGD stimulus, the levels of surface GluR2 decreased when hippocampal slices were submitted to the challenge, a result consistent with previous observations in cultured hippocampal neurons.

4.1. The phosphorylation of GluR4-T855 by JNK does not contribute to GluR4-containing AMPAR-mediated excitotoxic cell death

Recently, it was shown that GluR4 can be a JNK substrate under physiologic conditions (Thomas et al., 2008). In this work, the authors observed phosphorylation of Thr855 on GluR4 subunits both in GluR4-expressing HEK293 cells stimulated with sorbitol (used to activate JNK and other stress-related kinases) and in cortical neurons under physiologic conditions. The functional meaning of this occurrence, however, remains to be clarified. In our lab we observed that overactivation of Ca^{2+}-permeable AMPARs induced JNK activation and a significant increase in the phosphorylation levels of GluR4-T855. This was observed when HEK-GluR4 cells were stimulated with Glu in the presence of CTZ, at a time point coincident with maximal JNK activation (Vieira, M., unpublished data). Thomas et al. (2008) also demonstrated GluR2L-T912 as another JNK substrate, and showed that this phosphorylation site plays an important role in the regulation of AMPAR trafficking following changes in neuronal activity.

Given this evidence, we hypothesized that excitotoxicity-induced JNK-dependent phosphorylation of GluR4 could have a similar functional role, setting up a signal for increased insertion of GluR4-containing AMPAR receptors in the membrane, which might enhance Ca^{2+} influx and cell vulnerability to excitotoxic damage in HEK-GluR4 cells. The increase in GluR4-T855 phosphorylation mediated by overactivation of GluR4-containing Ca^{2+}-permeable AMPARs correlated with maximal JNK activation, and it is possibly due to JNK activity since selective JNK signaling pathway inhibitors decreased the phosphorylation signal (Figure 6). Thus, we investigated whether this phosphorylation could be involved in the cell death mechanism. HEK293A cells expressing the GluR4-T855 mutants, GluR4-T855A or GluR4-T855D, did not show different cell viability compared to that of the control cells, expressing the wild-type form of GluR4, upon excitotoxic stimulation (Figure 7). Taking together the results obtained by Vieira (Vieira, M., unpublished observations) and those presented in this work, we conclude that excitotoxic stimulation leads to the activation of JNK and to an increase in GluR4-T855 phosphorylation, but apparently phospho-GluR4-T855 is not involved in the death mechanism mediated by Ca^{2+}-permeable AMPARs.

4.2. OGD induces cell death in hippocampal neurons

To mimic brain ischemia conditions, several experimental models have been employed, such as middle carotid artery occlusion (MCAO), for *in vivo* studies, or oxygen-glucose deprivation (OGD), an *in vitro* model of global ischemia. Dissociated primary cultures (Rothman, 1984, Liu et al., 2004), acute hippocampal slice preparations (Dong et al., 1988) and organotypic hippocampal slice cultures (Rytter et al., 2003, Bonde et al., 2005) have been typically used in OGD studies. Ischemic cell

death *in vivo* is characterized by a selective and delayed neuronal damage in the hippocampus (Kirino, 2000), a feature that has also been observed in OGD studies, showing the suitability of this model to assess ischemia-induced cell death *in vitro* (Rytter et al., 2003, Liu et al., 2004, Bonde et al., 2005). Our first goal was to establish the period of challenge with OGD. Mature hippocampal cultures (14-15 DIV) were submitted to different times of OGD and neuronal viability was assessed 24h after the insult by the MTT test. Our results are consistent with previous studies, showing that OGD induces a decrease in cell viability in hippocampal cultures, and the extent of the lesion depended on the intensity of the OGD exposure (Figure 9A). Moreover, when OGD was extended to 1.5h, cell viability is decreased by 30%, whereas 2-2.5h of OGD corresponded to a 40% decrease and a 3h challenge reduced the energetic status of the cells by almost 60% (Figure 9A). As such, 2-2.5h of OGD might be the appropriated time of challenge to pursue further studies in hippocampal cultures.

We also observed that the MTT reduction in mature hippocampal cultures subjected to glucose withdrawal for a period corresponding to the longest time of the OGD challenge (3h) was similar to that observed in control neurons, kept in a glucose-containing saline buffer. This observation suggests that the absence of glucose is not enough to increase neuronal death by itself, at least for a period of 3h (Figure 9B), a result that is much intriguing, considering that glucose oxidation is thought to fuel most activity-associated energy metabolism in neurons. Neuronal survival in glucose-free media may be due to amino acid metabolism, a process that produces common metabolic intermediates, many of them part of, or linked to, the tricarboxylic acid (TCA) cycle. For instance, glutamate is an intermediate in the metabolism of glucose and amino acids. Serum glucose, through glycolysis and the TCA cycle, is converted to α-ketoglutarate which, through a transamination reaction, becomes converted into

glutamate (Figure 13). The amino acids alanine and aspartate provide the amino group to α-ketoglutarate, thus participating in the synthesis of glutamate. In these reactions, the TCA cycle intermediates pyruvate and oxaloacetate are also formed from alanine and aspartate, respectively. At this point, the TCA cycle is replenished with two important intermediates and the energetic metabolic reactions are allowed to continue. Moreover, the transamination reaction through which glutamate was formed can work in reverse, thus providing α-ketoglutarate to the TCA cycle. In fact, glutamate is continuously reconverted to α-ketoglutarate and metabolized through the TCA cycle in the brain (Hassel & Dingledine, in *Basic Neurochemistry,* 7th edition, 2004). Therefore, amino acids can replenish the TCA cycle with important intermediates, allowing the energetic reactions to continue in the cell (Hassel & Dingledine, in *Basic Neurochemistry,* 7th edition, 2004) (Figure 13).

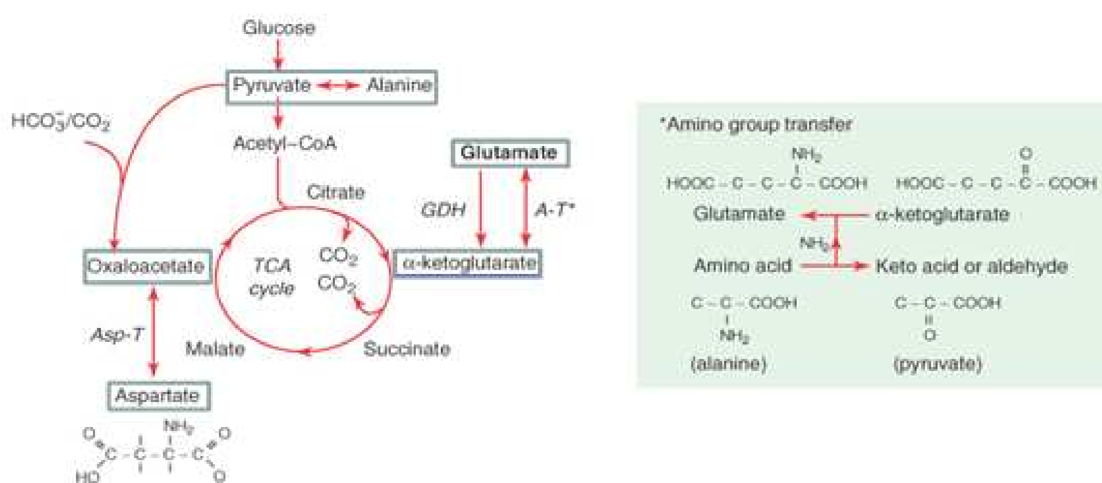


Figure 13. Formation of glutamate from α-ketoglutarate, a TCA cycle intermediate. α-Ketoglutarate is formed from glucose through glycolisys and the TCA cycle, and constitutes the carbon backbone of glutamate. The amino group, also required for the formation of glutamate, derives from another amino acid. The amino group donor can be aspartate, alanine or

other amino acids. After donation of its amino group, the donor becomes a keto acid or an aldeyde, such as pyruvate and oxaloacetate (as shown in the figure), both of which are TCA cycle intermediates. *Asp-T*, aspartate aminotransferase; *A-T*, aminotransferase reaction (Asp-T or other); *GDH*, glutamate dehydrogenase, *TCA cycle*, tricarboxylic acid cycle (adapted from Hassel & Dingledine, in *Basic Neurochemistry,* 7th edition, 2004).

4.3. Effect of the glutamate receptor antagonists against OGD-induced damage in young and mature cultures of hippocampal neurons

The lack of efficiency of a vast array of therapeutics in clinical trials contributed to the seeking of the understanding of neuronal cell death mechanisms and neurodegeneration. In this regard, we explored the mechanisms of neuronal death using the established model of *in vitro* ischemic injury, the oxygen-glucose deprivation (OGD) challenge and investigated the contribution of the different ionotropic glutamate receptors to cell death in cultures of hippocampal neurons submitted to OGD. We used two different approaches to evaluate cell viability in the presence of glutamate receptor antagonists in hippocampal cultures subjected to OGD. Although the MTT test provides information on the metabolic activity of the insulted neurons, it is generally accepted that a decrease in the MTT reduction relates with decreases in cell viability (Schelman et al., 2004, Araújo et al., 2003). Furthermore, this viability assay was complemented by the analysis of the nuclear morphology of the hippocampal neurons, which is a direct method to assess a parameter of cell death. The MTT test, performed 24h after the OGD stimuli in cultures with 14-15 DIV, showed no protective effect of the antagonists at any of the different OGD stimuli applied (Figure 10). However, the analysis of the nuclear morphology in cells stained with the DNA dye Hoescht 33342, showed an increase of

cell viability in neurons challenged by OGD for 2.5h in the presence of NBQX at 14 DIV (Figure 11A), and both MK-801 and NBQX protected neurons from this ischemic insult in cultures with 8 DIV (Figure 11B). These results can be due to several facts, mainly regarding the fundaments of the techniques used or differences in cell physiology of the cultures at a different age, as discussed below.

4.3.1. The MTT test *versus* analysis of nuclear morphology

The MTT-based colorimetric assay has been described as a sensitive, convenient, rapid and economical method for the measurement of *in vitro* citotoxicity and cell proliferation. This assay depends both on the number of cells present and on the energetic activity per cell. Also the sensitivity of cell types is relevant, since while some cell types give similar results, others may have a reduced sensitivity with MTT (Wan et al., 1994). The MTT test has its fundament on the metabolic conditions of the cell, since it is reported that both mitochondrial and cytoplasmatic dehydrogenases within viable cells are able to reduce the tetrazolium salt MTT into a blue coloured product (formazan). The pyridine nucleotide cofactors NADH and NADPH are involved in this redox reaction. The concentration of formazan will be proportional to the amount of metabolically viable cells present.

OGD can induce excessive formation of ROS by multiple mechanisms (Aarts et al., 2003). Thus, considering that these will alter the redox state of the cells, it is possible that the OGD-induced oxidative environment of the cell might disturb MTT reduction.

On the other hand, the analysis of the nuclear morphology of insulted neurons allow for a direct evaluation of cell viability. Apoptotic nuclei are hyperfluorescent,

condensed or fragmented, and smaller compared to normal nuclei (Bonfoco et al., 1997). However, detection of cell death based only on morphological criteria has its own limitations, since different cell types can have a different appearance in response to cell death stimuli or due to the fact that cells can condense and detach from plates when dying, thus limiting interpretation of the observations. However, the morphological analysis might detect differences that could be masked in the MTT test. This could explain why we observed no effect of the antagonists when cell viability was analyzed by the MTT assay, whereas analysis of nuclear condensation showed that at least NBQX protects cells from the OGD stimulus, in cultures with 14 DIV. Other approaches to address this problem would include assessment of cell viability by different methodologies, such as LDH release or analysis of the activation of specific proteases, such as calpains, calcium-activated neutral proteases known to participate in excitotoxic neuronal death (Saido et al., 1994; Brorson et al., 1995), including upon an OGD stimulus (Newcomb-Fernandez et al., 2001, Malagelada et al., 2005), and in several neurodegenerative conditions (Chan and Mattson, 1999).

4.3.2. Cell physiology of young and mature cultures: subunit composition of glutamate receptors

According to the results obtained by analysis of the nuclear morphology, in hippocampal neuronal cultures with 8 DIV, both NMDA and AMPA/KA receptors contribute to the excitotoxic response, whereas in mature cultures (14-15 DIV) only the AMPA/KA receptors are the main ionotropic glutamate receptors responsible for the OGD-induced excitotoxicity. This differential effect of glutamate receptor antagonists in blocking cell death might concern receptor subunit composition between young and

mature cultures, which has been proposed to underlie different brain susceptibility to excitotoxicity, as discussed below.

4.3.2.1. Developmental changes in subunit composition of NMDA receptors

In the adult rat hippocampus, NMDARs are constituted of NR1 subunits and at least one type of NR2 subunits. During development, a change in the composition of the NMDARs occurs and NR1/NR2B receptors are predominantly substituted by NR1/NR2A receptors (Liu, X. B. et al., 2004). It has been proposed that NMDAR can be differently linked to specific intracellular pathways and thus to different physiologic and pathological processes depending on whether they possess NR2A or NR2B subunits (Sattler & Tymiansky, 2001, Kim et al., 2005).

A study from Zhou and Baudry (2006) on the role of NMDARs in excitotoxicity has shown that slices of young, but not adult, rat hippocampus present high calpain activation and other cell death marks, indicating that changes in the mechanisms of NMDA-mediated neurotoxicity take place during the postnatal period. Moreover, the authors observed that excitotoxicity-mediated damage in slices from young rats was completely prevented when NR2B-containing receptors were selectively blocked, whereas NR2A-containing receptor antagonists had no effect on the slices. As such, the authors proposed that the switch in NMDA receptors from high NR2B and low NR2A expression at the first postnatal days to increased expression of NR1 and NR2A during postnatal development (Liu, X.B. et al., 2004) may be the reason why slices from young rat hippocampus are much more susceptible to excitotoxicity than slices from mature rat hippocampus. Curiously, in the same study, OGD treatment elicited a similar degree of

neurotoxicity in hippocampal slices from rats of different ages. However, the mechanisms by which OGD induces neurotoxicity are apparently different between young and old rats, since MK-801 could only partly block toxicity in slices from young rats, but had no effect on slices from adult rats. The cell death evaluation, by nuclear morphology analysis, that we performed in this work support those findings, since we observed that MK-801 protects 8 DIV neurons from OGD-induced toxicity (Figure 11B), whereas at 14 DIV MK-801 had no effect on neuronal damage. In these cultures, however, NBQX protected neurons from toxicity (Figure 11A), a result that supports a role for non-NMDA receptors involvement in OGD-induced cell death in older cultures. As such, both works can support the hypothesis that activation of NMDA receptors are involved in OGD-induced cell death in young but not mature rat hippocampal neurons. This difference in the protective role of the antagonists can also possibly be related to the location of the NR2A- or NR2B-containing receptors in the cell membrane (synaptic/extrasynaptic) or due to different intracellular pathways linked to each of the subunits (Zhou & Baudry, 2006).

4.3.2.2. Developmental changes in subunit composition of AMPA receptors

AMPA receptors can also have differential expression patterns during development (Talos et al., 2006a, Talos et al., 2006b). The hippocampus is one of the brain regions most enriched in GluR1-3 subunits, whereas GluR4 is present in lower amounts throughout the CNS, except in the cerebellum or the reticular thalamic nuclei (Santos et al., 2008). In order to understand how the protein levels of these subunits vary with the age of the culture, we performed a time-course analysis of the GluR1,

GluR2/3 and GluR4 protein patterns in primary hippocampal cultures with 7, 15 and 21 DIV. According to our results, cultures of hippocampal neurons at 15 DIV have the highest protein levels of GluR2/3, GluR4 and GluR1, whereas in older cultures with 21 DIV GluR2 and GluR4 become less abundant, with GluR1 still highly expressed (Figure 8A-C).

Previous studies in cultured hippocampal neurons have also shown developmental changes in the subunit composition of surface-expressed AMPA receptors. In fact, early in development a high proportion of AMPA receptors are present at synapses and do not possess GluR2, but after two weeks in culture, the GluR2 subunit is present in nearly every AMPA receptor-containing synapse (Pickard et al., 2000). The relative increase in synaptic GluR2 can reduce Ca^{2+} influx by forming Ca^{2+}-impermeable AMPA receptors on their own or in combination with other subunits, likely with GluR1 or GluR3 subunits in hippocampal neurons (Wenthold et al., 1996). These evidences suggest that older cultures might be less vulnerable to toxic stimulations than younger cultures due to a probable higher content of Ca^{2+}-impermeable AMPA receptors. In fact, our results show the total, and not synaptic, expression levels of GluR2, which is highly expressed after 7 and 15 DIV (Figure 8B); however, older cultures showed higher susceptibility to OGD than younger cultures (Figure 11 A, B). One hypothesis is that, the synaptic levels of GluR2 do not necessarily reflect the total amount of GluR2 in both cultures. In fact, the insertion and removal of GluR2-containing AMPA receptors are part of a highly regulated process (Hanley & Henley, 2005). Also, considering that the synaptic amount of GluR2 indeed reflects the intracellular levels of the protein, it is important to note that the machinery coupled to the receptors might also be different during development, leading to the activation of distinct signaling pathways between young or mature neuronal cultures.

4.4. OGD decreases the surface levels of GluR2

It is generally accepted that Ca^{2+} overload is involved in many cell death mechanisms, especially those that contribute for its excessive intracellular accumulation, such as ischemia. Under physiological conditions, the principal neurons of the hippocampus abundantly express GluR2-containing receptors, a subunit that determines Ca^{2+}-impermeability of AMPARs. As such, acute loss of GluR2 would be expected to confer enhanced pathogenicity of endogenous glutamate and susceptibility to neuronal insults. There are evidences showing that ischemic insults trigger downregulation of GluR2, at the mRNA and protein level, in the particularly vulnerable CA1 hippocampal neurons, inducing a long-lasting change in the subunit composition of AMPARs, from GluR2-containing Ca^{2+}-impermeable to GluR2-lacking Ca^{2+}-permeable receptors (Gorter et al., 1997, Ying et al., 1997, Liu et al., 2006). Research has been increasingly focused on the regulation of GluR2 expression in response to neuronal insults, and it was proposed a role for the gene-silencing transcription factor REST (repressor element-1 silencing transcription factor) in the switch for Ca^{2+}-permeable AMPA receptors. REST actively represses neuronal genes important to synaptic plasticity and remodeling, such as synaptic vesicle proteins, structural proteins and receptors, in progenitor and non-neuronal cells (Palm et al., 1998, Lunyak et al., 2005, Liu & Zukin, 2007). As differentiation takes place, REST is downregulated, an essential process for the maintenance of the neuronal phenotype. Neuronal insults, such as ischemia, activate REST in CA1 hippocampal neurons (Calderone et al., 2003). In these neurons destined to die, REST binds to the GluR2 promoter and suppresses gene expression. Knockdown of REST prevents GluR2 loss and protects neurons (Liu & Zukin, 2007).

Alternative mechanisms to explain the switch to Ca^{2+}-permeable AMPA receptors are related to receptor trafficking and mRNA editing, since both can be deregulated after neuronal insults. For instance, forebrain ischemia reduces the expression of ADAR2, an enzyme essential for GluR2 editing at the Q/R site, and receptors are then formed of unedit subunits (Peng et al., 2006). Rescue of ADAR2 levels in CA1 hippocampal neurons re-establishes GluR2 mRNA editing and protects susceptible neurons against global ischemia-induced damage. Therefore, neuron vulnerability to ischemia is also dependent of ADAR2 activity (Liu & Zukin, 2007).

In addition to delayed changes in AMPAR subunit composition as a result of altered GluR2 mRNA expression or GluR2 RNA editing, it was recently reported that in cultures of hippocampal neurons challenged by OGD, an *in vitro* model of brain ischemia, Ca^{2+}-permeable GluR2-lacking AMPARs are targeted to synaptic sites at very early times due to changes in the synaptic AMPA receptors traffic (Liu et al., 2006). A decrease in the surface levels of GluR2 in hippocampal cultures submitted to short OGD exposures was also reported (Dixon et al., 2009), similarly to the results that we obtained when hippocampal slices were challenged with OGD for a short period of time (Figure 12). This finding validates slices as a suitable model for the investigation of mild OGD-induced alterations in AMPAR number and subunit composition via alterations in receptor trafficking, as well as for the study of the proteins involved in this matter.

The finding that total cellular AMPA receptor subunit abundance is unchanged as late as 24h after insult (Liu et al., 2006) suggests that regulated receptor trafficking is likely to be an important mechanism mediating changes in synaptic AMPAR subunit composition at early times after ischemia.

4.4.1. Regulated AMPAR trafficking in ischemia

It has been proposed that the removal of GluR2-containing receptors from the plasma membrane is an endocytosis-dependent mechanism that is followed by insertion of GluR2-lacking receptors (Liu et al., 2006). The complete mechanism, though, remains to be clarified. It is known that under basal conditions, AMPARs suffer continuous cycles of endocytosis and reinsertion that allow for fast and effective control of synaptic receptor density (Hanley, 2008). There are many proteins that bind to AMPARs and participate in the regulation of LTD or LTP, activity-dependent forms of synaptic plasticity thought to underlie learning and memory. AMPAR endocytosis, exocytosis and subunit switching events are central to the necessary changes in synaptic plasticity. Given that ischemic insults promote targeting of GluR2-lacking AMPARs to synaptic sites and suppress GluR2 gene expression in vulnerable hippocampal neurons, these mechanisms could act synergistically to promote hippocampal neurons death.

The relatively rapid switch in subunit composition triggered by OGD is consistent with a role for regulated receptor trafficking. A possible scenario is that a pool of vesicular-associated GluR2-lacking AMPARs is docked beneath synaptic sites and is strategically located and available for activity or insult-driven insertion (Liu et al., 2006). It is also plausible that neuronal insults stabilize GluR2-lacking AMPARs expressed at low density on distal dendrites of CA1/CA3 pyramidal neurons under physiological conditions (Yin et al., 1999) while destabilizing GluR2-containing AMPARs. Future studies will be required to distinguish between these possibilities.

Under physiologic conditions, protein interacting with C kinase (PICK1) binds to AMPAR subunits GluR2 and 3, an interaction known to be required for AMPAR internalization from the synaptic membrane in response to Ca^{2+} influx in hippocampal

neurons (Hanley & Henley, 2005). In developing hippocampal CA1 synapses, GluR2-containing receptors are stabilized at the postsynaptic membrane by association with another protein thought to be involved in the receptor trafficking regulation, glutamate receptor-binding protein (GRIP). After an ischemic insult, protein kinase C (PKC) is activated and binds to PICK1 which, in turn, competes with GRIP for binding to GluR2 (Liu et al., 2006). When GRIP interaction with GluR2 is disrupted, PICK1 binds the subunit and promotes receptor internalization. These receptors are then substituted by GluR2-lacking receptors, rendering the cell more vulnerable to excitotoxic damage (Liu & Cull-Candy, 2005) (Figure 2). This suggests that GluR2-PICK1 interaction is a crucial mediator of OGD-induced AMPAR trafficking that results in an increased proportion of synaptic GluR2-lacking receptors and is also involved in OGD-induced cell death, since disruption of the PICK1-GluR2 binding with interfering peptides enhances cell viability during the stimulus (Liu et al., 2006, Dixon et al., 2009). However, the specific role of GluR2-lacking AMPARs brought about by PICK1-mediated trafficking at an earlier stage during OGD when compared with those expressed at later time points via altered gene expression is unclear. It is possible that the role of the early AMPAR-mediated calcium signal during OGD is to provide a trigger for signaling pathways that result in additional synaptic changes at a later time point, which may include altered *GluR2* gene expression (Dixon et al., 2009).

Interestingly, it has been shown that PICK1-GluR2 interaction in neurons is Ca^{2+}-sensitive, which allows PICK1 to respond to a local increase in Ca^{2+}, binding more strongly to GluR2 and consequently initiating AMPAR internalization. PICK1 binds Ca^{2+} ions directly (Henley, 2008) and overexpression of a Ca^{2+}-insensitive PICK1 mutant in hippocampal neurons leads to an occlusion of the activity-dependent AMPAR internalization from the plasma membrane, demonstrating that PICK1 plays a specific

role as a Ca^{2+} sensor to transduce Ca^{2+} influx directly into receptor trafficking (Hanley & Henley, 2005).

Besides the interaction with PICK1, GluR2 subunits also interact with GRIP (Hanley, 2008). A popular model is that GRIP maintains AMPARs at the synaptic plasma membrane, opposing the role of PICK1. It is thought that, in order for AMPAR trafficking to proceed, interactions with GRIP must be replaced by PICK1 at the GluR2 C-terminus. The dissociation of the GRIP-GluR2 interaction is subject to tight regulation with at least three mechanisms involved: phosphorylation of the GluR2 C-terminus, the ATPase activity of N-ethylmaleimide sensitive fusion protein (NSF) and a direct effect of Ca^{2+} ions (Hanley, 2008). PKC phosphorylates GluR2, promoting dissociation of GRIP from GluR2-lacking AMPARs and retrieval of receptors from synaptic sites. The binding of NSF to GluR2 disrupts the interactions between PICK1 and GluR2-containing AMPARs in order to associate GluR2 with GRIP and anchor the receptor at synapses (Gardner et al., 2005), a process described in cerebellar neurons. It has recently been demonstrated that the GluR2-NSF interaction is disrupted by direct binding of Ca^{2+} ions to NSF (Hanley, 2007), which suggests a mechanism whereby the dissociation of PICK1-GluR2 interactions by NSF is blocked in response to a Ca^{2+} signal, allowing PICK1-mediated AMPAR trafficking to proceed.

Taken together, these findings support that trafficking-mediated alterations in synaptic AMPAR subunit composition and/or number may represent a broad mechanism relevant to synaptic remodeling, as well as to neuronal death associated with a number of neurological disorders and diseases (Liu et al., 2006, Dixon et al., 2009).

4.5. Conclusion and future perspectives

Previous data from our laboratory showed that, in HEK-GluR4 cells, overactivation of Ca^{2+}-permeable AMPARs induces the activation of a toxic JNK signaling pathway (Santos et al., 2006, Vieira, M., unpublished data). Furthermore, it was observed that the excitotoxic activation of Ca^{2+}-permeable AMPARs increased phospho-GluR4 at T855 at a time point which correlates with maximal JNK activation after the excitotoxic stimulation (Vieira, M., unpublished data). In this work, we confirmed that Ca^{2+}-permeable AMPAR-induced phosphorylation of GluR4 at T855 is carried out by JNK, or by a kinase of the JNK pathway, since inhibition of the kinase activation decreased GluR4 phosphorylation levels at this residue. However, GluR4-T855 phosphorylation is apparently not contributing to the cell death mechanism, since mutation of this phosphorylation site had no effect on the stimulated cells. In the future, this result should also be validated in neurons submitted to OGD, in order to clarify whether GluR4 phosphorylation by JNK does not take a role on the excitotoxic response, or whether its phosphorylation is cell type and stimulus-specific.

Our work with hippocampal neurons challenged with OGD supports this *in vitro* model for global ischemia, since hippocampal neuronal cultures displayed the characteristic delayed cell death attributed to the ischemic insult, which was dependent on the stimulus intensity. Interestingly, when we used different cell viability assays to evaluate the viability of hippocampal neurons submitted to OGD in the presence of glutamate receptor antagonists, we observed some discrepancies concerning the neuroprotective effect of these compounds. Namely, we could not observe a protective effect of neither MK-801 nor NBQX when cell viability was assessed by the MTT test, although both compounds conferred protection against OGD when cell death was

evaluated by analysis of the nuclear morphology. These results suggest that, for the OGD stimulus, the direct analysis of the nuclear morphology of hippocampal neurons might be more adequate than the MTT test. Thus, it is important to attentively choose the approach to analyze OGD-triggered cell death, since techniques which have proven helpful in the analysis of the effect of other death stimuli might not be appropriate for the OGD stimulus. On the other hand, since we observed a distinct protective effect of MK-801 and NBQX in hippocampal cultures at different maturation stages, the OGD-induced cell death mechanisms might vary with neuronal development. In particular, differences in the cell biology of the cultures at different maturation stages, mainly related with glutamate receptors composition and a different coupling of the receptors to the cell death machinery, might be influencing the mechanisms of cell death. It is also important to note the OGD-mediated cell death might activate signaling pathways distinct to those mediated by NMDA receptors or AMPA receptors. In fact, some studies have reported the possible involvement of other receptors/channels in the excitotoxicity-related cell death, such as TRPM7 receptors (Aarts et al., 2003) or VGCC channels (Brewer et al., 2007).

Importantly, we have also shown that in hippocampal slices submitted to OGD there is a decrease in the superficial levels of GluR2, thus supporting the use of this biological preparation to study OGD-induced Ca^{2+}-permeable AMPA receptor-mediated cell death. As such, since the hippocampal slices allow the isolation of the distinct areas of the hippocampus, it would be interesting to use this system to analyze whether the decrease in the GluR2 surface level is an event that occurs preferentially in the CA1 hippocampal region, the vulnerable hippocampal region in transient global brain ischemia. Moreover, it would be worthy to investigate whether these changes in the surface content of GluR2 are developmentally regulated, thereby influencing the

excitotoxic response mechanism induced by OGD at different stages of neuronal development.

Although the mechanism of removal of GluR2-containing AMPARs from the synapses has been extensively studied (Liu & Cull-Candy, 2005), the mechanism by which Ca^{2+}-AMPARs are inserted and stabilized at the synapse remains to be clarified. Besides the participation of PICK1 and GRIP, AMPAR trafficking is also regulated by other scaffolding proteins that interact with these receptors, such as the transmembrane AMPA receptor-regulatory proteins (TARPs) (Payne, 2008). Stargazin, a TARP-related protein, binds the receptors early in the synthetic pathway, ensures their proper maturation and promotes their surface expression (Tomita et al., 2003). Stargazin also binds to the scaffolding protein PSD-95, and this interaction translocates surface AMPARs to the synapse (Chen et al., 2000). It is known that the phosphorylation status of stargazin influences its regulatory action on the synaptic strength (Tomita et al., 2005). TARP γ-8, which has a high homology with stargazin, is preferentially expressed in the mouse hippocampus, where it controls AMPAR protein levels and extrasynaptic surface expression (Rouach et al., 2005). Recently it was shown that mice lacking TARP γ-8 are less vulnerable to kainate-induced AMPAR-mediated toxicity (Tomita et al., 2007). Nevertheless, the effect of ischemic insults on these protein levels, or in their phosphorylation status, has never been addressed. As such, in order to check whether this TARP contributes to the insertion and stabilization of Ca^{2+}-AMPARs at the surface membrane, it would be interesting to examine the protein/phosphorylation levels of TARP γ-8 in OGD-challenged hippocampal neurons.

The understanding of the pathways that link the OGD-induced overactivation of glutamate receptors to cell death is of extremely importance. The approaches focused in our work, both in HEK293 cells and hippocampal neurons, were carried out as part of a

project that aims to dissect the molecular mechanisms linking excitotoxic activation of Ca^{2+}-permeable AMPARs to cell death. Understanding such pathways can be of outstanding relevance to the development of therapeutic strategies that target protein interactions mediated by ischemic insults, while sparing the physiological activity of AMPA receptors. In the future, such accomplishment may have a promising role in treatments for transient cerebral global ischemia episodes.

Chapter 5 References

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