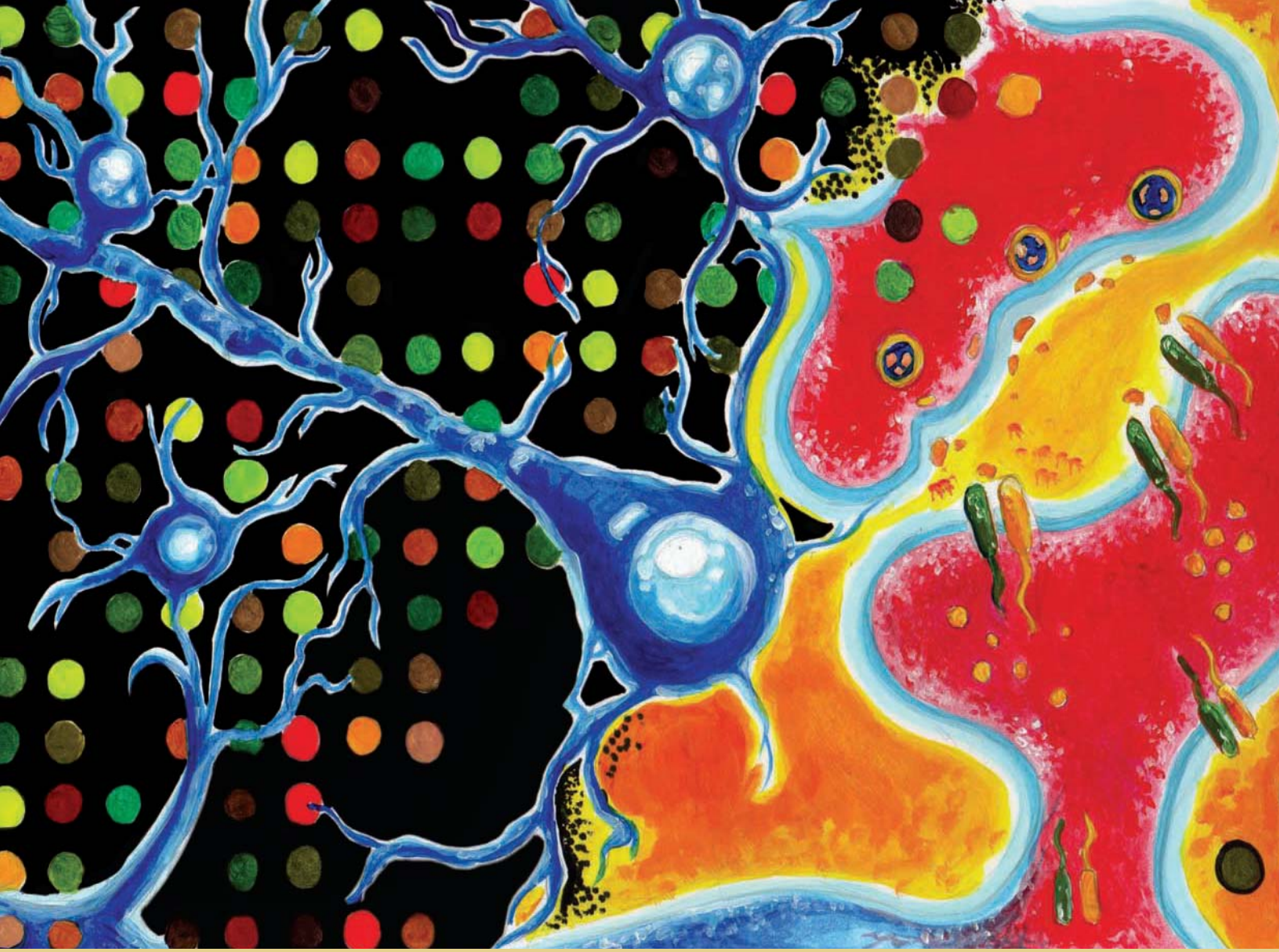

Joana Filipa Coelho Fernandes

In vitro ischemia-induced changes in the transcriptome of hippocampal neurons

Neuroprotective pathways in brain ischemia

Tese de Doutoramento em Ciências e Tecnologias da Saúde,
especialidade de Biologia Celular e Molecular
orientada por Ana Luísa Carvalho e Armanda Emanuela Castro e Santos
e apresentada à Faculdade de Farmácia da Universidade de Coimbra

2014

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Universidade de Coimbra

In vitro ischemia-induced changes in the transcriptome of hippocampal neurons

Neuroprotective pathways in brain ischemia

Alterações no transcriptoma de neurónios do hipocampo induzidas por isquémia *in vitro*

Vias de neuroprotecção em isquémia cerebral

Tese apresentada à Faculdade de Farmácia da Universidade de Coimbra para a prestação de provas de Doutoramento em Ciências e Tecnologias da Saúde, especialidade de Biologia Celular e Molecular

Joana Filipa Coelho Fernandes

Coimbra, 2014

Capa e projecto gráfico: Joana Fernandes e Ana Dias. “Microarray signals in the neurons of the hippocampus: implications for the study of the synapse and cell biology”.

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“It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of light, it was the season of darkness, it was the spring of hope, it was the winter of despair.”

Charles Dickens, *A Tale of Two Cities*

"Nothing in this world can take the place of persistence. Talent will not: nothing is more common than unsuccessful men with talent. Genius will not: unrewarded genius is almost a proverb. Education will not: the world is full of educated derelicts. Persistence and determination alone are omnipotent."

Calvin Coolidge

“My experience is that there is, you know, surprisingly, always hope.”

11th Doctor, *Vincent and the Doctor*

Agradecimentos

Se vi mais longe foi porque estava aos ombros de gigantes, Isaac Newton

O Professor Doutor Alexandre Quintanilha disse uma vez numa entrevista que é preciso ter-se uma *determinada personalidade* para se prosperar numa carreira científica. Nunca mais me esqueci das suas palavras e, durante muito tempo, achei que estava prestes a descobrir que não a tinha. Mais tarde percebi que estava no caminho normal: faz parte de um Doutoramento ter momentos em que sentimos que não estamos a prosperar e que seríamos mais eficientes numa cadeia de *fast-food*. Mas, muitas vezes é nesses momentos que, tendo vontade de viver e crescer, desenvolvemos essa *determinada personalidade* e lutamos ao máximo para fazer as coisas acontecer. É, no entanto, preciso uma outra coisa: estar rodeado das pessoas certas. Este é o momento de agradecer a essas pessoas que, ao longo dos últimos quatro anos, me ajudaram a desenvolver não apenas o trabalho do meu Doutoramento, mas também a minha capacidade, a minha personalidade e a minha independência. Aos vossos ombros vi melhor e mais longe.

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Geronimo!

Joana Fernandes, 23 de Fevereiro de 2014

Table of Contents

Abbreviations

ADAR2	Adenosine deaminase, RNA-specific, B1
AIF	Apoptosis-inducing factor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor
AP-1	Activator Protein-1
Apaf-1	Apoptotic peptidase activating factor 1
APV	(2R)-amino-5-phosphonopentanoate
ASICs	Acid-sensing Ion Channels
Bcl2	B-cell lymphoma 2
Bcl-XL	B-cell Lymphoma-extra large
BID	BH3 interacting-domain death agonist
CA1	Cornu ammonis 1
Ca^{2+}	Calcium ion
CA3	Cornu ammonis 3
CaM	Calmodulin
CaMKII	Ca^{2+}/Calmodulin Kinase II
CaN	Calcineurin
CHO	Chinese Hamster Ovary
Cl^-	Cloride ion
CLIC1	Chloride intracellular channel 1
CLICs	Chloride Intracellular Channels
Clstn	Calsyntenin
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
CREB	cAMP response element-binding protein
DIV	Days *in vitro*
DREAM	Downstream regulatory element antagonist modulator
ER	Endoplasmic reticulum
FMRP	Fragile X mental retardation protein
G	Glycine
GABA	γ-Amino-butyric Acid
$GABA_A$	γ-Amino-butyric acid type A
GRIP1	Glutamate receptor interacting protein 1
GSH	Reduced glutathione
GST	Glutathione-S-transferase
GYKI52466	4-(8-Methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepin-5-yl)-benzenamine dihydrochloride
HMGB1	High mobility Group Box 1
IAA-94	R(+)-[(6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)-oxy] acetic acid
IEGs	Immediate early gene
iGluR	Ionotropic glutamate receptor
IL-6	Interleukin 6
IP3	Inositol-1,4,5-trisphosphate
ITGB6	Integrin B-6
JNK	c-Jun N-terminal kinase
LDH	Lactate Dehydrogenase

LTD	Long-term Depression
LTP	Long-term Potentiation
MAPK	Mitogen-activated protein kinase
MeCP2	Methyl CpG-binding Protein
Mg^{2+}	Magnesium ion
mGluR	Metabotropic glutamate receptors
MK801	(5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine Maleate
MMP	Matrix metallopeptidase
mSin3A	Mammalian SIN3 transcription regulator family member A
mtPTP	Mitochondrial permeability transition pore
Na^{+}	Sodium ion
Naspm	N-[3-[[4-[(3-Aminopropyl)amino]butyl]amino]propyl]-1-naphthaleneacetamide trihydrochloride
NKCC1	Na-K-2Cl-Cotransporter
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NSF	N-ethylmaleimide-sensitive factor
OGD	Oxygen and glucose deprivation
PARP	Poly-(ADP-ribose) polymerase
PICK1	Protein interacting with C kinase
PKC	Protein kinase C
PP1γ	Protein phosphatase 1γ
PSD	Post-synaptic Density
Q	Glutamine
qPCR	Quantitative PCR
R	Arginine
RE1	Repressor Element-1
REST/NRSF	RE1 silencing transcripton factor/Neuron-restrictive silencer factor
ROS	Reactive oxygen species
SAPAP2	Synapse-associated protein 90/postsynaptic density-95-associated proteins, encoded by Dlgap2
SBDPs	Spectrin Breakdown products
siRNA	Small Interfering RNA
Smac/DIABLO	Second mitochondria activator of caspases/Direct IAP binding protein with low pI
SNAP-25	Synaptosomal-associated protein, 25kDa
SNARE	N-ethylmaleimide-sensitive factor attachment protein receptors
TARP	Transmembrane AMPAR regulatory protein
TNFα	Tumor necrosis factor α
TRPC	Transient receptor potential cation channel, subfamily Canonical
TRPM	Transient receptor potential cation channel, subfamily Melastatin
Unc13b	Unc 13 homolog B
VRAC	Volume-regulated anion channel
VSCCs	Voltage-sensitive Ca2+ channels
VSOR	Volume-sensitive outwardly rectifying
Zn^{2+}	Zinc ion

Resumo

A isquémia cerebral global devido à interrupção do fluxo sanguíneo no cérebro leva à privação de oxigénio e glicose nas células, reduzindo a energia disponível para manutenção do funcionamento celular. Os neurónios são especialmente sensíveis a esta falha energética, o que pode levar à activação de vias moleculares de morte celular. A região CA1 do hipocampo é particularmente vulnerável à isquémia global. Contudo, os sinais de morte celular surgem apenas horas ou dias após o insulto, criando uma janela temporal na qual podem ser aplicadas estratégias terapêuticas com vista à diminuição dos danos neurológicos causados pelo insulto. Pensa-se que este intervalo se deve a modificações transcripcionais que podem promover a sobrevivência ou a morte neuronal. No entanto, apesar do trabalho desenvolvido na identificação de genes cuja expressão está alterada após a isquémia global, os mecanismos responsáveis pela vulnerabilidade dos neurónios do hipocampo continuam por esclarecer. Assim, este trabalho teve como objectivo a identificação de mecanismos moleculares diferentemente regulados em culturas primárias de neurónios do hipocampo submetidos a um modelo *in vitro* para a isquémia global, a privação de oxigénio e glicose (do inglês, *oxygen and glucose deprivation* – OGD).

Começámos por estabelecer o protocolo para análise da expressão genética usando este modelo. Verificámos que o estímulo de OGD induz um aumento na morte celular em função da duração do insulto, e que esta é significativa 24h após o estímulo, mostrando que este modelo induz morte celular retardada. Utilizando antagonistas selectivos para os receptores de glutamato do tipo AMPA e NMDA vimos que o bloqueio destes receptores teve um efeito neuroprotector, confirmando o seu papel na componente excitotóxica da morte por OGD. Vimos ainda a activação de calpaínas, proteases ligadas a vias de morte celular.

De seguida investigámos as alterações no transcriptoma dos neurónios do hipocampo submetidos a OGD utilizando *microarrays*. Para isso, o RNA total das células foi extraído 7h e 24h após o estímulo com o objectivo de identificar genes envolvidos na resposta imediata e mais tardia à isquémia. Vimos que às 7h após OGD há uma maior repressão, enquanto 24h após o insulto há uma maior indução, na expressão de genes. A análise da ontologia genética mostrou que genes relacionados com stress oxidativo, metabolismo, apoptose, sinapse e actividade de canais iónicos apresentam diferentes níveis de expressão após OGD. Tanto quanto sabemos, este é o primeiro estudo a combinar *microarrays* e OGD como ferramenta para estudar alterações no perfil genético de neurónios do hipocampo a diferentes tempos de

recuperação. Os resultados obtidos nos *microarrays* foram validados através da análise de qPCR para genes selecionados, pertencentes a diferentes grupos ontológicos.

Observámos também que vários genes codificantes para proteínas da sinapse apresentaram diminuição na sua expressão em neurónios submetidos a OGD, bem como um aumento dos níveis de REST, um factor de transcrição que reprime a expressão de genes codificantes de proteínas neuronais sinápticas, como alguns dos que apresentam diminuição da expressão após OGD. Vimos ainda que este estímulo provoca a diminuição nos níveis de mRNA e proteína da subunidade GluA1 dos receptores AMPA, bem como das subunidades GluN2A e GluN2B e o aumento dos níveis de mRNA da subunidade GluN3A dos receptores NMDA, o que pode levar à alteração da composição destes receptores em neurónios do hipocampo. Estes resultados sugerem que o estímulo de OGD leva à activação de um programa transcripcional que causa uma repressão da expressão de proteínas sinápticas.

Por fim, investigámos os níveis de expressão de genes codificantes para canais iónicos e, nomeadamente, a contribuição do canal de cloreto CLIC1 para a morte induzida por OGD, uma vez que o mRNA do CLIC1 está aumentado em neurónios submetidos a OGD. O papel do CLIC1 em neurónios é desconhecido, embora se pense que a activação deste canal em micróglia leva à morte de neurónios em condições tóxicas. Contudo, os nossos resultados mostram que a sobre-expressão do CLIC1 torna os neurónios menos vulneráveis ao dano induzido por OGD, e resultados preliminares sugerem que a expressão do CLIC1 pode estar aumentada em regiões do hipocampo mais resistentes à isquémia global. Os nossos resultados sugerem que o CLIC1 faz parte de um mecanismo intrínseco de protecção activado em neurónios após o insulto isquémico.

Os resultados obtidos caracterizam o perfil global de expressão genética induzido por OGD em neurónios do hipocampo, permitindo o estudo de dois mecanismos distintos que podem contribuir para a sobrevivência neuronal: a diminuição da expressão de componentes sinápticos, nomeadamente dos que estão envolvidos na excitotoxicidade, e o aumento na expressão do CLIC1. Estes mecanismos podem ser explorados no sentido de desenvolver estratégias terapêuticas para o tratamento da isquémia cerebral.

Abstract

Cerebral global ischemia due to interruption of blood supply to the brain results in oxygen and glucose deprivation of brain cells, reducing the energy available to maintain normal cellular functions. Neurons are especially sensitive to this energetic insufficiency and consequently fail to maintain the ionic gradients necessary for cellular function and homeostasis, which ultimately leads to the activation of several molecular pathways that impair neuronal function or may lead to cell death. The neurons of the CA1 region of the hippocampus are particularly vulnerable to global ischemia. However, signs of cell death arise only hours to days after the insult, providing a temporal window in which therapeutical approaches to prevent the delayed neurological and cognitive deficits triggered by ischemia can be employed. This delay is thought to include transcriptional changes that can either prime cell survival or enhance neuronal death. However, despite the effort to identify genes differently expressed after ischemia, the mechanisms responsible for the selective vulnerability of hippocampal neurons to global ischemia remain elusive. As such, the present work was aimed at investigating which transcription-dependent molecular mechanisms are differently regulated in hippocampal neuronal cultures subjected to oxygen and glucose deprivation (OGD), an *in vitro* model for cerebral global ischemia.

Initially, we established the experimental set-up for the analysis of gene expression in this model. We observed an increase in OGD-induced neuronal death as a function of OGD time length, but neuronal death was only significant 24h after the stimulus, showing that OGD induced delayed neuronal death. Selective antagonists of the AMPA and NMDA glutamate receptors were neuroprotective, confirming the contribution of these receptors to the excitotoxic component of this *in vitro* ischemic model. OGD also triggered the activation of calpains, proteases known to mediate many deleterious effects in post-ischemic neurons.

We then used microarray technology to study changes in the transcriptome of rat hippocampal neurons submitted to OGD. For that purpose, total RNA was extracted 7h and 24h after OGD and used in a whole-genome RNA microarray to identify genes related to an early and a delayed ischemic response. At 7h of recovery there is a general repression of genes, while at 24h there is a general induction of gene expression. Analysis of the gene ontology showed that genes related with a variety of cellular functions, such as the response to oxidative stress, metabolism, apoptosis, synapse and ion channel activity, were differently regulated after OGD. As far as we know, no previous study has used microarray technology and the OGD model as a tool to

investigate ischemia-induced changes in the transcriptome of hippocampal neurons at different periods of recovery. The validity of the microarray data was confirmed by qPCR analysis of selected genes from different functional groups.

According to the microarray data, several synaptic protein genes were down-regulated after OGD. We also observed that OGD triggers the up-regulation of REST, a transcription factor that represses the expression of genes related with the synaptic function. Additionally, OGD decreased the mRNA and protein levels of the GluA1 AMPAR subunit as well as the GluN2A and GluN2B subunits of NMDARs, but increased the mRNA expression of the NMDAR subunit GluN3A, which might lead to a change in the composition of ionotropic glutamate receptors in hippocampal neurons. These results suggest that OGD activates a transcriptional program leading to a general repression of proteins present in the synapse.

Moreover, we analyzed OGD-induced changes in the expression levels of ion channel protein genes. In particular, we pursued the contribution of the chloride channel CLIC1 to the OGD-mediated neuronal response. The role of CLIC1 in neurons is largely unknown but it has been suggested to contribute to neuronal death when activated in microglia under toxic conditions. However, we observed that neurons over-expressing CLIC1 are less vulnerable to OGD-induced cell death, and preliminary results suggest that CLIC1 may be up-regulated in the hippocampal regions that are more resistant to ischemia. Overall, these results suggest that CLIC1 can be part of an intrinsic protective mechanism activated in neurons after an ischemic insult.

The results obtained in this work present the global expression profile elicited by *in vitro* ischemia in hippocampal neurons, and help to investigate two different mechanisms that may contribute to neuronal survival in neurons submitted to OGD: decrease in the expression of synaptic components, namely those involved in excitotoxicity, and up-regulation of the chloride channel CLIC1. The targets that we identified may be explored to develop attractive therapeutic strategies for the treatment of cerebral ischemia.

Chapter I

Introduction

Cerebral ischemia

Cerebral ischemia is the condition in which brain tissues are subjected to hypoxia or to an impairment in blood supply, leading to biochemical alterations that may culminate in cell death. The deficiency in cerebral blood flow restricts the delivery of substrates such as oxygen and glucose, thus reducing the energy available for the maintenance of functional brain cells.

Brain tissue has a relatively high consumption of oxygen and glucose and depends almost exclusively on the oxidative phosphorylation metabolic pathway for energy production (Verweij *et al.*, 2007). This renders brain cells particularly susceptible to ischemia-induced energetic deficiencies and therefore unable to maintain the ionic gradients necessary for normal cellular function and homeostasis (Hansen and Nedergaard, 1988; Pundik *et al.*, 2012; Song and Yu, 2013). Under ischemic conditions, ATP depletion results in loss of ionic gradients and therefore in the dissipation of the membrane potential, which promotes cell depolarization. One of the most remarkable consequences of this phenomenon is the increase in the intracellular levels of sodium (Na^+), calcium (Ca^{2+}), zinc (Zn^{2+}) and chloride (Cl^-) ions, which accumulate in the intracellular space via ion channels/receptors or via specific transporters, leading to water flow into the cells and causing cellular edema (Kahle *et al.*, 2009; Song and Yu, 2013).

The excessive depolarization of neurons contributes to an increased release of glutamate in excitatory synapses. Also, the re-uptake of excitatory neurotransmitters, which is coupled to the Na^+ gradient, is compromised by the energetic failure, further contributing to the accumulation of glutamate in the extracellular space (Sheldon and Robinson, 2007; Wang and Qin, 2010). This process results in the overactivation of synaptic and extrasynaptic glutamate receptors and other ion channels, which further contributes to ionic imbalance. The increased influx of Ca^{2+}, for instance, causes the activation of several signaling pathways with deleterious effects on cell viability, including the activation of proteases such as calpains and caspases, as well as endonucleases and kinases (Szydlowska and Tymianski, 2010; Wang and Qin, 2010).

The increased accumulation of Ca^{2+} can also lead to an overproduction of free radicals, malfunction/damage of the mitochondria and the endoplasmic reticulum (ER), acidosis, cell swelling, cytoskeletal breakdown, disruption of cell membranes and DNA fragmentation. Such severe damage on many different cellular systems impairs

neuronal function and might lead to cell death (Szydlowska and Tymianski, 2010; Wang and Qin, 2010).

The severity and duration of ischemia determine the number and type of cells that may be affected, as well as the timing of various injury mechanisms and their relative contribution to cell death. Neuronal depolarization is generally accepted as a critical point that influences the neuronal outcome to ischemic insults. When ischemic depolarization is brief (1-2 minutes), restoration of blood flow (reperfusion) allow neuronal survival, as well as in the activation of mechanisms that render neurons more resistant to subsequent more severe ischemic episodes. This phenomenon is referred to as ischemic tolerance or ischemic preconditioning (Tapuria *et al.*, 2008; Narayanan *et al.*, 2013). If ischemic depolarization is of an intermediate range (5-30 minutes), after reperfusion a complex cascade of molecular events develops, during hours to days, and will determine whether neurons survive or die. Prolonged depolarization (over 30 minutes) likely results in neuronal death in any case, even if reperfusion occurs (Neumar, 2000).

Etiology of cerebral ischemia

Cerebral ischemia can be focal or global. Focal ischemia is most commonly the result of stroke, cerebral hemorrhage or traumatic brain injury. A stroke can be due to the blockade of a blood vessel supplying the brain by a blood clot (most frequent kind of stroke) or by the bursting of a blood vessel that bleeds into the brain. Stroke is the third leading cause of death in the Western world and in many cases, it triggers neurological and cognitive deficits that may become chronic in the individuals that survive. Reduction of cerebral blood flow is generally more intense in the *core* of the affected area, whereas less severe hypoperfusion occurs in the surrounding region, termed the *penumbra*, whose tissue might be perfused from collateral blood vessels. While in the core area cells die mostly by necrosis, cells in the penumbra area can present signs of cell death at later time points after the insult and can sometimes be rescued if reperfusion takes place before the energetic deficiency is profoundly aggravated (Zukin et al., 2004).

Global ischemia arises when blood flow to the entire brain is transiently blocked and is most commonly observed in patients of cardiorrespiratory arrest, cardiac surgery or near-drowning. Although impairment of blood flow restricts the supply of oxygen and glucose to the whole brain during the ischemic insult, only selective neuronal

subpopulations are affected. In fact, neurons located in the hippocampus seem to be particularly susceptible, as well as neurons from the cerebellum, striatum and cortex (Pulsinelli, 1985). In the hippocampus, the pyramidal neurons of the cornu ammonis 1 (CA1) region are the most vulnerable to ischemic insults, whereas neurons from the CA3 and the dentate gyrus subregions are more resistant (Kirino and Sano, 1984; Tasker, 2001; Crepel *et al.*, 2003). While after focal ischemia neuronal damage can usually be observed soon after the insult (in the core area), the first signs of neuronal death after global ischemia are not observed until two or three days after the end of the insult (Petito *et al.*, 1987; Bottiger *et al.*, 1998). In fact, despite the normal morphology of the cells and the normal levels of intracellular Ca^{2+} observed early after reperfusion, there is a late rise in the intracellular free Zn^{2+} and Ca^{2+} concentrations, in part due to their influx through ionotropic glutamate receptors, that has been related to neuronal damage in the susceptible neurons of the CA1 region (Calderone *et al.*, 2004; Stork and Li, 2006; Li *et al.*, 2007; Stork and Li, 2009; Lau and Tymianski, 2010; Szydlowska and Tymianski, 2010). The substantial delay between the ischemic insult and the onset of cell death, a process known as *delayed neuronal death*, is thought to be dependent on transcriptional changes triggered by the ischemic insult, and, importantly, it provides a temporal window in which therapeutical approaches directed to block the signaling mechanisms leading to cell death can be undertaken (Zukin *et al.*, 2004).

Experimental models for cerebral ischemia

In order to investigate the molecular mechanisms of disease triggered by cerebral ischemia, several experimental *in vivo* and *in vitro* models have been developed. While *in vivo* models have greater clinical relevance, because the effects induced by cerebral ischemia develop in an intact animal (where the neuronal circuitry is preserved, thus allowing comparison between different brain regions), *in vitro* models are mostly used to examine the mechanisms underlying the fate of the insulted neurons at a more molecular level.

Focal ischemia is most frequently studied by performing a permanent or temporary occlusion of the middle cerebral artery (MCAO), thereby mimicking a stroke or cerebral infarction in humans (DeGirolami *et al.*, 1984; Nagasawa and Kogure, 1989). This *in vivo* model induces an ischemic core with cells that present the features of necrotic cell death and whose damage is irreversible, and a penumbra in which cell death can still be inhibited (Zukin *et al.*, 2004).

Global ischemia is commonly studied using both *in vivo* and *in vitro* models. The most common models of global ischemia are the four-vessel occlusion, typically performed in rats (Pulsinelli and Brierley, 1979; Pulsinelli and Buchan, 1988; Deng and Xu, 2009) and the two-vessel occlusion (Smith *et al.*, 1984; Raval *et al.*, 2009), that has been undertaken in gerbils and mice. In these models, brief ischemic episodes affect the entire brain but elicit neuronal death specifically in the pyramidal neurons of the CA1 region of the hippocampus and the hilar neurons of the dentate gyrus. In these models, the morphological signs of cell death are not manifested until 24-72h after the insult (Zukin *et al.*, 2004).

The *in vitro* model for global ischemia consists in oxygen and glucose deprivation (OGD), performed in primary cell cultures or brain slices of different brain regions, such as the hippocampus, the cortex or the cerebellum (Goldberg and Choi, 1993; Taylor *et al.*, 1999; Bonde *et al.*, 2005; Rau *et al.*, 2012; Lalonde and Mielke, 2014). In this model, mature cultures (usually cells have 14-15 days *in vitro* (DIV)), the culture medium is substituted for a glucose-free salt solution and the cells placed in an oxygen-free chamber during the insult. The OGD model requires a longer insult to induce cell death, but ensues many of the features characteristic of the *in vivo* models. These include the selective neuronal damage in the CA1 neurons of the hippocampus, observed when OGD is performed in organotypic hippocampal slices, in which the hippocampal areas are still intact, and delayed cell death in both neuronal cultures and organotypic slices, which is usually assayed from 24h to 72h after the insult (Taylor *et al.*, 1999; Arai *et al.*, 2001a; Arai *et al.*, 2001b; Ahlgren *et al.*, 2011). These biological models can be particularly easy to manipulate, namely for experiments of knock-down or overexpression of genes or proteins of interest. The relevance of the study is enhanced when organotypic cultures of brain slices are used, in which the neuronal circuitry of a certain region of the brain is preserved.

Glutamate-mediated component of cerebral ischemia physiopathology

Excitatory synapses and 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. 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 the Krebs cycle (Hara and Snyder, 2007).

At chemical synapses, the glutamate 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, glutamate is highly involved in cognitive functions like learning and memory (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 into astrocytic glia. This latter mechanism is the most predominant in the reuptake of glutamate (Hara and Snyder, 2007).

The post-synaptic cell accommodates a multiprotein complex harboring different receptor complexes, a region termed the *post-synaptic density* (PSD). In addition to glutamate receptors, the PSD contains hundreds of other proteins that directly participate in synaptic transmission, such as kinases, phosphatases, adhesion molecules and scaffold proteins, which, by bringing together various signaling components, provide a rapid and efficient signal transduction (Sheng and Hoogenraad, 2007). A prominent organizing protein is PSD-95, which couples glutamate receptors to intracellular proteins and signaling enzymes (Sheng and Hoogenraad, 2007).

When released from presynaptic vesicles, 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 that pass electric current in response to glutamate binding and comprise three functionally distinct subtypes: α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainate (KA) and *N*-methyl-D-aspartate (NMDA) receptors. The distinct subtypes were initially distinguished by the different affinity of the various glutamate analogues on receptor binding and their different electrophysiological properties. These receptors are mainly concentrated at postsynaptic sites, where they contribute to a variety of different functions (Cull-Candy *et al.*, 2006; Santos *et al.*, 2009; Sanz-Clemente *et al.*, 2013).

NMDA receptors (NMDARs) are activated by the glutamate analogue NMDA and possess a recognition site for glycine or for D-serine, that both act as co-agonists and are required for the receptor activation (Papouin *et al.*, 2012). When activated, NMDARs open a channel that conducts Na^+ and Ca^{2+}. Under basal conditions, the channel is blocked by Mg^{2+}. However, this blockade is removed by depolarization. Continuous strong stimulation increases NMDAR activation and plays an important role in long-term potentiation (LTP). AMPA receptors (AMPARs), another class of ionotropic glutamate receptors, respond selectively to glutamate derivatives such as AMPA and kainate, opening a channel that allows Na^+ and, under specific conditions, Ca^{2+} influx, causing cell membrane to depolarize. Both NMDARs and AMPARs are localized in the same cells in close proximity. Thus, when Na^+ ions flow through AMPARs, triggering cell depolarization, the Mg^{2+} blockade of NMDARs is relieved and the receptors are activated, leading to Ca^{2+} entry. Finally, kainate receptors, a third class of ionotropic glutamate receptors, bind 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 events, 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 (Traynelis *et al.*, 2010).

Excitotoxicity

Many neurotransmitters likely participate in signaling events that influence neurotoxicity, but glutamate appears to be the principal one. In fact, by the same time glutamate was considered the most prevalent excitatory neurotransmitter in the central nervous system (CNS), there was evidence that glutamate could also be a potent brain neurotoxin (Lucas and Newhouse, 1957; Olney, 1969; Olney and Sharpe, 1969). The toxic role of glutamate was found to be mediated by glutamate receptors since application of glutamate receptor antagonists attenuated synaptic transmission, acting as neuroprotective agents (Simon *et al.*, 1984). The pathological process whereby overactivation of glutamate receptors by excitatory amino acids produce neurodegeneration is referred to as *excitotoxicity*. Under normal conditions, neurons sense the presence of too much glutamate in the extracellular space and activate glutamate transporters on the cell membrane that siphon glutamate back into the cells from which it was released, in a process dependent on the Na^+ gradient. However, this protective pumping process cannot cope with situations in which extracellular levels of

glutamate rise sharply. During an ischemic insult, there is massive release of synaptic glutamate, reversal of glutamate transporters and inhibition of glutamate re-uptake mechanisms, which contribute to the extracellular accumulation of glutamate (Takahashi *et al.*, 1997; Rossi *et al.*, 2000). The damaging power of excessive glutamate is manifested by an overexcitation of the cell, resulting in the excessive opening of cell pores and consequent entrance of large quantities of ions, such as Ca^{2+} or Cl^-, that would otherwise enter only in limited and controlled amounts. For instance, NMDARs have been extensively studied due to their key role in mediating at least certain aspects of glutamate neurotoxicity, owing to their high Ca^{2+} permeability (Lai *et al.*, 2013).

Besides cerebral ischemia, excitotoxicity is associated with many diseases, like spinal cord injury, brain trauma, epileptic seizures, and neurodegenerative diseases of the CNS such as multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease and Huntington's disease (Mehta *et al.*, 2013). Neurodegenerative disorders associated with excitotoxicity share a common pathogenesis mechanism involving impairment of intracellular Ca^{2+} homeostasis, nitric oxide (NO) synthesis and generation of free radicals, culminating in programmed cell death, which leads to progressive neurodegeneration (Mehta *et al.*, 2013).

Molecular mechanisms of ischemic neuronal injury

Calcium

Calcium is one of the most important intracellular messenger molecules and can mediate a variety of cellular responses. Consequently, homeostatic mechanisms exist to maintain a low intracellular Ca^{2+} concentration so that Ca^{2+} signals remain spatially and temporally localized. Free cytosolic Ca^{2+} concentrations are maintained mainly by the ER pump and by extrusion through the plasma membrane Ca^{2+}-ATPase and the Na^+-Ca^{2+} exchanger (NCX) (Hara and Snyder, 2007). During ischemia, ATP depletion results in cessation of energy-dependent mechanisms located in the mitochondria, ER and plasma membrane to remove Ca^{2+} from the cytosol (Neumar, 2000). Indeed, Ca^{2+} is excessively increased in neurons exposed to ischemic insults, an effect observed both *in vivo* (Silver and Erecinska, 1992; Nakamura *et al.*, 1999; Calderone *et al.*, 2003) or *in vitro* (Lobner and Lipton, 1993; Martinez-Sanchez *et al.*, 2004). Disruption of Ca^{2+} homeostasis occurs at multiple stages during cerebral ischemia and reperfusion. For instance, reduced levels of ATP cause depolarization of neurons that ultimately results in the activation of voltage-gated Ca^{2+} and Na^+ channels, as well as glutamate receptors,

that contribute to the influx of Ca^{2+} (Szydlowska and Tymianski, 2010). Also, Na^{+} influx by AMPARs causes dissipation of the Na^{+} gradient, thus leading to the reversal of the NCX and further increasing Ca^{2+} influx. Upon its release, glutamate also acts on metabotropic receptors, which can lead to production of inositol-1,4,5-trisphosphate (IP3). IP3 binds to the IP3 receptor on the ER causing release of Ca^{2+} from this intracellular compartment into the cytosol (Neumar, 2000).

Elevated levels of cytosolic Ca^{2+}, exceeding the capacity of intracellular Ca^{2+}-regulatory mechanisms, promote derangement of cell systems and activation of cell death pathways, mainly by triggering lipolysis, proteolysis, NO production, endonuclease-mediated DNA degradation and kinase/phosphatase activation that may signal for changes in gene expression, thus contributing to neurodegeneration through necrosis or apoptosis (Neumar, 2000; Szydlowska and Tymianski, 2010) **(Figure 1.1)**.

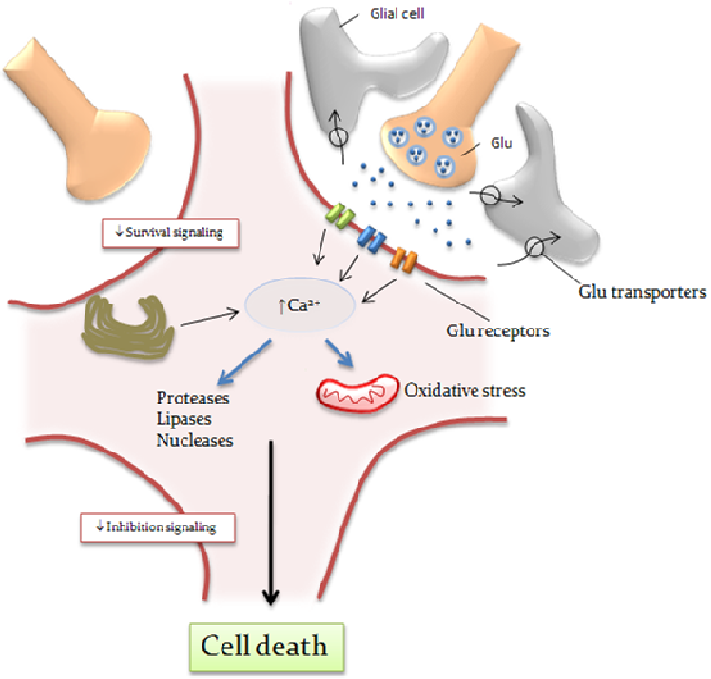


Figure 1.1. Effects of intracellular Ca^{2+} accumulation in neurons after ischemia. Several mechanisms are activated when cytoplasmic Ca^{2+} levels rise above physiological levels to remove it. However, under excitotoxic conditions, accumulation of Ca^{2+} exceeds the buffering capacity of neurons. This leads to the activation of several signaling pathways related with apoptotic or necrotic cell death, including the activation of calpains, caspases, among other proteases, endonucleases and kinases. Altogether, this derangement in cell signaling results in the overproduction of free radicals, malfunction/damage of mitochondria and endoplasmic reticulum, acidosis, cytoskeletal breakdown, cell membranes disruption, and DNA fragmentation, which, ultimately, leads neurons towards cell death.

Deleterious mechanisms triggered by Ca^{2+}: calpain activation, disruption of mitochondrial activity and oxidative stress

One class of proteases activated by Ca^{2+} are the calpains, a family of cysteine proteases that trigger substrate-specific proteolysis that may contribute to neuronal death (Bevers and Neumar, 2008). Their activation as been observed after both *in vivo* (Garcia-Bonilla *et al.*, 2006; Clinkinbeard *et al.*, 2013) and *in vitro* ischemia (Newcomb-Fernandez *et al.*, 2001; Zhou and Baudry, 2006; Lobo *et al.*, 2011). Calpains can cleave many proteins responsible for Ca^{2+} homeostasis, such as the NCX (Bano *et al.*, 2005), L-type calcium channels (De Jongh *et al.*, 1994), IP_3 receptors (Kopil *et al.*, 2012), as well as NMDA and AMPA receptor subunits (Simpkins *et al.*, 2003; Yuen *et al.*, 2007). Cytosolic enzymes such as Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and the protein phosphatase calcineurin (CaN), other important players in regulating Ca^{2+} homeostasis, can also be cleaved after calpain activation (Vosler *et al.*, 2008). Calpains also promote cleavage-mediated activation of BH3 interacting-domain death agonist (BID), a member of the family of pro-apoptotic B cell lymphoma 2 (Bcl-2) proteins (Chen *et al.*, 2001).

Additionally, excessive Ca^{2+} loading causes mitochondrial Ca^{2+} accumulation, which is an early event in excitotoxic neuronal death (Racay *et al.*, 2009). In isolated mitochondria, Ca^{2+} overload can evoke sustained mitochondrial permeability transition pore (mtPTP) opening and cytochrome c release. Opening of the mtPTP causes a massive swelling of the mitochondria coupled with collapse of the mitochondrial membrane potential, whereas blocking mtPTP prevents apoptotic neurodegeneration, which suggests a potential strategy for neuroprotection (Sullivan *et al.*, 2005). In pathological conditions such as ischemia, an excessive loading of Ca^{2+} into the mitochondria induces apoptosis by stimulating the release of apoptosis-promoting factors like cytochrome c, apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pl (Smac/DIABLO) and pro-caspases from the mitochondrial inter-membrane space into the cytoplasm (Shibata *et al.*, 2002). Once in the cytoplasm, cytochrome c interacts with apoptotic peptidase activating factor 1 (Apaf-1) and procaspase-9, forming the so-called apoptosome and initiating the caspase-dependent mitochondrial pathway (Chinnaiyan, 1999; Hill *et al.*, 2004).

Oxidative and nitrosative stress are also thought to have crucial deleterious effects in neurons following ischemic insults. In fact, an abnormal increase in Ca^{2+} concentration, such as that elicited by an ischemic insult, can lead to an accumulation

of NO and reactive oxygen species (ROS), such as superoxide, which contribute to neuronal derangement and demise (Michel and Feron, 1997). Nitric oxide is produced by NO synthase (NOS) from oxygen and arginine and has the ability to interact with a plethora of molecules, resulting in molecular modifications that have toxic effects in neurons, such as lipid peroxidation, DNA damage and changes in enzyme activity (Saito *et al.*, 2005). In a context of cerebral ischemia, the toxic effects of NO have been reported since inhibition of nNOS (the neuronal form of NOS) have been demonstrated to reduce CA1 neuronal death after global ischemia in gerbils (Caldwell *et al.*, 1994; Kohno *et al.*, 1996; O'Neill *et al.*, 1996; Nakagomi *et al.*, 1997) and rats (Nanri *et al.*, 1998), and to decrease lipid peroxidation (Caldwell *et al.*, 1995). The activation of nNOS can be regulated by Ca^{2+} through activation of the Ca^{2+}/calmodulin complex and, given the close proximity to the NMDARs, is implicated in one of the molecular pathways leading to cell death activated by these receptors under ischemic conditions. The mechanism by which NO exacerbates cell death occurs through deregulation of intracellular Ca^{2+} stores, namely at the level of the mitochondria and ER (Kohno *et al.*, 1997). Additionally, mutant mice for nNOS have increased resistance to global ischemic insults (Panahian *et al.*, 1996). The production of free radicals, or ROS, can occur through activation of different pathways, but the primary endogenous source of ROS is the mitochondria, where they are produced via metabolism of oxygen through the electron transport chain. In fact, exposure of isolated mitochondria to increased levels of Ca^{2+} and Na^{+} result in a feed-forward system of increasing free radical accumulation (Dykens, 1994), whereas removal of NMDAR-mediated Ca^{2+} influx attenuates free radical production (Dugan *et al.*, 1995). Damage from free radicals is normally controlled by endogenous protective mechanisms such as antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) (Warner *et al.*, 2004). However, after an ischemic insult, free radical production overcomes these protective mechanisms causing damage to DNA, proteins and lipids. Moreover, cytoplasmic free radicals such as superoxide can interact with other radicals, such as NO, to form powerful oxidants (Squadrito and Pryor, 1998).

The "source-specificity" hypothesis

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). For instance, previous studies have shown that whereas Ca^{2+} loading through L-type

voltage-sensitive Ca^{2+} channels (VSCCs) was nontoxic to cells, similar Ca^{2+} loads produced via NMDARs was highly neurotoxic (Tymianski *et al.*, 1993; Sattler *et al.*, 1998). 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 (Szydlowska and Tymianski, 2010).

Thus, researchers have been focusing as to how the signaling mechanisms activated by Ca^{2+} entry through different glutamate and non-glutamate receptors would influence neuronal cell death during an excitotoxic insult.

Zinc

Zinc 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. Zinc ions accumulate in hippocampal neurons during ischemia and the addition of Zn^{2+} chelators has proven to be neuroprotective both upon *in vivo* (Koh *et al.*, 1996; Calderone *et al.*, 2004) or *in vitro* (Yin *et al.*, 2002; Stork and Li, 2006; Stork and Li, 2009) ischemia. Curiously, Zn^{2+} accumulation is more potent at inducing cell damage than Ca^{2+}, acting by disruption of the mitochondrial activity leading to an increase in the production of ROS (Sensi *et al.*, 1999; Sensi *et al.*, 2000; Jiang *et al.*, 2001; Sensi and Jeng, 2004; Stork and Li, 2006; Medvedeva *et al.*, 2009; Stork and Li, 2009). Zinc ions can also cause the activation of poly (ADP-ribose) polymerase (PARP) activation, a protein involved in many cellular processes such as DNA repair and programmed cell death (Kim and Koh, 2002).

Although the precise mechanism by which Zn^{2+} induces neuronal damage is not yet known, *in vitro* studies such as those using OGD, have contributed to uncover the characteristics of the toxic effect mediated by Zn^{2+}. Indeed, regarding the route through which Zn^{2+} entries into neurons, it has been proposed that Ca^{2+}-permeable AMPARs might mediate the principal contribution to the accumulation of these ions in neurons submitted to OGD, since specific blockade of these receptors attenuates both Zn^{2+} accumulation and subsequent neuronal death, contrary to the effect of the inhibition of VSCCs or NMDARs (Yin *et al.*, 2002; Kwak and Weiss, 2006). In fact, it is considered that NMDARs are an improbable route of entry for Zn^{2+}, given their poor permeability to these ions and the potential blockade Zn^{2+} can exert on these receptors. Another possible route of entry for Zn^{2+} is the transient receptor potential melastatin 7 (TRPM7)

ion channel, which has been shown to be highly permeable to Zn^{2+} and participate in Zn^{2+}-mediated injury (Monteilh-Zoller *et al.*, 2003; Inoue *et al.*, 2010).

Role of the ionotropic glutamate receptors in cerebral ischemia

NMDA receptors

Structure and diversity

NMDA receptors are the most extensively studied class of glutamate receptors due to their high permeability to Ca^{2+} and Na^{+}. In normal conditions, activation of these receptors contributes to cell depolarization and intracellular Ca^{2+} transients that are responsible for the maintenance of synaptic plasticity both in the developing and adult brain.

At resting membrane potential, the pore of the NMDAR channel is blocked by physiological levels of extracellular Mg^{2+}. Given that this blockade is voltage-dependent, activation of NMDARs requires membrane depolarization (Mayer *et al.*, 1984; Nowak *et al.*, 1984). As such, NMDARs are considered molecular coincidence detectors, since their activation requires postsynaptic depolarization that coincides with presynaptic release of glutamate.

Typical NMDARs are di-heteromers composed of two obligatory GluN1 subunits and two GluN2 (GluN2A-D) or GluN3 (GluN3A-B) subunits, that assemble as a tetrameric complex. However, NMDARs can also assemble as tri-heteromers. For instance, GluN1/GluN2B/GluN3A or GluN1/GluN2B/GluN2D complexes are expressed at early stages of development, whereas GluN1/GluN2A/GluN2B or GluN1/GluN2A/GluN2C are more expressed in adulthood (Low and Wee, 2010). Alternative splicing of the NMDAR subunits provides additional heterogeneity (Paoletti, 2011). Each NMDAR subunit is composed of three transmembrane domains (M1, 3 and 4) and one re-entrant loop (M2). Glutamate binds in the pocket created by the extracellular regions (S1-2) present in the N-terminal tail and the loop between the M3 and M4 domains, respectively. The C-terminus is cytoplasmic and varies in length between the different subunits **(Figure 1.2)**. NMDA receptors are activated by the simultaneous release of glutamate and a different endogenous co-agonist, D-serine and glycine, that gate synaptic and extrasynaptic NMDARs, respectively (Papouin *et al.*, 2012). The GluN1 subunits can bind both glycine and D-serine, GluN3 subunits can

only be gated by glycine, and glutamate can only bind to GluN2 subunits (Sanz-Clemente *et al.*, 2013).

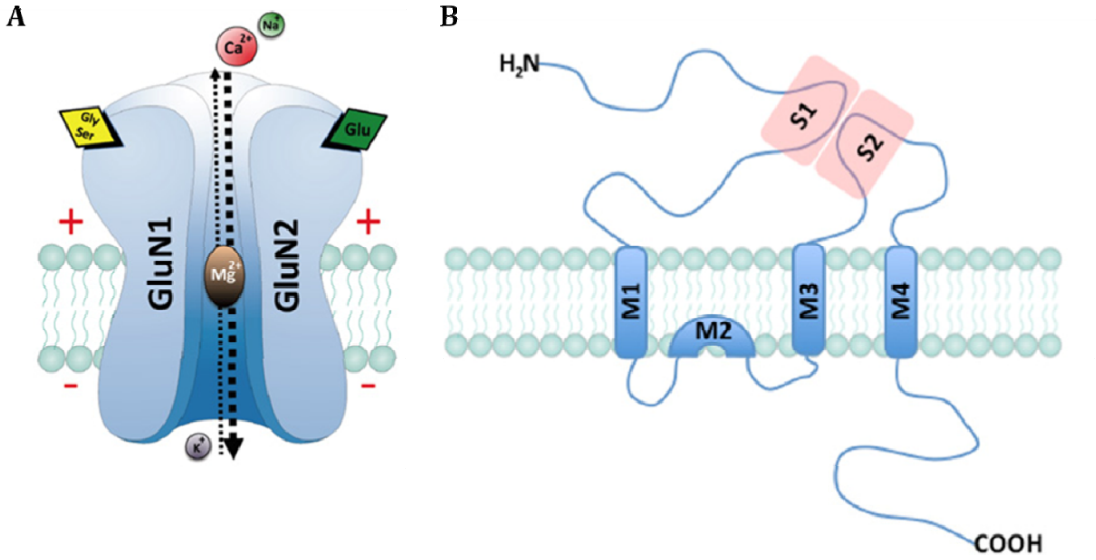


Figure 1.2. Model of a typical NMDA receptor, comprising GluN1/GluN2 subunits. **(A)** The channel pore is blocked by Mg^{2+} in a voltage-dependent manner. Depolarization of neurons relieves this blockade and glutamate and glycine/D-serine binding cause the channel to open, allowing Ca^{2+}, Na^{+} or K^{+} influx. **(B)** Each NMDAR subunit consists of three transmembrane domains (M1, 3 and 4) and one re-entrant loop (M2). Glutamate binding site is the pocket created by the extracellular regions S1 in the N-terminal tail and the loop between M3 and M4 (S2) (Adapted from Sanz-Clemente et al., 2013).

NMDAR composition versus localization

During development, a change in the composition of the NMDARs occurs and the predominant GluN1/GluN2B receptors are in part substituted by GluN1/GluN2A receptors (Liu *et al.*, 2004b; Zhou and Baudry, 2006; Gambrill and Barria, 2011). It has been proposed that NMDARs can be differently linked to specific intracellular pathways and thus to different physiological and pathological processes depending on whether they possess GluN2A or GluN2B subunits (Kim *et al.*, 2005; Szydlowska and Tymianski, 2010). Due to the different characteristics of the GluN2A and GluN2B subunits, such as channel kinetics and their distinct interaction with different signaling molecules (Paoletti, 2011; Sanz-Clemente *et al.*, 2013), it is tempting to speculate that these two NMDAR subtypes have distinct functions. Indeed, there has been evidence suggesting that it is the subunit composition that determines whether the receptors will

activate cell survival (GluN2A) or cell death (GluN2B) cascades (Liu *et al.*, 2007; Chen *et al.*, 2008).

However, another perspective to explain the dual roles of these receptors is related with the sub-cellular localization of NMDARs. As such, GluN2A-containing NMDARs, which are predominantly synaptic, were mainly linked to the activation of signaling cascades and genes related with the survival program of neurons, whereas extrasynaptic GluN2B-containing NMDARs would activate signaling cascades and genes that promote cell death (Hardingham *et al.*, 2002; Zhang *et al.*, 2007; Xu *et al.*, 2009; Hardingham and Bading, 2010). However, other studies show that both GluN2A and GluN2B subunits are present at synaptic and extrasynaptic sites (Groc *et al.*, 2006; Harris and Pettit, 2007) and can trigger cell death and neuroprotective signaling (Dawson *et al.*, 1991; von Engelhardt *et al.*, 2007; Martel *et al.*, 2009b), suggesting that the subunit composition does not explain thoroughly the dichotomous nature of NMDAR signaling. Recently, Martel et al. (2012) demonstrated that the C-terminal of the GluN2 subunits contributes critically to excitotoxicity, and that NMDAR subunits containing the GluN2B C-terminal are more lethal than those containing the GluN2A tails, regardless of location (Martel *et al.*, 2012). This observation suggests that the subunit/C-terminal domain composition may represent an additional factor that determines the level of excitotoxicity following chronic NMDAR activation.

Strategies to overcome ischemia-induced overactivation of NMDARs

The overactivation of the NMDARs has long been considered a hallmark in the process of excitotoxic-related ischemic neuronal death. However, clinical trials have shown that the blockade of their activity, using selective antagonists such as (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK801) or (2*R*)-amino-5-phosphonopentanoate (APV), does not ameliorate neuronal damage due to the adverse side effects associated with blocking the physiologic activity of the receptors (Chen and Lipton, 2006). As such, research has rather focused on the identification of signaling cascades and specific protein interactions mediating cell death downstream of NMDARs as possible targets for therapeutic interventions. To date, many GluN1 or GluN2 subunit binding partners have been identified in the postsynaptic density besides PSD-95, such as calmodulin (CaM) (Ehlers *et al.*, 1996; Akyol *et al.*, 2004), CaMKII (Leonard *et al.*, 2002), α-actinin (Wyszynski *et al.*, 1997; Merrill *et al.*, 2007), tubulin (van Rossum *et al.*, 1999) and spectrin (Wechsler and Teichberg, 1998), among others.

Regarding the involvement of PSD-95 in NMDAR-mediated cell death, it has been shown that PSD-95 effectively mediates the connection between NMDARs and nNOS, which is preferentially activated by Ca^{2+} influx through these receptors (Sattler *et al.*, 1999; Arundine and Tymianski, 2004). The NMDAR-nNOS interaction promotes the production of NO, that participates in NMDAR-dependent excitotoxicity (Dawson *et al.*, 1991; Sattler *et al.*, 1999). The possibility that the NMDAR/PSD-95 interaction might constitute a therapeutic target for diseases that involve excitotoxicity was considered attractive as an alternative therapeutic approach that could avoid the negative consequences of blocking general NMDAR function. However, PSD-95 allows the interaction between many different molecules, rendering the deletion of this protein an impractical therapy for brain injury. Alternatively, using a peptide that competes with the GluN2 subunit specifically for the binding site in PSD-95 has been shown to suppress ischemia-induced excitotoxicity (Aarts *et al.*, 2002; Martel *et al.*, 2009a; Fan *et al.*, 2010; Doucet *et al.*, 2012). This strategy was effective when applied either before or 1h after the *in vitro* and *in vivo* insult (Aarts *et al.*, 2002) or 3h after the *in vivo* insult (Sun *et al.*, 2008).

Another strategy based on the use of interaction-specific peptides that can work as a potential therapeutic approach against NMDARs-mediated neuronal injury involves the enhancement of endogenous NMDAR-activity modulators. For instance, the downstream regulatory element antagonist modulator (DREAM) has been shown to act as a negative modulator of NMDAR activity (Zhang *et al.*, 2010). As such, a peptide was generated according to the critical binding site of DREAM in the GluN1 subunit, mimicking the interaction of DREAM with the NMDARs. The application of this peptide has shown to be protective against NMDAR-induced neuronal injury in cultured hippocampal neurons submitted to OGD, as well as in mice subjected to focal ischemia, either before or after the ischemic insult (Zhang *et al.*, 2010).

Together, these studies open the possibility that the development of similar strategies, either based on the disruption of specific deleterious molecular pathways linked to the activation of NMDARs or on the enhancement of regulatory proteins that modulate these receptors and counteract overactive NMDAR function, may be employed to study and manage signaling pathways responsible for ischemic neuronal injury.

Since NMDARs are important targets for therapeutic intervention, and their pharmacological diversity is largely determined by the GluN2 subunit (GluN2A or

GluN2B) present in the heteromeric receptors, information concerning the post-ischemic expression levels and subunit composition of the NMDARs is necessary to guarantee specificity and efficacy of potential neuroprotective strategies against neuronal death signaling activated by these receptors.

Expression levels of NMDAR subunits after cerebral ischemia

In vivo studies of global ischemia have been able to detect changes in the regulation of the NMDAR subunit levels in the CA1 region at early times after the insult, but different studies reported distinct observations. For instance, whereas some studies show a decrease in the expression levels of GluN2A and GluN2B subunits (Zhang *et al.*, 1997; Hsu *et al.*, 1998), contradictory data shows an increase in the immunoreactivity of both subunits (Won *et al.*, 2001). The OGD model has also been used to assess changes in the expression of NMDAR genes induced by ischemia, but has not been able to clarify the controversy between different observations. For instance, it has been observed that a brief OGD insult can trigger an immediate down-regulation in the mRNA levels of GluN1, GluN2A and GluN2B subunits, with partial recovery after 3h, and that this process seems to be independent of the Ca^{2+} influx through both AMPARs and NMDARs, given that these changes were not altered in the presence of the receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and MK801, respectively (Dos-Anjos *et al.*, 2009). Another study shows that the OGD insult triggers a decrease in synaptic proteins such as PSD-95, CaMKII and NMDAR subunits GluN1 and GluN2B in neurons of the hippocampal CA3 region 24h after the insult (Jung *et al.*, 2012). However, in contrast with these observations, Ahlgren and co-workers (2011) showed that OGD stimulation failed to induce significant changes in the mRNA levels of NMDAR subunits in the hippocampus as far as 24h after the insult (Ahlgren *et al.*, 2011). Although the aforementioned *in vitro* studies were performed in organotypical hippocampal slices, it is possible that the opposing results are due to differences in the duration of the OGD insult and post-stimulation period, which influence the regions affected by the ischemic insult: short stimuli ensure a delayed and CA1 region-specific cell death, whereas longer periods of OGD lead to unselective cell death in the whole slice (Zukin *et al.*, 2004).

Less common NMDAR subunits can also be targets of differential regulation induced by ischemia. For instance, it has been reported that a brief OGD stimulus leads to an increase in the mRNA and protein levels of the GluN2C subunit after 90 minutes of recovery, whereas GluN2A and GluN2B levels remain unaltered (Small *et al.*, 1997).

In the adult hippocampus, GluN2C subunit expression is normally absent and restricted to glial cells. Moreover, GluN2C subunits are associated with a smaller single channel conductance and shorter mean open time (Ebralidze *et al.*, 1996; Rossi *et al.*, 2002). Therefore, an OGD-induced increase in the protein levels of GluN2C might confer resistance to neurons. However, the changes in the expression levels of GluN2C were not region-specific, rendering any conclusions about its role in neurodegeneration unclear.

Intriguingly, the expression levels of GluN3A also seem to be altered following ischemic insults. Normally, the expression of GluN3A subunits in the brain is regionally and temporally restricted and in the adult hippocampus the expression levels are very low (Wong *et al.*, 2002). When present, the GluN3A subunit decreases the conductance and Ca^{2+} permeability of GluN1/GluN2 channels and renders NMDARs insensitive to Mg^{2+} (Henson *et al.*, 2010) **(Figure 1.3)**. Accordingly, GluN3A knock-out neurons have a larger amplitude of NMDAR whole-cell currents and Ca^{2+} conductance than that of wild-type neurons (Mosbacher *et al.*, 1994; Das *et al.*, 1998), whereas transgenic mice expressing exogenous GluN3A subunits in the adult brain present reduced Ca^{2+} permeability of NMDA-evoked currents (Shi *et al.*, 2001). As such, the GluN3A subunit has been suggested to act as an inhibitory molecule to counteract the overactivation of NMDARs. In fact, it has been observed that exogenously added GluN3A increases neuronal survival in mice subjected to focal ischemia (DeGracia and Hu, 2007). Noteworthy, it appears that *in vitro* and *in vivo* ischemic insults can induce the up-regulation of endogenous GluN3A, rendering neurons resistant to excitotoxicity and reducing the contribution of NMDARs to the generation of toxic free radicals (Krause and Tiffany, 1993).

Concluding remarks on the regulation of NMDARs after cerebral ischemia

According to the aforementioned studies, besides leading to the overactivation of NMDARs, it appears that ischemic insults can also trigger the hypoactivity of NMDARs, whether it is by down-regulating NMDAR subunits or by increasing the expression levels of subunits that diminish NMDAR activity, such as the GluN2C and the GluN3A. It is probable that these opposing modifications in NMDAR activation take place at distinct phases, with the overactivation of the receptors occurring during or immediately after the ischemic event, and the reduction in NMDARs activity occurring at a later phase after the insult. Nevertheless, there are some topics that

remain to be clarified. For instance, it is still not clear whether such changes occur prior or after the onset of cell death, or whether upon global ischemia they are region-specific and thus related to the susceptibility or resistance of different areas in the hippocampus.

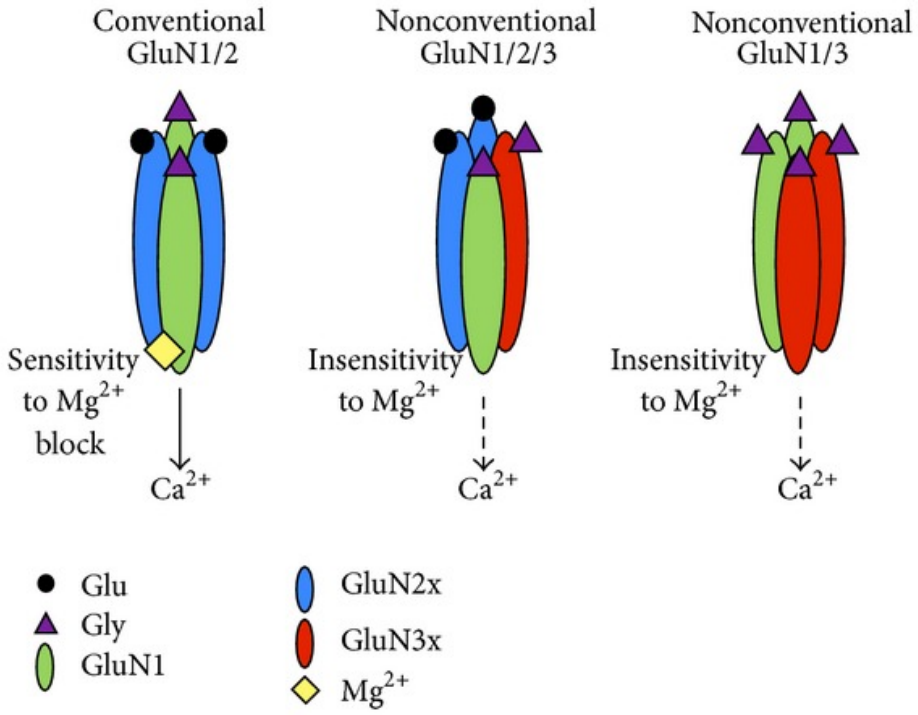


Figure 1.3. Schematic diagram showing the relative differences between conventional GluN1/GluN2 NMDA receptors (left), heterotrimeric GluN1/GluN2/GluN3 receptors (center) and heterodimeric GluN1/GluN3 glycine-activated receptors (right). The major differences between these receptors are the relative sensivity to Mg^{2+}, capacity to bind glutamate or glycine and relative permeability to Ca^{2+}. The GluN3 subunit thus has a dominant-negative effect on NMDAR activity (From Kehoe *et al.*, 2013).

AMPA receptors

Structure of AMPA receptors

AMPA receptors constitute the class of ionotropic glutamate receptors that mediates the fast depolarization in glutamate-induced neurotransmission, playing a key role in the synaptic plasticity of the vertebrate CNS. Changes in synaptic strength,

such as those occurring on LTP and long-term depression (LTD), which are thought to be involved in learning and memory formation, are associated with changes in AMPAR distribution and phosphorylation. Furthermore, deregulation of AMPAR activity is related to many pathologic brain injuries (Santos *et al.*, 2009).

AMPA receptors are homo- or heteromeric assemblies of four subunits, GluA1-4, which are encoded by separate genes. AMPA receptor mRNA can be detected at very early stages of development, although the expression of the subunits is developmentally regulated (Palmer *et al.*, 2005), and can also be found in glial cells (Gallo and Russell, 1995). The mRNAs for GluA1, 2 and 3 are largely expressed throughout the CNS, whereas GluA4 is expressed in a restricted spatial and temporal fashion (Santos *et al.*, 2009).

AMPA receptor subunits have a similar topology to that of the NMDARs, with four hydrophobic membrane domains with an extracellular N-terminal domain and an intracellular C-terminal tail, which is different among the subunits **(Figure 1.4)**.

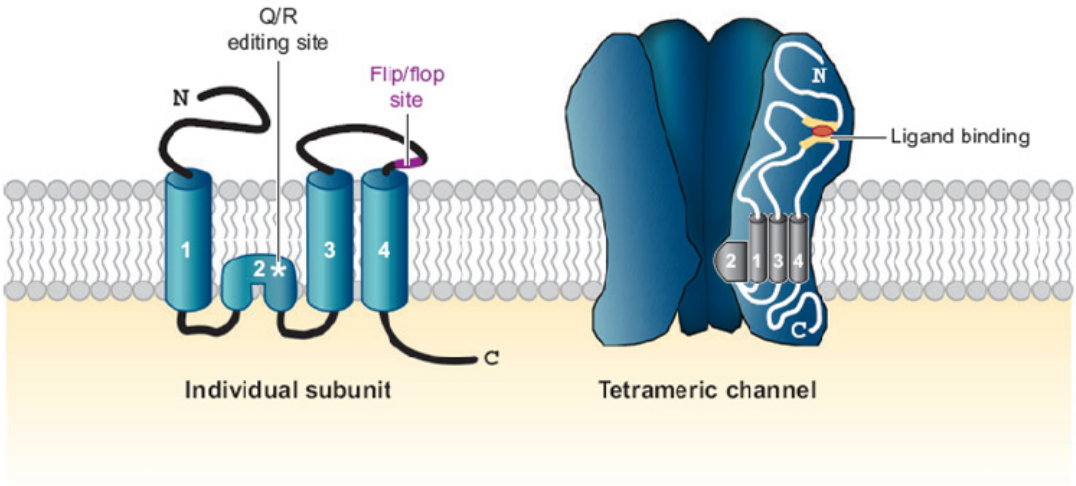


Figure 1.4. Structure of the AMPA receptor subunits and the tetrameric channel. The individual subunits are formed of four transmembrane domains. The channel comprises four subunits, generally two dimers which, in turn, are usually two different subunits (GluA1/2 or GluA2/3, for example) (From Shepherd and Huganir, 2007).

Diversity of AMPA receptor subunits: the role of GluA2

The ability of Ca^{2+} to enter the cell through AMPARs is determined by the GluA2 subunit, which is preferentially incorporated into the receptor (Sans *et al.*, 2003).

When AMPARs are assembled from combinations of GluA1, GluA3 and GluA4, the receptors are highly permeable to Ca^{2+}. However, when GluA2 is present, Ca^{2+}-permeability is significantly reduced (Hollmann *et al.*, 1991). Since the majority of AMPARs are hetero-oligomers containing GluA1/GluA2 or GluA2/GluA3 subunits, these receptors are usually impermeable to Ca^{2+} (Wenthold *et al.*, 1996). As such, the GluA2 subunit is a key component of AMPAR function. Indeed, the presence of GluA2 in heteromeric AMPAR assemblies determines not only the permeability to cation ions like Ca^{2+} or Zn^{2+}, but also channel kinetics, conductance, receptor assembly, among other properties (Liu and Zukin, 2007). AMPA receptors are assembled in the ER and are trafficked to the plasma membrane. Their presence at the synapse results from a controlled and dynamic process between insertion (exocytosis) and removal (endocytosis) (Malinow and Malenka, 2002; Man, 2011). The subunit composition of AMPARs also influence their trafficking (Malinow and Malenka, 2002). The presence of the GluA2 subunit is important for the stability and trafficking of AMPARs within the synapse, though it is not essential (Panicker *et al.*, 2008).

A wide variety of functionally distinct AMPARs 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. We will briefly describe the RNA splicing of AMPARs and the editing process of GluA2, since this subunit controls Ca^{2+} influx through the receptors and we are particularly interested in Ca^{2+}-permeable AMPARs, the subtype of AMPARs more relevant to transient global ischemia-induced neuronal death.

RNA splicing

Alternative RNA splicing increases the molecular diversity of AMPAR subunits. For instance, GluA1, GluA4 and an alternative splicing form of GluA2, GluA2L, have longer cytoplasmic tails, whereas GluA2, GluA3 and an alternative form of GluA4, GluA4c, present a short cytoplasmic domain (Santos *et al.*, 2009). Interactions with specific proteins through the C-terminal tail of each subunit have an important role in controlling the trafficking and stabilization of AMPARs at the synapses, including after neuronal insults. As such, AMPARs composed of GluA2 and 3 constitutively replace existing synaptic receptors, but, in contrast, those constituted by GluA1 and 2L or GluA2 and 4 are targeted to synapses during plasticity (Shi *et al.*, 2001).

Moreover, all the subunits are expressed in two distinct isoforms, *flip* and *flop*, which are generated by alternative splicing of a region near the fourth transmembrane

domain **(Figure 1.4)**. Despite these 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). For instance, homomeric AMPAR channels formed by GluA4$_{flop}$ subunits desensitize four times faster than GluA4$_{flip}$ channels, a difference also found between GluA3$_{flop}$ and GluA3$_{flip}$ channels (Mosbacher *et al.*, 1994).

GluA2 RNA editing

RNA editing, a process that consists in the enzymatic modification of the DNA encoded information, is an important mechanism of receptor regulation. In the AMPAR family, desensitization kinetics is controlled by editing of GluA2, GluA3 and GluA4 at a site preceding the fourth transmembrane region, the R/G site, in which an arginine (R) is substituted by a glycine residue (G). This process is 80-90% complete in the adult rat brain (Barbon and Barlati, 2011).

Another editing event occurs in the GluA2 subunit, in which a glutamine (Q) codon is substituted by an arginine (R) codon in the region of the second membrane-associated segment (Q/R site), which forms the pore of the receptor **(Figure 1.4)**. Unedited subunits, containing a neutral glutamine residue, consist of Ca^{2+}-permeable AMPARs, 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). In neonatal and adult rat brain, nearly 100% of GluA2 mRNA is edited at the Q/R site, rendering GluA2-containing AMPARs impermeable to Ca^{2+}, whereas the unedited form is detectable only in the embryonic brain, in a small percentage (1%) of total GluA2 mRNA (Wright and Vissel, 2012). Thus, the majority of AMPARs in the CNS contain the GluA2(R) subunit and are, therefore, impermeable to Ca^{2+}. However, a significant amount of Ca^{2+}-permeable AMPARs 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 (Liu and Zukin, 2007; Wright and Vissel, 2012).

Role of Ca^{2+}-permeable AMPARs in cerebral ischemia

The levels of Ca^{2+} -permeable AMPARs are regulated in response to neuronal activity, through mechanisms that involve particular protein-protein interactions with

GluA2 and that regulate the insertion of GluA2-lacking and GluA2-containing receptors in the membrane (Hanley, 2013). It has been found that two proteins, both containing GluA2-interacting PDZ domains, the protein interacting with C-kinase-1 (PICK1) (Hanley and Henley, 2005; Hanley, 2006; Cao *et al.*, 2007; Jaafari *et al.*, 2012) and the N-ethylmaleimide sensitive fusion protein (NSF) (Braithwaite *et al.*, 2002; Hanley *et al.*, 2002; Hanley, 2007), are required for the activity-dependent trafficking of the receptors. Both proteins participate in a dynamic process of regulation and control of the GluA2-content at the cell surface, thus indirectly controlling Ca^{2+} permeability of synaptic receptors.

Previous studies using cultured hippocampal neurons and the OGD stimulus showed early effects at the level of AMPAR traffic. A brief period of OGD stimulation caused the internalization of synaptic GluA2-containing AMPARs in hippocampal neurons (Liu *et al.*, 2006; Dixon *et al.*, 2009). The decrease in GluA2 surface levels shortly after the onset of the insult is mediated by activation of a regulated receptor internalization pathway identical to that of the physiologic transport of AMPARs in response to neuronal activity. In hippocampal neurons, GluA2-containing AMPARs are anchored at the synapse by glutamate receptor interacting protein (GRIP), but following OGD, protein kinase C (PKC) is activated and phosphorylates GluA2, causing the disruption of the GluA2 binding to GRIP. PICK1 then competes with GRIP for binding to GluA2 and the association of GluA2-containing AMPARs with PICK1 promotes receptor internalization, whereas GluA2-lacking AMPARs are inserted in the synapse (Liu *et al.*, 2006; Dixon *et al.*, 2009) **(Figure 1.5)**. Binding of PICK1 with GluA2 is a crucial step in this process, given that disruption of this interaction blocks the retrieval of GluA2 from the cell membrane and protects neurons from the delayed cell death triggered by the OGD insult (Dixon *et al.*, 2009). Thus, it is believed that the replacing of GluA2-containing AMPARs by GluA2-lacking AMPARs may account for the selective vulnerability of hippocampal CA1 neurons.

Besides the changes in the trafficking of AMPARs, another mechanism contributing to the increase in the Ca^{2+}-permeable AMPARs content is the decrease of GluA2 gene expression upon an ischemic insult. Global ischemia triggers the reduction of GluA2 expression in the pyramidal neurons of the CA1 region of the hippocampus, both at the mRNA (Pellegrini-Giampietro *et al.*, 1992; Gorter *et al.*, 1997) and protein levels (Opitz *et al.*, 2000; Noh *et al.*, 2005), resulting in increased levels of Ca^{2+}-permeable AMPARs. Insulted neurons show increased amplitude in the AMPAR-mediated currents, which can be blocked in the presence of the specific inhibitor of Ca^{2+}-permeable AMPARs, Naspm (Noh *et al.*, 2005). Also, in these neurons, acute loss of GluA2 enhances vulnerability to glutamate-induced pathogenecity (Liu and Zukin,

2007). Additionally, it has been reported that the suppression in GluA2 gene expression is dependent of ischemia-induced activation of the gene encoding the repressor element-1 silencing transcription factor/neuron restrictive silencer factor (REST/NRSF) (Calderone *et al.*, 2003; Noh *et al.*, 2012).

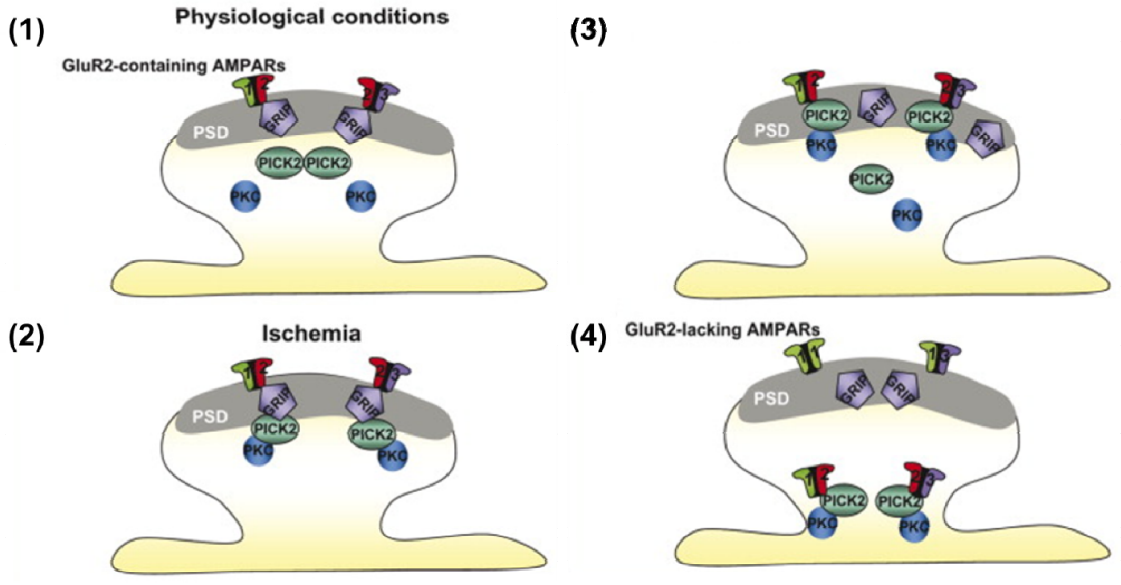


Figure 1.5. Ischemia-induced switch in AMPA receptor subtypes at CA1 synapses. **(1)** In the basal state, GluA2-containing AMPARs are stabilized at the postsynaptic membrane by association with GRIP. **(2)** Ischemic insults activate PKC and promote binding of PKC to PICK1. The PICK1–PKC complex binds to the GRIP–GluA2 complex. **(3)** PICK1 competes with GRIP for binding to GluA2, leading to disruption of GluA2 binding to GRIP. **(4)** Association of GluA2-containing AMPARs with PICK1 promotes receptor internalization, while synaptic incorporation of GluA2-lacking AMPARs occurs, thereby replacing GluA2-containing AMPARs at insulted CA1 synapses (Adapted from Liu & Zukin, 2007).

Indeed, Ca^{2+}-permeable AMPARs have been increasingly believed to be important mediators of ischemia-induced neuronal death (Buchan *et al.*, 1991; Pellegrini-Giampietro *et al.*, 1992; Gorter *et al.*, 1997; Opitz *et al.*, 2000; Liu *et al.*, 2004a; Noh *et al.*, 2005; Liu *et al.*, 2006; Dixon *et al.*, 2009). Promoting the expression of AMPARs impermeable to Ca^{2+} enhances protection of CA1 neurons after transient global ischemia whereas overexpression of the unedited form of GluA2, GluA2(Q), causes the death of hippocampal CA3 neurons and dentate gyrus cells, which otherwise would be resistant to ischemia-triggered damage (Liu *et al.*, 2004a). Altogether, these

results pointed out that AMPARs permeable to Ca^{2+} (GluA2-lacking) may play a significant role in ischemia-induced neurotoxicity.

Moreover, recent evidence suggests that changes in GluA2-containing AMPARs can be related with the resistance shown in neurons from the hippocampal CA3 region. According to Dennis et al. (2011), the OGD insult induces internalization and degradation of GluA2 and GluA1 proteins in the neurons of the CA3 region soon after the insult, which seems to be responsible for the nearly complete depression of AMPAR synaptic transmission in this area of the hippocampus. Similar to the mechanism proposed for the removal of GluA2 from the CA1 synapses, this process also seems to require the activation of PKC and the involvement of PICK1 (Dennis *et al.*, 2011). According to the authors, the removal of AMPARs in the CA3 region results from a neuroprotective response against ischemia, thus explaining the different vulnerability between these neurons and those of the CA1 region.

Additionally, the GluA2 mRNA editing process can also be affected by cerebral ischemia, since it was shown that transient global ischemia causes a cell-specific reduction in the expression of adenosine deaminases acting on RNA-2 (ADAR2), the enzyme normally responsible for GluA2 Q/R editing in the CA1 hippocampal neurons (Peng *et al.*, 2006). Consequently, the GluA2 subunits that form the receptors are predominantly unedited, which, in addition to a reduced GluA2 expression, further contributes to the up-regulation of Ca^{2+}-permeable AMPARs after cerebral ischemia (Peng *et al.*, 2006).

Concluding remarks on ischemia-induced regulation of AMPARs

Taken together, these observations suggest that there are three mechanisms responsible for the changes in AMPARs, occurring at different moments after the insult: the event of receptor trafficking, taking place at early times after ischemia, the delayed decrease in GluA2 expression levels mediated by activation of REST and co-repressors, such as mSin3A (mammalian Sin3 transcriptional regulator family member A) and CoREST **(Figure 1.6)** and the increase in GluA2 (Q) unedited subunits, responsible for a long-lasting change in AMPAR subunit composition and function. It is believed that the Ca^{2+} signaling provided by the initial GluA2-lacking receptors may represent an early mechanism responsible for long-term changes in the transcriptional program, relevant to synaptic remodeling, including the altered GluA2 gene expression (Liu and Zukin, 2007).

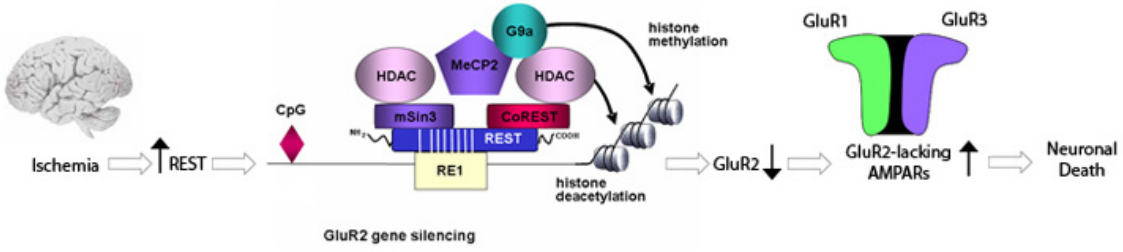


Figure 1.6. Schematic figure showing REST binding to the GluA2 gene promoter and silencing of GluA2 gene expression. Ischemia induces activation of REST, promoting REST binding to the responsive element-1 (RE1) sequence present in the GluA2 promoter, thereby silencing GluA2 transcription. Other proteins such as mSin3A and CoREST, recruited by REST, and histone deacetylases, also bind to the promoter and participate in gene silencing of GluA2. CoREST also recruits methyl-CpG binding protein 2 (MeCP2) and site-specific histone methyltransferase G9a, which promotes DNA and histone methylation. Down-regulation of GluA2 expression reduces the amount of total GluA2 protein available to compose the receptors, resulting in the assembly and insertion of GluA2-lacking AMPARs at CA1 synapses of the post-ischemic hippocampus (Adapted from Liu & Zukin 2007).

However, given the data obtained regarding the influence of ischemic insults in AMPAR trafficking and subunit composition, it remains to be clarified whether such changes are region-specific and the influence of AMPARs for the final outcome of each of the different regions of the hippocampus after the ischemic insult.

Non-glutamate-mediated component of cerebral ischemia physiopathology

Despite the strong evidence showing that excitotoxicity is responsible for neuronal demise after brain ischemia, due to the overactivation of glutamate receptors and their participation in triggering molecular pathways leading to cell death (Szydlowska and Tymianski, 2010), clinical trials using antagonists of these receptors have failed as a neuroprotective strategy (Lai *et al.*, 2013). This is probably due to the severe side effects that arise from blocking the physiologic activity of such important receptors to brain function, but it also suggests that there may exist mechanisms of neurotoxicity that operate independently of, or in parallel with, excitotoxicity

(Szydlowska and Tymianski, 2010). Given that brain ischemia causes great ionic imbalance during and after the insult (Pundik *et al.*, 2012), it is increasingly evident that many of the events triggered by ischemic insults center on the regulation of different ion channels that may be coupled to distinct signaling pathways (Song and Yu, 2013), in which some may be detrimental, and their prevention potentially beneficial, and some may be important to enhance cell resistance to ischemia.

Several studies have shown the involvement of a variety of ion channels that may contribute to ionic imbalance during an ischemic insult, such as the NCXs (Czyz and Kiedrowski, 2002; Annunziato *et al.*, 2004; Bano *et al.*, 2005), the Ca^{2+}- permeable acid-sensing ion channels (ASICs) (Xiong *et al.*, 2004), the Na^{+}–K^{+}–Cl^{-} co-transporter isoform 1 (NKCC1) (Chen *et al.*, 2005), the Na^{+}–H^{+} exchanger (Sheldon *et al.*, 2004; Chesler, 2005; Luo *et al.*, 2005) and members of the transient receptor potential (TRP) channels (McNulty and Fonfria, 2005). It is crucial to understand the contribution of each one of these types of ion channels since they can mediate opposing effects in the ischemic response, as is the case of certain members of the TRP channel family, for instance. The TRP channels are a group of ion channels mostly located on the cell membrane of various types of cells and are grouped into six sub-families (Gees *et al.*, 2012). The TRPMs ("melastatin") is one of these sub-families and include a group of eight distinct members, but so far only TRPM2 and TRPM7 have been shown to be important in neuronal death after *in vitro* and *in vivo* ischemia. TRPM2 and TRPM7 are both non-selective cation channels (Gees *et al.*, 2012) and have been shown to further increase oxidative stress and intracelullar Ca^{2+} accumulation induced by ischemia (Aarts *et al.*, 2003; Aarts and Tymianski, 2005; Jia *et al.*, 2011; Naziroglu, 2011; Verma *et al.*, 2012; Alim *et al.*, 2013; Nakayama *et al.*, 2013; Shimizu *et al.*, 2013; Song and Yu, 2013). On the other hand, TRPC6, which belongs to the "canonical" sub-family of TRPs, has been shown to have a protective role during ischemic insults. In fact, overexpression of TRPC6 prevented cell death by activation of the cAMP response element–binding protein (CREB) signaling pathway (Du *et al.*, 2010) and by inhibiting Ca^{2+}-currents mediated by NMDAR activation (Li *et al.*, 2012). The distinct contribution of different ion channels to the ischemic injury suggests that an efficient therapy for brain ischemia may rely on the targeting of multiple receptors or ion channels and regulatory pathways (Song and Yu, 2013).

Chloride ions and brain ischemia

Cation channels (namely Ca^{2+} channels) are not the only channels disrupted by the energetic failure in the ischemic brain. Ionic imbalance can also include other channels, such as the volume-regulated anion channels (VRACs), a group of widely expressed Cl^- channels that regulate cell volume under physiologic and pathologic conditions (Nilius and Droogmans, 2003; Inoue *et al.*, 2005). A moderate activation of VRACs enables restoration of cell volume in the face of osmotic stress, but overactivation of these channels can result in neuronal death, depending on the severity of the insult (Nilius and Droogmans, 2003; Inoue *et al.*, 2005). In fact, several studies show that significant alterations in Cl^- homeostasis can contribute to neuronal death. For instance, excitotoxic insults induce somatic and dendritic swelling mediated by Cl^- influx (Rothman, 1985; Olney *et al.*, 1986; Choi, 1987; Hasbani *et al.*, 1998; Inoue and Okada, 2007). The γ-aminobutyric acid type A ($GABA_A$) receptors seem to be partially responsible for this effect since pharmacological inhibition of these receptors abolished intracellular Cl^- accumulation and partially blocked excitotoxic injury (Hasbani *et al.*, 1998; Chen *et al.*, 1999; Babot *et al.*, 2005). Also, *in vivo* ischemia and OGD cause neuronal VRAC activation and subsequent neuronal death; however, once again, pharmacological inhibition of VRACs was shown to be neuroprotective, further supporting that activation of chloride channels contributes to ischemic neuronal damage (Feustel *et al.*, 2004; Zhang *et al.*, 2011; Alibrahim *et al.*, 2013). Other anion channels, such as the volume-sensitive outwardly rectifying (VSOR) Cl^- channels, are activated under excitotoxic or ischemic conditions and may also participate in toxic chloride accumulation (Inoue and Okada, 2007; Zhang *et al.*, 2011).

Despite the accumulating evidence showing the influence of impaired Cl^- homeostasis in brain ischemia, the specific molecular targets of the general inhibitors of VRAC activity remain to be identified (Zhang et al., 2011). However, one of the inhibitors that protected neurons from excitoxicity and OGD- induced VRAC activation and neuronal cell death is IAA-94 (R(+)-[(6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)-oxy] acetic acid) (Inoue and Okada, 2007; Zhang *et al.*, 2011), which has been shown to selectively block intracellular Cl^- currents mediated by the chloride intracellular channel 1 (CLIC1) (Valenzuela *et al.*, 2000; Novarino *et al.*, 2004; Milton *et al.*, 2008; Averaimo *et al.*, 2010).

The chloride intracellular channel CLIC1

The chloride intracellular channels (CLICs) are a recently identified class of chloride channel proteins that includes seven members (p64, parchorin, CLIC1-5) (Redhead *et al.*, 1992; Fernandez-Salas *et al.*, 2002; Littler *et al.*, 2010). The CLIC proteins are characterized by the presence of a conserved CLIC module of ~240 amino acids, present in each member of the family, although several members may contain additional unrelated N-terminal domains. Most CLICs are localized in intracellular membranes and have been related to functions including apoptosis, regulation of pH and the cell cycle (Valenzuela *et al.*, 1997; Valenzuela *et al.*, 2000; Fernandez-Salas *et al.*, 2002). These anion channels do not fit the paradigm set by classical ion channel proteins because some soluble, signal peptide-free CLICs bypass the classical secretory pathway and auto-insert directly into membranes, where they have ion channel activity (Cromer *et al.*, 2002; Tulk *et al.*, 2002; Littler *et al.*, 2010). These proteins can therefore coexist in both soluble and integral membrane forms and are considered as "metamorphic proteins" (Murzin, 2008). Other cases of metamorphic proteins are, for instance, some bacterial toxins and some intracellular proteins including Bcl-XL (B-cell lymphoma-extra large) and the annexins (Gouaux, 1997).

CLIC1 is the most studied member of this family of proteins (Averaimo *et al.*, 2010; Littler *et al.*, 2010). It has a wide tissue and sub-cellular distribution in mammalian cells (Ulmasov *et al.*, 2007), although CLIC1 homologues can also be found in invertebrates (Littler *et al.*, 2008) and plants (Elter *et al.*, 2007). Under reducing conditions, CLIC1 exists largely as a soluble, monomeric intracellular protein that is structurally homologous to the glutathione S-transferase (GST) superfamily, with a redox active site resembling glutaredoxin (Dulhunty *et al.*, 2001; Harrop *et al.*, 2001). However, under certain conditions, CLIC1 can suffer major conformational changes in the protein structure that allow membrane insertion (Averaimo *et al.*, 2010). In the monomeric form, CLIC1 has a binding site for the reduced form of glutathione (GSH) in its N-terminal domain (Dulhunty *et al.*, 2001; Harrop *et al.*, 2001; Littler *et al.*, 2004). According to the line of evidence accumulated during the last years, it is believed that, under oxidation, CLIC1 suffers drastic conformational changes, causing the detaching of GSH from its binding site that is consequently rearranged and, together with the amino terminal of the protein, probably constitutes the hydrophobic region that interacts with the cell membrane. CLIC1 could, therefore, act as a sensor of cell oxidation (Littler *et al.*, 2004; Novarino *et al.*, 2004; Milton *et al.*, 2008; Goodchild *et al.*, 2009). Moreover, the membrane insertion process of CLIC1 is also regulated by intracellular pH. Experiments in artificial membranes have shown that CLIC1 insertion

into lipid membranes increases not only in acidic pH (Warton *et al.*, 2002) but also in basic conditions (Tulk *et al.*, 2002).

Nevertheless, the specific mechanism by which CLIC1 inserts into biological membranes is still not completely understood. Littler and colleagues (2004) observed that *in vitro* CLIC1 undergoes structural changes upon oxidation, exposing hydrophobic residues that promote the dimerization of the protein, which minimizes the contact with the aqueous environment of the cytoplasm. The authors suggest that, *in vivo*, this exposed hydrophobic region could also act as a membrane-docking region, which allows the insertion of the CLIC1-dimer into membranes. Based on these observations, Littler and colleagues proposed a model for the insertion of CLIC1 in biological membranes where oxidative conditions are essential for the transition between the soluble and membrane forms of the protein (Littler *et al.*, 2004).

Another hypothesis for the mechanism of CLIC1 insertion into biological membranes came from Goodchild and colleagues (2009), who suggested that the dimerization process taking place during oxidation is not necessary for the insertion of the protein into membranes. In this model, the CLIC1 monomer could initially interact with the membrane surface and only then oxidation would promote the structural changes that allow the protein to cross the membrane and form a functional ion channel. This hypothesis is supported by the evidence that both monomeric and dimeric forms of CLIC1 are able to form functional ion channels in artificial membranes (Goodchild et al 2009). However, if it is true that monomeric CLIC1 has a GSH binding site, it remains to be explained how CLIC1 can interact with the lipid membrane before oxidation-mediated changes in the structure of the protein. Recent data also suggest that CLIC1 likely dimerizes to form a membrane-competent protopore complex (Peter *et al.*, 2013) and that between six to eight CLIC1 proteins can assemble into an oligomer to form an ionic channel (Goodchild *et al.*, 2011) **(Figure 1.7)**.

Membrane-bound CLIC1 exposes the N-terminal to the extracellular side, whereas the C-terminal remains on the intracellular side, as observed in overexpression experiments of recombinant CLIC1 with a flag epitope at the amino terminal or at the C-terminal (Tonini *et al.*, 2000).

CLIC1 is highly conserved among species, suggesting a very important cellular role, but its molecular function is still not well known (Littler *et al.*, 2010). Although CLIC1 was initially found to localize to the cell nucleus and intracellular vesicles of chinese hamster ovary (CHO) cells transfected with CLIC1-GST fusion protein (Valenzuela *et al.*, 1997), further studies have shown that the subcellular distribution of

this protein varies according to the cell type (Averaimo *et al.*, 2010). Thus, it is not inappropriate to think that the molecular role of CLIC1 can also be different among cell types. In fact, CLIC1 has been implicated in a number of different fundamental cell processes such as cell cycle (Valenzuela *et al.*, 1997), cell differentiation (Yang *et al.*, 2009) and sperm mobility (Myers *et al.*, 2004).

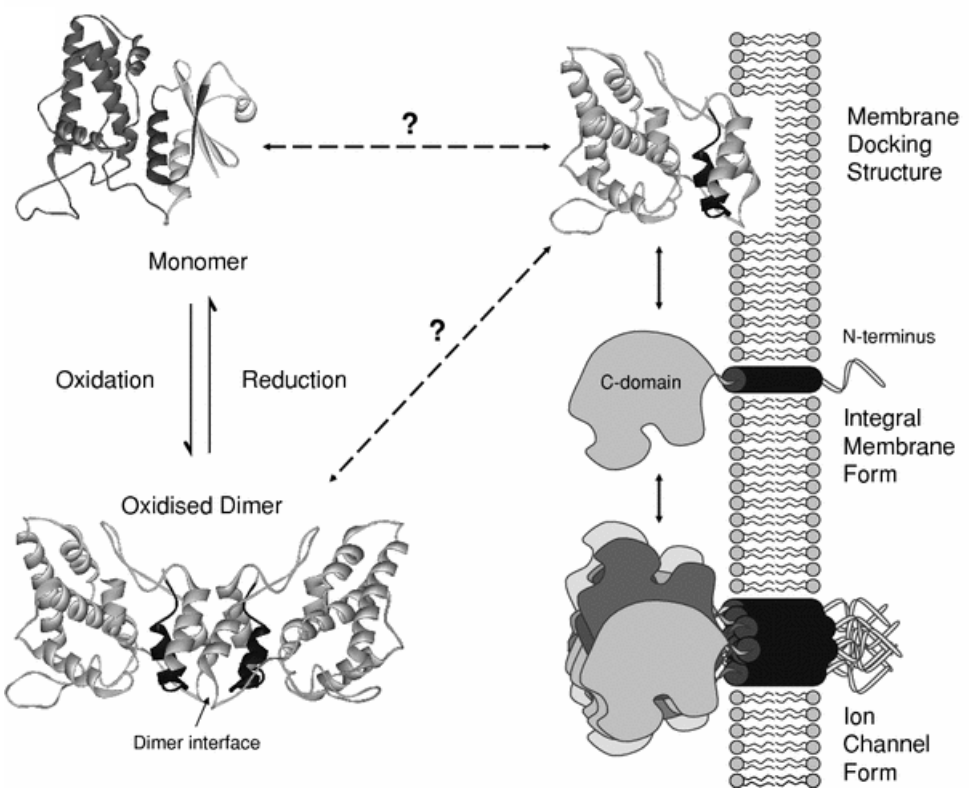


Figure 1.7. Model of CLIC1 insertion into the membrane. Under oxidative conditions, the soluble CLIC1 monomer suffers a structural transition to an all α-helical, non-covalent dimer in solution, and an hydrophobic surface is exposed. This hydrophobic surface may serve as an interface that allows dimerization or, when in the presence of a lipid bilayer, to initiate the process of membrane-docking. Nevertheless, it is still unclear whether formation of dimers is required or whether oxidation of a single CLIC1 monomer can trigger the conformational changes needed for membrane docking. After binding to the membrane, the N-terminal and the putative transmembrane domain are inserted across the bilayer, whereas the C-terminal remains in the cytoplasm. Several CLIC1 subunits bound to the membrane then converge to form the active ion channel, likely by oligomerization (Adapted from Goodchild et al., 2009).

The role of CLIC1 in neurons is completely unknown. The only data concerning CLIC1 activity in brain cells come from studies in cocultures of microglia with neuronal cells, in which toxic Aβ stimulation induces CLIC1 insertion into the membrane of microglial cells, acting as a chloride channel and inducing the production of ROS and the release of tumor necrosis factor α (TNF-α), that cause neuronal apoptosis (Novarino *et al.*, 2004; Milton *et al.*, 2008). Both the Aβ-induced Cl^- conductance and ROS generation were prevented by the selective CLIC1 blocker IAA-94 and by small interfering RNA (siRNA) knockdown (Novarino *et al.*, 2004; Milton *et al.*, 2008), implicating CLIC1 in neuronal damage mechanisms.

As mentioned above, oxidative stress is one of the factors that can control the transition between the soluble and the integral membrane form of CLIC1. Production of ROS and oxidative stress are known contributors to neuronal damage under ischemic conditions (Abramov *et al.*, 2007; Zhou *et al.*, 2007; Wang *et al.*, 2009; Zhou and Baudry, 2009; Liu *et al.*, 2011), thus it would be very interesting to analyze whether CLIC1 can also participate in the neuronal response to ischemic insults, either as a soluble protein or as a chloride ion channel.

Transcriptional modifications induced by cerebral ischemia

Altered gene expression and protein synthesis

As discussed above, cerebral ischemia triggers a multifaceted cascade of physiopathologic events, some of which are believed to be mediated in part by alterations of transcriptional and translational activities. Strong evidence has demonstrated that several gene families are expressed differentially during cerebral ischemia, including, for instance, transcription factors, heat shock proteins, adhesion molecules, receptors, neurotransmitters, growth factors and regulators of apoptosis (Jin *et al.*, 2001; Kim *et al.*, 2002; Schmidt-Kastner *et al.*, 2002; Tang *et al.*, 2002; Kawahara *et al.*, 2004; Lu *et al.*, 2004; Buttner *et al.*, 2009; Ramos-Cejudo *et al.*, 2012). However, the first group of genes to have increased expression levels during an ischemic insult are the immediate early genes (IEGs). IEGs are transcription factors that regulate subsequent expression of various genes that will mediate and regulate the activation of pro-survival or pro-death cascades. The most common IEGs are c-fos and

c-jun, that encode proteins that regulate the transcription of a large number of genes by binding to a specific activator protein-1 (AP-1) transcription activation site in their promoter region (Rauscher *et al.*, 1988), mediating long-term responses to signals that regulate many cellular processes, including differentiation, apoptosis, survival and cell growth (Vesely *et al.*, 2009). It has been shown that cerebral ischemia induces a rapid and transient synthesis of both c-fos and c-jun in the hippocampus after ischemia (Kogure and Kato, 1993). The synthesis of c-fos is prominent in the dentate gyrus and CA3, which are resistant to ischemia, but is minimal in the CA1 region, which is vulnerable to ischemia, thereby suggesting a neuroprotective role for this transcription factor. The neuroprotective role of c-fos is further confirmed by an up-regulation of this transcription factor after treatment with NMDA, which reduces neuronal damage following OGD (Cho *et al.*, 2001). However, it can also promote neuronal death following OGD; in fact, knockdown of this protein significantly protects neurons in this model of neuronal death (Cardoso *et al.*, 2010). Taken together, these observations further support that changes in gene expression may be either beneficial (friend) or detrimental (foe), and likely play a major role in neuronal outcome after cerebral ischemia (Akins *et al.*, 1996).

The events of altered gene expression can occur after the exposure to ischemia and continue after the insult, which may explain the delay between the insult and the first signs of cell death (Neumar, 2000). However, increased mRNA transcription does not guarantee increased levels of the corresponding protein since an overall depression in protein synthesis takes place in post-ischemic cells, which is transient in neurons that survive but permanent in neurons that will die (Thilmann *et al.*, 1986; Araki *et al.*, 1990; Krause and Tiffany, 1993; DeGracia and Hu, 2007). This causes a generalized decrease in gene expression that may inhibit the cell ability to replace damaged proteins necessary for survival as well as to translate newly formed mRNAs. Although generally viewed as detrimental, transient protein synthesis depression may protect neurons by preventing the expression of genes that cause programmed cell death, for instance. As such, alterations in gene expression induced by ischemic insults can be a double-edged sword. If transient inhibition of protein synthesis may theoretically reduce expression of proteins that signal for and/or execute post-ischemic injury pathways, pro-survival proteins will also have impaired synthesis (Neumar, 2000).

It is therefore important to consider the global depression of protein synthesis in the post-ischemic brain when studying the impact of changes in mRNA translation. The effect of new mRNA translation might be expected to be minimal under conditions where protein synthesis is less than 10% of baseline. However, there is clear evidence that newly expressed mRNAs are being translated into proteins. After transient

ischemia, most brain regions recover their protein synthesis capability; however, recovery in the selectively vulnerable areas is poor (Krause and Tiffany, 1993), as is the case, for instance, of the neurons of the hippocampal CA1 after transient global ischemia (Thilmann *et al.*, 1986; Araki *et al.*, 1990), which show increased levels of neuronal damage. These findings suggest that neurons that are unable to synthesize new proteins needed to survive show greater vulnerability to an ischemic insult, whereas cells that have successfully induced this response are resistant to subsequent lethal ischemic insults. In fact, in many tissues, brief episodes of ischemia impart resistance to subsequent prolonged ischemia that would normally cause cell death, a phenomenon referred to as ischemic preconditioning. The molecular mechanisms of ischemic preconditioning may help to identify endogenous protective mechanisms. *In vitro*, new mRNA and protein synthesis has been shown to be required for the development of ischemic preconditioning (Tapuria *et al.*, 2008; Narayanan *et al.*, 2013).

In summary, the induction of certain transcription factors, such as the IEGs, will lead to a multifaceted series of events that include the induction and repression of specific genes, some of which coding for proteins that may be part of a survival or death response that will influence the outcome of brain cells after the insult. As such, modulating the function of newly expressed proteins can be a potential therapeutic strategy against ischemia-induced neuronal damage. However, the success of this approach requires knowledge of which potentially injurious proteins are being expressed, or which protective proteins are failing to be up-regulated, under ischemic conditions. Mapping the time course of gene response is therefore an important approach to understand the functional interactions of different molecular pathways in brain ischemia. One good approach to gain insight into which genes are altered after brain ischemia is the microarray technology, which allows a genome-wide survey of gene expression changes.

The use of the microarray technology to investigate changes in gene expression after cerebral ischemia

Several studies investigated changes in the transcriptome of the ischemic brain using either human brain cells or animal models of ischemia induced by permanent MCAO or global ischemia **(Table 1.1)**. The OGD model has also been used to infer about changes in gene expression associated with pre- and post-conditioning-derived neuroprotection (Benardete and Bergold, 2009; Prasad *et al.*, 2012) **(Table 1.1)**.

Together, these studies have greatly contributed to identify some of the genes that have different expression levels after an ischemic insult and also to classify them into ontological families according to their function, which has helped to obtain a general perspective of the major cellular functions that might be modified after cerebral ischemia. However, while it is accepted that several cellular functions are compromised, our understanding of how this correlates with the selective vulnerability and delayed death of hippocampal neurons observed under global ischemia is still unclear. As such, the combined use of the OGD insult and the microarray technology may be a useful tool to investigate changes in the transcriptome of hippocampal neurons and correlate them with the physiopathologic events taking place after brain ischemia.

Table 1.1. Previous studies employing microarray approaches to study cerebral ischemia

	Jin *et al.*, 2001	Kim *et al.*, 2002	Tang *et al.*, 2002	Schmidt-Kastner *et al.*, 2002	Kawahara *et al.*, 2004	Lu *et al.*, 2004	Benardete *et al.*, 2009	Buttner *et al.*, 2009	Ramos-Cejudo *et al.*, 2012	Prasad *et al.*, 2012
Model	Adult male rat	Adult male rat	Adult male rat	Adult male rat	Adult male rats	Adult male rat	Hippocamp. slices	Adult male rat	Adult rat	Rat cortical cultures
Ischemia model	15' Global ischemia	PermanentMCAO	Permanent MCAO	2h MCAO	2' and 6' Global ischemia	30' or 2h MCAO	5'OGD (Pre-C)	15' Global ischemia	60' MCAO	OGD protocols for Pre-C and Post-C
Time points for RNA extraction	4, 24 and 72h	6h	24h	3h	1,3,12,24 and 48h	30', 4h, 8h, 24h, 3 and 7 days	3, 6 and 12h	1h, 6h or 24h	24h or 3 days	3h after OGD protocols
Gene Chip	Selected ODNs of human genes	Atlas Rat cDNA Array	Rat U34A array (Affymetrix)	Mouse UniGene 1 microarray (Incyte Genomics)	Rat U34A array (Affymetrix)	Rat U34 ODN array (Affymetrix)	Rat 230 2.0 DNA array (Affymetrix)	Rat 230 2.0 DNA array (Affymetrix)	Whole-Rat Genome Microarrays 4x44K (Agilent)	Whole-Rat Genome Microarrays 4x44K (Agilent)
No. of genes/ESTs /probes	374 genes	1176 genes	~8000 ESTs	9044 genes	~8000 ESTs	1322 genes	~30.000 ESTs	~30.000 ESTs	20.395 probes	26.930 genes
Cut-off value	1.7-fold	2.0-fold	2.0-fold	1.7-fold	2.0-fold	2.0-fold	1.2, 1.5 and 2.0-fold	2.0-fold	2.0-fold	1.5-fold
Validation technique	WB, IHC	qPCR	qPCR	qPCR	In situ hybridizat.	qPCR	qPCR	qPCR	qPCR	qPCR

Legend: ESTs (Expressed sequence tag); IHC (Immunohistochemistry); MCAO (middle cerebral artery occlusion); ODNs (oligodeoxynucleotides); Post-C (Postconditioning); Pre-C (Preconditioning), qPCR (quantitative real-time PCR); WB (Western Blot);

Objectives

Transient global cerebral ischemia induces the delayed death of the pyramidal hippocampal neurons of the CA1 region, which occurs only hours to days after the insult (Petito *et al.*, 1987; Bottiger *et al.*, 1998). This time-window between the end of the transient ischemic insult and the first signs of neuronal demise is believed to be associated with the activation of competing programs of gene expression, in which some will facilitate cell survival, whereas others will contribute to neuronal death (Papadopoulos *et al.*, 2000). However, the mechanisms responsible for the selective vulnerability of CA1 hippocampal neurons to global ischemia remain to be clarified.

Therefore, the present work was globally aimed at investigating changes in gene expression induced by an ischemic insult in order to identify new genes that may have a relevant role in cell death or in cell survival pathways specifically triggered in hippocampal neurons. For that purpose, we used primary cultures of rat hippocampal neurons subjected to oxygen and glucose deprivation (OGD), an established *in vitro* model for cerebral global ischemia that is suitable for investigations at the molecular level (Zukin *et al.*, 2004).

The first objective of the present work (Chapter III) was the characterization of alterations in the global gene expression profile of hippocampal neurons exposed to OGD. For this purpose we characterized the time-course of cell death mediated by OGD and the excitotoxic component of OGD-induced neuronal death, and performed microarray analysis to unveil genes whose expression was significantly altered in cultured hippocampal neurons at 7h and 24h after the insult, a time point prior to and after the onset of cell death. Analysis of the microarray results revealed a general down-regulation of a group of synaptic protein genes in hippocampal neurons after the OGD insult. Since important biochemical modifications have been shown to take place at the synaptic level of post-ischemic neurons, the second objective of this study (Chapter IV) was to pursue the analysis of OGD-induced down-regulation in synaptic protein genes, namely in genes encoding subunits of the AMPAR and NMDAR glutamate receptors. Finally (Chapter V), we identified OGD-induced changes in the expression levels of ion channel protein genes in hippocampal neurons subjected to OGD and analyzed the role of the intracellular chloride channel CLIC1 to the ischemic response.

The results obtained within the scope of this study may help to elucidate the pro-death and pro-survival mechanisms activated in post-ischemic neurons, therefore

unveiling endogenous molecular mechanisms and pathways that may be valuable for the development of therapeutic strategies for the treatment of cerebral ischemia.

Chapter II

Materials & Methods

Primary hippocampal neuronal cultures

Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin (0.06%, 15 min, 37°C; GIBCO Invitrogen) 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 $Na_2HPO_4.2H_2O$, 5 glucose, 1 sodium pyruvate, 10 HEPES and 0.001% phenol red). The hippocampi were then washed with HBSS containing 10% fetal bovine serum (GIBCO Invitrogen), to stop trypsin activity, and transferred to Neurobasal medium (GIBCO Invitrogen), supplemented with SM1 supplement (1:50 dilution; Stem Cell Technologies), 25 μM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin (Sigma-Aldrich). The cells were dissociated in this solution and were then plated in 6-well microplates (MW6) (for Western blot and real-time PCR experiments) or 24-well microplates (MW24) (for cell death assays), previously coated with poly-D-lysine (0.1 mg/mL, Sigma-Aldrich) or on poly-D-lysine coated glass coverslips, at a density of $85.0x10^3$ cells/cm^2. The cultures were maintained in a humidified incubator with 5% CO_2/95% air, at 37°C, for 14-15 days. All animal procedures were reviewed and approved by Direcção Geral de Veterinária (DGAV), Portugal.

Oxygen-Glucose Deprivation (OGD)

For the OGD challenge, hippocampal cultures were incubated in a glucose–free saline buffer (in mM: 10 HEPES, 116 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1 NaH_2PO_4, 1.8 $CaCl_2$, 25 $NaHCO_3$, 25 sucrose, pH 7.3) in an anaerobic chamber (Forma Anaerobic System, Thermo Fisher Scientific), at 37 °C, for the indicated periods of time. Control neurons were placed in a similar saline buffer with 25 mM glucose instead of sucrose and kept in an air/CO_2 incubator, at 37°C, for the same period of time. After the stimulation periods, the saline buffers were replaced by the conditioned medium and the cultures returned to the air/CO_2 incubator, where they were left to recover for the indicated times.

In studies performed in the presence of the NMDA receptor antagonist MK-801 (10 μM), the AMPA receptor antagonist GYKI 52466 (4-(8-Methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepin-5-yl)-benzenamine dihydrochloride, 50 μM) and the selective Ca^{2+}-permeable AMPARs (CP-AMPARs) antagonist Naspm (N-[3-[[4-[(3-Aminopropyl)amino]butyl]amino]propyl]-1-naphthaleneacetamide trihydrochloride, 50 μM), a pre-incubation of 15 minutes was done and the antagonists were present

during both the insult and the recovery period. MK-801, GYKI 52466 and Naspm were purchased from Tocris Cookson Ltd. For the studies with the CLIC1 channel protein, hippocampal neurons were treated with 50 μM CLIC1 inhibitor R(+)-[(6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5yl)-oxy] acetic acid (IAA-94, Sigma-Aldrich), which was present only during the stimulation period.

Transient Global Cerebral Ischemia in the Rat

Experiments were carried out in accordance with the Guidelines of the European Union Council (86/609/EU), following Spanish regulations (RD 1201/2005, BOE 252/34367–91, 2005) for the use of laboratory animals, and were approved by the Scientific Committee of the University of León. All efforts were made to minimize animal suffering and to reduce the number of animals used. Global cerebral ischemia was performed as previously described (Montori et al., 2010), with slight modifications.

Eight 3-month-old Sprague–Dawley male rats weighing 350–450 g were housed under standard temperature (22±1ºC) in a 12h light/dark controlled environment with free access to food and water. The animals were divided randomly into ischemic and sham groups. Rats were placed in the anesthesia induction box supplied with 4% isoflurane (IsoFlo, Abbott Laboratories Ltd) at 3 L/min in 100% oxygen. Animals were maintained under anesthesia with a flux of 1.5–2.5% isoflurane at 800 ml/min in 100% oxygen, delivered through a face mask. Both common carotid arteries were exposed through a midline incision and transient global ischemia was induced by bilateral common carotid artery occlusion for 15 min with atraumatic aneurysm clips and a moderate hypotension (40–50 mm Hg). Hypotension was carried out by exsanguination, the femoral artery was exposed and catheterized thus allowing continuous recording of arterial blood pressure. 50 UI heparin/kg were supplied to the animal through this catheter and 50 UI heparin were maintained in 3 ml saline in the syringe. Then, about 8 ml of blood was slowly extracted (1 ml/min) through the catheter and collected in the syringe until reaching the desired hypotension that was maintained introducing or extracting blood in the artery. Rectal temperature was controlled at 36 ± 1ºC during surgery with a feedback regulated heating pad. After ischemia, blood was returned to the animal at 1 ml/min until the animal arterial blood pressure recovered. Then the catheter was removed and the animal was sutured. After regaining consciousness, animals were maintained in an air-conditioned room at 22 ± 1ºC during 48h. For sham-operated rats, all procedures were performed exactly as for ischemic animals with the exception that the carotid arteries were not clamped.

Two days after the ischemic insult, the animals were decapitated and their brains were rapidly removed. The CA1, the CA3, the striatum and the cerebral cortex were obtained using a dissecting microscope, frozen in liquid nitrogen and stored at -80ºC until use.

Analysis of the nuclear morphology

For analysis of the nuclear morphology, neurons were fixed 24h after OGD at room temperature in 4% sucrose/ 4% paraformaldehyde in PBS, washed with PBS and incubated with the fluorescent dye Hoechst 33342 (1 μg/ml, Molecular Probes Europe) for 10 min. The coverslips were mounted on glass slides with Dako mounting medium (Thermo Scientific) and examined with a Zeiss Axiovert 200 fluorescence microscope (40× objective). The cell-permeable DNA stain Hoescht 33342 presents blue fluorescence. 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 duplicate and approximately 400 cells were counted per coverslip in 6-10 randomly selected different optical fields. Cell death is expressed as the percentage of dead cells relatively to the total number of scored cells.

LDH release assay

For the assessment of LDH release, the culture conditioned medium was collected after the indicated times of recovery. The LDH activity was assayed using a commercial kit (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI), and determined as indicated in the manufacturer's protocol. The percentage of LDH release was determined as the ratio between LDH activity in the extracellular medium and total LDH activity, obtained after complete cell lysis with Triton X-100. The percentage of cell death was calculated relatively to cells treated with lysis buffer, which were considered as 100%. All experiments were carried out in duplicate or triplicate, for each independent experiment.

Total RNA isolation, RNA Quality and RNA Concentration

Seven and 24 hours after the OGD challenge, total RNA was extracted from cultured hippocampal neurons with TriZol reagent (Invitrogen, Barcelona, Spain), following the manufacturer's specifications. Briefly, 1 ml of TRIzol was added to each

well of a 6-well plate and the content of each experimental condition (two wells) was collected. Chloroform was then added for phase separation and the RNA precipitated by isopropanol addition. The precipitated RNA was washed with 75% ethanol, centrifuged, air-dried and ressuspended in 20 µl of RNase-free water (GIBCO Invitrogen). RNA quality and integrity were evaluated using the Experion automated gel-electrophoresis system (Bio-Rad, Amadora, Portugal). RNA concentration was determined using a NanoDrop 2000c/2000 UV-Vis Spectrophotomer (Thermo scientific). The samples were stored at -80°C until further use.

Microarray hybridization

For the microarray analysis, total RNA from rat hippocampal neuronal cultures submitted to control or OGD conditions was collected after 7h and 24h of recovery. RNA from three independent cultures was used as biological replicates. Equal amounts of RNA extract (200 ng) from each replicate were amplified and Cy-3-labeled using the Low Input Quick Amp Labeling kit (Agilent Technologies). Hybridizations were carried out following Agilent Technologies instructions for One-Color Microarray-Based Gene Expression Analysis (Agilent Technologies, Santa Clara, CA, USA), using whole-genome Rat GE 4x44K v3 Microarrays. Images were obtained using the Agilent G2565AA microarray scanner and fluorescence quantization was performed using the Agilent Feature Extraction 10.5.1.1 software and the GE1_105_Dec08 protocol. The signal intensity was aligned and normalized between microarrays by centering the median of the signal distribution using BRB-ArrayTools v3.8.1. The microarray data was submitted to GEO database and has been given the accession number GSE54037.

Microarray data analysis

The TIGR MultiExperiment Viewer (MeV) v4.6 was used for statistical analysis of the data. Student's t-tests were used to determine differentially expressed genes, with a *p*-value cut-off of 0.05. Of these, only genes with a fold change above 2.0 were considered differentially expressed and included in further analyses.

For gene ontology analyses, the lists of differentially expressed genes from all conditions were imported to GoMiner™. Ontological classes were selected manually and, for the production of the pie-charts, the number of genes for each class divided by the sum of the total number of genes in the selected classes, as indicated in figure captions.

Primer Design

Primers for target genes were designed using the "Beacon Designer 7" software (Premier Biosoft International), with the following specifications: (1) GC content about 50%; (2) Annealing temperature (Ta) between 55 ± 5°C; (3) Secondary structures and primer-dimers were avoided; (4) Primers length between 18-24 bp; (5) Final product length between 100-200 bp. All primers used in this work are listed in the **Table 2.1.**

Quantitative real-Time PCR

For cDNA synthesis, 1 μg of total RNA was used with the iScript™ cDNA Synthesis Kit (BioRad), according to the manufacturers' instructions. For quantitative real-time PCR (qRT-PCR) 20 μl reactions were prepared with 2 μl of 1:10 diluted cDNA, 10 μl of 2x iQ™ SYBR® Green Supermix (BioRad) and specific primers at 250 nM. The fluorescent signal was measured after each elongation step of the PCR reaction, in the iQ5 Multicolor Real-Time PCR Detection System (BioRad), and was used to determine the threshold cycle (C_t), as previously described (Manadas et al., 2009). Melting curves were performed in order to detect non-specific amplification products, a non-template control was included in all assays, and for each set of primers a standard curve was performed to assess primer efficiency. Reactions were run in duplicate. For each gene, average Ct was calculated as the mean of five biological replicates for each condition. The expression level of each gene was normalized to the expression level of the gene in the corresponding control condition. All C_t values were normalized to two internal control genes, Actb and Gapdh (shown not to change between control and OGD conditions), using the GenEx software (MultiD Analyses). Fold change values above 1.0 indicate an up-regulation relative to the control condition, whereas fold change values below 1 indicate a down-regulation relative to the control condition. All values are indicated as log-transformed data.

Preparation of total protein extracts

For the analysis of the total protein of AMPARs and NMDARs, hippocampal neuronal cultures were washed twice with ice-cold PBS and 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) supplemented with 1 mM DTT and a mixture of protease inhibitors: 0.1 mM PMSF and CLAP (1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, and 1 μg/ml pepstatin; Sigma Aldrich) was added. Samples were frozen twice at -80°C, after

which the total protein was quantified using the BCA method (Thermo Scientific). Samples were then denatured with 2x concentrated denaturing buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue) at 95°C for 5 min.

For the CLIC1 studies, different protocols were used to obtain total cell extracts from different types of material. Total protein extracts from cultured N9 cells (microglial cells) were prepared by lysing cells at 4°C in RIPA lysis buffer (in mM: 50 Tris pH 8.0, 150 NaCl, 50 EDTA, 0.5% sodium deoxycholate, 1% Triton X-100), supplemented with a protease inhibitor cocktail (Sigma), 2 mM DTT and 0.1 mM PMSF. Also, extracts of adult rat brain, liver and kidney were prepared using the same lysis buffer. Fifty μg of total protein were resuspended in 2x denaturing buffer (62,5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol, added fresh) and were separated in SDS-PAGE. To analyze total protein levels of CLIC1 in hippocampal neuronal cultures, at different periods after OGD (0h, 24h and 48h), cells were washed with ice-cold PBS and lysed in a mixture containing an equal amount of 1x denaturing buffer indicated above and lysis buffer (in mM: 50 Tris-HCl pH 7.4, 5 EGTA, 1 DTT). Cells were then scraped and the extracts were sonicated. The concentration of protein lysates was determined using the Bio-Rad protein assay (Bio-Rad). One hundred μl of each protein extract were separated by SDS-PAGE. Finally, for the analysis of total levels of CLIC1 in samples of hippocampus (CA1 and CA3 regions), striatum and cortex of rats submitted to global ischemia, after thawing the samples, tissue sections were weighted and 1.5 mg of tissue from each area of the hippocampus and the striatum were homogenized with 125 μl, whereas 5 mg from the cortex was homogenized in 250 μl of the same lysis buffer indicated above for total protein extracts in hippocampal cultures, supplemented with protease inhibitors and 1% DOC, incubated for 30 min incubation on ice and frozen at -80°C. After thawing, cellular extracts were sonicated, followed by centrifugation at 18,000 g for 30 min at 4°C and the pellet was discarded. The concentration of protein lysates was determined using the Bio-Rad protein assay (Bio-Rad). One hundred μg of each sample were used for SDS-PAGE after denaturation with 2x concentrated denaturing buffer, indicated above.

Biotinylation assay

Biotinylation assays were performed 24h after the OGD insult. Cells were washed twice with PBS with calcium and magnesium (PBS/Ca^{2+}/Mg^{2+}; in mM: 137

NaCl, 2.7 KCl, 1.8 KH_2PO_4, 10 Na_2POH_4, plus 0.5 $MgCl_2$, 1 $CaCl_2$, pH 7.4), followed by incubation with 0.25 mg/ml NHS-SS-Biotin (Thermo Scientific) for 15 min at 4ºC under mild shaking. Cells were then washed twice with PBS/Ca^{2+}/Mg^{2+} supplemented with glycine (100 mM) and incubated in this solution for 15 min, at 4ºC under mild shaking. For analysis of the surface AMPARs, cells were lysed in the lysis buffer indicated above for total protein extracts, supplemented with protease inhibitors, followed by 30 min incubation on ice, and frozen at -80ºC. After thawing, cellular extracts were centrifuged at 18 000 g for 30 min at 4ºC and the pellet was discarded. Fifty µg of each protein extract was used for the input and 150 µg was used for incubation with NeutrAvidin beads. For analysis of the surface NMDARs, cells were incubated with lysis buffer (in mM: 50 Tris-HCl, pH 7.4, 5 EGTA, 1 DTT), supplemented with protease inhibitors, for 30 min at 4ºC under mild shaking, after which samples were collected and briefly sonicated. Cellular extracts were then incubated with 1% DOC, pH 9.0, 1h at 37ºC, centrifuged at 18,000 g for 30 min at 4ºC and the pellet was discarded. One hundred µg of each protein extract was used for the input and 400 µg was used for incubation with NeutrAvidin beads.

In both cases, NeutrAvidin beads (Pierce) were added in equal amounts of the supernatant fluid (2.5 µl/10 µg total protein) and incubated for 2h at 4ºC in an orbital shaker. The beads were washed four times with the correspondent lysis buffer. The samples were then eluted with 2x denaturating buffer, boiled at 95ºC for 5 min and centrifuged into a tube collector with a 0.45 µm filter.

Preparation of nuclear protein extracts

Nuclear extracts of hippocampal neurons were prepared 24h after the OGD insult. Cells were washed with ice-cold PBS and solubilized in ice-cold buffer 1 (in mM: 10 HEPES, 10 NaCl, 3 $MgCl_2$, 1 EGTA and 0.1% Triton X-100, pH 7.5) for 30–40 min. The nuclei were pelleted by centrifugation at 2 400 g for 10 min at 4ºC and then resuspended in ice-cold buffer 2 (in mM: 25 HEPES, 300 NaCl, 5 $MgCl_2$, 1 EGTA and 20% glycerol, pH 7.4) for 1h, after which they were centrifuged at 12 000 g for 20 min at 4ºC. The supernatants (nuclear extracts) were collected and stored at -80ºC until use. Both buffers were supplemented with 0.1 mM PMSF and 1 µg/ml CLAP, as well as with 1 mM DTT before use. Protein concentration of the extracts was measured using the Bradford assay. Samples were then denatured with 2x concentrated denaturing buffer at 95ºC for 5 min, and 100 µl of each sample were used for SDS-PAGE.

SDS-PAGE and Western Blotting

Protein samples were separated by SDS-PAGE in 7.5 % (REST), 8% (NMDA receptor subunits and CLIC1) or 10% (AMPA receptor subunits) polyacrylamide gels, transferred to PVDF membranes (Millipore) and immunoblotted. The membranes were blocked with Tris-buffered saline-Tween (TBS-T) (in mM: 20 Tris, 137 NaCl, pH 7.6, and 0.1 % Tween20) with 5% non-fat milk, for 1h at room temperature, and then incubated with the primary antibody in TBS-T 5% milk, overnight at 4ºC. Incubation with antibodies against β-actin (Sigma-Aldrich) and α-spectrin (Millipore) were performed for 1h at room temperature, whereas antibodies against REST, GluA1, GluA2, GluN1, GluN2A (Millipore), GluN2B (BD Biosciences) and CLIC1 (Millipore) were incubated overnight at 4ºC. After extensive washing, membranes were incubated with the secondary antibody conjugated with alkaline phosphatase for 1h at room temperature. After additional washes, the membranes were developed using the enhanced chemifluorescence (ECF) substrate, and scanned on the Storm 860 Gel and Blot Imaging System (Amersham Biosciences). The density of the bands was analyzed with ImageQuant 5.0 software. When reprobing was necessary, the membranes were stripped of antibody with NaOH 0.2 M for 20 minutes, blocked again and incubated with the appropriate antibodies.

Cell transfection and immunocytochemistry

For CLIC1 overexpression studies, neurons were cultured in 12 MW plates and were transfected at DIV 13 using the calcium phosphate co-precipitation method. Briefly, precipitates containing 1.5 µg of pEBG-eGFP (control plasmid encoding EGFP) or 3 µg of pIRES1-eGFP-Clic1, a bicistronic plasmid encoding both EGFP and CLIC1, were prepared using the following solutions: TE (in mM: 1 Tris-HCl pH 7.5, 1 EDTA pH 8.0), 2x HEBS (in mM: 12 Dextrose, 50 HEPES, 10 KCl, 280 NaCl and 1.5 $Na_2HPO_4 \cdot 2H_2O$) and $CaCl_2$ (2.5M $CaCl_2$ in 10 mM HEPES) and allowed to develop for 30 min. The precipitates were added to the neurons, in the presence of 2 mM kynurenic acid (Sigma-Aldrich), and incubated for 3h at 37ºC. After the transfection period, cells were washed with an acidic solution of Neurobasal medium containing 2 mM kynurenic acid to dissolve the precipitates and then placed in conditioned medium until DIV 15. The pIRES1-eGFP-Clic1 plasmid was a generous gift from Doctor Michele Mazzanti, from Università “La Sapienza”, Rome, Italy.

Transfected hippocampal neurons were labeled with an anti-GFP antibody (1:500 - #598, MBL International, Woburn, MA), to detect transfected neurons. Briefly,

following 4% paraformaldehyde/4% sucrose fixation (10 min at room temperature), the coverslips were incubated with 10% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hour at 37°C to prevent nonspecific antibody binding, and incubated overnight with the primary antibodies for GFP (1:500, Millipore) in 3% BSA at 4°C. The secondary antibody anti-rabbit Alexa 488 (Jackson ImmunoResearch, Suffolk, UK) was also prepared in 3% BSA (1:500) and incubated at 37°C for 1 hour. The coverslips were extensively washed with PBS after antibody incubations. Additionally, cells were stained with Hoechst 33342 in order to visualize cell nuclei and, after washing, mounted in fluorescence mounting medium from DAKO (Glostrup, Denmark).

Statistical Analysis

Results are presented as means ± S.E.M. of the number of experiments indicated. The normality of the data was assessed using the Kolmogorov-Smirvov test. Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by the Bonferroni's test or by Student´s *t*-test, as indicated in the figure captions. These statistical analyses were performed using the software package GraphPad Prism 5.

Table 2.1. Primer sequences used in quantitative real-time PCR (qPCR) analyses.

Chapter	Gene	Primers	Anneling Temperature
House-keeping genes (Chapters III-V)	Actb	Fwd: CGTCACCTACTCTAACCG Rvs: CTTGTGCTATCTGCTCATC	52,8
	Gapdh	Fard: AACCTGCCAAGTATGATG Rvs: GGAGTTGCTGTTGAAGTC	53,4
Chapter III	Batf3	Fwd: ATGATGACAGGAAGGTTC Rvs: CTCCAGACTCTCATGTTC	58
	Hmgb1	Fwd: AATACGGATTGCTCAGGAA Rvs: GGACAACTGGTACTAATATGC	55
	Nfil3	Fwd: CTCTCCCTGAAATTAAAGTTTG Rvs: GCTGTGGAATTACTGAGTT	55
	Itgb6	Fwd: ATAAGCCTCTCAGCGTAG Rvs: CTCAACTTAAGAACCAAGC	54.3
	Gadd45g	Fwd: GAATCTTTACTTGCCCTC Rvs: TTCTTCCAGAGTCATTGT	56
	Prkcε	Fwd: ATACTTACACTTGTGGAT Rvs: AATAGTTCGAGACATTCT	57.2
	Prkcδ	Fwd: GTGTGTGCAGTATTTCCT Rvs: TCATAGTTGGGAACATGG	56.6
	Mmp25	Fwd: CAAGAGGTGGATTCTCAG Rvs: GTAACTGTCTTGGTGGTA	56.3
	Mmp3	Fwd: AATTGTTAAGAAGATCCATG Rvs: AGAGTAAGGAAACCACTT	55.4
	Adamts5	Fwd: GCAACAGACCCAACTAAAG Rvs: TTGCTGCTGTGGCTAATG	56.4
	Adamts7	Fwd: ACATATTCAGAGGAAGAG Rvs: AGGAACAACTAAGACTAC	55
Chapter IV	Pick1	Fwd: TGGATGTGAAGTTTGAGTA Rvs: GTATAGCGGCTCTCCTAG	57
	Grip1	Fwd: GAAGCAAGAAATCAAGGA Rvs: TGCCAGAGTCTTTGTATA	54.7
	Cacgn3	Fwd: TTTGGAGCCTTCTCTTTC Rvs: GCTGATGCTTCTCAATATAG	55.5
	Cacgn8	Fwd: TGGAATCATTGAAACGCT Rvs: ATGGTGGTCAGTAGTACC	55.9
	Sypl2	Fwd: TACATAGTGGTCATCTGG Rvs: GTACATTGAGCATTCCTAA	56.8
	Snap25	Fwd: TACATAGTGGTCATCTGG Rvs: GTACATTGAGCATTCCTAA	54.7
	Clstn2	Fwd: CAGATCAAGTGTTCAGAG Rvs: ATGTTCCGTATCAGAGAC	55.6
	Clstn3	Fwd: TAACACCATTCAGAACGA Rvs: AGTAGAGAAAGGCGATTC	55.7
	Dlgap	Fwd: ACATGGACCACATCCCAC Rvs: AAATGAACAGACAGTAAGGG	57.9
	Fmr1	Fwd: GTCTTTCTGGGTAAATCACAT Rvs: AATGGCACAGCACTTCAT	54.9
	Gria1	Fwd: GAACCATCCGTGTTTGTTCG Rvs: TTCCTGTCTGCTCCAGTTAC	57
	Gria2	Fwd: GAAGCCTTGTGACACCATGA Rvs: AGCCTTGCCTTGCTCCTCAT	57
	Grin1	Fwd: TACACTGCCAACTTGGCAGCTTTC Rvs: CATGAAGACCCCTGCCATGTT	58

	Grin2a	Fwd: TGGCTGCCTTCATGATCC Rvs: TGCAGCGCAATTCCATAG	58
	Grin2b	Fwd: GGATCTACCAGTCTAACATG Rvs: GATAGTTAGTGATCCCACTG	58
	Grin3a	Fwd: TCCTCTGCCACCTCAGTAA Rvs: TCTCACTTGGCTGGCTTCT	58
	Rest	Fwd: TAAGCCATGCCAGTATGA Rvs: AACTTCTTAGCACTGTGAAC	57
Chapter V	Clca2	Fwd: GTCTGCCTGTCAACAATT Rvs: AGAGACCAAATGAGCAAATG	54,8
	Clic1	Fwd: GGGATAAGGGAAAGATTAGAGGA Rvs: ACTGAAATACGTTGTCTTACCC	54,3
	Clic3	Fwd: GATGTGCTGAAGGACTTC Rvs: TTCCAGAAACTCCTCAATC	57,7
	Clic5	Fwd: CGTCTGACCCGATTTCTG Rvs: AATGGTGAAGAAGAGTCTGTAG	55,8
	Trpc5	Fwd: CCTGTTCCAGCTCTCTTC Rvs: CGTCCTCTGATGAGTCTAA	56,2
	Trpc6	Fwd: GAAGAAGGTTGGCTAATCG Rvs: CGTTCCTCCTCAATAGACA	55,5
	Trpc7	Fwd: AACAAGGGCAAGGACATT Rvs: GACATTTGGTCTGGCTTT	56,9
	Trpm2	Fwd: AGAACATCAAGAAGAAGGAAT Rvs: TAACCACACTCACACACTA	55,4

Chapter III

Profile of ischemia-induced changes in the transcriptome of hippocampal neurons

Abstract

Transient global cerebral ischemia triggers a complex cascade of molecular events that unfolds between hours to days after the insult. It is commonly accepted that many changes associated with post-ischemic neuronal injury include a profound alteration in the gene expression profile of brain cells that influences the response to ischemia. Although changes in the expression of many functional groups of genes related to inflammation, metabolism, production of reactive oxygen species and different signaling pathways have been previously described, our understanding of how these pathways correlate with the selective and delayed death of hippocampal neurons, a characteristic feature of global cerebral ischemia, remains incomplete.

Thus, to investigate the molecular mechanisms that may account for the vulnerability of hippocampal neurons to global ischemia, we submitted primary hippocampal neuronal cultures to oxygen-glucose deprivation (OGD), an *in vitro* model of global ischemia. The OGD protocol we established recapitulates features of global cerebral ischemia, namely ischemia-induced delayed cell death in hippocampal neurons and activation of ionotropic glutamate receptors, such as NMDA and AMPA receptors, that play an essential role in the excitotoxic events evoked by the ischemic insult. Therefore, we then investigated the changes in the transcriptome of hippocampal neurons induced by OGD. For that purpose, total RNA was extracted 7h and 24h after OGD and used in a whole-genome RNA microarray to identify genes related to an early and a delayed ischemic response. Analysis of gene ontology showed that OGD followed by 7h or 24h of recovery induces changes in the expression levels of genes related with inflammation, response to oxidative stress, metabolism, apoptosis, synaptic proteins and ion channels. Importantly, genes that show different expression levels are mainly specific to one of the two time points of recovery analyzed. The expression levels of several genes were confirmed by qPCR and were in good agreement with the microarray data, showing that the combined use of the OGD model and the microarray technology can be a useful tool to study molecular mechanisms contributing to the neuronal demise after transient global ischemia, in particular to elucidate in the development of therapeutic strategies aimed at improving neuronal outcome after cerebral ischemia.

Introduction

Global cerebral ischemia is a pathological condition in which brain tissue is subjected to reduced levels of oxygen and glucose due to an impairment in blood supply to the entire brain, causing biochemical modifications in the normal functioning of neurons that can lead to injury in specific neuronal subpopulations. Early after the onset of the ischemic insult, the decrease in ATP levels results in the disruption of ionic gradients, loss of membrane potential and enhanced neuronal activity, due to the depolarization of neurons. Excessive amounts of glutamate accumulate in the synapses and lead to overactivation of glutamate receptors, such as AMPA and NMDA receptors, which will contribute to the accumulation of Ca^{2+}, Na^{+} and even Zn^{2+} in the cytosol of neurons. Ca^{2+}, for instance, is at least partially responsible for long-term damaging effects in neurons, due to its capacity to activate enzymes and signal transduction pathways, including calpains, caspases, endonucleases and kinases that are responsible for triggering cell death (Szydlowska and Tymianski, 2010).

One of the main features of transient global cerebral ischemia is the delayed death of the hippocampal neurons of the CA1 region, which occurs only hours to days after the insult (Petito *et al.*, 1987; Bottiger *et al.*, 1998). This time-window between the end of the transient ischemic insult and the first signs of neuronal demise is believed to be associated with the activation of competing programs of gene expression, in which some will facilitate cell survival, whereas others will contribute to neuronal death (Papadopoulos *et al.*, 2000). A great effort has been put into identifying genes that participate in the response of hippocampal cells to global cerebral ischemia *in vivo* (Jin *et al.*, 2001; Kawahara *et al.*, 2004; Buttner *et al.*, 2009). However, while it is accepted that several cellular functions are compromised, our understanding of how this correlates with the selective and delayed death of hippocampal neurons is still unclear. This analysis requires investigations at the molecular level, more easily performed using *in vitro* models. In the present study we used microarray technology to identify genes whose expression is significantly altered in hippocampal neuronal cultures submitted to oxygen and glucose deprivation (OGD), an established *in vitro* model for cerebral global ischemia (Zukin *et al.*, 2004). To the best of our knowledge no large scale study was developed so far using an OGD insult as a tool to study ischemia-induced changes in the transcriptome of hippocampal neurons at different periods of recovery. In accordance to what has been previously observed in models of global and focal ischemia (Jin *et al.*, 2001; Kim *et al.*, 2002; Schmidt-Kastner *et al.*, 2002; Tang *et al.*, 2002; Kawahara *et al.*, 2004; Lu *et al.*, 2004; Buttner *et al.*, 2009; Ramos-Cejudo

et al., 2012), OGD induced changes in the expression levels of genes related with a variety of functions within neurons, such as metabolic processes, response to oxidative stress, inflammation, apoptosis, signaling pathways, synapse, ion channel activity, among others. Therefore, this model, when combined with the microarray technology, can serve as a useful tool to obtain a comprehensive characterization of the changes in gene expression induced by ischemia in a particular type of cells, and opens the way to studying to which extent these changes influence the fate of neuronal cells exposed to ischemia.

Results

OGD induces delayed neuronal death in mature hippocampal neurons

In order to characterize the neuronal injury induced by *in vitro* oxygen and glucose deprivation, primary hippocampal neuronal cultures were subjected to different periods of OGD, followed by 24h incubation in culture conditioned medium, after which cell viability was evaluated by analysis of the nuclear morphology. Periods of OGD ≥ 1h 30 min resulted in a decrease in cell viability of nearly 20% **(Figure 3.1A)**. Therefore, in all forthcoming experiments the cells were subjected to OGD for 2h and further incubated in culture conditioned medium for the indicated periods of time.

To test whether OGD causes delayed neuronal death, we assessed cell viability at different periods after the insult, by quantification of the LDH release **(Figure 3.1B)**. We observed that at post-incubation periods of up to 18 h after OGD there was no detectable change in cell viability, whereas at 24h after the insult LDH release was increased, thus confirming that the OGD challenge induces delayed neuronal death, similarly to the ischemic insult *in vivo* (Petito *et al.*, 1987; Bottiger *et al.*, 1998; Calderone *et al.*, 2003; Zukin *et al.*, 2004).

Previous studies have indicated that the intracellular effects of ischemia include the activation of calpains, a family of calcium-activated cysteine proteases which trigger substrate-specific proteolysis that may contribute to neuronal death (Bevers and Neumar, 2008), after both *in vivo* (Garcia-Bonilla *et al.*, 2006; Clinkinbeard *et al.*, 2013) and *in vitro* ischemia (Newcomb-Fernandez *et al.*, 2001; Zhou and Baudry, 2006; Lobo *et al.*, 2011). Calpain activation (usually detected by the breakdown products of a preferred substrate, spectrin) can therefore serve as an indirect indicator of the induction of cell

death. As expected, no significant OGD-induced calpain activation was observed 7h after subjecting hippocampal neurons to the ischemic injury (when there is still no significant cell death, as assessed by quantifying LDH release, **Figure 3.1B**); however, at 24h after injury, significant activation of these proteases was observed, as indicated by the formation of 145 kDa spectrin cleavage products **(Figure 3.1C)**. No 120 kDa cleavage products were detected, indicating that caspase-3 does not contribute to spectrin cleavage under the experimental conditions used (Wang, 2000). Taken together, these results show that incubation under OGD (2h) results in significant cell death in primary hippocampal neurons, 24h after the insult. Therefore, this protocol can be used to investigate the ischemic response at earlier (7h) or later (24h) recovery periods after the insult.

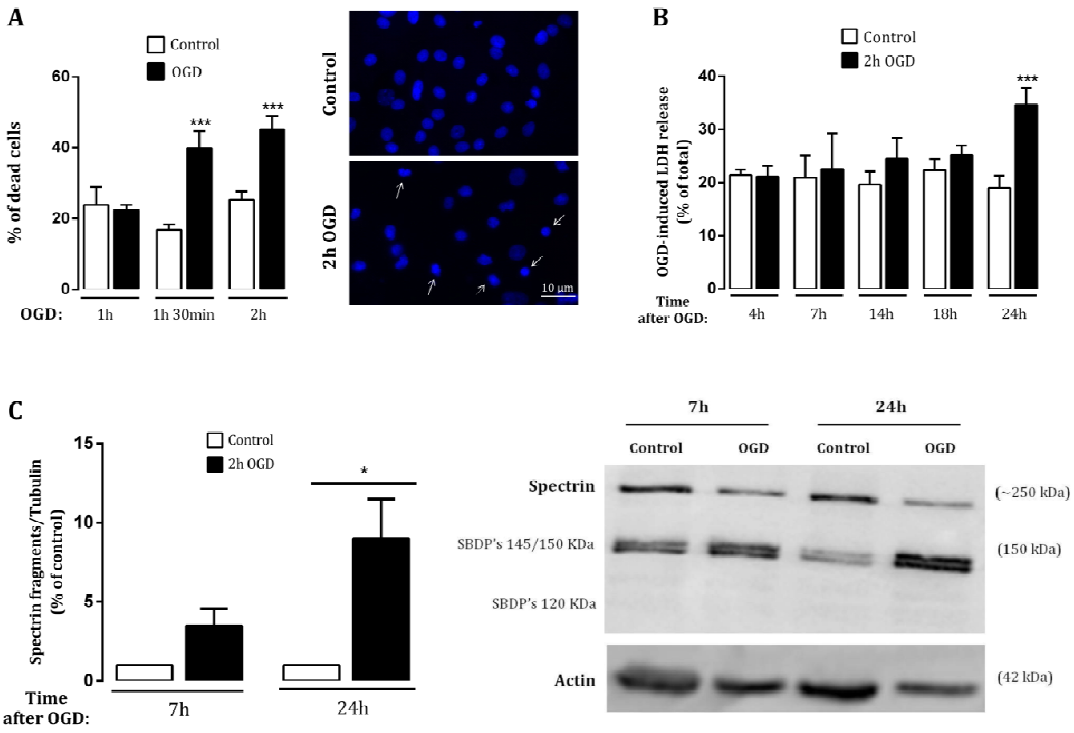


Figure 3.1. OGD induces delayed neuronal death of mature hippocampal neurons in culture. (A) OGD causes hippocampal neuronal cell death, as determined by analysis of nuclear morphology. After incubation under OGD conditions for 1h (n = 3), 1h30 (n = 12) and 2h (n = 10), cells were returned to the 5% CO2 incubator for 24h. Cell viability was then assessed by analysis of the nuclear morphology. Pyknotic nuclei (arrows) were counted as dead cells. The results were expressed as the percentage of dead cells relatively to the total cell number. The right panel depicts nuclear morphology of neurons subjected to control and 2h OGD. **(B)** Time-course of OGD-induced neuronal death, as determined by LDH release. Cells

were subjected to OGD for 2h and the LDH release was assessed 0h (n = 6), 4h (n = 5), 7h (n = 3), 14h (n = 6), 18h (n = 6) and 24h (n = 13) after the stimulus. **(C)** OGD induces cleavage of spectrin and the formation of spectrin breakdown products (SBDPs). SBDPs protein levels were analyzed by Western blot 7h and 24h after 2h of OGD. Actin was used as loading control. The results are representative of four-five independent experiments, performed in distinct preparations. Bars represent the mean ± SEM of the indicated number of independent experiments. *$p<0.05$, ***$p<0.001$, as determined by the Student's *t*-test.

OGD-induced hippocampal neuronal death is prevented by glutamate receptor antagonists

Glutamate toxicity due to overactivation of glutamate receptors, or *excitotoxicity*, has been previously related to cerebral ischemia and is mediated by overactivation of glutamate receptors, since the application of glutamate receptor antagonists attenuates synaptic transmission and neuronal death, acting as neuroprotective (Kwak and Weiss, 2006; Lau and Tymianski, 2010). To analyze the contribution of NMDA and AMPA ionotropic glutamate receptors to neuronal death elicited by OGD, hippocampal neuronal cultures were submitted to OGD in the absence or in the presence of MK801 (selective NMDAR antagonist), GYKI 52466 (4-(8-Methyl-9H-1,3-dioxolo[4,5-h][2,3]benzodiazepin-5-yl)-benzenamine dihydrochloride, selective AMPAR antagonist) or Naspm (N-[3-[[4-[(3-Aminopropyl)amino]butyl]amino] propyl]-1-naphthaleneacetamide trihydrochloride, selective Ca^{2+}-permeable AMPAR antagonist). All antagonists were added prior to OGD and kept during the stimulation and post-stimulation periods. Cell viability was assessed 24h after the stimulus by analysis of nuclear morphology **(Figure 3.2A-C)** and by quantification of LDH release **(Figure 3.2D-F)**. Both assays showed that cell death induced by OGD was prevented by the NMDA and AMPA receptor antagonists. Also, the selective CP-AMPARs antagonist Naspm inhibited the increase in LDH release, indicating the partipation of this subtype of AMPARs in the neuronal death elicited by OGD. Taken together, these results confirm that both AMPA and NMDA receptors are involved in OGD-induced neuronal death and mediate the main excitotoxic component of this *in vitro* model of cerebral ischemia.

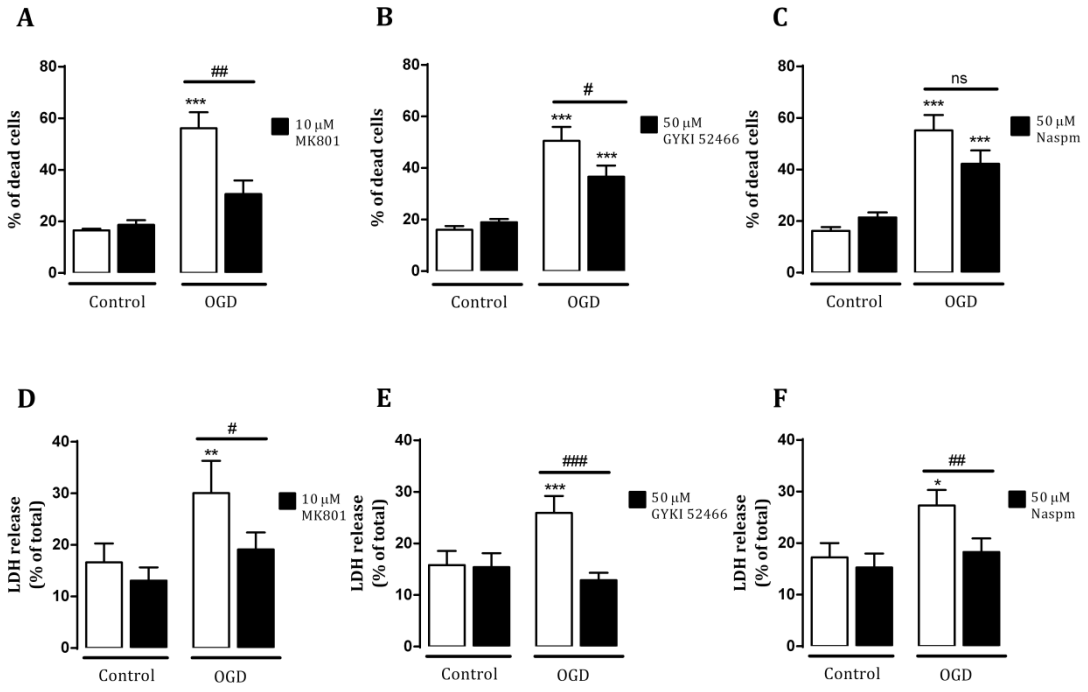


Figure 3.2. Inhibition of glutamate receptors protects hippocampal neurons against OGD-induced cell death. Mature hippocampal neurons were subjected to 2h OGD in the absence or presence of glutamate receptor antagonists. When present, the antagonists were added 15 min prior to stimulation and kept during the stimulation and post-stimulation periods. Cell viability was assessed 24h after the stimulus by analysis of the nuclear morphology **(A-C)** and by determination of the LDH release **(D-F)**. Bars represent the mean ± SEM of 4-9 independent experiments, performed in distinct preparations. Statistical analysis was performed using One-way ANOVA followed by Bonferroni's Multiple Comparison Test: *p<0.05, **p<0.01, ***p<0.001, relative to control; #p<0.05, ##p<0.01, ###p<0.001 relative to OGD condition. MK-801, selective NMDAR antagonist; GYKI 52466, selective AMPAR antagonist; Naspm, selective CP-AMPAR antagonist. The LDH studies were performed by Marta Vieira.

OGD induces large-scale regulation of hippocampal gene expression

To investigate the molecular mechanisms involved in the neuronal response mediated by the OGD insult, we performed a whole-rat genome Agilent microarray analysis. Total RNA from rat hippocampal neuronal cultures submitted to control conditions or OGD were analyzed after 7h and 24h incubation in culture conditioned medium, in order to compare gene expression profiles at a time point prior to and after the onset of cell death. All experimental conditions were performed with three independent biological replicates. Student's *t*-test was used to determine the genes

whose expression was significantly different between cells subjected to ischemic injury and the correspondent control. Only genes with $p<0.05$ and with an expression fold change of 2.0 relatively to the control condition were considered differentially expressed and selected for further analyses. From the approximately 44 000 probes present on each array, the expression levels of 4 506 transcripts were altered 7h after OGD, whereas 1 922 transcripts were differently expressed at 24h after OGD, when compared to their respective controls ($p<0.05$, Student's *t*-test). Of these, we observed that at 7h and 24h of incubation after OGD, the levels of a total of 413 and 499 transcripts were more than two fold altered in response to OGD, respectively **(Figure 3.3A)**.

Figure 3.3B-C shows the number of genes that were exclusively altered at 7h or 24h after OGD, as well as the number of genes affected at both time points. Of all the transcripts up-regulated after OGD, the expression levels of 333 transcripts were increased specifically 7h after injury, 419 transcripts were exclusively increased after 24h after the insult and a total of 80 transcripts were found to be up-regulated after both time periods of incubation **(Figure 3.3B)**. On the other hand, 360 transcripts were exclusively down-regulated at 7h after OGD, whereas only 64 transcripts had reduced expression levels specifically at 24h after OGD. A total number of 27 transcripts were down-regulated both at 7h and 24h after OGD **(Figure 3.3C)**. These results indicate that whereas at 7h after *in vitro* ischemia the changes in the transcriptome are related to both an up- and down-regulation of gene expression, at 24h after the insult there is mainly up-regulation of gene expression.

Most altered genes in response to OGD

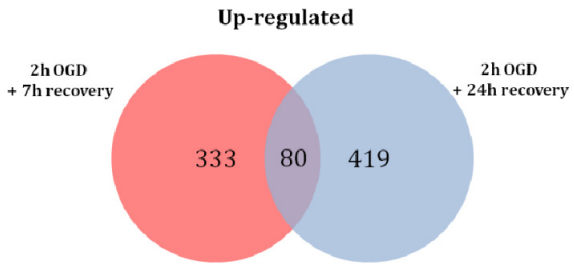
Table 3.1 indicates the most up- and down-regulated genes at both times of recovery, *i.e.*, genes that are regulated as a constitutive response to the OGD insult. The maximum expression change was found for Il1rl1, which increased from ~13 (7h) to 47-fold (24h) **(Table 3.1A)**. Other strongly up-regulated genes code for inflammatory response-related Reg3b, enzyme inhibitor Tfpi2, inflammatory chemokine Cxcl1, hormone Gal and protease inhibitor serpine1. All these genes had an increase >5-fold and, with the exception of Gal, which peaked at 7h, the transcript levels of these genes continuously increased up until 24h of recovery. The most down-regulated **(Table 3.1B)** gene at both times of recovery encodes the extracellular matrix protease Mmp28, with a fold change of ~0.2. Other genes had expression levels decreased by a factor of 3 or more, and encode for RGD1559748 (unknown function), calcium and DAG-regulated

enzyme Rasgrp1, hypothetical protein LOC100360071 (Neuropeptidase S-like), extracellular matrix-related protein C1qtnf1, and Ube2ql1 **(Table 3.1B)**.

A

Comparison	Total number of genes with altered expression (p<0.05)	Up-regulated genes (*Fold change ≥ 2*)	Down-regulated genes (*Fold change ≥ 2*)
Ctrl 7h *vs* OGD 7h	4 506	413	387
Ctrl 24h *vs* OGD 24h	1 922	499	91

B



C

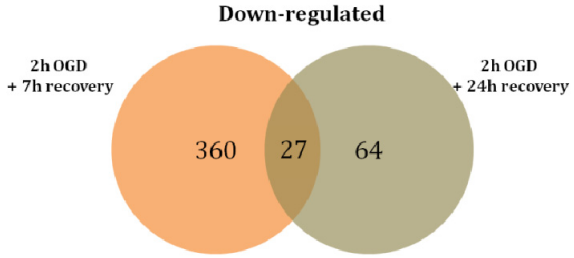


Figure 3.3. Summary of gene expression changes at 7h and 24h after OGD. (A) Student's *t*-test analysis was applied to the microarray data to identify all genes whose expression was significantly different between conditions (p<0.05). Up-regulated and down-regulated genes include those whose expression levels had a fold change ≥ 2.0. **(B and C)** Number of up-regulated **(B)** or down-regulated **(C)** transcripts at 7h and 24h after 2h OGD. The intersection represents the number of transcripts whose transcription was changed in both recovery periods. The transcripts used in this analysis were considered differentially expressed after using two cut-off criteria: a p-value <0.05 and a fold change of 2.0. The VENNY informatic tool was used to compare the lists of transcripts to obtain the Venn Diagrams.

Other genes were found to be strongly regulated specifically at one of the two periods of recovery considered in this work. Specifically at 7h of recovery (**Table 3.2**), the most strongly up-regulated gene was found to be the apoptosis-related Mageb3, which increased ~11-fold **(Table 3.2A)**. Other genes were strongly increased in

expression at this point of recovery, with expression levels >8-fold **(Table 3.2A)**. According to **Table 3.2B**, Agtr1b was found to be the gene most down-regulated at 7h, with a 5-fold decrease compared to the control. Other genes were also strongly down-regulated at this point of recovery **(Table 3.3B)**.

At 24h of recovery **(Table 3.3)**, the most strongly up-regulated gene was the translation initiation factor Eif4g2, whose expression was ~12-fold higher compared to the control **(Table 3.3A)**. Other genes were found to be strongly increased at this point of recovery **(Table 3.3A)**, with expression levels increased by 6 to 12-fold. The most down-regulated gene at 24h after the OGD insult was found to be RGD1559536 (similar to vitellogenin-like 1 precursor), a putative protease inhibitor whose expression levels were 5-fold lower compared to the control **(Table 3.3B)**. The expression levels of other down-regulated genes were also found to be highly decreased (2.5 to 5-fold) **(Table 3.3B)**.

Transcriptional adaptations induced by OGD

To study the changes induced by the OGD insult in terms of functional gene groups, we next considered all the transcripts that displayed a significant expression increase or decrease at 7h and 24h of incubation after injury (or at both time points of recovery, where indicated) by gene ontology categories using the informatics tool GoMiner™. **Figure 3.4** shows the number of genes up- or down-regulated at 7h and 24h after OGD for different ontological classes. We found that genes related with metabolic processes, signaling pathways, receptor activity, transcription, RNA biosynthesis and apoptosis were the most altered after OGD. Genes included in other categories such as ion transmembrane transporter activity, inflammation and synapse were also highly regulated by the OGD challenge. Whereas the most up-regulated classes (and the number of genes with increased expression included in each one) are similar between 7h and 24h of recovery, the pattern of down-regulation is different. It is clear that at 7h after OGD there are more genes whose expression is decreased than at 24h, in all the classes considered **(Figure 3.4)**.

Table 3.1. Most strongly up-regulated **(A)** and down-regulated **(B)** genes at 7h after OGD (fold change ≥ 2).

A. Most up-regulated genes after 7h of recovery				
Gene Symbol	**Gene Name**	**Function**	**Fold Change 7h**	***p*-value**
Mageb3	Melanoma antigen family B, 3	Apoptosis	10.82	0.05
Pax3	Paired box 3	Transcription factor	10.65	0.04
LOC685371	Hypothetical protein LOC685371	Unknown	10.19	0.03
Evc2	Ellis van Creveld syndrome 2 homolog (human)	Organ development	9.86	0.04
RGD1309808	Similar to apolipoprotein L2, apolipoprotein L-II	Lipid transporter activity	9.66	0.04
Mkx	Mohawk homeobox	Transcription factor	9.17	0.04
Mcc	Mutated in colorectal cancers	Unknown	9.17	0.04
Krt80	Keratin 80	Cytoskeleton	8.68	0.04
Ckm	Creatine kinase, muscle	Metabolic process	8.50	0.04
Fam155b	Family with sequence similarity 155. member B	Unknown	8.18	0.03
B. Most down-regulated genes after 7h of recovery				
Gene Symbol	**Gene Name**	**Function**	**Fold Change 7h**	***p*-value**
Agtr1b	Angiotensin II receptor, type 1b	Receptor activity	0.21	0.002
Clrn1	Clarin 1	Cytoskeleton	0.22	0.0002
Dclk3	Doublecortin-like kinase 3	Kinase activity	0.23	0.02
RGD1305627	Hypothetical LOC314467	Protease inhibitor	0.23	0.01
Ttc22	Tetratricopeptide repeat domain 22	Unknown	0.23	0.01
Mmp9	Matrix metallopeptidase 9	Extracellular matrix	0.23	0.03
Nostrin	Nitric oxide synthase trafficker	DNA binding	0.24	0.03
Cga	Glycoprotein hormones, alpha polypeptide	Hormone activity	0.25	0.02
Ccl1	Chemokine (C-C motif) ligand 1	Inflammation	0.25	0.04
RGD1562683	Rgd1562683	Unknown	0.25	0.03

Table 3.2. Most strongly up-regulated **(A)** and down-regulated **(B)** genes at 24h after OGD (fold change ≥ 2).

A. Most up-regulated genes after 24h of recovery

Gene Symbol	Gene Name	Function	Fold change 24h	*p*-value
Eif4g2	Eukaryotic translation initiation factor 4, gamma 2	Translation initiation factor	12.23	0.005
Arhgap24	Rho gtpase activating protein 24	Enzyme	11.98	0.001
Slfn2	Schlafen 2	Cell cycle	11.20	0.02
LOC100365145	Hypothetical protein LOC100365145	Unknown	10.47	0.05
Ctgf	Connective tissue growth factor	Growth factor activity	9.84	0.01
Cxcl2	Chemokine (C-X-C motif) ligand 2	Inflammation	8.61	0.05
S100a6	S100 calcium binding protein A6	Calcium binding	7.79	0.03
Cxcl10	Chemokine (C-X-C motif) ligand 10	Inflammation	7.57	0.02
Ptgr1	Prostaglandin reductase 1	Apoptosis	7.03	0.03
Hmga2	High mobility group AT-hook 2	Transcription	6.99	0.02

B. Most down-regulated genes after 24h of recovery

Gene Symbol	Gene Name	Function	Fold change 24h	*p*-value
RGD1559536	Similar to vitellogenin-like 1 precursor	Protease inhibitor	0.21	0.04
Ifi203	Interferon activated gene 203	Transcription factor	0.24	0.05
Agbl1	ATP/GTP binding protein-like 1	Signaling	0.27	0.02
Gpr83	G protein-coupled receptor 83	Receptor activity	0.30	0.03
F2	Coagulation factor II (thrombin)	Protease activity	0.30	0.01
Cd27	CD27 molecule	Apoptosis	0.31	0.04
Hapln1	Hyaluronan and proteoglycan link protein 1	Extracellular matrix	0.32	0.01
Grin1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	Receptor activity	0.39	0.04
LOC499418	Similar to Putative protein c21orf56 homolog	Unknown	0.40	0.02
Wdr63	WD repeat domain 63	Cytoskeleton	0.40	0.01

Table 3.3. Most strongly up-regulated **(A)** and down-regulated **(B)** genes at both recovery periods (7h and 24h) after OGD (fold change ≥ 2).

A. Most up-regulated genes at both periods of recovery (7h and 24h) after OGD						
Gene Symbol	**Gene Name**	**Function**	**Fold change 7h**	***p*-value**	**Fold change 24h**	***p*-value**
Il1rl1	Interleukin 1 receptor-like 1	Inflammation	13.01	0.03	47.02	0.001
Reg3b	Regenerating islet-derived 3 beta	Inflammation	17.43	0.01	29.00	0.01
Tfpi2	Tissue factor pathway inhibitor 2	Enzyme inhibitor	7.24	0.05	22.64	0.01
Cxcl1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	Inflammation	8.35	0.002	11.99	0.01
Gal	Galanin prepropeptide	Hormone activity	9.39	0.004	5.60	0.03
Serpine1	Serpine peptidase inhibitor. clade E (nexin. plasminogen activator inhibitor type 1), member1	Protease inhibitor	5.03	0.02	9.77	0.03
B. Most down-regulated genes at both periods of recovery (7h and 24h) after OGD						
Gene Symbol	**Gene Name**	**Function**	**Fold change 7h**	***p*-value**	**Fold change 24h**	***p*-value**
Mmp28	Matrix metallopeptidase 28	Extracellular matrix	0.20	0.02	0.24	0.01
RGD1559748	Similar to Palate lung and nasal carcinoma-like protein precursor (Tongue plunc-like protein)	Unknown	0.29	0.001	0.25	0.01
Rasgrp1	RAS guanyl releasing protein 1 (calcium and DAG-regulated)	Enzyme activity	0.25	0.003	0.30	0.03
LOC100360071	Neuropeptide S-like	Signaling	0.19	0.0002	0.38	0.05
C1qtnf1	C1q and tumor necrosis factor related protein 1	Extracellular matrix	0.28	0.002	0.30	0.01
Ube2ql1	Ubiquitin-conjugating enzyme E2Q family-like 1	Metabolic process	0.31	0.01	0.36	0.04

We then selected the functional gene classes with potential relevance to the excitotoxic process, comprising a total of 586 and 403 genes that were altered at 7h and 24h after OGD, respectively, and analyzed changes in gene expression among genes belonging to those classes. In **Figure 3.5** we indicate the percentage of altered genes in ontology groups regulated exclusively at 7h (A), 24h (B) or regulated at both periods of recovery (C). The categories of metabolic processes and signaling pathways contained the largest percentage of regulated (up- or down-regulated) genes at both periods of incubation after OGD, both constitutively or time point-specific regulated genes.

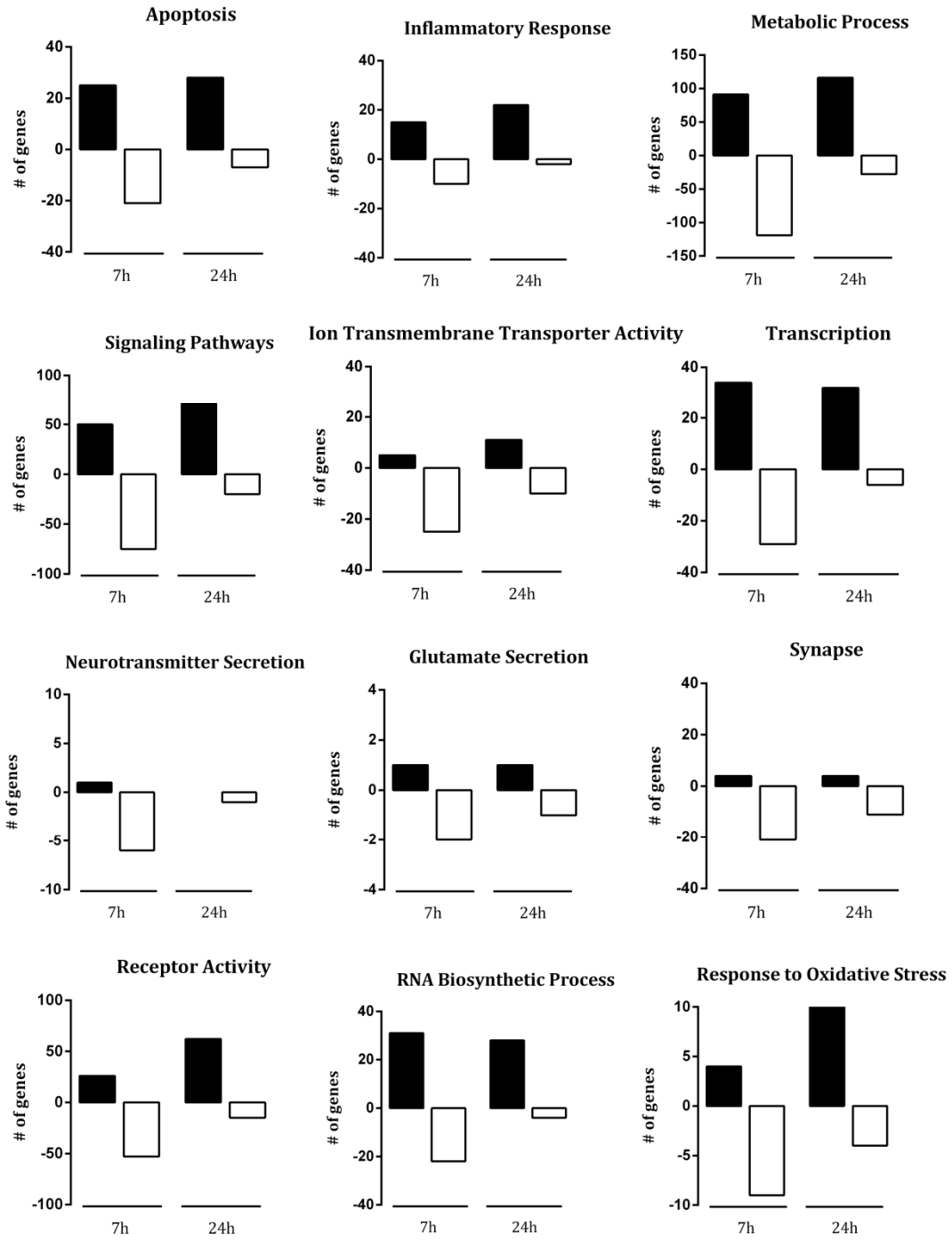


Figure 3.4. Time course analysis of OGD-induced differential gene expression within each functional gene category. The number of genes that were up-regulated (black bars) or down-regulated (white bars) at each period of recovery after the OGD insult is plotted for each functional gene category. Gene ontology analyses were performed using GoMiner™ and functional groups were selected manually.

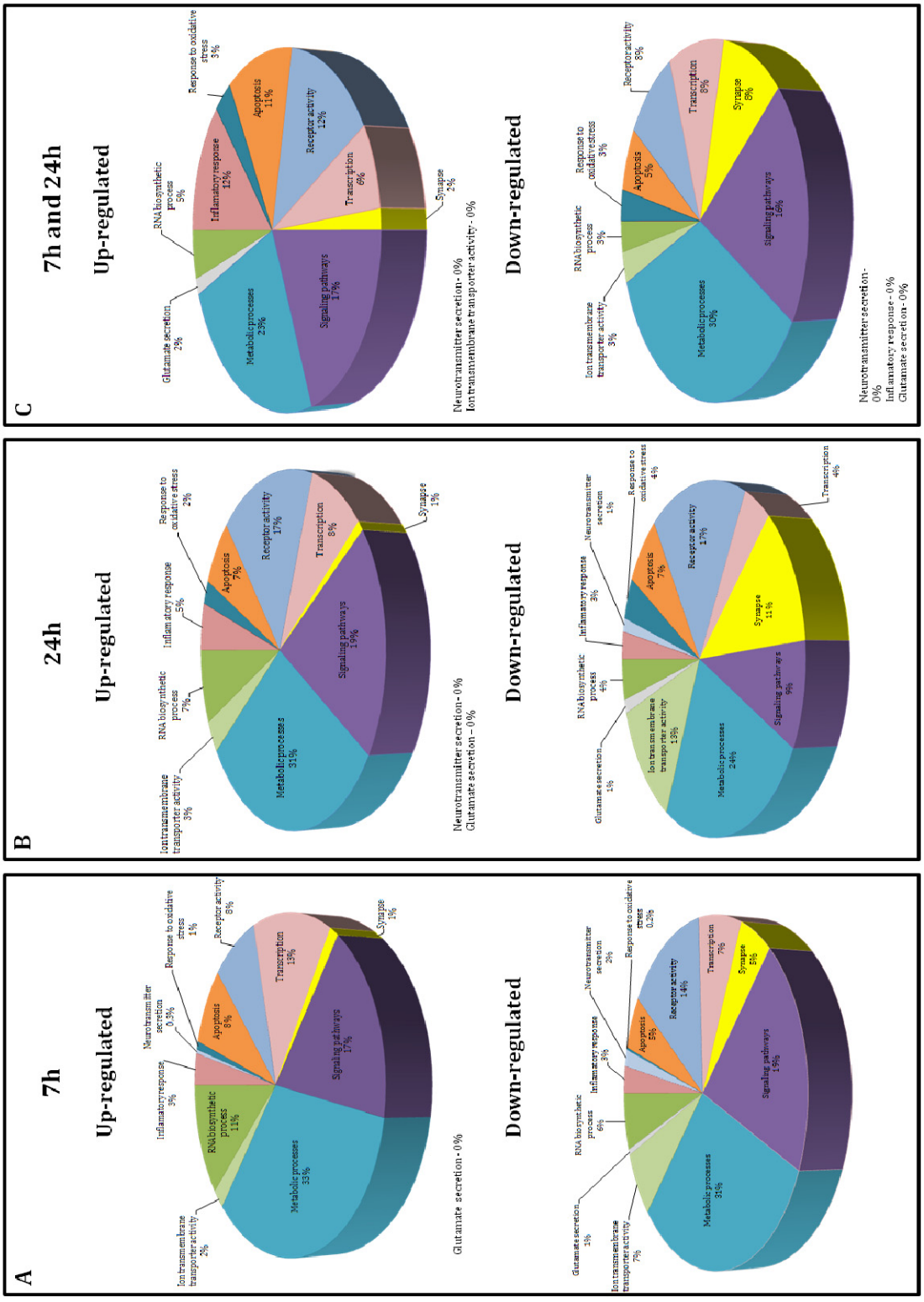

A
7h
Up-regulated
Metabolic processes 33%
Signaling pathways 17%
Transcription 15%
Receptor activity 8%
Synapse 1%
Response to oxidative stress 1%
Neurotransmitter secretion 0.3%
Inflammatory response 3%
RNA biosynthetic process 11%
Apoptosis 8%
Ion transmembrane transporter activity 2%
Glutamate secretion - 0%
Down-regulated
Metabolic processes 31%
Signaling pathways 19%
Synapse 5%
Transcription 7%
Receptor activity 14%
Apoptosis 5%
Response to oxidative stress 0.2%
Neurotransmitter secretion 2%
Inflammatory response 3%
RNA biosynthetic process 6%
Ion transmembrane transporter activity 7%
Glutamate secretion 1%
B
24h
Up-regulated
Metabolic processes 31%
Signaling pathways 19%
Transcription 8%
Receptor activity 17%
Synapse 1%
Response to oxidative stress 2%
Inflammatory response 5%
Apoptosis 7%
RNA biosynthetic process 7%
Ion transmembrane transporter activity 3%
Neurotransmitter secretion - 0%
Glutamate secretion – 0%
Down-regulated
Metabolic processes 24%
Signaling pathways 9%
Synapse 11%
Transcription 4%
Receptor activity 17%
Response to oxidative stress 4%
Neurotransmitter secretion 1%
Apoptosis 7%
Inflammatory response 3%
RNA biosynthetic process 4%
Ion transmembrane transporter activity 13%
Glutamate secretion 1%
C
7h and 24h
Up-regulated
Metabolic processes 23%
Signaling pathways 17%
Transcription 6%
Receptor activity 12%
Synapse 2%
Response to oxidative stress 3%
Inflammatory response 11%
Apoptosis 11%
RNA biosynthetic process 5%
Glutamate secretion 2%
Neurotransmitter secretion - 0%
Ion transmembrane transporter activity - 0%
Down-regulated
Metabolic processes 30%
Signaling pathways 16%
Synapse 8%
Transcription 8%
Receptor activity 8%
Response to oxidative stress 3%
Apoptosis 5%
RNA biosynthetic process 3%
Ion transmembrane transporter activity 3%
Neurotransmitter secretion - 0%
Inflamatory response - 0%
Glutamate secretion - 0%

Figure 3.5. Ontology of genes differentially expressed at 7h and 24h of incubation after 2h of OGD. Gene ontology analyses included genes that had a *p*-value <0.05 and a fold change of 2.0 and were performed using GoMiner™. Classes were selected manually and the number of genes for each class divided by the sum of the total number of genes in the selected classes (586 genes at 7h and 403 genes at 24h). Note that some genes are included in more than one class. **(A)** Ontology of genes up-regulated (upper) and down-regulated (lower) relatively to the control at 7h of incubation in culture conditioned medium after 2h OGD. In this analysis, the total numbers of up-regulated and down-regulated genes were 232 and 354, respectively. **(B)** Ontology of genes up-regulated (upper) and down-regulated (lower) relatively to the control at 24h of incubation in culture conditioned medium after 2h OGD. In this analysis, the total numbers of up-regulated and down-regulated genes were 333 and 70, respectively. **(C)** Ontology of genes up-regulated (upper) and down-regulated (lower) at 7h and 24h of incubation in culture conditioned medium after 2h OGD. In this analysis, the total numbers of up-regulated and down-regulated genes were 60 and 31, respectively.

Additionally, differential effects were observed when comparing the expression profiles at 7h and 24h after injury. For example, at 24h there was an increase in the percentage of up-regulated genes that code for proteins involved in the response to oxidative stress and receptor activity, whereas fewer genes were up-regulated in classes such as transcription and RNA synthesis, when compared to what is observed 7h after OGD. Also, at 24h more genes were down-regulated in classes such as response to oxidative stress and synapse, whereas genes coding for proteins related with transcription, RNA synthesis, metabolism and signaling pathways were less down-regulated. The complete lists of genes for every ontology group considered at 7h, 24h and both time points used in these analyses are shown in **Supplemental Tables 1-3.**

Taken together, these results demonstrate that the OGD model induces similar changes in functional groups of genes that have been shown to be differently regulated after *in vivo* ischemia (Jin *et al.*, 2001; Kim *et al.*, 2002; Schmidt-Kastner *et al.*, 2002; Tang *et al.*, 2002; Kawahara *et al.*, 2004; Lu *et al.*, 2004; Buttner *et al.*, 2009; Ramos-Cejudo *et al.*, 2012). Moreover, although many genes were differentially expressed at both time points of recovery, most genes were exclusively altered at only one of the post-injury periods tested, suggesting that specific molecular pathways can be regulated at an early or late response to OGD.

Confirmation of the microarray results by quantitative polymerase chain reaction (qPCR)

To validate the gene expression profiles obtained in the microarray experiment, we performed qPCR analyses for 11 selected genes. We chose differentially regulated (up- and down-regulated) genes from different functional categories **(Table 3.4)**.

Table 3.4. Microarray data of the genes selected for validation through qPCR analyses.

	Gene Name	Function	Status in microarrays	Fold change in microarrays
Prkcε	Protein kinase C epsilon	Protein kinase C	Down-regulated at 24h after OGD	0.46
Prkcδ	Protein kinase C delta	Protein kinase C	Up-regulated at 24h after OGD	2.20
Mmp25	Matrix metallopeptidase 25	Extracelular matrix protease	Up-regulated at 24h after OGD	2.27
Mmp3	Matrix metallopeptidase 3	Extracelular matrix protease	Up-regulated at 24h after OGD	2.93
Adamts5	ADAM metallopeptidase with thrombospondin type 1 motif 5	Extracelular matrix protease	Up-regulated at 24h after OGD	2.24
Adamts7	ADAM metallopeptidase with thrombospondin type 1 motif 7	Extracelular matrix protease	Up-regulated at 7h and 24h after OGD	2.28 (7h) / 2.64 (24h)
Batf3	Basic leucine zipper transcription factor, ATF-like 3	Transcription factor	Up-regulated at 7h after OGD	2.94
Hmgb1	High mobility group box 1	Transcription factor	Up-regulated at 7h after OGD	2.00
Nfil3	Nuclear factor, interleukin 3 regulated	Transcription factor	Down-regulated at 24h after OGD	0.44
Itgb6	Integrin beta 6	Cell adhesion	Up-regulated at 7h after OGD	3.74
Gadd45g	Growth arrest and DNA-damage-inducible, gamma	Cell death	Up-regulated at 7h after OGD	2.08

We observed that the mRNA of most of the selected genes had a similar variation as that deduced by the microarray experiment in terms of the direction of the differential expression **(Figure 3.6)**. Also, in general, the observed changes evaluated

by qPCR were much greater than those obtained with the microarray data analysis. The qPCR results for Prkcδ and Batf3 were in excellent agreement with the microarray results. Also, Gadd45g mRNA levels tended to increase after 7h of recovery, which would also agree with the microarray results, although no statistical significance was found. In some cases, qPCR analysis allowed the detection of changes in the mRNA levels at both time points of recovery rather than just the one indicated in the microarray data: Mmp25, Mmp3, Adamts5 and Adamts7 were up-regulated, whereas Prkcε was down-regulated at both 7h and 24h after OGD. Among the selected genes analyzed, only three genes had different results between the two techniques. Whereas Hmgb1 and Nfil3 were expected to be up- and down-regulated, respectively, at 7h of recovery according to the microarray data, none of these genes were found to have different expression levels when compared to the control when assessed by qPCR. Also Itgb6 was expected to be up-regulated at 7h, but qPCR analysis revealed changes in the mRNA levels only at 24h of recovery.

The expression levels of other genes with a particular interest to our work, namely synaptic protein genes and ion channel genes, were also validated by qPCR analyses. The implications of their regulation after the OGD challenge will be discussed in Chapters IV and V, respectively.

Discussion

Transient global cerebral ischemia induces a profound change in the transcriptome of brain cells, which is partially associated with the induction or repression of specific genes that influence the outcome of the ischemic insult. However, the mechanisms responsible for the selective vulnerability of hippocampal neurons to global ischemia remain to be clarified. As such, in this study we have subjected mature primary cultures of hippocampal neurons to OGD, a well-established *in vitro* model for global ischemia, in order to identify molecular changes that may be involved in the response of hippocampal neurons to an ischemic insult. The OGD challenge induced delayed neuronal death in hippocampal cultures and activated an excitotoxic component mediated by NMDA and AMPA receptors as observed in previous studies (Bonde *et al.*, 2005; Liu *et al.*, 2006; Zhou and Baudry, 2006; Dixon *et al.*, 2009). OGD also induced the activation of calpains, which corroborates previous studies showing that these proteases can play an important role in the enhancement of cell death

induced by *in vitro* ischemic insults (Newcomb-Fernandez *et al.*, 2001; Zhou and Baudry, 2006; Lobo *et al.*, 2011).

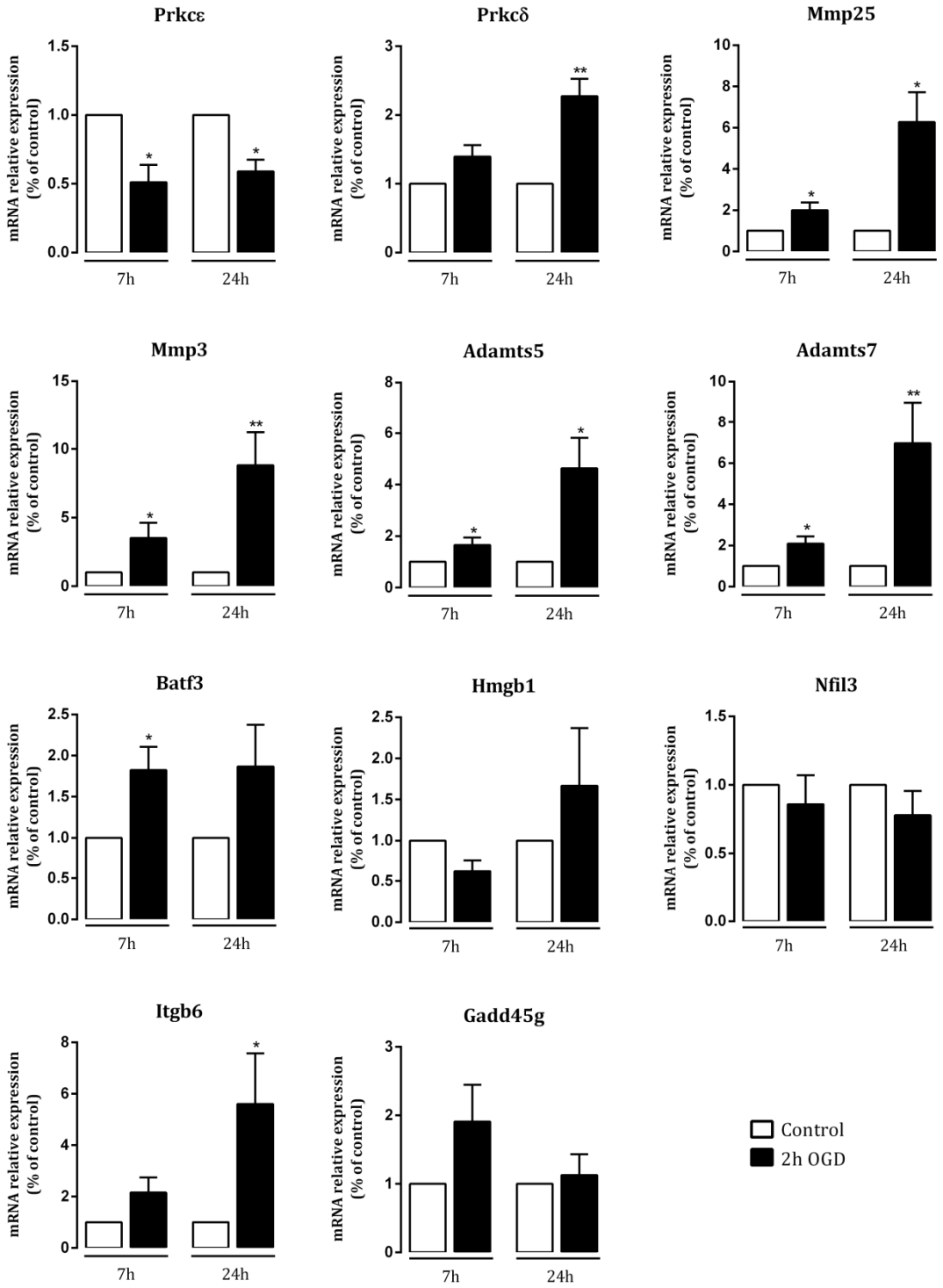


Figure 3.6. Effect of OGD followed by 7h or 24h of incubation in culture conditioned medium in the mRNA levels of 11 selected genes, compared to the respective control. Total RNA was extracted with TriZol at 7h and 24h after OGD. Quantitative PCR analysis was performed using cDNA prepared from 1 μg of total RNA and specific primers for each selected gene. Fold change in mRNA levels was normalized to Gapdh and Actb. Bars represent the mean ± SEM of 5 independent experiments, performed in different preparations. *$p<0.05$, **$p<0.01$, as determined using the Student's *t*-test on log-transformed data.

Although the OGD model has been used to infer about changes in gene expression associated with pre- and post-conditioning-derived neuroprotection (Benardete and Bergold, 2009; Prasad *et al.*, 2012), to the best of our knowledge no similar large scale study was developed so far using this insult as a tool to study ischemia-induced changes in the transcriptome of hippocampal neurons at different periods of recovery. Although our method does not take into account the interference of other hippocampal cells such as astrocytes, it has allowed the detection of a large number of genes related with the specific response of neurons to the ischemic insult. The validity of our approach was confirmed by performing qPCR analysis of many selected genes, belonging to different functional groups, which were in good agreement with the microarray data.

Notably, our results show that, although there can be some overlap between the genes differently regulated after 7h and 24h of recovery, each particular interval is characterized by the induction or suppression of distinctive sets of genes, showing that OGD induces a specific response at an early and a later time point. In fact, at an early recovery time point (7h) after OGD there are more differently regulated transcripts than at 24h, which corroborates the notion that the response to ischemia is a dynamic and coordinated process that starts soon after the insult, but is extended until later time points of recovery. In fact, this is supported by the observation that genes related with functions such as transcription and RNA biosynthesis are highly regulated at both periods of recovery. We also noted that the response at 7h of recovery is more associated with a general repression of genes than at 24h, at which point there is a general induction of gene expression. This observation further supports the idea that the response to ischemia at a delayed time point is still part of an active process that rather involves the induction than the repression of genes, and excludes the possibility that an unspecific down-regulation of genes could be associated with an increased rate of cell death.

Consistent with the results obtained using gene ontology analysis after *in vivo* ischemia (Jin *et al.*, 2001; Kim *et al.*, 2002; Schmidt-Kastner *et al.*, 2002; Tang *et al.*, 2002; Kawahara *et al.*, 2004; Lu *et al.*, 2004; Buttner *et al.*, 2009; Ramos-Cejudo *et al.*, 2012), we found that genes related with metabolic processes, signaling pathways, receptor activity, transcription, inflammation, neurotransmitter/glutamate secretion, RNA biosynthesis and apoptosis were differentially regulated after OGD. These categories were chosen due to their relevance in identifying the molecular pathways involved in the selective vulnerability of hippocampal neurons to ischemic insults.

Metabolism, inflammation and response to oxidative stress

As expected, changes in the expression levels of genes related with metabolism, oxidative stress and inflammation are part of the response of neurons to OGD, as these cellular events are closely influenced by one another. Neurons have a very high metabolic rate, consuming high amounts of both glucose and oxygen. Thus, since the deficiency in these components caused by the ischemic insult results in ATP depletion and anoxic depolarization, it is predictable that gene expression associated with the primary metabolism of neurons is influenced after the OGD challenge.

In global cerebral ischemia, reduction in the levels of ATP contributes to the abnormal extracellular concentration of glutamate, resulting in the overactivation of glutamate receptors, namely NMDARs and AMPARs, which contributes to the toxic accumulation of Ca^{2+} in neurons (Zukin *et al.*, 2004). In addition to the activation of Ca^{2+} channels, the disruption of Ca^{2+} homeostasis is operated through the deregulation of intracellular buffering mechanisms, namely at the level of the mitochondria and the endoplasmic reticulum (ER). The influx of Ca^{2+} mediated by NMDARs leads to the dissipation of the mitochondrial membrane potential, which further reduces ATP synthesis and increases the production of free radical species. Similar to what occurs *in vivo*, the OGD challenge can also induce a decrease in the ATP levels in neurons, with partial recovery during the reoxygenation period (Almeida *et al.*, 2002; Iijima *et al.*, 2003; Martinez-Sanchez *et al.*, 2004; Zhou *et al.*, 2007) and the dissipation of the mitochondrial membrane potential (Almeida *et al.*, 2002; Iijima *et al.*, 2003). Moreover, the deregulation of Ca^{2+} homeostasis has other deleterious effects by mediating the activation of several intracellular pathways, which will ultimately contribute to cell death (Martinez-Sanchez *et al.*, 2004; Szydlowska and Tymianski, 2010; Vs *et al.*, 2013).

Neurons are normally exposed to a baseline level of oxidative stress, since reactive oxygen species (ROS) are a normal product of mitochondrial oxidative metabolism. However, following an ischemic insult, there is a burst of free radical production that is thought to have crucial deleterious effects in neurons. For example, free radicals species react with membrane lipids inducing lipid peroxidation, which causes a decrease in membrane fluidity and subsequent neuronal death (Zhou and Baudry, 2009). The production of free radicals is closely related with mitochondrial Ca^{2+} overload, which stimulates mitochondrial superoxide and nitric oxide (NO) production (Neumar, 2000). *In vitro*, the generation of free radicals occurs both during the stimulus and the recovery period. The generation of free radicals on the latter phase is also dependent on Ca^{2+} influx, either through activation of glutamate receptors (Abramov *et al.*, 2007) or other ionic channels such as the transient receptor potential melastatin (TRPM) channels (Aarts *et al.*, 2003). According to our results, the regulation of genes involved in the response to oxidative stress is different depending on the recovery interval after OGD **(Supplemental Tables 1-3, Response to Oxidative Stress category)**. While after 7h there is a general repression of genes that code for proteins that may enhance the production of free radicals after brain ischemia, such as the metallopeptidase 9 (MMP9) (Jian Liu and Rosenberg, 2005; Yang *et al.*, 2010) or TRPM2 (Hara *et al.*, 2002; McNulty and Fonfria, 2005; Alim *et al.*, 2013), after 24h the response is more associated with the up-regulation of genes encoding proteins that may have a protective role against ROS. These include, for instance, α-synuclein (Seo *et al.*, 2002) and annexin 1, a known inhibitor of cytosolic phospholipase A2 (Kim *et al.*, 2001), which contributes to oxidative stress after cerebral ischemia (Kishimoto *et al.*, 2010) and OGD (Paramo *et al.*, 2010).

The inflammatory response combines the expression of pro- and anti-inflammatory genes **(Supplemental Tables 1-3, Inflammation category)**. However, whereas early inflammatory processes may exacerbate injury, late inflammation may be essential for the recovery of neurons (Neumar, 2000; Iadecola and Anrather, 2011). In fact, while at 7h after OGD there is a general increase in the expression of genes whose proteins are involved in pro-inflammatory events, such as cytokine interleukin 6 (IL-6), high-mobility group box 1 (HMGB1), integrin beta-6 (ITGB6) and CD44, at 24h of recovery there is an increased expression of genes related with vasodilation (Calca) and cell migration (Cxcl10 and Ccl5), which may reflect the anti-inflammatory component involving the migration of macrophages, which are known to be active in a later response to ischemia (Neumar, 2000). Although some inflammatory events are reproducible *in vitro* using OGD, such as the increased cytokine expression levels, this model can be limited, especially when used in

dissociated cultures, due to the absence of immune cells, as well as astrocytes and microglia, that can influence the anti-inflammatory response in the brain, which is the case of this work. The alteration in expression levels of genes related with migration of macrophages or vasodilation might therefore result from an innate response of neurons when affected by an ischemic insult, but that cannot be studied under a context of the *in vitro* model used in this work. Hence, although the OGD model has the ability to reproduce many of the changes induced by global ischemia, it does not account for the majority of the inflammatory reactions.

Glutamate release, ion channels and synaptic proteins

It has been well-documented that the disruption of ionic gradients and loss of membrane potential disrupts normal electrical signaling at the initial stage of an ischemic insult, causing deregulation in neurotransmitter release and leading to excitotoxicity (Lau and Tymianski, 2010; Mehta *et al.*, 2013). Hence, we analyzed gene expression related with neurotransmitters release to uncover possible changes associated with this process during recovery after the OGD challenge. We observed that the majority of genes related to that function that were differently expressed after OGD were down-regulated, namely at 7h of recovery **(Supplemental Tables 1-3, Neurotransmitter Secretion and Glutamate Secretion categories)**. Our results suggest that, at this point of recovery, neurons activate a program to decrease the expression of genes coding for proteins involved in the machinery of synaptic vesicle exocytosis, such as synaptosomal-associated protein, 25 kDa (SNAP-25), unc-13 homolog B (Unc13b) or synaptotagmin 3, which could contribute to reduce neurotransmitter release, namely glutamate, and, by extent, excitotoxicity.

We also observed that OGD evoked a significant down-regulation in most ion channel genes and synaptic protein genes, which has also been reported in studies employing *in vivo* ischemic insults (Lu *et al.*, 2004; Buttner *et al.*, 2009). Given that excessive intracellular accumulation of Ca^{2+} seems to be a common trigger for distinct mechanisms that lead to cell death (production of ROS, DNA cleavage, activation of proteases and death genes expression), the down-regulation of genes that code for proteins associated with Ca^{2+} entry in neurons may play a key role in the recovery from ischemic injury. In fact, many studies have shown that genetic or pharmacological inhibition of certain ion channels can exert a neuroprotective effect in injured neurons (Szydlowska and Tymianski, 2010; Zhang *et al.*, 2011). The regulation of ion channel genes after the OGD challenge will be discussed in more detail in Chapter V.

Different studies have also recognized the down-regulation of various neurotransmitter receptor genes after ischemic insults, such as those encoding for subunits of the NMDA receptors (Hsu *et al.*, 1998; Friedman *et al.*, 2001; Lu *et al.*, 2004), AMPA receptors (Calderone *et al.*, 2003; Liu *et al.*, 2006; Dixon *et al.*, 2009; Dennis *et al.*, 2011) or γ-aminobutyric acid (GABA) receptors (Francis *et al.*, 1999; Mele *et al.*, 2014). The results of the present study show that, while at 7h the majority of genes that are down-regulated are related with receptor trafficking (Pick1), synaptic vesicle transport (Svop) and fusion (Synpr), and synaptic transmission mediated by cholinergic receptors (Chrng and Chrna4), at 24h after OGD there seems to be a repression of proteins of excitatory (NMDAR subunits) and inhibitory (GABAR subunits) synapses. However, the functional significance of this down-regulation has yet to be clarified. For instance, whereas down-regulation of the NMDARs could account for a decrease in Ca^{2+} permeability in affected neurons, it could also cause impairment in the normal signaling of neurons. Likewise, changes in the GABAergic transmission may have distinct implications. For instance, it has been postulated that ischemic insults lead to an increase in GABA levels, which could reflect an intrinsic protective mechanism to counteract excitotoxicity and inhibit glutamatergic transmission (Clarkson *et al.*, 2010). However, there is evidence that glutamate-evoked depolarization of neurons during ischemia induces a substantial increase in the intracellular levels of chloride ions (Cl^-) (Schwartz-Bloom and Sah, 2001; Allen *et al.*, 2004). On the other hand, ischemic insults cause down-regulation in the $GABA_A$ receptors levels, which could potentially reduce damage from depolarization and swelling. However, Cl^- entry is not exclusive to $GABA_A$ receptor activation, thus inactivation of these receptors alone will not completely abolish cell damage (Nilius and Droogmans, 2003). Moreover, inhibition of the ischemia-induced reduction in $GABA_A$ receptor levels has shown to be neuroprotective, suggesting that these receptors may after all be necessary to neuronal survival (Smith *et al.*, 2012; Mele *et al.*, 2014).

The regulation of other synaptic protein genes, namely those encoding the subunits of the AMPA and NMDA receptors after the OGD insult will be discussed in further detail in Chapter IV.

Signaling pathways and apoptosis: mediation of cell death

Other genes that showed a different regulation upon the OGD challenge can be linked to a variety of functions such as cell signaling or apoptosis. For instance, at 7h

after OGD many genes involved in cell signal transmission mediated by G-coupled receptors, small GTPases and receptor tyrosine kinases have reduced expression levels. In particular, several genes related with mitogen-activated protein kinase (MAPK) signaling, such as Gadd45g, Taok3/Jik, Madd, Jip2 and Mapk10/Jnk3 are altered following the OGD challenge. 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. The activation of the stress kinases p38 and c-Jun N-terminal kinase (JNK) has been reported under ischemic conditions (Lai *et al.*, 2013). However, we observed both up- and down-regulation in genes related with these signaling cascades. For instance, while there is an increase in the mRNA levels of Gadd45g (confirmed by qPCR in the present work), whose respective protein can increase JNK activation (Takekawa and Saito, 1998), and Taok3/Jik, whose protein activates the p38 cascade (Yustein *et al.*, 2003), there is a decrease in the expression levels of Madd, whose protein is a known JNK substrate that translocates to the nucleus upon ischemic insults (Zhang *et al.*, 1998), and Jip2, that encodes a scaffold protein for both JNK and p38 pathways (Whitmarsh, 2006). Likewise, at 24h of recovery after OGD, we observed an increased expression of Gadd45α, whose protein can be increased due to JNK activation (Yin *et al.*, 2004) and was reported to increase after cerebral ischemia (Hou *et al.*, 1997; Zhu *et al.*, 1997; Laabich *et al.*, 2001), but a decrease in the mRNA levels of Mapk10/Jnk3, which encodes the JNK isoform expressed in the brain.

Naturally, since many of the aforementioned signaling pathways are intrinsically associated with neuronal death, apoptotic genes were also subject to different regulation by OGD. Similar to *in vivo* studies, genes involved in pro- and anti-apoptotic pathways were found to have altered expression levels at both recovery periods, which points out the complexity of programmed cell death after an ischemic insult. Notably, while at 7h pro-apoptotic gene encoding caspase-8 has reduced mRNA levels, at 24h we found up-regulation of other members of the caspase family, such as caspase-1 and caspase-14. Caspase-1 was found to have increased mRNA levels after MCAO and inhibition of its activation has shown to be neuroprotective (Shi *et al.*, 2013).

Taken together, the results obtained in this part of the work show that OGD not only mimics cell death events induced by cerebral ischemia, but can also serve, when combined with the microarray technology, as a useful tool to gain insight into particular cellular and molecular mechanisms evoked by global ischemia in hippocampal neurons. The extent to which the post-ischemic alterations identified in this work influence the

fate of neuronal cells exposed to ischemia can now be addressed, and may result in the identification of attractive therapeutic targets for the treatment of cerebral ischemia.

Chapter IV

Down-regulation of synaptic protein genes induced by ischemia in hippocampal neurons

Abstract

Excitotoxicity, caused by overactivation of glutamate receptors, is implicated in the neuronal death observed in global cerebral ischemia, which leads to the selective and delayed death of hippocampal neurons. However, despite the strong evidence collected over the past decades about the crucial role of the ionotropic glutamate receptors such as the AMPARs and NMDARs under ischemic conditions, clinical trials using antagonists for these receptors have not been able to reduce neuronal death. Synaptic failure is one of the earliest events of cerebral ischemia leading to impaired neurotransmission, which might be related with the biochemical alterations that occur in post-ischemic neurons, which include changes in the expression levels and the molecular composition of proteins related with synaptic transmission.

As such, the aim of this part of the study was to analyze changes in the synaptic protein genes under ischemic conditions. The present chapter follows the results obtained in the microarray experiment to identify synaptic protein genes whose expression was significantly altered in hippocampal neuronal cultures submitted to oxygen and glucose deprivation (OGD). Gene ontology analysis showed that synaptic protein genes, such as those encoding for PICK1, GRIP1, TARPγ3, calsyntenin-2/3, SAPAP2 and SNAP-25, were down-regulated after OGD. Additionally, OGD lead to the decrease in the expression of the GluA1 subunit of the AMPARs as well as the GluN2A and GluN2B subunits of NMDARs, but may promote the expression of the GluN3A subunit, thus altering the composition of the receptors in post-ischemic neurons.

Together, our results indicate that OGD activates a transcriptional program leading to down-regulation in the expression of genes coding for synaptic proteins, suggesting that the synaptic proteome may change after ischemia. This can be of particular interest to guarantee specificity of neuroprotective strategies, in particular those concerning the activation and further downstream signaling mediated by glutamate receptors.

Introduction

During brain ischemia, the increased levels of glutamate in the synaptic cleft induce overstimulation of glutamate receptors such as the N-methyl-D-aspartate receptors (NMDARs) and the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPARs). This overactivation of glutamate receptors is commonly referred

to as excitotoxicity and is assumed to be a critical mechanism contributing to pathological synaptic plasticity and the subsequent activation of cell death signaling pathways elicited by global ischemia (Lau and Tymianski, 2010; Mehta *et al.*, 2013). Failure in synaptic activity is one of the earliest events in cerebral ischemia, due to the energetic imbalance that occurs during an ischemic insult and that leads to neuronal depolarization and impaired neurotransmission (Hofmeijer and van Putten, 2012). Moreover, there are biochemical alterations that occur in neurons submitted to ischemic insults, which include changes in the expression levels and the molecular composition of proteins related with synaptic transmission, such as the ionotropic glutamate receptors of the AMPAR and the NMDAR types, among other proteins (Gascon *et al.*, 2005; Liu *et al.*, 2006; Dixon *et al.*, 2009; Jung *et al.*, 2012) that can be implicated in the mechanisms promoting either cell death or cell survival.

Different studies have obtained distinct results regarding the expression levels of these receptors under ischemic conditions; however, despite the controversy, ischemia-induced changes in the protein levels of synaptic components seem to take place shortly after the insult and prior to neurodegeneration, suggesting that they could be implicated in the mechanisms leading to cell death.

Moreover, the activation of specific transcription factors may account for the altered expression levels of synaptic proteins under ischemic conditions, as is the case of the transcriptional repressor element-1 (RE1) silencing transcription factor (REST)/neuron-restrictive silencer factor (NRSF). REST is a gene silencing transcription factor that, when active, represses the expression of genes critical to the elaboration of the neuronal response to brain ischemia. In adult neurons, REST is inactivated, an essential process for the maintenance of the neuronal phenotype, but neuronal insults re-activate REST in CA1 hippocampal neurons. In fact, REST has been shown to contribute to neuronal death by silencing the AMPAR GluA2 subunit expression, thus changing the pattern of gene expression in neurons destined to die (Calderone *et al.*, 2003; Noh *et al.*, 2012).

As such, the aim of this study was to determine the mRNA and protein levels of synaptic proteins under ischemic conditions. The present study has used microarray technology to identify synaptic protein genes whose expression was significantly altered in hippocampal neuronal cultures submitted to oxygen and glucose deprivation (OGD), an established *in vitro* model for cerebral global ischemia. In particular, we observed that OGD up-regulates REST expression, triggers a transcriptional program that down-regulates synaptic protein-encoding genes and induces changes in the subunit

composition of the AMPAR and the NMDAR subtypes. These results help elucidate how synapses are changed in post-ischemic neurons.

Results

Synaptic protein genes are down-regulated after OGD

According to the microarray data (see Chapter III), genes coding for several synaptic proteins were differentially regulated after the OGD challenge (**Supplemental Tables 1-3, Synapse category)**. We chose to validate the gene expression changes obtained in the microarray assay for 15 distinct synaptic protein genes **(Table 4.1)**. These genes were of particular interest to our work, since they code for proteins involved in the glutamatergic neurotransmission, whose imbalance contributes to ischemic injury. Thus, the expression levels of genes encoding proteins related with AMPAR trafficking (Pick1, Grip1, Cacgn3 and Cacgn8), pre- and post-synaptic compartments (Sypl2, Snap25, Clstn2, Clstn3, Dlgap2 and Fmr1) and subunits of the AMPA (Gria1 and Gria2) and NMDA receptors (Grin1, Grin2a and Grin2b) were analyzed by quantitative PCR (qPCR) **(Figure 4.1)**. Notably, most of these genes were down-regulated after the OGD insult, as indicated by the microarray experiment **(Table 4.1)** and confirmed by qPCR analyses **(Figure 4.1)**. For instance, Cacgn3, Clstn2 and Clstn3 were in excellent agreement with the microarray data, since their expression levels were reduced at the same time points of recovery as in the microarray assay. Sypl was also in agreement with the microarray data, and it was the only synaptic protein gene confirmed to be up-regulated in response to the OGD insult, since the up-regulation of Frm1 7h after OGD indicated in the microarray data was not confirmed by qPCR. In some cases, qPCR analysis allowed the detection of a decrease in the mRNA levels of genes at earlier (Gria1 and Grin2a), later (Pick1, Snap25 and Dlgap2) or at both (Grip1, Grin1 and Grin2b) time points of recovery rather than just the one indicated in the microarray data. The down-regulation of Cacgn8, as indicated by the microarray data, was not detected by qPCR analysis, whereas Gria2 was found to have reduced mRNA levels at 24h of recovery, which was undetected in the microarray experiment.

Table 4.1. Microarray data showing the effect of OGD in the mRNA levels of genes encoding synaptic proteins.

	Gene	Gene Name	Protein	Status	Fold Change
AMPAR trafficking	Pick1	Protein interacting with PRKCA 1	PICK1	Down-regulated at 7h	0.48
	Grip1	Glutamate receptor interacting protein 1	GRIP1	Down-regulated at 7h and 24h	0.62 (7h)/0.64 (24h)
	Cacng3	Calcium channel, voltage-dependent, gamma subunit 3	TARP γ3	Down-regulated at 7h	0.41
	Cacng8	Calcium channel, voltage-dependent, gamma subunit 8	TARP γ8	Down-regulated at 24h	0.64
Pre- and post-synaptic compartment	Sypl2	Synaptophysin-like 2	SYPL2	Up-regulated at 7h and 24h	2.61 (7h)/2.63 (24h)
	Snap25	Synaptosomal-associated protein 25	SNAP-25	Down-regulated at 7h	0.43
	Clstn2	Calsyntenin 2	Calsyntenin 2	Down-regulated at 24h	0.44
	Clstn3	Calsyntenin 3	Calsyntenin 3	Down-regulated at 7h and 24h	0.44 (7h)/0.38 (24h)
	Dlgap2	Discs, large (Drosophila) homolog-associated protein 2	SAPAP2	Down-regulated at 7h and 24h	0.48 (7h)/ 0.4 (24h)
	Fmr1	Fragile X mental retardation 1	FMRP	Up-regulated at 7h	2.34
AMPAR subunits	Gria1	Glutamate receptor, ionotropic, AMPA 1	GluA1	Down-regulated at 24h	0.55
	Gria2	Glutamate receptor, ionotropic, AMPA 2	GluA2	No change	--
NMDAR subunits	Grin1	Glutamate receptor, ionotropic, *N*-methyl-D-aspartate 1	GluN1	Down-regulated at 24h	0.39
	Grin2a	Glutamate receptor, ionotropic, *N*-methyl-D-aspartate 2A	GluN2A	Down-regulated at 24h	0.44
	Grin2b	Glutamate receptor, ionotropic, *N*-methyl-D-aspartate 2B	GluN2B	Down-regulated at 24h	0.44

In general, the qPCR analysis confirmed the OGD-induced changes in the expression levels of synaptic protein genes detected with the microarray data analysis. As such, our results show that OGD activates a transcriptional program leading to the repression of genes related with the synaptic function in hippocampal neurons, suggesting that changes in the synapse take place after the ischemic event.

The expression levels of the silencing transcription factor REST increase after OGD

The gene-silencing transcription factor REST actively represses neuronal genes important for synaptic plasticity and remodeling, such as synaptic vesicle proteins, synaptic structural proteins and receptors, in progenitor and non-neuronal cells (Palm *et al.*, 1998; Lunyak and Rosenfeld, 2005; Liu and Zukin, 2007). As neuronal differentiation takes place, REST is down-regulated, an essential process for the maintenance of the neuronal phenotype. Neuronal insults, such as transient global cerebral ischemia, activate REST in CA1 hippocampal neurons destined to die (Calderone *et al.*, 2003; Noh *et al.*, 2012). We therefore tested whether the OGD insult also triggers the induction of REST. In the microarray data the expression fold change for Rest was 1.63 at 7h after OGD (p=0.016). We also analyzed Rest mRNA expression in hippocampal cultures submitted to OGD by qPCR, and observed that even though at 7h after the insult the mRNA levels of REST were not significantly different from the control, a significant increase was observed at 24h **(Figure 4.2A)**. Consistently, the increase in the mRNA levels of Rest translated in the induction of the REST protein levels at the same time-point after OGD **(Figure 4.2B)**. These results corroborate previous observations suggesting that REST may be one of the transcriptional factors mediating the transcriptional response to ischemia. Indeed, 9 out of the 15 genes encoding synaptic proteins that were down-regulated after OGD contain putative REST-binding sites (**Figure 4.2C**, according to (Otto *et al.*, 2007)) and may therefore be regulated by REST.

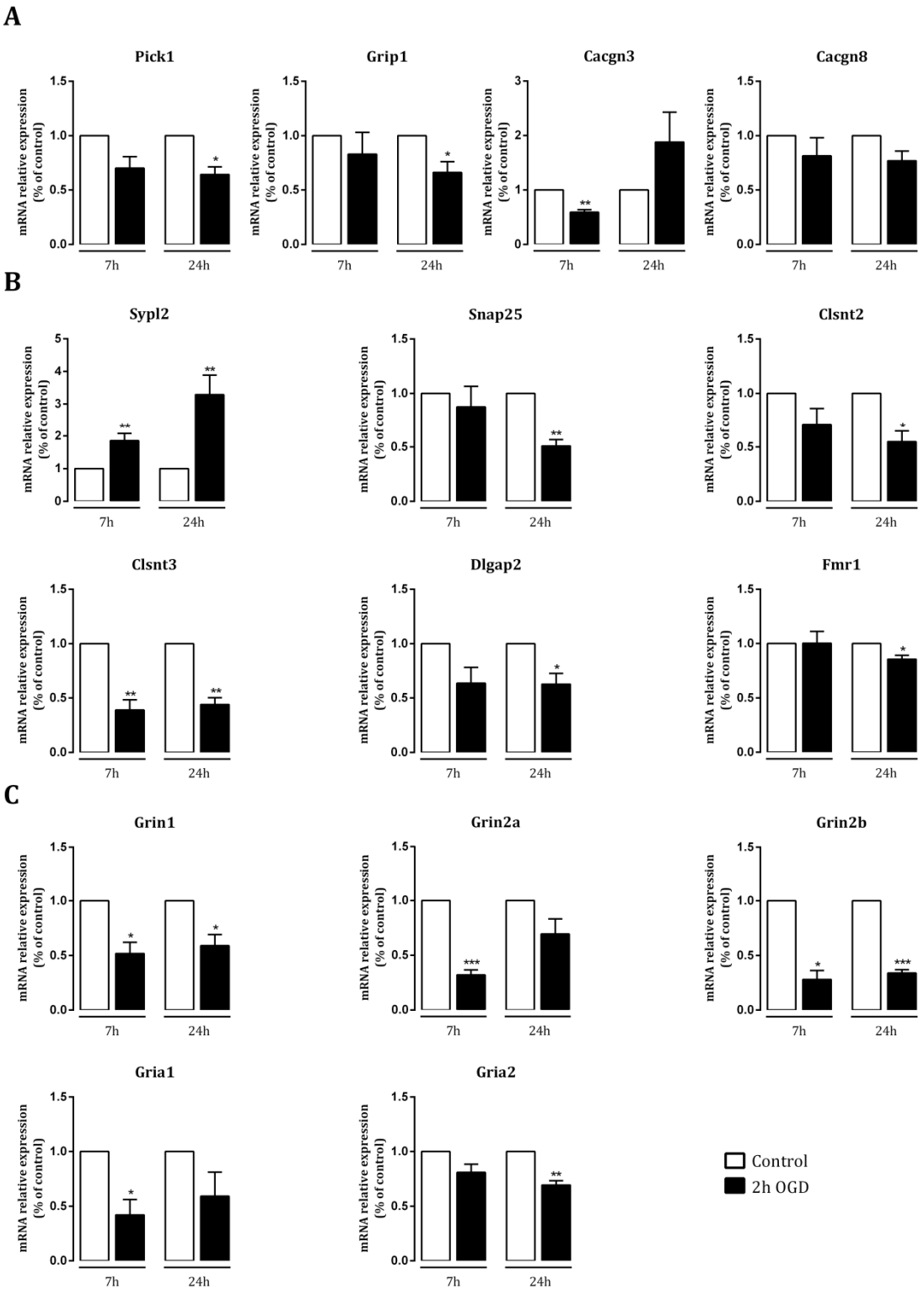


Figure 4.1. OGD induces changes in the mRNA levels of transcripts encoding synaptic proteins. Total RNA was extracted with TriZol 7h and 24h after the OGD insult. Quantitative PCR analysis was performed using cDNA prepared from 1 µg of total RNA and specific primers for each selected gene. Fold change in mRNA levels was normalized to Gapdh and Actb. Quantitative PCR analysis showed that genes encoding proteins associated with

AMPAR trafficking **(A)** and pre- and post-synaptic compartments **(B)**, as well as subunits of the AMPA and NMDA receptors **(C)** were mostly down-regulated (with the exception of Sypl2, which had increased expression levels) after OGD, at least at one of the time points analyzed after OGD. Bars represent the mean ± SEM of 5 independent experiments, performed in distinct preparations. $*p<0.05$, $**p<0.01$, $***p<0.001$, as determined by the Student's *t*-test on log-transformed data.

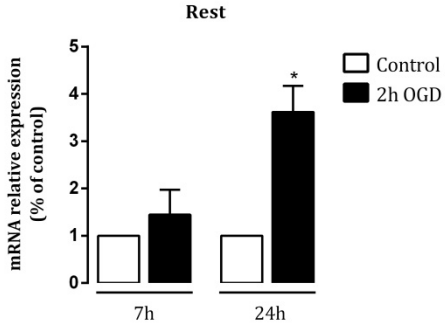
We next analyzed whether genes that have previously been described to have REST enrichment at their promoters after transient global brain ischemia in rats (Noh *et al.*, 2012) were differently expressed after OGD (**Figure 4.2D**). Interestingly, analysis of the microarray data showed that several genes with enriched REST (Noh *et al.*, 2012) have decreased expression levels at 7h or 24h of recovery after OGD; in the case of Snap25, Grin1, Grin2a and Grin2b, the down-regulation in their mRNAs was further confirmed by qPCR analysis in the present work (**Figure 4.1**). Gria2, the gene encoding GluA2, has been proven to be a REST target gene in the post-ischemic CA1 hippocampal region (Noh *et al.*, 2012). Although our microarray analysis did not detect significant differences in GluA2 expression, qPCR analysis showed down-regulation of GluA2 mRNA levels 24h after OGD (**Figure 4.1C**). Collectively, these observations support previous evidence (Calderone *et al.*, 2003; Noh *et al.*, 2012) and the hypothesis that REST is activated under ischemic conditions. REST activation may be responsible for the repression in the transcription of some of the synaptic protein genes that we observed to be down-regulated under OGD.

OGD down-regulates total and cell surface GluA1 protein levels

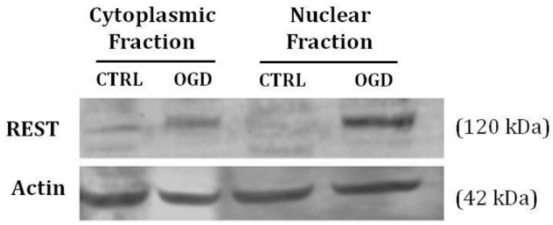
Previous studies indicate that both transient global cerebral ischemia and *in vitro* ischemic insults can down-regulate the AMPAR subunit GluA2, both at the mRNA and protein levels, in hippocampal neurons (Pellegrini-Giampietro *et al.*, 1992; Gorter *et al.*, 1997; Opitz *et al.*, 2000; Calderone *et al.*, 2003; Noh *et al.*, 2005; Liu *et al.*, 2006; Dixon *et al.*, 2009; Noh *et al.*, 2012), thus inducing a switch from GluA2-containing/Ca^{2+}-impermeable AMPAR to GluA2-lacking/Ca^{2+}-permeable AMPAR. However, according to our qPCR data, the mRNA levels of both GluA1 (Gria1) and GluA2 (Gria2) subunits was decreased after the OGD insult **(Figure 4.1C)**. We therefore tested whether total protein levels of GluA1 and GluA2 varied in the same direction as their respective mRNAs, at 7h and 24h after OGD. Despite the reduction in

the mRNA levels of GluA1 observed after 7h after injury, the decrease in the protein levels was detected only 24h after OGD. Curiously, GluA2 protein levels remained unaltered after OGD, despite the reduction in the mRNA levels that was observed even at 24h after the insult **(Figure 4.3A)**.

A



B



C

RE-1consensus sequence: TTCAGCACCACGACAGCGCC		
Gene	**Protein**	**Putative RE-1 sequence(s)**
Grin1	GluN1	TTC AGC ACC TCG GAC AGC ACC TTC ATC AAC ACG AAC AGC GGC CTC AGC ACC ACT GCG TCT GGC GGC GCC TCC AGC ACC CAG GAC AGC TCT
Grin2a	GluN2A	TTC AGA GCC AAG GTC AGA GCC CTC AGC ACC ACC AAA GGG GAA AGA GCA TAT GCT CCA CTC CGG ACA GCG GC TTC AGA ACC TGG ACA GAA GC
Dlgap2	SAPAP2	CTC AGT AGC AAG GAG AGC GCC GCC AGG ACC AAG GAC AGC ACC GTC AGC ACC AAG GAC AGC ACC GTC AGC ACC AAG GAC AGC ACC
Clstn2	Calsyntenin 2	TTC AGA ACC ATG GCT AGC ACC TTC AGC ACC AAA GAC TGA TCC
Gria1	GluA1	TTC AGC TCT GGG GAC AGA GCC TTC AGC ACC TGA TGT GGC GTG ACA GC TCC TTG AGC ACT TCA GCT CTG GGG ACA GA GCC
Gria2	GluA2	TTT AGC ACC GCG GAC AGC GCT TTT AGC ACC GCG GAC AGC GCT
Sypl2	Synaptophysin-like 2	TTC AAC TCC ACA GAC AGT GTC
Cacgn3	TARPg3	TTC AGC ACC ACG GAC CTC CAA CTC AGC ACC ACG GAC CTC AGC TTC ACC CCC ATG TAC ATC GCC
Grip1	GRIP1	TTT AGC ACC TCA GAC ACC TCC

D

Genes with REST enrichment at promoter sites after ischemia (according to Noh *et al.*, 2012)	Protein	Fold Change at 7h after OGD	*p*-value	Fold Change at 24h after OGD	*p*-value
C1qtnf1	C1qTNF1	0.28	0.002	0.30	0.006
Chrnb2	nAChRB2	0.71	0.02	--	--
Ece1	ECE1	1.83	0.003	--	--
Grin1*	GluN1	--	--	0.39	0.03
Grin2a *	GluN2A	--	--	0.44	0.03
Grin2b *	GluN2B	0.71	0.03	0.44	0.02
Grin2c	GluN2C	--	--	0.50	0.03
LOC680885	--	--	--	2.08	0.04
Mtp18	MTP18	0.59	0.03	--	--
Ndor1	Ndor1	0.79	0.04	--	--
Neurod1	NeuroD	1.79	0.01	--	--
Nppa	Natriuretic peptide A	0.45	0.01	--	--
Rnf215	Ring finger protein 215	0.74	0.04	--	--
Scg2	Secretogranin II	--	--	0.64	0.05
Snap25*	SNAP-25	0.43	0.02	--	--
Snrpn	snRPN	0.65	0.03	--	--
Zfp653	Zinc finger protein 653	0.76	0.01	--	--
* Genes also found to be down-regulated by qPCR analysis in the present work					

Figure 4.2. OGD increases REST expression in mature hippocampal neurons. (A) Quantitative PCR analysis showed the OGD insult induced a marked increase in Rest mRNA. Total RNA was extracted with TriZol 7h and 24h after the OGD insult. Quantitative PCR analysis was performed using cDNA prepared from 1 μg of total RNA and specific primers for each selected gene. Fold change in mRNA levels was normalized to Gapdh and Actb. Bars represent the mean ± SEM of 3 independent experiments, performed in distinct preparations. *Significantly different from control (*p<0.05, Student's t-test on log-transformed data). **(B)** Representative Western blot shows a marked increase in REST protein levels, both in the cytoplasmic and nuclear fractions of hippocampal neurons submitted to OGD followed by 24h of incubation in culture conditioned medium (n = 3). Actin was used as loading control. **(C)** Putative RE-1 sequence(s)/REST-binding site(s) in synaptic genes (according to Otto et al. (2007)) found to be down-regulated after OGD. **(D)** Genes with enrichment of REST after ischemia (according to Noh et al., 2012) found to be differently expressed after OGD.

We then biotinylated cell surface proteins in hippocampal neuronal cultures 24h after incubation under sham or OGD conditions, and purified biotinylated proteins by affinity chromatography to analyze the cell surface content of both subunits of AMPAR **(Figure 4.3B)**. We observed that, consistent with the results obtained for the total protein levels, surface GluA1 was reduced 24h after OGD, whereas surface GluA2 expression was unaffected. These results show that AMPAR present at the cell surface in a mixed population of hippocampal neurons subjected to OGD have a decreased

content on GluA1 when evaluated 24h after the insult, resulting from a delayed reduction in the expression levels of GluA1 in these experimental conditions. The reduction in AMPAR GluA1 content that we detected may represent a neuroprotective mechanism occurring in specific neuronal subtypes in the preparation.

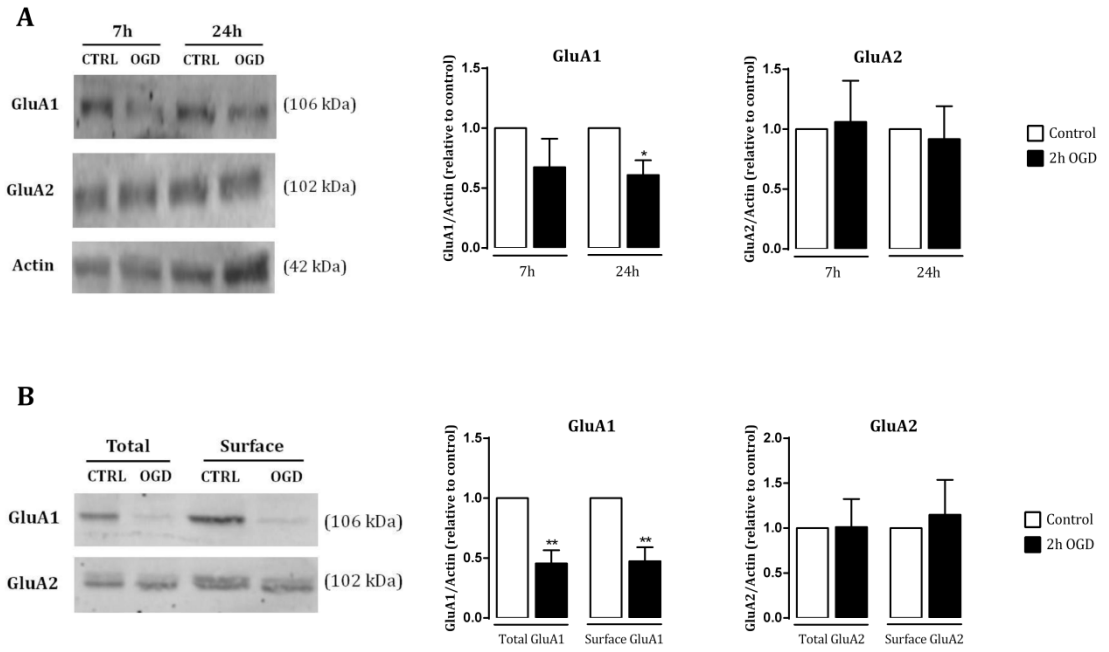


Figure 4.3. Ischemic insults affect the protein levels of the AMPAR subunits GluA1 and GluA2. (A) Total protein extracts were prepared 7h and 24h after the OGD insult and Western blot analysis were performed. Total GluA1 is decreased after 24h, whereas GluA2 levels remain unaltered following both periods of recovery. The left panel shows a representative Western blot for total GluA1 and GluA2 present in cell lysates after OGD. The right panel represents the quantification of the Western blots. Bars represent the mean ± SEM of 5 independent experiments, performed in distinct preparations. **(B)** GluA1 and GluA2 surface levels were analyzed after biotinylation of cultured hippocampal neurons, 24h after the OGD insult. At this time point after OGD GluA1 is removed from the surface, whereas GluA2 surface levels remain unaltered. The left panel shows a representative Western blot for surface GluA1 and GluA2 after the insult. Total actin (from the total extract) was used as loading control. Bars represent the mean ± SEM of 3-4 independent experiments, performed in distinct preparations. *$p<0.05$, as determined by the Student's *t*-test.

OGD down-regulates GluN2 subunits and increases the expression levels of GluN3A

The NMDA subtype of ionotropic glutamate receptors has been extensively studied in ischemic conditions due to their overactivation by glutamate and subsequent contribution to the activation of pathologic molecular pathways (Hardingham and Bading, 2010; Sanz-Clemente *et al.*, 2013). In the *in vitro* ischemia model used in the present work, cell death is also dependent on the activation of NMDA receptors **(Figure 2.2A, D)**. According to our qPCR data, the mRNA levels of GluN1 (Grin1) and GluN2B (Grin2b) were down-regulated both at 7h and 24h of incubation after OGD, whereas GluN2A (Grin2a) was down-regulated only at 7h after the ischemic injury **(Figure 4.1C)**. We then analyzed the total protein levels of these subunits by Western blot **(Figure 4.4A)**. At 24h after OGD, protein levels for both GluN2A and GluN2B were decreased, whereas GluN1 was not significantly changed, despite a clear tendency for increased expression. Together, these results indicate that OGD leads to the down-regulation of the most abundant GluN2 subunits in the hippocampus, thereby influencing the subunit composition of NMDARs.

Given that the total protein levels of GluN1 failed to accompany the decrease on protein levels found for both GluN2A and GluN2B subunits, we hypothesized that OGD may influence the expression levels of the GluN3 subunit, which can also promote GluN1 trafficking to the cell surface (McIlhinney *et al.*, 1998; Perez-Otano *et al.*, 2001). In fact, analysis of the mRNA levels of the GluN3 subunit revealed that GluN3A displayed a remarkable increase in the mRNA expression 24h after OGD, whereas GluN3B mRNA levels remained unaltered at both periods of recovery **(Figure 4.4B)**. This suggests that OGD specifically induces the expression of GluN3A, but not GluN3B, thus promoting the presence of GluN1-GluN3A receptors in neurons submitted to ischemic insults. This result is in accordance with recent observations indicating that GluN3A can be up-regulated in response to ischemic insults and might therefore exert a neuroprotective effect against neuronal damage (Wang *et al.*, 2013).

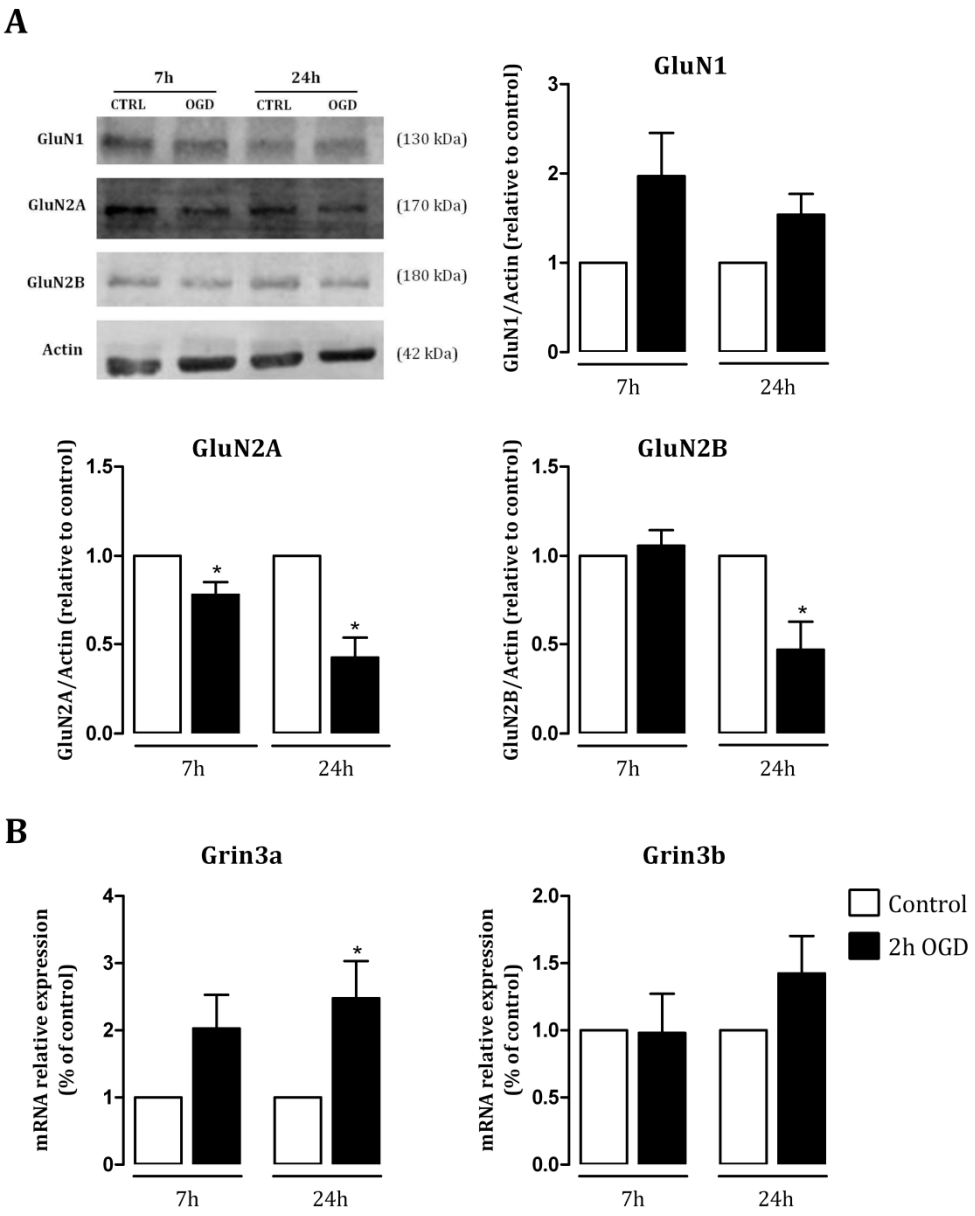


Figure 4.4. OGD affects the expression levels of NMDAR subunits. (A) Total protein extracts were collected 7h and 24h after OGD and Western blot analysis was performed. Whereas total GluN1 levels tend to increase after the insult, a decrease in total GluN2A was observed after both periods of recovery while GluN2B decreased at 24h after the OGD insult. The left panel shows a representative Western blot for GluN1, GluN2A and GluN2B levels after OGD. The right panel represents the quantification of the Western blots. Actin was used as loading control. Bars represent the mean ± SEM of 5-9 independent experiments, performed in distinct preparations. **(B)** OGD induces a marked increase in the mRNA levels of GluN3A but not in GluN3B. Bars represent the mean ± SEM of 3-5 independent experiments, performed in distinct preparations. *$p<0.05$, as determined by the Student's *t*-test.

Discussion

OGD activates a transcriptional program to down-regulate synaptic genes

According to our microarray and qPCR data, most of the synaptic protein genes considered in this work showed down-regulation during the recovery periods after the OGD insult. As far as we know, this is the first study to detect changes in the expression levels of an extensive group of genes coding for synaptic protein genes upon ischemia. Down-regulation of some synaptic protein genes (such as Snap25, Gria1,2, and Grin1, 2a) has been reported previously in studies regarding changes in gene expression after focal (Lu *et al.*, 2004) and global (Kawahara *et al.*, 2004) ischemia, but the other synaptic protein genes that we identified had not been previously reported to be altered after ischemic insults. Different studies have also recognized the down-regulation of various neurotransmitter receptor genes after ischemic insults, such as those encoding for subunits of the NMDA receptors (Zhang *et al.*, 1997; Hsu *et al.*, 1998; Friedman *et al.*, 2001; Lu *et al.*, 2004; Gascon *et al.*, 2005; Dos-Anjos *et al.*, 2009a; Jung *et al.*, 2012) and of the AMPA receptors (Liu *et al.*, 2006; Dixon *et al.*, 2009; Dennis *et al.*, 2011; Noh *et al.*, 2012). However, as far as we know, this is the first study to detect changes in the expression levels of an extensive group of synaptic protein genes and most of the synaptic protein genes selected for verification by the qPCR method had not been previously reported to have altered expression levels after ischemic insults.

Although cerebral ischemia can induce a reduction of spines and synapses as neurons degenerate (Kovalenko *et al.*, 2006; Jung *et al.*, 2012), the down-regulation of specific synaptic protein genes suggests the initiation of an adaptive response to hyperexcitability in post-ischemic neurons. The group of genes involved in synaptic function that we identified code for proteins that can be involved in many regulatory processes, *i.e.* the cycling of synaptic vesicles and neurotransmitter release at nerve terminals (Snap25), trafficking and stabilization of glutamate receptors at the cell surface (Pick1, Grip1, Cacgn3 and 8) and propagation of neuronal signal impulses upon cell stimulation (AMPAR and NMDAR subunits).

SNAP-25 (synaptosomal-associated protein, 25kDa) is involved in synaptic vesicle membrane docking and fusion mediated by N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), therefore contributing to the regulation of neurotransmitter release in presynaptic terminals. Moreover, SNAP-25 has been shown to have a critical role in protein kinase C (PKC)-induced, SNARE-dependent insertion of NMDAR at synaptic sites, a mechanism relevant to synaptic plasticity (Lau *et al.*,

2010). A decrease in the mRNA levels of SNAP-25 at a later time point after the ischemic insult, as our data shows, might consist of a protective strategy of neurons to reduce neuronal hyperexcitability by reducing the release of glutamate as well as the insertion of NMDAR at the plasma membrane. PICK1 (protein interacting with C kinase) and GRIP1 (glutamate receptor interacting protein) are both involved in the trafficking of AMPARs (Liu and Cull-Candy, 2005). Under physiologic conditions PICK1 binds to AMPAR subunits GluA2, an interaction known to be required for AMPAR internalization (Hanley and Henley, 2005). In hippocampal neurons subjected to OGD, rapid PICK1-mediated GluA2 internalization during the OGD insult was found to contribute to cell death mediated by GluA2-lacking Ca^{2+}-permeable AMPARs (Dixon *et al.*, 2009). A delayed decrease in the levels of PICK1 following the reduction in its mRNA levels would probably account for the maintenance of the GluA2 subunit at the cell surface of post-ischemic neurons, as observed in our work. Interestingly, the gene coding for TARPγ3 (transmembrane AMPAR regulatory protein γ3) was also shown to be down-regulated after OGD, suggesting another mechanism through which trafficking of AMPAR to the cell surface is changed after ischemia. In fact, it has been shown that genetic deletion of TARPγ8 selectively abolishes sustained depolarizations in hippocampus mediated by kainate activation of AMPARs (Tomita *et al.*, 2007). Therefore, it would be interesting to confirm whether the protein levels of TARPγ3 or TARPγ8 follow the same pattern of their respective mRNAs and, if so, their implication for the transport of AMPAR in ischemia. FMRP (Fragile X mental retardation protein) is a dendritic modulator of mRNA transport and translation repression, and knock-out mice present reduced levels of many postsynaptic proteins (Schutt *et al.*, 2009). SAPAP2 (Synapse-associated protein 90/postsynaptic density-95-associated proteins, encoded by Dlgap2) is a member of the SAPAP family of postsynaptic proteins that can interact with various synaptic components, including the NMDARs (Kim *et al.*, 1997; Naisbitt *et al.*, 1997; Takeuchi *et al.*, 1997). Since both the genes encoding for FMR1 and SAPAP2 were down-regulated after OGD, a decrease in the respective proteins could hypothetically play a role in the re-organization of functional multiprotein units at post-ischemic synapses. Additionally, calsyntenin-3 (Clstn3), which is dramatically reduced after OGD, has been recently attributed a function in promoting synapse development, with $Clstn3^{-/-}$ mice showing compromised inhibitory and excitatory neurotransmission (Pettem *et al.*, 2013). Overall, our results suggest that neurons can activate a program to decrease the expression of genes coding for proteins involved in the machinery of synaptic transmission, which might contribute to reduce glutamate-mediated signaling and excitotoxicity.

We also obtained evidence that the transcription factor REST is induced upon an OGD insult to cultured hippocampal neurons, in agreement with previous studies showing an increase of REST mRNA in the CA1 region of organotypic hippocampal slices (Calderone *et al.*, 2003; Noh *et al.*, 2012). Noteworthy, knockdown of REST protected CA1 neurons from OGD-induced death, thus suggesting that expression of REST is causally related with neuronal death (Calderone *et al.*, 2003). One of most studied targets of REST repressive activity is the AMPAR subunit GluA2, to whose promoter REST can bind, thus suppressing GluA2 expression (Liu and Zukin, 2007). Other genes have been identified with REST-occupied target sequences (RE1 sites) after a genome-wide approach using serial analysis of chromatin occupancy in HEK cells (Otto *et al.*, 2007), among which some of the synaptic protein-encoding genes analyzed in the present work **(Figure 4.2C)**. Interestingly, we found that several genes previously described to have REST enrichment at their promoters after *in vivo* ischemia (Noh *et al.*, 2012) have reduced mRNA levels after the OGD insult **(Figure 4.2D)**, thus providing a new list of REST targets with a potential role in ischemia. Additionally, since several of the synaptic protein genes we found to be down-regulated have putative REST-binding sites, it will be of interest to confirm that REST can indeed bind to the promoter of these genes under ischemic conditions.

Interestingly, ischemia-induced changes in the protein levels of synaptic components suggest that important alterations occur at the synapses of post-ischemic neurons. In particular, our results support the hypothesis that transcription-dependent mechanisms take place in insulted neurons that promote the reduction of neuronal activity through down-regulation of the expression of central synaptic players. In particular, given that NMDAR and AMPAR have been long considered important targets for therapeutical intervention, information concerning their post-ischemic expression levels is of the upmost interest.

Our results indicate that at 24h after a 2h OGD insult, AMPARs present at the cell surface of cultured hippocampal neurons have a decreased content of GluA1 subunits, whereas GluA2 protein levels are unchanged. Previous studies using cultured hippocampal neurons and the OGD stimulus showed early effects at the level of AMPAR traffic. Brief OGD caused the internalization of synaptic GluA2-containing AMPARs in hippocampal neurons (Liu *et al.*, 2006; Dixon *et al.*, 2009). Other studies indicated that global ischemia triggers the reduction of GluA2 expression in the hippocampus CA1 region neurons, both at the mRNA (Pellegrini-Giampietro *et al.*, 1992; Gorter *et al.*, 1997) and protein levels (Opitz *et al.*, 2000; Noh *et al.*, 2005), resulting in increased levels of Ca^{2+}-permeable AMPARs. In the present study, the

antagonist for Ca^{2+}-permeable AMPAR, Naspm, protected hippocampal neurons from OGD-induced cell death (**Figure 2F**), supporting, along with other studies (Noh *et al.*, 2005), a function for Ca^{2+}-permeable AMPAR in ischemia-induced cell death. However, in cultured hippocampal neurons we failed to detect changes in GluA2 expression and traffic reported by others, presumably because these changes are cell-type specific and become diluted in a mixed population of hippocampal neurons. Also, the intensity of the ischemic insult might influence transcriptional and post-transcriptional regulatory mechanisms which may explain different results obtained in distinct works. Nevertheless, our study has shown for the first time a dramatic decrease in the total mRNA and protein levels of GluA1 24h after a 2h OGD insult. The decreased levels of GluA1-containing AMPARs likely result in a depression of synaptic transmission, with consequences similar to the increased internalization of AMPARs found in CA3 pyramidal neurons following a 15 min OGD protocol (Dennis *et al.*, 2011). Both the endocytosis of AMPAR (Dennis *et al.*, 2011) and the delayed decrease on GluA1 expression after OGD that we describe here are potentially neuroprotective mechanisms.

Furthermore, we found that the subunit composition of NMDARs is altered after the OGD insult, which is in agreement with previous studies showing a down-regulation of genes encoding for subunits of the NMDARs after *in vivo* (Hsu *et al.*, 1998; Friedman *et al.*, 2001; Lu *et al.*, 2004; Dos-Anjos *et al.*, 2009b) and *in vitro* (Dos-Anjos *et al.*, 2009a; Jung *et al.*, 2012) ischemia. In our experimental system, OGD decreased the total mRNA and protein levels of the GluN2 subunits without affecting the protein level of the GluN1 subunit (**Figure 4.4**). Additionally, we investigated the role of GluN3 subunit (GluN3A and GluN3B) expression in hippocampal neurons submitted to OGD since the GluN3 subunits are now recognized players in the modulation of NMDAR activity (Pachernegg *et al.*, 2012). Interestingly, GluN3A expression is induced by OGD (**Figure 4.4B**), corroborating the results obtained in a recent study showing that GluN3A protein levels increase in neurons submitted to OGD and focal ischemia (Wang *et al.*, 2013), but in disagreement with an earlier report showing that focal ischemia down-regulates GluN3A at the mRNA and protein levels 24h after the insult (Zhu *et al.*, 2012). Although GluN3 subunits can be found in many brain tissues including the hippocampus (Wong *et al.*, 2002; Wee *et al.*, 2008), the expression of these subunits is temporally restricted. Whereas GluN3A is predominantly expressed during early development and diminishes to lower levels in adulthood, GluN3B levels are low during early development and gradually increase into adulthood (Henson *et al.*, 2010). Compared with NMDAR comprised of GluN1/GluN2 subunits, GluN3-containing NMDARs exhibit several different properties, including

lower amplitude currents, lower Ca^{2+} permeability and reduced Mg^{2+} sensitivity (Das *et al.*, 1998; Nishi *et al.*, 2001; Chatterton *et al.*, 2002; Matsuda *et al.*, 2002; Henson *et al.*, 2010; Low and Wee, 2010; Pina-Crespo *et al.*, 2010). As such, GluN3 subunits are considered to have a dominant-negative effect upon NMDAR activity, which can be an advantage under pathologic conditions. Indeed, the neuroprotective role of GluN3A against excitotoxicity and ischemia-mediated injury has been already shown (Nakanishi *et al.*, 2009; Wang *et al.*, 2013). Cultured neurons from GluN3A knock-out mice display greater vulnerability to toxic NMDA application, whereas neurons expressing transgenic GluN3A are more resistant to NMDA-mediated neurotoxicity and focal ischemia than wild-type neurons (Nakanishi *et al.*, 2009). Although some evidence suggests that the protective role of GluN3A is associated with a decrease in Ca^{2+} permeability and in the production of ROS mediated by NMDARs (Wang *et al.*, 2013), further studies are required to confirm the up-regulation of GluN1/GluN3A receptors upon ischemic insults and to understand the role mediated by these receptors in post-ischemic neurons. Importantly, as no significant changes were observed for the GluN3B subunit, neither at the mRNA (the present study) or protein (Wang *et al.*, 2013) levels after ischemic insults, it is probable that this subunit is not involved in the pathological response mediated by ischemia, at least in hippocampal neurons.

In conclusion, our results suggest that OGD induces the activation of a transcriptional program that represses the expression of many synaptic protein genes that encode for proteins mainly associated with glutamatergic neurotransmission. In particular, we have shown that OGD alters the expression of AMPA and NMDA receptor subunits that modulate the ion channel activity of the receptors, namely Ca^{2+}-permeability. Given that NMDA and AMPA receptors have long been considered important targets for therapeutical intervention, information concerning the post-ischemic expression levels of these receptor subunits is crucial to guarantee efficacy of potential neuroprotective strategies concerning the activation and further downstream signaling mediated by these receptors.

Chapter V

Uncovering the role of CLIC1 in the ischemia-triggered response in hippocampal neurons

Abstract

Although the application of selective antagonists of NMDA and AMPA receptors has been shown to be neuroprotective in *in vitro* and *in vivo* studies of brain ischemia, clinical trials using the same approach have not been successful, either due to the side effects that arise from blocking the physiologic function of these receptors or to the inefficacy in improving the outcome of ischemic stroke. Indeed, increasing evidence shows that ischemic insults promote the activation of multiple signaling pathways beyond the component of excitotoxicity mediated by glutamate receptors. In fact, other ion channels can also contribute to ionic imbalance in post-ischemic neurons.

Therefore, we analyzed the microarray data concerning changes in the expression of genes encoding ion channel proteins, induced by ischemic conditions, in order to identify a new possible target for brain ischemia treatment. Gene ontology analysis showed that genes encoding for several non-selective cation channels of the transient receptor potential (TRP) family were up-regulated (Trpc5 and Trpc7) or down-regulated (Trpc6 and Trpm2) after OGD. Additionally, OGD lead to the increase in the expression levels of several chloride channels (Clca2, Clic1, Clic3 and Clic5).

CLIC1 is the most studied member of the CLIC family of chloride channels, but its role in the brain is still largely unknown. Our results indicate that *in vivo* ischemic insults increase the protein levels of CLIC1 in specific areas of the rat brain and that, when overexpressed in hippocampal neurons, CLIC1 protects against OGD-induced cell death. Our data strongly suggest that CLIC1 up-regulation is part of an intrinsic protective mechanism activated in neurons subjected to ischemic conditions.

Introduction

Excitotoxicity has long been established as the predominant mechanism responsible for neuronal demise under conditions such as brain ischemia, due to the overactivation of glutamate receptors, that leads to excessive excitability of neurons and activation of molecular pathways leading to cell death (Szydlowska and Tymianski, 2010). The N-methyl D-aspartate receptors (NMDARs) have been considered a key player in excitotoxic cell death, mainly due to intracellular Ca^{2+} overload mediated by these receptors. However, the blockade of glutamate receptors as a neuroprotective strategy has failed in clinical trials (Lai *et al.*, 2013), partly due to the severe side effects

that arise from blocking the physiologic activity of such important receptors to the brain. Thus, it has become necessary to look for other mechanisms that might involve non-glutamate-mediated, non-excitotoxic pathways of neuronal damage.

Given that brain ischemia leads to great ionic imbalance, either during the insult or during recovery (Pundik *et al.*, 2012), it is increasingly evident that many of the events triggered by ischemic insults center on the regulation of ion channel signaling pathways (Song and Yu, 2013), in which some may be detrimental, and their prevention potentially beneficial, and some may be important to enhance cell resistance to ischemia. For instance, whereas the activation of the TRPM2 and TRPM7 channels further increased ischemia-induced oxidative stress and intracelullar Ca^{2+} accumulation (Aarts *et al.*, 2003; Aarts and Tymianski, 2005; Jia *et al.*, 2011; Naziroglu, 2011; Verma *et al.*, 2012; Alim *et al.*, 2013; Nakayama *et al.*, 2013; Shimizu *et al.*, 2013; Song and Yu, 2013), overexpression of TRPC6, another Ca^{2+} channel, prevented cell death by activation of the cAMP response element–binding protein (CREB) signaling pathway (Du *et al.*, 2010) and by inhibiting Ca^{2+}-currents mediated by NMDAR activation (Li *et al.*, 2012). Therefore, an efficient therapy for brain ischemia may rely on the targeting of multiple receptors or ion channels and regulatory pathways (Song and Yu, 2013).

Despite the increase in intracellular Ca^{2+} accumulation in the ischemic brain, other channels might contribute to ischemia-induced ionic imbalance, such as the volume-regulated anion channels (VRACs). VRACs consist of a group of widely expressed Cl^- channels that regulate cell volume under physiologic and pathologic conditions (Nilius and Droogmans, 2003; Inoue *et al.*, 2005). In fact, ischemic insults lead to VRAC activation in neurons, whereas pharmacological inhibition of VRACs is neuroprotective, showing that activation of VRACs contributes to ischemic neuronal damage (Feustel *et al.*, 2004; Zhang *et al.*, 2011; Alibrahim *et al.*, 2013). Little is known about the molecular targets of VRAC pharmacological inhibition, but one of the inhibitors that protected neurons from excitoxicity- and oxygen-glucose deprivation (OGD)-induced VRAC activation and neuronal cell death was IAA-94 (R(+)-[(6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)-oxy] acetic acid) (Inoue and Okada, 2007; Zhang *et al.*, 2011), a known blocker for the chloride intracellular channel 1 (CLIC1) (Valenzuela *et al.*, 2000; Novarino *et al.*, 2004; Milton *et al.*, 2008; Averaimo *et al.*, 2010).

CLIC1 is a member of a family of intracellular chloride ion channels (Valenzuela *et al.*, 1997; Averaimo *et al.*, 2010; Littler *et al.*, 2010). CLIC1 is considered a "metamorphic protein" because it exists as a soluble, cytoplasmic and nucleoplasmic

protein, but can also insert in lipid membranes where it functions as a chloride ion channel (Tulk *et al.*, 2002; Littler *et al.*, 2004; Averaimo *et al.*, 2013). Certain stimuli, such as oxidation (Littler *et al.*, 2004; Goodchild *et al.*, 2009) or low pH (Tulk *et al.*, 2002; Warton *et al.*, 2002), can induce major conformational changes in the protein structure that allow membrane insertion, therefore controlling the transition of CLIC1 between these two forms (Averaimo *et al.*, 2010). In the monomeric form, CLIC1 is structurally similar to the proteins of the Glutathione-S-transferase (GST) superfamily, with a thioredoxin N-terminal domain resembling glutathione and an all alpha-helical C-terminal domain, typical of these proteins (Cromer *et al.*, 2002; Littler *et al.*, 2010). The structure and stoichiometry of the integral membrane form of CLIC1 is still unclear. Recent data suggest that CLIC1 likely dimerizes to form a membrane-competent protopore complex (Peter *et al.*, 2013) and that between six to eight CLIC1 molecules can assemble into an oligomer to form an ionic channel (Goodchild *et al.*, 2011).

The role of CLIC1 in neurons is largely unknown. It has been shown that, in cocultures of microglia with neuronal cells, toxic Aβ stimulation induces insertion of CLIC1 into the membrane of microglial cells, acting as a chloride channel and inducing the release of reactive oxygen species (ROS) and tumor necrosis factor-α (TNF-α), that cause neuronal apoptosis (Novarino *et al.*, 2004; Milton *et al.*, 2008). However, neuronal demise was prevented by the selective CLIC1 blocker IAA-94 and by siRNA knockdown of CLIC1 (Novarino *et al.*, 2004; Milton *et al.*, 2008).

The present study has used microarray technology to identify ion channel protein genes whose expression was significantly altered in hippocampal neuronal cultures submitted to OGD, an established *in vitro* model for cerebral global ischemia. We observed that OGD causes the up-regulation of several ion channel protein genes, among which is Clic1. As such, the aim of this part of the study was to determine the role of CLIC1 in defining the neuronal outcome under ischemic conditions. Neurons over-expressing CLIC1 were less vulnerable to OGD-induced cell death, but the IAA-94 compound, that inhibits the channel activity of CLIC1, did not affect OGD-induced neuronal death in hippocampal cultures, indicating that the protective effect of CLIC1 is independent of its channel activity, and possibly due to the GST-like activity of the soluble form of the protein. Our results suggest, therefore, that CLIC1 can be part of an intrinsic protective mechanism and a potential target to enhance neuronal survival after an ischemic insult.

Results

OGD induces up-regulation of several calcium and chloride ion channel protein genes

The microarray data comparing gene expression profiles in control and OGD-submitted hippocampal neurons (see Chapter III) showed that genes coding for several ion channel proteins are differentially regulated after the OGD challenge (**Supplemental Tables 1-3, Ion Channel Transmembrane Activity category)**. We chose to validate the gene expression changes obtained in the microarray assay for 8 distinct genes encoding several ion channels **(Table 5.1)**.

Table 5.1. Microarray data showing the effect of OGD in hippocampal neurons in the mRNA levels of genes encoding ion channel proteins.

	Gene	Gene Name	Protein	Status	Fold Change
Non-selective cation channels	Trpc5	Transient receptor potential cation channel, subfamily c, member 5	TRPC5	Down-regulated at 24h	0.413
	Trpc6	Transient receptor potential cation channel, subfamily c, member 6	TRPC6	Down-regulated at 7h	0.366
	Trpc7	Transient receptor potential cation channel, subfamily c, member 7	TRPC7	Down-regulated at 7h	0.34
	Trpm2	Transient receptor potential cation channel, subfamily m, member 2	TRPM2	Down-regulated at 7h	0.489
Chloride channels	Clca2	Chloride channel calcium activated 2	CLCA2	Up-regulated at 24h	4.66
	Clic1	Chloride intracellular channel 1	CLIC1	Up-regulated at 24h	3.039
	Clic3	Chloride intracellular channel 3	CLIC3	Down-regulated at 7h	0.422
	Clic5	Chloride intracellular channel 5	CLIC5	Up-regulated at 24h	2.26

These genes were of particular interest to our work, since they encode proteins involved in the ionic homeostasis of neurons, which is known to be profoundly altered after ischemic insults and whose imbalance largely contributes to ischemic injury (Kahle *et al.*, 2009; Song and Yu, 2013). Thus, the expression levels of genes encoding proteins related with non-selective cation channels (Trpc5, Trpc6, Trpc7 and Trpm2) and chloride channels (Clca2, Clic1, Clic3 and Clic5) were analyzed by quantitative real-time PCR (qPCR) **(Figure 5.1)**. For instance, Trpc6, Trpm2, Clca2 and Clic1 were in excellent agreement with the microarray data, since their expression levels varied accordingly at the same time points of recovery as in the microarray assay. The result for Clic5 was also in agreement with the microarray data, since it had increased expression at 24h of recovery, but the qPCR analysis allowed the detection of this increase also at 7h of recovery. However, for three genes the qPCR analysis contradicted the microarray data: Trpc5 was expected to be down-regulated at 24h after OGD, but was shown instead to be up-regulated at this time point and Trpc7 and Clic3, which were both expected to have lower expression levels at 7h of recovery, were found to be up-regulated at both 7h and 24h after OGD.

Notably, most of the genes considered were up-regulated after the OGD insult, suggesting that OGD activates a transcriptional program that promotes the expression of genes related with cellular homeostasis in affected neurons.

Changes in CLIC1 protein levels after ischemia

CLIC1 has been shown to migrate as a ~27 kDa protein in microglia lysates run in SDS-PAGE [(Novarino *et al.*, 2004; Milton *et al.*, 2008), **Figure 5.2**]. However, we observed that in total extracts from rat kidney, liver and brain, CLIC1 has a slower migration and can be detected as a ~150 kDa band, **(Figure 5.2A)**. This observation raises the possibility that in these tissues CLIC1 may exist predominantly in an oligomeric form.

To test whether the protein levels of CLIC1 varied in the same direction as the respective mRNAs, total levels of CLIC1 were evaluated by Western blot in whole cell lysates prepared at different time points after OGD (immediately after the end of the 2h OGD insult, and 24h or 48h of recovery after OGD). We observed that, despite the increase in Clic1 mRNA at 24h of recovery, we could not detect changes in the protein levels at any of the time points of recovery considered **(Figure 5.2B)**.

However, when we analyzed CLIC1 protein levels in brain tissue obtained from rats submitted to transient global cerebral ischemia, after 48h of reperfusion, we observed that, whereas in the CA1 region of the hippocampus CLIC1 levels remained unaltered, there is a tendency to increased expression of CLIC1 in the hippocampal CA3 region and in the cortex **(Figure 5.3C)**. *In vivo* ischemia did not induce a significant change in the protein levels of CLIC1 in the striatum.

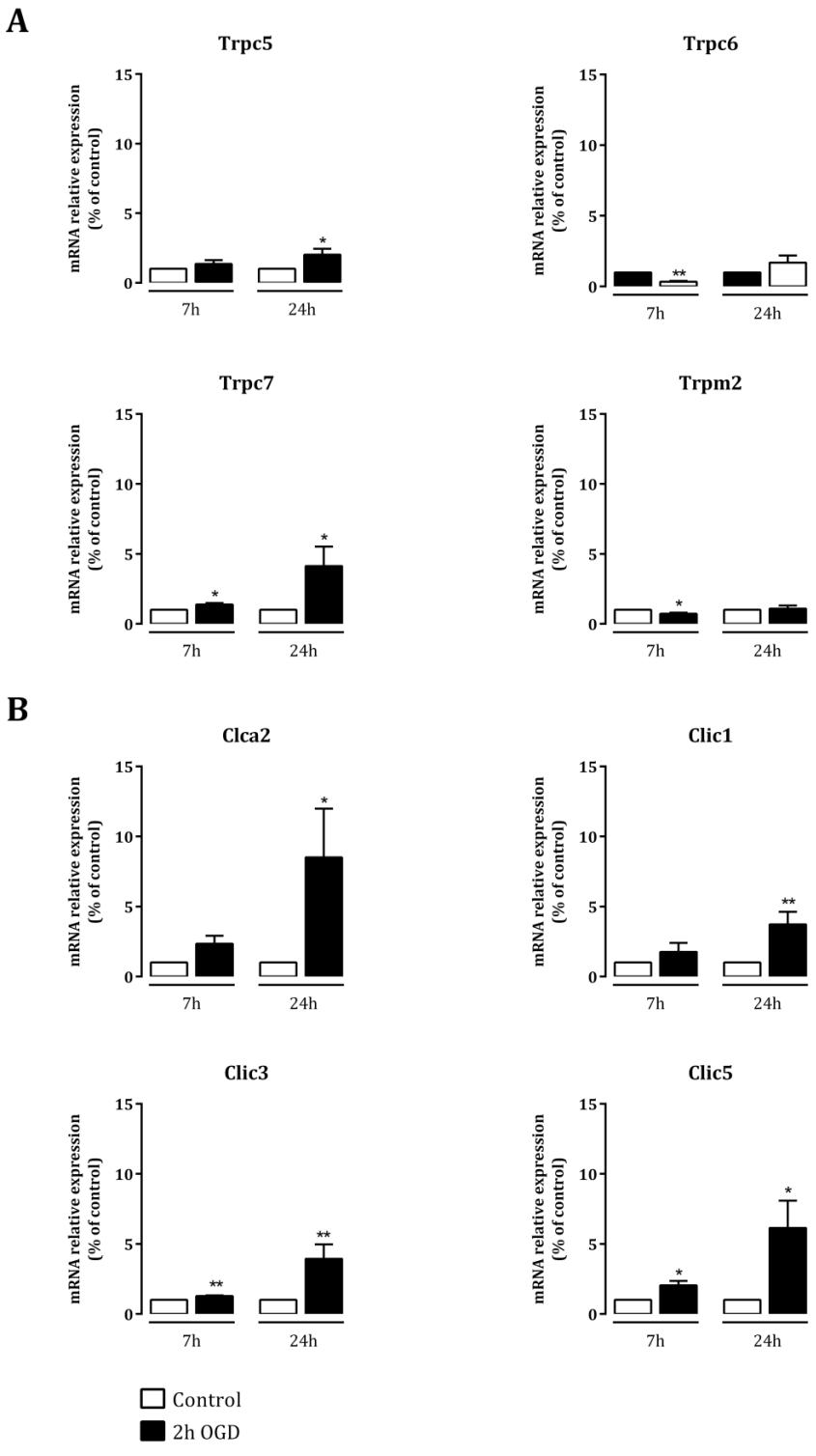


Figure 5.1. OGD induces changes in the mRNA levels of transcripts encoding ion channel proteins. Total RNA was extracted with TriZol 7 and 24 hours after the OGD insult. Quantitative PCR analysis was performed using cDNA prepared from 1 μg of total RNA and specific primers for each selected gene. Fold change in mRNA levels was normalized to Gapdh and Actb. qPCR analysis showed that genes encoding proteins associated with non-selective cation channels **(A)** and chloride ion channels **(B)** were mostly up-regulated after OGD (with the exception of Trpc6 and Trmp2, which had decreased expression levels at 7h of recovery). Bars represent the mean ± SEM of 5 independent experiments. *Significantly different from control (*p<0.05, **p<0.01, ***p<0.001, Student's t-test on log-transformed data).

Taken together, our results show that although mRNA levels for CLIC1 were increased after OGD in hippocampal neurons, changes in the CLIC1 protein levels were not detected within 48 h after the ischemic challenge. However, *in vivo* global ischemia may induce changes in the expression levels of CLIC1, detected at 48h of reperfusion. Since CLIC1 protein levels seem to be up-regulated in brain regions that are more resistant to ischemic insults, in particular the CA3 region of the hippocampus (Kirino and Sano, 1984; Tasker, 2001; Crepel *et al.*, 2003), it is important to investigate how CLIC1 expression affects neuronal death in neurons exposed to ischemia.

Overexpression of CLIC1 prevents OGD-induced neuronal death

To determine whether an increased amount of CLIC1 influences the susceptibility of hippocampal neurons to the ischemic insult, we studied the effect of CLIC1 overexpression in hippocampal neuronal survival following OGD. For that purpose, hippocampal neuronal cultures at DIV 13 were transfected with pEBG-eGFP (control plasmid encoding EGFP) or pIRES1-eGFP-Clic1, a bicistronic plasmid encoding both EGFP and CLIC1. Forty-eight hours later, neuronal cultures were submitted to the 2h OGD insult and cell death was assessed 24h later by analysis of the nuclear morphology of transfected cells, identified by GFP expression. We observed that overexpression of CLIC1 significantly reduced neuronal death elicited by the OGD insult **(Figure 5.3)**, thus suggesting that an increased availability of CLIC1 can have a protective effect in neurons submitted to OGD.

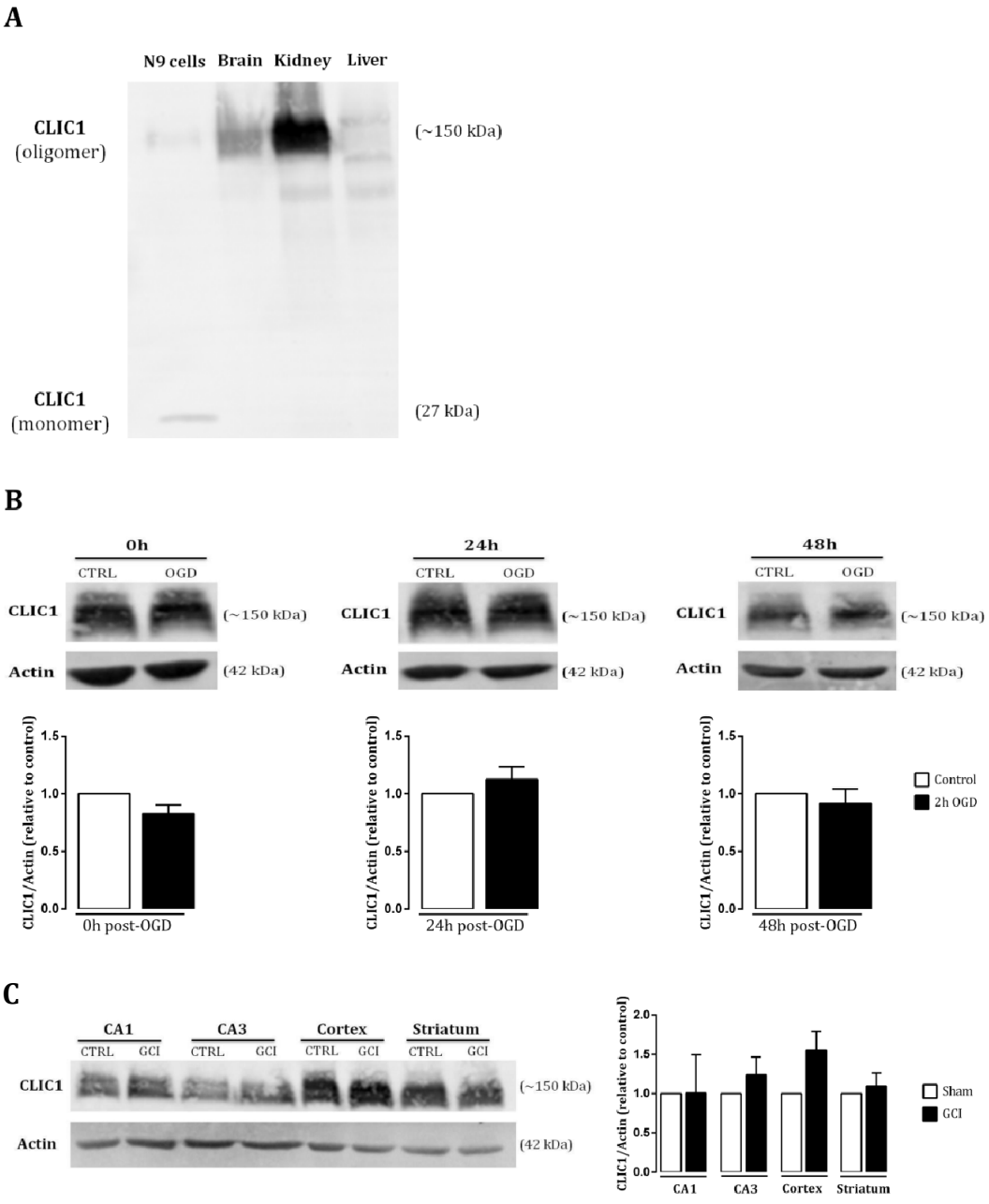


Figure 5.2. Expression of CLIC1 in different tissues and hippocampal neuronal cultures. (A) Western blot analysis of CLIC1 in protein lysates from microglia (N9 cells), kidney, liver and brain. In all cell types, except in the microglia, CLIC1 appears as a ~150 kDa protein. **(B)** CLIC1 protein levels are not changed after the OGD insult. Total protein extracts were prepared immediately after the OGD insult (0h) and at 24h and 48h of recovery. Total CLIC1 levels remain similar to control at all time points considered. Upper panels show a representative Western blot for total CLIC1 present in total cell lysates at 0h (n=5), 24h (n=9) and 48h (n=5) of recovery after the 2h OGD insult. Lower panels represent the quantification of the respective Western blots. Total actin was used as loading control. Bars represent the mean ± SEM of 5 independent experiments. **(C)** CLIC1 is differently regulated after global ischemia in different brain regions. Total protein extracts from different brain regions were prepared 48h

after transient global cerebral ischemia (GCI) in order to analyze the protein levels of CLIC1 in different brain areas. CLIC1 is unchanged in the CA1 region of the hippocampus, but increases in the hippocampal CA3 region and the cortex in brain samples from animals subjected to global ischemia, compared to control animals. The left panel shows a representative Western blot for total CLIC1 present in cell lysates of the CA1 and CA3 hippocampal regions, cortex and striatum of post-ischemic and control brains. The right panel represents the quantification of the Western blots. Total actin was used as loading control. Bars represent the mean ± SEM of 3 independent experiments. *Significantly different from control (*p<0.05, Student's t-test).

Pharmacological inhibition of CLIC1 channel activity does not affect OGD-induced neuronal death

The CLIC1 protein has been extensively studied in the last few years (Averaimo *et al.*, 2010; Littler *et al.*, 2010). Recent evidence shows that CLIC1 plays an important role in mediating cell death in a model of Alzheimer's disease, since neuronal death could be prevented by pharmacological inhibition of this protein in microglia cells (Novarino *et al.*, 2004; Milton *et al.*, 2008). Given that Clic1 mRNA levels were up-regulated upon OGD **(Figure 5.1)**, we tested the effect of the Cl^- channel activity inhibitor of CLIC1, IAA-94, on OGD-induced cell death in hippocampal neurons.

For that purpose, hippocampal neuronal cultures were submitted to 2h OGD in the absence or in the presence of IAA-94, which has been considered to specifically inhibit the channel activity of CLIC1 (Valenzuela *et al.*, 2000; Novarino *et al.*, 2004; Milton *et al.*, 2008). The inhibitor was present only during the stimulation period and cell viability was assessed 24h after the stimulus by quantification of the LDH release **(Figure 5.4)**. We observed that cell death induced by OGD was unaffected by the application of IAA-94, showing that the ion channel form of CLIC1 is not implicated, during the ischemic insult, in defining the neuronal response to ischemia. We also tested whether incubation of hippocampal neurons with 30 μM IAA-94, both during the ischemic stimulus and the recovery period, could change the levels of cell death after OGD, but found no significant differences in OGD-triggered cell death in IAA-94-treated neurons (19.43 ± 8.3 in neurons submitted to OGD, 16.87 ± 4.7 in IAA-94-treated neurons submitted to OGD). These observations suggest that the putative protective role of CLIC1 against OGD-induced neuronal damage, suggested by the protection afforded by CLIC1 overexpression in neurons **(Figure 5.3)**, may be mediated by the soluble, cytoplasmic form of the protein.

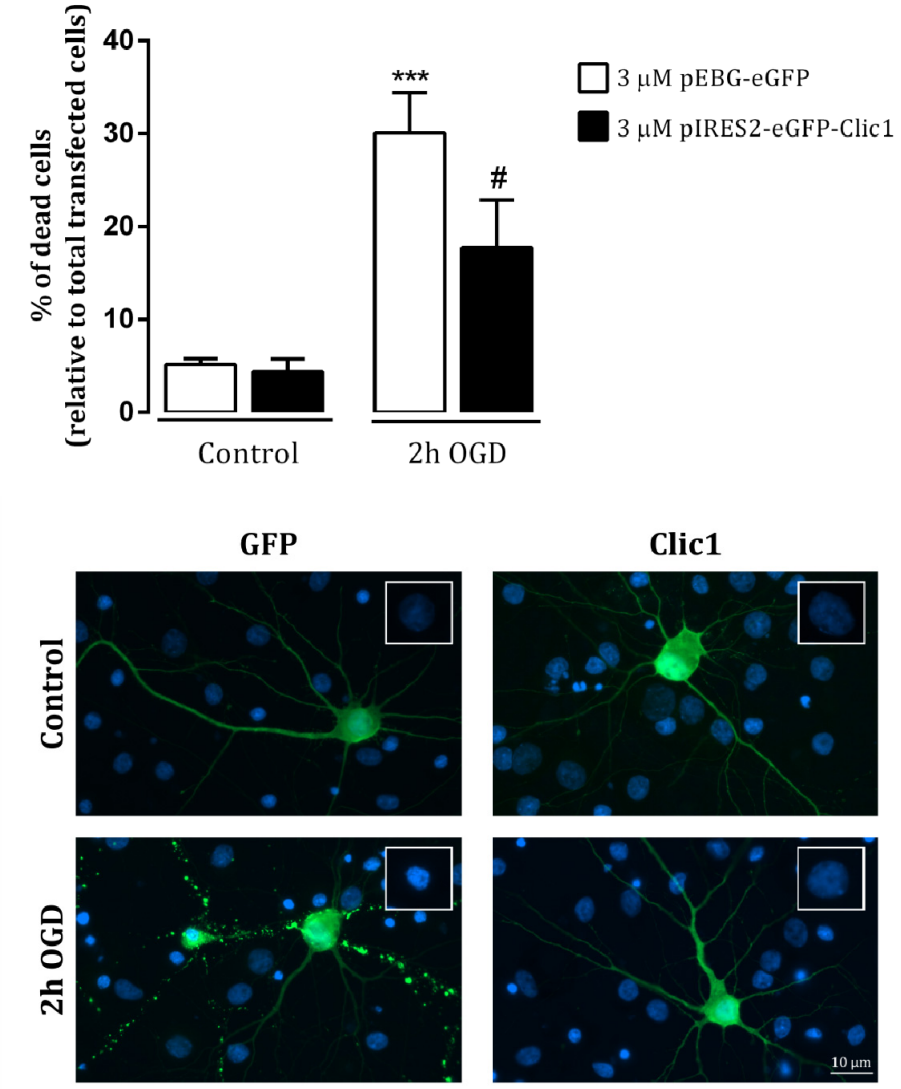


Figure 5.3. Overexpression of the CLIC1 protein significantly reduces the extent of OGD-induced neuronal death. Mature hippocampal cultures were transfected at DIV 13 with the indicated constructs using the calcium phosphate protocol. At DIV 15 cells were submitted to 2h OGD. Cell viability was assessed 24h later by analysis of the nuclear morphology of GFP-positive, transfected cells. Cells with pyknotic nuclei were counted as dead cells. The results were expressed as the percentage of dead cells relatively to the total transfected (GFP-positive) cell number. The lower panel depicts representative images of transfected neurons subjected to control conditions or 2h OGD in the presence of control or CLIC1-encoding plasmids. Inserts show Hoechst staining for transfected cells. Scale bar – 20 µm; Insert – 10 µm. Bars represent the mean ± SEM of 9 independent experiments. *Significantly different from control (***p<0.001, #p<0.05, One way ANOVA followed by Bonferronni's multiple comparison test).

Discussion

Brain cells require a high consumption of oxygen and glucose to maintain ATP synthesis, necessary to support ionic gradients across the cell membrane. During the acute phase of brain ischemia, the energetic insufficiency that results from the deprivation of oxygen and glucose leads to the failure of a plethora of ATP-dependent cellular processes crucial for cellular homeostasis and function (Hansen and Nedergaard, 1988; Song and Yu, 2013). One of the immediate consequences of ATP depletion is the loss of membrane electrochemical gradients, which promotes cell depolarization. Excessive Na^+, Ca^{2+} and Cl^- accumulate in the intracellular space via ion channels/receptors, disrupting cellular homeostasis, and water flows into the cells causing cellular edema (Kahle *et al.*, 2009; Song and Yu, 2013). Depolarization further contributes to excessive glutamate release, which results in overactivation of glutamate receptors and additional accumulation of intracellular Ca^{2+}, causing a derangement in cell signaling (e.g., activation of transcription factors, lipases, endonucleases and proteases) that accentuates neuronal damage (Szydlowska and Tymianski, 2010).

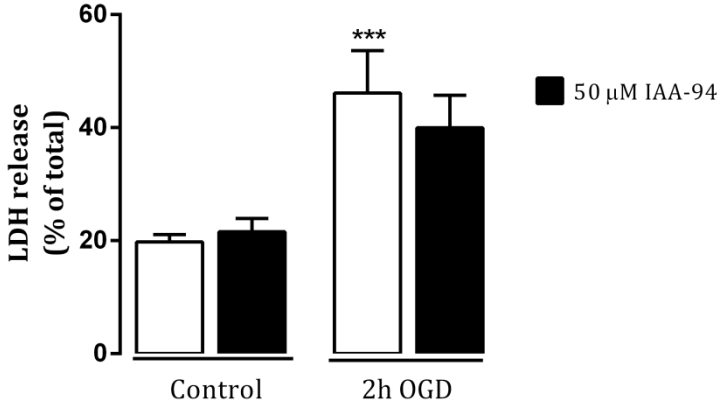


Figure 5.4. Inhibition of CLIC1 channel activity does not affect OGD-induced cell death in hippocampal neurons. Mature hippocampal neurons were subjected to 2h OGD in the absence or presence of the specific CLIC1 channel inhibitor, IAA-94 (50 μM). The inhibitor was present only during the stimulation period. Cell viability was assessed 24h after the stimulus by determination of the LDH release. Bars represent the mean ± SEM of 6 independent experiments. *Significantly different from the respective control (***p<0.001, Student's t-test).

According to our microarray data, many genes encoding ion channels are differentially expressed after the OGD insult, showing that ischemic insults not only regulate ion channel activity, but also change their expression levels. In fact, after qPCR

analyses, we observed that most of the ion channel protein genes had increased expression levels after 24h of recovery from OGD. This supports the idea that, despite increased cell death at this time point (see Chapter III), neurons are still undergoing a dynamic process of gene regulation in response to the ischemic insult. Curiously, the up-regulation of these genes seems to start before the onset of cell death, as shown in the cases in which there is a tendency to or a significant increase in the expression levels at 7h after OGD **(Figure 5.1)**. The first question that arises is whether this general up-regulation of ion channels contributes to neuronal demise, further contributing to impaired homeostasis, or whether it is part of an intrinsic protective mechanism important to promote cell survival.

Regulation of ion channel protein genes after OGD: TRP receptors

Among the selected ion channel encoding genes, only two were found to be down-regulated with the qPCR analysis, both at 7h of recovery after OGD: Trpc6 and Trpm2. TRPs are a group of ion channels mostly located on the cell membrane of various types of cells and are grouped into six sub-families, including the TRPC ("canonical") and TRPM ("melastatin") families (Gees *et al.*, 2012). TRPC6 is a non-selective cation channel with a slightly higher permeability to Ca^{2+} and has been linked to dendritic growth, neuronal survival and synapse formation (Gees *et al.*, 2012). Consistent with our own observation, a recent work has shown that ischemic insults caused the down-regulation of TRPC6 prior to and during neuronal death, whereas inhibition of TRPC6 degradation reduced the levels of cell death, suggesting that preventive strategies against brain ischemia might include enhancement of TRPC6 expression levels (Du *et al.*, 2010; Li *et al.*, 2012).

The TRPM2 channels are permeable to Na^+, K^+ and Ca^{2+} and can be activated by oxidative stress and TNFα (Miller, 2006; Naziroglu, 2011). Interestingly, TRPM2 has been considered as an important mediator of cell death induced by oxidative stress under ischemic conditions. Indeed, pharmacological inhibition and knockdown of TRPM2 channels is neuroprotective to male cortical neurons submitted to OGD and MCAO, showing that impairment of TRPM2 activity may represent a potential therapeutic target to decrease neuronal damage after ischemia, at least in the male brain (Jia *et al.*, 2011; Naziroglu, 2011; Verma *et al.*, 2012; Nakayama *et al.*, 2013; Shimizu *et al.*, 2013). Recently, it has been suggested that TRPM2 is required for the up-regulation of the NMDAR subunit GluN2B and the down-regulation of the GluN2A subunit (Alim *et al.*, 2013). Since a number of studies have identified that GluN2A and

GluN2B subunits activate pro-survival and pro-death pathways, respectively (Hardingham and Bading, 2010), the resistance observed in neurons of TRPM2$^{-/-}$ mice (both female and male) submitted to MCAO may be due to an enhancement of pro-survival cascades mediated by GluN2A which, in these neurons, cannot be blocked by the presence of TRPM2 (Alim *et al.*, 2013). As far as we know, our study is the first showing TRPM2 expression levels can be differentially regulated by an ischemic insult. In fact, considering the strong evidence about a pro-death role of TRPM2, our observation suggests that, at an early time point after the insult, neurons may present reduced levels of TRPM2 as part of a protective pathway to reduce oxidative stress-induced damage.

The other genes encoding for TRP channels considered for qPCR confirmation (Trpc5 and Trpc7) were significantly up-regulated at 24h after OGD **(Figure 5.1)**. TRPC5 and TRPC7 also belong to the "canonical" sub-family of TRP receptors and are both non-selective cation channels (Gees *et al.*, 2012). TRPC5 has been associated with neurite extension and growth cone morphology in young hippocampal neurons (Bezzerides *et al.*, 2004). Moreover, Ca^{2+} influx through the TRPC5 channels expressed in growth cones and synapses activates Ca^{2+}/CaM kinase kinase (CaMKK) and its downstream target Ca^{2+}/CaM kinase I (CaMKI), which subsequently promotes axon formation (Davare *et al.*, 2009). TRPC7 is a constitutively active channel, whose activity can be further enhanced by stimulation of G protein-coupled receptor or DAG analogs (Okada *et al.*, 1999). There is no evidence showing any involvement of these two channels in post-ischemic neurons, but given the significant increase in their respective mRNA levels after OGD, especially in the case of Trpc7 (4 fold increase), it might be that the encoded channels represent important contributors to the ischemic response.

Regulation of chloride channels after OGD: CLIC1 as a putative mediator of a pro-survival mechanism

OGD induced the up-regulation of several genes encoding Cl^- channels (Clca2, Clic1, Clic3 and Clic5). Excitotoxic insults have been shown to induce somatic and dendritic swelling mediated by Cl^- influx (Rothman, 1985; Olney *et al.*, 1986; Choi, 1987; Hasbani *et al.*, 1998; Inoue and Okada, 2007). Cl^- entry is often related with the activation of γ-amino-butyric acid type A ($GABA_A$) receptors, since $GABA_A$ receptor inhibitors partially block excitotoxic injury (Hasbani *et al.*, 1998; Chen *et al.*, 1999;

Babot *et al.*, 2005), but Cl^- influx can also be mediated by other anion channels activated under excitotoxic or ischemic conditions (Inoue and Okada, 2007; Zhang *et al.*, 2011).

Clca2 encodes for a chloride channel that belongs to the family of CLCA (chloride channel, calcium activated) proteins (Hartzell *et al.*, 2005) and, in our work, was the most up-regulated Cl^- channel at 24h after OGD. Although CLCA2 has never been reported to have a role in brain ischemia, there is evidence showing that the expression levels of another member of this family of channels, Clca1, increases in cultured hippocampal neurons exposed to excitotoxic stimulation of NMDARs (Zhang *et al.*, 2007), as well as after OGD and forebrain ischemia (Wahl *et al.*, 2009). Interestingly, the increase in Clca1 expression was observed after specific stimulation of extrasynaptic NMDARs and the overexpression of this gene leads to neuronal death (Zhang *et al.*, 2007). These observations therefore indicate that one mechanism through which extrasynaptic NMDAR activation damages neurons may involve the activation of Clca1 as a putative pro-death gene. It would therefore be very interesting to understand whether Clca2 is also a mediator of neuronal death under ischemic conditions.

Clic1, Clic3 and Clic5 encode for chloride channels that belong to the CLIC (chloride intracellular channel) family of proteins. These channels do not fit into the paradigm of classical ion channel proteins, since they can exist in a soluble form or integrated into the cell membrane with an ion channel function (Littler *et al.*, 2010). None of these genes have been reported to have a role in brain ischemia.

CLIC1 has a wide tissue and sub-cellular distribution in mammalian cells (Ulmasov *et al.*, 2007) and has become the most studied member of this family (Averaimo *et al.*, 2010; Littler *et al.*, 2010). CLIC1 can adopt at least two stable structures, since it undergoes a structural rearrangement that allows the transition from the soluble form to a membrane-bound form, where it works as a chloride channel, making it a metamorphic protein (Murzin, 2008). The transition between these two states of CLIC1 can be controlled by redox conditions (Goodchild *et al.*, 2009). Given that production of ROS and oxidative stress are known contributors to ischemic neuronal damage (Abramov *et al.*, 2007; Zhou *et al.*, 2007; Wang *et al.*, 2009; Zhou and Baudry, 2009; Liu *et al.*, 2011), we hypothesize that the strong up-regulation of the Clic1 gene, observed at 24h after OGD, may reflect a mechanism activated in response to ischemia-induced oxidative conditions.

CLIC1 has been described as a 27 kDa protein in protein extracts from microglia, the only brain cells in which the role of CLIC1 has been addressed (Novarino *et al.*, 2004; Milton *et al.*, 2008). We looked for the expression of CLIC1 in several tissues (brain, liver and kidney) and found that CLIC1 migrates as a ~150 kDa protein in Western blot analysis **(Figure 5.2A)**, suggesting that in these tissues CLIC1 may exist predominantly in an oligomeric form. We next tested whether the protein levels reflected the up-regulation in OGD-induced CLIC1 mRNA levels observed in hippocampal neurons. Once again, we observed that CLIC1 migrates as a high molecular weight protein when we perform Western blot analysis of protein lysates of hippocampal neurons. CLIC1 levels remained similar to control when analyzed immediately (0h), 24h or 48h after OGD **(Figure 5.2B)**. However, in protein extracts from animals submitted to global ischemia, we observed that CLIC1 protein levels may be differentially regulated in different brain regions. In particular, it is interesting to note that CLIC1 showed a tendency to increase in the CA3 region, but remained unaltered in the CA1 region of the hippocampus in brain samples from animals subjected to global ischemia, when compared to samples from control animals **(Figure 5.2C)**. The differences observed between the *in vitro* and *in vivo* experiments can be due to the presence of other brain cells in the *in vivo* samples and to the fact that in hippocampal cultures region-specific effects may become diluted in the mixed population of neurons. However, further work will be necessary to confirm that global ischemia differently regulates the levels of CLIC1 in the sub-regions of the hippocampus.

Given that OGD up-regulates the mRNA levels of Clic1, we tested how CLIC1 availability affected the response to ischemic insults by overexpressing CLIC1 in hippocampal neuronal cultures prior to the OGD stimulation. According to previous studies, CLIC1 can insert into the cell membrane where it functions as Cl^- channel that, in certain conditions, contributes to neuronal death (Novarino *et al.*, 2004; Milton *et al.*, 2008). However, we observed that, when over-expressed in neurons, CLIC1 contributes to a significant reduction in OGD-induced cell death **(Figure 5.3)**. To explore the possibility that the protective role of CLIC1 could be mediated by its membrane-bound form, we incubated hippocampal cultures with the selective CLIC1 inhibitor IAA-94, previously shown to block the Cl^- current mediated by CLIC1 (Valenzuela *et al.*, 2000; Novarino *et al.*, 2004; Milton *et al.*, 2008) and submitted neurons to the OGD stimulus. Pharmacological inhibition of CLIC1 did not affect OGD-induced cell death, showing that blocking the putative contribution of ion channel activity of CLIC1 under ischemic conditions is neither toxic nor protective to neurons **(Figure 5.4)**.

Collectively, our data suggest that, when available in neurons, CLIC1 exerts a protective mechanism that is probably mediated by its soluble form, with no contribution from its ion channel activity. Many possible functions have been hypothesized for the intracellular activity of the soluble form of CLIC1 (Littler *et al.*, 2010). The hypothesis of CLIC1 having an enzymatic activity derives from the observation that CLIC1 has a binding site for reduced glutathione (GSH) in its N-terminal domain (Averaimo *et al.*, 2010) and also adopts the canonical structure fold of the GST superfamily (Cromer *et al.*, 2002). GSTs are known for their detoxification activity by catalysing GSH conjugation to many endogenous and exogenous compounds, as, for instance, products of oxidative stress (Oakley, 2005). The possibility of CLIC1 working as an enzyme against ischemia-mediated production of ROS as part of a pro-survival mechanism is therefore plausible and tempting.

CLIC1 has also been considered to be a putative scaffolding protein, as various interactions with members of the cytoskeleton have been identified (Littler *et al.*, 2010). In spermatozoa, CLIC1 was co-purified with a testis-specific isotype of protein phosphatase 1γ (PP1γ) and protein 14-3-3 (Myers *et al.*, 2004). It would be interesting to test these interactions in neurons, considering that PP1γ may play a role in neuronal resistance to global ischemia (Horiguchi *et al.*, 2002) and that 14-3-3γ (an isotype of the 14-3-3 protein family) is thought to have anti-apoptotic functions and to promote neuronal survival after brain ischemia (Dong *et al.*, 2010). Both these proteins could, therefore, be part of the protective signaling induced by the non-channel activity of CLIC1. Unfortunately, little is known about the enzymatic or scaffolding functions of CLIC1, which have been barely addressed (Littler *et al.*, 2010). As such, further work is required to understand the exact mechanism by which CLIC1 mediates neuronal survival under ischemic conditions.

Altogether, our results suggest a novel role for CLIC1, a protein whose function in neurons, or in brain ischemia, has never been addressed before. Further work will be necessary to understand the specific contribution of this protein to the ischemic response, but our data strongly suggests that CLIC1 may be part of an intrinsic protective mechanism activated in neurons.

Chapter VI

Conclusions

Cerebral ischemia is the third leading cause of death in the Western world and, among the survivors it is a main cause of permanent disability. Global cerebral ischemia, resulting from impairment in blood supply to the brain, induces the selective neurodegeneration of the CA1 region of the hippocampus, which occurs only hours to days after the insult (Petito *et al.*, 1987; Bottiger *et al.*, 1998). This time-window between the end of the ischemic insult and the first signs of neuronal demise is believed to be associated with the activation of competing programs of gene expression, which will be protective or detrimental to neuronal outcome (Papadopoulos *et al.*, 2000).

One major goal of the therapy in global cerebral ischemia is to prevent the delayed neurological and cognitive deficits that are triggered by the ischemic accident. However, despite the intensive work in the field, no efficient treatment has yet been provided. In fact, the molecular mechanisms responsible for the selective vulnerability of hippocampal neurons to global ischemia are still not completely understood. As such, the goal of the present study was to investigate molecular mechanisms differently regulated after ischemia in order to identify potential targets for the treatment of global ischemia. For that purpose, our experiments were conducted in hippocampal neuronal cultures subjected to an *in vitro* model for cerebral global ischemia, OGD.

In the first chapter, we set up the protocol for the OGD insult and analyzed changes in the transcriptome of hippocampal neurons induced by OGD. We started by showing that 2h OGD followed by 24h of recovery results in delayed cell death in the hippocampal neuronal cultures. Excitotoxicity is considered to be one of the first events during an ischemic insult, as an excessive amount of glutamate, accumulating in the extracellular space, causes the overactivation of glutamate receptors and, consequently, of molecular cascades that culminate in neuronal damage. As such, to analyze the contribution of AMPA and NMDA glutamate receptors to OGD-induced death, we exposed hippocampal cultures to 2h OGD in the absence or presence of selective AMPAR and NMDAR antagonists. We observed that, when present, the antagonists conferred protection to neurons, showing that OGD-induced neuronal death has an excitotoxic component mediated, at least in part, by activation of AMPARs and NMDARs, as suggested in previous studies (Bonde *et al.*, 2005; Liu *et al.*, 2006; Zhou and Baudry, 2006; Dixon *et al.*, 2009). Furthermore, OGD induced the activation of calpains, a family of proteases that have been shown to contribute to the progression of cell death after *in vitro* ischemic insults (Newcomb-Fernandez *et al.*, 2001; Zhou and Baudry, 2006; Lobo *et al.*, 2011). With this first set of experiments we therefore characterized OGD-mediated cell death, confirming that OGD is suitable as an *in vitro*

model for global ischemia, and established the experimental set-up for the analysis of gene expression in this model.

We then investigated changes in the transcriptome in cultures of hippocampal neurons submitted to OGD. For that purpose, total RNA was extracted 7h and 24h after OGD and used in a whole-genome RNA microarray to identify genes related to an early and a delayed ischemic response. Analysis of gene ontology showed that genes related with metabolic processes, signaling pathways, receptor activity, transcription, inflammation, neurotransmitter/glutamate secretion, RNA biosynthesis and apoptosis were differentially regulated after OGD. This observation demonstrates that the OGD model induces similar changes in functional groups of genes that have been shown to be differently regulated after *in vivo* ischemia (Kim *et al.*, 2002; Tang *et al.*, 2002; Lu *et al.*, 2004; Buttner *et al.*, 2009; Ramos-Cejudo *et al.*, 2012).

Notably, although many genes were differentially expressed at both time points of recovery, most genes were exclusively altered at 7h or at 24h, suggesting that distinctive sets of genes are specifically regulated in response to OGD at an early or late time point after the insult. In fact, at 7h after OGD there are more differently regulated transcripts than at 24h, which supports the idea that transcriptional changes occur after the exposure to ischemia and continue after the insult, and strongly explains the delay between the insult and the first signs of cell death (Neumar, 2000). Also, whereas changes in the transcriptome detected at 7h of recovery were more associated with a general down-regulation of genes, at 24h there was more up-regulation of genes. This observation shows that, although at a delayed time point after the insult there is already an increasing rate of cell death, other neurons are still undergoing an active process in response to OGD that is essentially mediated by induction of gene expression. However, increased mRNA transcription does not necessarily translate into increased amounts of the encoded protein. In fact, ischemic insults can cause a general depression in protein synthesis, that is transient in neurons that survive but permanent in neurons that go on to die (Thilmann *et al.*, 1986; Araki *et al.*, 1990; Liu *et al.*, 2004; Vesely *et al.*, 2009). This suggests that one factor contributing to the vulnerability of neurons may be the incapacity to produce new or replace damaged proteins necessary for survival, due to an impairment in protein synthesis. Although most brain regions recover the capacity to synthesize proteins after transient ischemic insults, the recovery in the CA1 neurons in the gerbil hippocampus is poor (Thilmann *et al.*, 1986; Araki *et al.*, 1990). This suggests that neurons that are unable to synthesize new proteins important for the progression of survival cascades are more vulnerable to an ischemic insult.

Although a genomic approach can provide global information on ischemia-induced changes in the profile of insulted neurons, pinpointing related cellular events occurring at different phases of OGD-induced injury, interpretation of the microarray data should be made with careful attention. For instance, the use of neuronal cultures submitted to an *in vitro* model of ischemia can be a limitation due to the impossibility to examine the response of other types of brain cells (e.g. astrocytes, microglia) to the insult. However, this can be considered an advantage when the main objective is the detection of changes associated with the specific response of certain brain cells, such as neurons. In our work, we pursued the study of the response of hippocampal neurons, which have been shown to be one of the most vulnerable brain cells to ischemic injury. Moreover, although the regulation of genes related with opposing cascades, e.g. pro- and anti-apoptosis, represent both sides of the response activated by OGD (the balance towards one or another will be dependent on certain aspects, such as duration of the insult), it might also reflect the individual response of neurons that have been irreversibly injured and that of surviving neurons.

To obtain conclusive results, it is critical to verify the microarray data by independent techniques. In this study, confirmation of the expression levels of several different genes was performed by qPCR analyses and was overall in good agreement with the microarray data, further supporting the combined use of the OGD model and microarray technology to gain insight into particular cellular and molecular mechanisms evoked by global ischemia in hippocampal neurons. This part of the study allowed the unveiling of post-ischemic alterations that can influence the fate of neuronal cells exposed to ischemia, and may result in the identification of attractive therapeutic targets for the treatment of cerebral ischemia.

In the second part of our work, we observed that OGD induces changes in the expression levels of several synaptic protein genes, such as those encoding for PICK1, GRIP1, TARPγ3, calsyntenin-2/3, SAPAP2, SNAP-25 and subunits of the AMPA and NMDA glutamate receptors. We therefore suggest that OGD activates a transcriptional program that results in the repression of proteins related with glutamatergic transmission. In particular, we show that AMPARs present at the cell surface of cultured hippocampal neurons have a reduction in the content of GluA1 subunits, whereas GluA2 protein levels were unchanged. In fact, as far as we know, this is the first study to report a dramatic decrease in the total mRNA and protein levels of GluA1 at 24h after a 2h OGD insult, but correlates well with a previous study showing that a 15 min OGD insult leads to the internalization of AMPARs in neurons from the CA3 region of the hippocampus (Dennis *et al.*, 2011). Ischemic insults also trigger a reduction in

the levels of the GluA2 subunit in neurons of the hippocampal CA1 region (Pellegrini-Giampietro *et al.*, 1992; Gorter *et al.*, 1997; Opitz *et al.*, 2000; Noh *et al.*, 2005; Liu *et al.*, 2006; Dixon *et al.*, 2009), resulting in increased levels of Ca^{2+}-permeable AMPA receptors in these cells; however, in this study we were not able to detect those changes, probably due to the presence of neurons of the different hippocampal subregions. We also observed that the subunit composition of the NMDARs is altered after OGD, since the GluN2 (A and B) subunits are decreased both at the mRNA and protein levels, whereas GluN3A has increased mRNA expression. We therefore propose that the delayed decrease on GluA1 expression after OGD (and possibly of the GluA2 subunit as well) and the increase in Glu3A mRNA are potentially part of a neuroprotective mechanism that results from modifications taking place at the synaptic level, and that can protect neurons from hyperexcitability of post-ischemic neurons. Since different subunits can activate different molecular pathways, information concerning the post-ischemic composition of glutamate receptors can be very useful for the design of drugs aimed to block the activation and signaling mediated by these receptors.

Finally, analyses of the OGD-induced changes in the transcriptome of hippocampal neurons lead to the identification of many genes encoding ion channels. We observed a general up-regulation of these genes at 24h after OGD, which correlate with previous studies showing the involvement of different ion channels in the ionic imbalance triggered by ischemia (Song and Yu, 2013). In particular, we observed that OGD promotes the up-regulation of Clic1, a gene that encodes the intracellular Cl^- channel CLIC1 (Valenzuela *et al.*, 1997; Averaimo *et al.*, 2010; Littler *et al.*, 2010). The role of CLIC1 in neurons is completely unknown; in fact, our study is the first to address the role of CLIC1 in hippocampal neurons after an ischemic insult. We observed that overexpression of CLIC1 results in protection of neurons against OGD-induced damage. Although in our experiments we could not discern between the two possible forms of the protein (soluble or integral-membrane ion channel), our results unveil a novel role for CLIC1 as part of a protective mechanism activated in post-ischemic neurons. Further work is now necessary to pursue the role of CLIC1 in determining the neuronal outcome after ischemia, and to determine whether differential CLIC1 up-regulation in different subregions of the hippocampus can account for differences in the susceptibility to ischemia.

In summary, our microarray data provided lists of genes that are differently regulated after OGD and, in particular, allowed us to uncover two different mechanisms that we now propose to be part of an intrinsic protective program activated in insulted neurons. On one hand, neurons promote the down-regulation of several components of

the synaptic machinery, namely by reducing the content and changing the subunit composition of the AMPA and NMDA glutamate receptors, which can protect neurons against post-ischemic injury. On the other hand, neurons promote the up-regulation of CLIC1 that protects neurons against OGD-induced damage. However, it is important to note that, in parallel with pro-survival mechanisms, OGD also induces the activation of molecular cascades that lead neurons towards death. In fact, the microarray data also helped us to identify and study the development of signaling pathways leading to cell death, activated after OGD (Vieira, Fernandes et al., under revision). The identification of pro-survival and pro-death signaling modules thus account for the accuracy of the OGD model as a useful tool to study global ischemia. Understanding the implications of the mechanisms that we described here may help to understand the different degree of vulnerability shown in the subregions of the hippocampus and therefore account for potential therapeutic strategies aimed at improving neuronal outcome after cerebral ischemia.

Chapter VII

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Supplemental Tables

Supplemental Table 1. List of genes up-regulated and down-regulated at 7h after OGD for different ontological classes. Gene ontology analyses included genes that had a *p*-value <0.05 and a fold change of 2.0 and were performed using GoMiner™. Classes were selected manually. Note that some genes are included in more than one class.

		Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value
Apoptosis	**Up-regulated**	**Tcf7l2**	Transcription factor 7-like 2 (T-cell specific, HMG-box)	NM_001191052	7.39	0.050
		Il6	Interleukin 6	NM_012589	7.19	0.022
		Bmp2	Bone morphogenetic protein 2	NM_017178	4.00	0.003
		Hspa1b	Heat shock 70kd protein 1B (mapped)	NM_212504	3.05	0.044
		Adm	Adrenomedullin	NM_012715	2.67	0.029
		Igfbp3	Insulin-like growth factor binding protein 3	NM_012588	2.44	0.000
		Wwox	WW domain-containing oxidoreductase	NM_001106188	2.38	0.038
		Myc	Myelocytomatosis oncogene	NM_012603	2.38	0.014
		Sox7	SRY (sex determining region Y)-box 7	NM_001106045	2.37	0.044
		Pvr	Poliovirus receptor	NM_017076	2.33	0.021
		Cd44	Cd44 molecule	NM_012924	2.28	0.041
		Tgfb1	Transforming growth factor, beta 1	NM_021578	2.27	0.047
		Set	SET nuclear oncogene	NM_001012504	2.13	0.007
		Gadd45g	Growth arrest and DNA-damage-inducible, gamma	NM_001077640	2.08	0.014
		Cx3cr1	Chemokine (C-X3-C motif) receptor 1	NM_133534	2.06	0.041
		Sin3a	SIN3 homolog A, transcription regulator (yeast)	NM_001108761	2.03	0.004
		Ppp1r15a	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	NM_133546	2.02	0.035
		Hmgb1	High mobility group box 1	NM_012963	2.00	0.009
	Down-regulated	**Gene Symbol**	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
		Agtr1b	Angiotensin II receptor, type 1b	NM_031009	0.21	0.002
		Mmp9	Matrix metallopeptidase 9	NM_031055	0.23	0.027

		Tp63	Tumor protein p63	NM_019221	0.25	0.000
		Pou4f3	POU class 4 homeobox 3	NM_001108889	0.26	0.013
		Cidec	Cell death-inducing DFFA-like effector c	NM_001024333	0.32	0.014
		Il4	Interleukin 4	NM_201270	0.34	0.002
		Esr1	Estrogen receptor 1	NM_012689	0.34	0.002
		Fgf8	Fibroblast growth factor 8	NM_133286	0.35	0.004
		Prkcz	Protein kinase C, zeta	NM_022507	0.37	0.012
		Adcyap1	Adenylate cyclase activating polypeptide 1	NM_016989	0.40	0.002
		Casp8	Caspase 8	NM_022277	0.40	0.028
		Lck	Lymphocyte-specific protein tyrosine kinase	NM_001100709	0.41	0.006
		Dbh	Dopamine beta-hydroxylase (dopamine beta-monooxygenase)	NM_013158	0.41	0.046
		Foxl2	Forkhead box L2	ENSRNOT00000023091	0.44	0.004
		Ache	Acetylcholinesterase	NM_172009	0.44	0.015
		Snca	Synuclein, alpha (non A4 component of amyloid precursor)	S73008	0.45	0.030
		Madd	MAP-kinase activating death domain	NM_053585	0.46	0.019
		Apbb1	Amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65)	NM_080478	0.47	0.017
		Plekhg5	Pleckstrin homology domain containing, family G (with rhogef domain) member 5	NM_201272	0.48	0.003

		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
Inflammatory Response	**Up-regulated**	**Il6**	Interleukin 6	NM_012589	7.19	0.022
		Bmp2	Bone morphogenetic protein 2	NM_017178	4.00	0.003
		Cd55	Cd55 molecule	NM_022269	3.79	0.011
		Itgb6	Integrin, beta 6	NM_001004263	3.75	0.003
		Tlr2	Toll-like receptor 2	NM_198769	3.58	0.005
		Cd44	Cd44 molecule	NM_012924	2.28	0.041
		Tgfb1	Transforming growth factor, beta 1	NM_021578	2.27	0.047

		Hmgb1	High mobility group box 1	NM_012963	2.00	0.009

		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
	Down-regulated	**Ptafr**	Platelet-activating factor receptor	NM_053321	0.30	0.025
		Il4	Interleukin 4	NM_201270	0.34	0.002
		Afap1l2	Actin filament associated protein 1-like 2	XM_001064140	0.36	0.002
		Adcyap1	Adenylate cyclase activating polypeptide 1	NM_016989	0.40	0.002
		Masp1	Mannan-binding lectin serine peptidase 1	NM_022257	0.40	0.024
		Ache	Acetylcholinesterase	NM_172009	0.44	0.015
		F12	Coagulation factor XII (Hageman factor)	NM_001014006	0.45	0.011
		Tac1	Tachykinin 1	NM_012666	0.49	0.010
		Itgb2	Integrin, beta 2	NM_001037780	0.49	0.035
		Tnfrsf11a	Tumor necrosis factor receptor superfamily, member 11a	XM_001063501	0.49	0.005

		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
Ion Transmembr. Transporter Activity	**Up-regulated**	**Slc26a3**	Solute carrier family 26, member 3	NM_053755	4.34	0.013
		Kcnj8	Potassium inwardly-rectifying channel, subfamily J, member 8	NM_017099	3.32	0.032
		Slc1a5	Solute carrier family 1 (neutral amino acid transporter), member 5	NM_175758	2.59	0.007
		Slc15a3	Solute carrier family 15, member 3	NM_139341	2.35	0.031
		Glra2	Glycine receptor, alpha 2	NM_012568	2.24	0.028

		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
	Down-regulated	**Trpc7**	Transient receptor potential cation channel, subfamily C, member 7	NM_001191691	0.34	0.031
		Kcnj4	Potassium inwardly-rectifying channel, subfamily J, member 4	NM_053870	0.36	0.012
		Trpc6	Transient receptor potential cation channel, subfamily C, member 6	NM_053559	0.37	0.009
		Chrng	Cholinergic receptor, nicotinic, gamma	NM_019145	0.38	0.035

		Cacna2d3	Calcium channel, voltage-dependent, alpha2/delta subunit 3	NM_175595	0.40	0.028
		Atp9b	Atpase, class II, type 9B	NM_001106130	0.40	0.008
		Slc13a1	Solute carrier family 13 (sodium/sulfate symporters), member 1	NM_031651	0.40	0.026
		Atp5d	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	BC161836	0.41	0.000
		Cacng3	Calcium channel, voltage-dependent, gamma subunit 3	NM_080691	0.41	0.009
		Clic3	Chloride intracellular channel 3	NM_001013080	0.42	0.039
		Nox1	NADPH oxidase 1	NM_053683	0.43	0.002
		Snap25	Synaptosomal-associated protein 25	NM_030991	0.43	0.027
		Chrna4	Cholinergic receptor, nicotinic, alpha 4	NM_024354	0.43	0.025
		Slc30a2	Solute carrier family 30 (zinc transporter), member 2	NM_001083122	0.44	0.005
		Cftr	Cystic fibrosis transmembrane conductance regulator homolog (human)	NM_031506	0.44	0.013
		Svop	SV2 related protein	NM_134404	0.44	0.033
		Slc6a5	Solute carrier family 6 (neurotransmitter transporter, glycine), member 5	NM_203334	0.45	0.021
		Cacnb2	Calcium channel, voltage-dependent, beta 2 subunit	NM_053851	0.45	0.003
		Slc17a7	Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7	NM_053859	0.45	0.022
		Atp6v0a4	Atpase, H+ transporting, lysosomal V0 subunit A4	NM_001106591	0.46	0.028
		Kcnk6	Potassium channel, subfamily K, member 6	NM_053806	0.46	0.009
		Kcns1	Potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1	NM_053954	0.46	0.042
		Kctd2	Potassium channel tetramerisation domain containing 2	XM_001081684	0.48	0.023
		Trpm2	Transient receptor potential cation channel, subfamily M, member 2	NM_001011559	0.49	0.002

		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
	Up-regulated	**Pax3**	Paired box 3	NM_053710	10.65	0.044
		Rgd1309808	Similar to apolipoprotein L2; apolipoprotein L-II	NM_001134801	9.66	0.042
		Mkx	Mohawk homeobox	ENSRNOT00000025623	9.17	0.040
		Ckm	Creatine kinase, muscle	NM_012530	8.50	0.035

Metabolic Process		**Tcf7l2**	Transcription factor 7-like 2 (T-cell specific, HMG-box)	NM_001191052	7.39	0.050
		Il6	Interleukin 6	NM_012589	7.19	0.022
		Ereg	Epiregulin	NM_021689	6.11	0.002
		Fst	Follistatin	NM_012561	4.89	0.020
		Nr4a3	Nuclear receptor subfamily 4, group A, member 3	NM_017352	4.54	0.003
		Dio3	Deiodinase, iodothyronine, type III	NM_017210	4.47	0.003
		Adamts1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	NM_024400	4.32	0.001
		Ucn2	Urocortin 2	NM_133385	4.06	0.018
		Rgd1561667	Similar to putative protein kinase	XM_001054195	4.05	0.026
		Rgd1565390	Similar to putative protein kinase	XM_344843	3.28	0.018
		Bmp2	Bone morphogenetic protein 2	NM_017178	4.00	0.003
		Runx1	Runt-related transcription factor 1	NM_017325	3.90	0.000
		Alpl	Alkaline phosphatase, liver/bone/kidney	NM_013059	3.89	0.000
		Car13	Carbonic anhydrase 13	NM_001134993	3.81	0.048
		Cd55	Cd55 molecule	NM_022269	3.79	0.011
		Loc365499	Similar to KP78b CG17216-PA	XM_001053352	3.60	0.050
		Tlr2	Toll-like receptor 2	NM_198769	3.58	0.005
		Map3k8	Mitogen-activated protein kinase kinase kinase 8	NM_053847	3.43	0.001
		Phlda1	Pleckstrin homology-like domain, family A, member 1	NM_017180	3.13	0.008
		Plaur	Plasminogen activator, urokinase receptor	NM_134352	3.13	0.006
		Loc689299	Similar to serine/threonine kinase	XM_001070335	3.08	0.049
		Areg	Amphiregulin	NM_017123	3.07	0.017
		Hspa1b	Heat shock 70kd protein 1B (mapped)	NM_212504	3.05	0.044
		Loc681776	Similar to High mobility group protein 1 (HMG-1) (High mobility group protein B1) (Amphoterin) (Heparin-binding protein p30)	XM_002725357	3.03	0.018
		Loc681117	Similar to ribosomal protein L6	XM_001060376	3.03	0.026

		Sfrs11	Splicing factor, arginine/serine-rich 11	NM_001035255	2.99	0.031
		Batf3	Basic leucine zipper transcription factor, ATF-like 3	NM_021865	2.95	0.005
		Uncx	UNC homeobox	NM_017179	2.90	0.001
		Usp36	Ubiquitin specific peptidase 36	NM_001107069	2.88	0.023
		Foxe3	Forkhead box E3	ENSRNOT00000010208	2.85	0.017
		Hlx	H2.0-like homeobox	NM_001077674	2.83	0.000
		Hmga1	High mobility group AT-hook 1	NM_139327	2.82	0.011
		Sgsm1	Small G protein signaling modulator 1	ENSRNOT00000056824	2.81	0.038
		Adm	Adrenomedullin	NM_012715	2.67	0.029
		Nasp	Nuclear autoantigenic sperm protein (histone-binding)	NM_001005543	2.60	0.042
		Pax1	Paired box 1	NM_001107787	2.59	0.030
		Hbegf	Heparin-binding EGF-like growth factor	NM_012945	2.56	0.010
		Prss23	Protease, serine, 23	NM_001007691	2.49	0.037
		Erc1	ELKS/RAB6-interacting/CAST family member 1	NM_170788	2.47	0.033
		Taok3	TAO kinase 3	NM_001024254	2.46	0.036
		Igfbp3	Insulin-like growth factor binding protein 3	NM_012588	2.44	0.000
		Rnf144b	Ring finger protein 144B	NM_001108881	2.43	0.006
		Hdc	Histidine decarboxylase	NM_017016	2.40	0.027
		Wnt6	Wingless-type MMTV integration site family, member 6	NM_001108226	2.39	0.048
		Wwox	WW domain-containing oxidoreductase	NM_001106188	2.38	0.038
		Myc	Myelocytomatosis oncogene	NM_012603	2.38	0.014
		Sox7	SRY (sex determining region Y)-box 7	NM_001106045	2.37	0.044
		Eid3	EP300 interacting inhibitor of differentiation 3	NM_001044304	2.37	0.027
		Tgm1	Transglutaminase 1, K polypeptide	NM_031659	2.36	0.022
		Slc15a3	Solute carrier family 15, member 3	NM_139341	2.35	0.031
		Fmr1	Fragile X mental retardation 1	NM_052804	2.34	0.027
		Npm3	Nucleophosmin/nucleoplasmin, 3	ENSRNOT00000023963	2.29	0.012
		Ptx3	Pentraxin related gene	NM_001109536	2.29	0.023

		Cd44	Cd44 molecule	NM_012924	2.28	0.041
		Tgfb1	Transforming growth factor, beta 1	NM_021578	2.27	0.047
		Junb	Jun B proto-oncogene	NM_021836	2.26	0.001
		Ctrc	Chymotrypsin C (caldecrin)	NM_001077649	2.25	0.039
		Rgd1561672	Similar to novel protein	XM_001065540	2.24	0.014
		Klf5	Kruppel-like factor 5	NM_053394	2.23	0.012
		Rgd1564342	Similar to hypothetical protein FLJ32685	ENSRNOT00000030991	2.21	0.044
		Atf3	Activating transcription factor 3	NM_012912	2.20	0.021
		Rxfp3	Relaxin/insulin-like family peptide receptor 3	NM_001008310	2.15	0.018
		Fzd6	Frizzled homolog 6 (Drosophila)	NM_001130536	2.15	0.005
		Zfp217	Zinc finger protein 217	NM_001107813	2.14	0.003
		Cym	Chymosin	NM_020091	2.11	0.038
		Pdgfc	Platelet derived growth factor C	NM_031317	2.11	0.044
		Gtpbp4	GTP binding protein 4	NM_053689	2.09	0.003
		Gadd45g	Growth arrest and DNA-damage-inducible, gamma	NM_001077640	2.08	0.014
		Hsp90aa1	Heat shock protein 90, alpha (cytosolic), class A member 1	NM_175761	2.07	0.018
		Sbk2	SH3-binding domain kinase family, member 2	NM_001127539	2.04	0.023
		Sin3a	SIN3 homolog A, transcription regulator (yeast)	NM_001108761	2.03	0.004
		Ppp1r15a	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	NM_133546	2.02	0.035
		Hmgb1	High mobility group box 1	NM_012963	2.00	0.009
		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
	Down-regulated	**Hes5**	Hairy and enhancer of split 5 (Drosophila)	NM_024383	0.18	0.033
		Tgm5	Transglutaminase 5	XM_001080153	0.20	0.001
		Mmp9	Matrix metallopeptidase 9	NM_031055	0.23	0.027
		Nostrin	Nitric oxide synthase trafficker	NM_001024260	0.24	0.029
		Cga	Glycoprotein hormones, alpha polypeptide	NM_053918	0.25	0.024

		Cdkn1c	Cyclin-dependent kinase inhibitor 1C	NM_182735	0.25	0.005
		Tp63	Tumor protein p63	NM_019221	0.25	0.000
		Pou4f3	POU class 4 homeobox 3	NM_001108889	0.26	0.013
		Adam2	ADAM metallopeptidase domain 2	NM_020077	0.26	0.001
		Tmprss11d	Transmembrane protease, serine 11d	NM_022630	0.27	0.027
		Adamdec1	ADAM-like, decysin 1	NM_001106046	0.28	0.003
		Aicda	Activation-induced cytidine deaminase	NM_001100779	0.29	0.015
		Ptafr	Platelet-activating factor receptor	NM_053321	0.30	0.025
		Cpxm2	Carboxypeptidase X (M14 family), member 2	NM_001106306	0.31	0.029
		Cyp27a1	Cytochrome P450, family 27, subfamily a, polypeptide 1	NM_178847	0.32	0.025
		Neu4	Sialidase 4	NM_001108234	0.32	0.020
		Htr4	5-hydroxytryptamine (serotonin) receptor 4	NM_012853	0.32	0.002
		Cyp11b1	Cytochrome P450, family 11, subfamily b, polypeptide 1	NM_012537	0.32	0.003
		Tyrp1	Tyrosinase-related protein 1	NM_001106664	0.33	0.012
		Tbx1	T-box 1	NM_001108322	0.33	0.037
		Fancd2	Fanconi anemia, complementation group D2	NM_001001719	0.34	0.044
		Il4	Interleukin 4	NM_201270	0.34	0.002
		Esr1	Estrogen receptor 1	NM_012689	0.34	0.002
		Fgf8	Fibroblast growth factor 8	NM_133286	0.35	0.004
		Pcsk2	Proprotein convertase subtilisin/kexin type 2	NM_012746	0.35	0.020
		Rgd1304879	Similar to Zinc finger protein 398 (Zinc finger DNA binding protein p52/p71)	NM_001014056	0.35	0.004
		Uox	Urate oxidase	NM_053768	0.36	0.030
		Ctsll3	Cathepsin L-like 3	ENSRNOT00000061398	0.36	0.002
		Afap1l2	Actin filament associated protein 1-like 2	XM_001064140	0.36	0.002
		Acan	Aggrecan	NM_022190	0.36	0.036
		Camkk1	Calcium/calmodulin-dependent protein kinase kinase 1, alpha	NM_031662	0.36	0.004
		Prkcz	Protein kinase C, zeta	NM_022507	0.37	0.012

		Gstm6	Glutathione S-transferase, mu 6	NM_001109192	0.38	0.007
		Pkm2	Pyruvate kinase, muscle	NM_053297	0.38	0.035
		Adcyap1	Adenylate cyclase activating polypeptide 1	NM_016989	0.40	0.002
		Acot12	Acyl-coa thioesterase 12	NM_130747	0.40	0.033
		Masp1	Mannan-binding lectin serine peptidase 1	NM_022257	0.40	0.024
		Casp8	Caspase 8	NM_022277	0.40	0.028
		Atp9b	Atpase, class II, type 9B	NM_001106130	0.40	0.008
		Gna15	Guanine nucleotide binding protein, alpha 15	NM_053542	0.40	0.001
		Hoxc6	Homeo box C6	XM_001069410	0.40	0.015
		Dmrtc1a	DMRT-like family c1a	NM_001025288	0.40	0.008
		Atp5d	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	BC161836	0.41	0.000
		Dclk3	Doublecortin-like kinase 3	NM_001191800	0.41	0.000
		Lck	Lymphocyte-specific protein tyrosine kinase	NM_001100709	0.41	0.006
		Dbh	Dopamine beta-hydroxylase (dopamine beta-monooxygenase)	NM_013158	0.41	0.046
		P4htm	Prolyl 4-hydroxylase, transmembrane	ENSRNOT00000027466	0.41	0.013
		Plagl1	Pleiomorphic adenoma gene-like 1	NM_012760	0.41	0.015
		Mas1	MAS1 oncogene	NM_012757	0.42	0.030
		Calcr	Calcitonin receptor	NM_053816	0.42	0.040
		Prmt8	Protein arginine methyltransferase 8	XM_002726433	0.42	0.023
		Nox1	NADPH oxidase 1	NM_053683	0.43	0.002
		Wnt3	Wingless-type MMTV integration site family, member 3	NM_001105715	0.43	0.003
		Chrna4	Cholinergic receptor, nicotinic, alpha 4	NM_024354	0.43	0.025
		Hcfc1	Host cell factor C1	NM_001139507	0.43	0.005
		Foxl2	Forkhead box L2	ENSRNOT00000023091	0.44	0.004
		Egr4	Early growth response 4	NM_019137	0.44	0.008
		Cftr	Cystic fibrosis transmembrane conductance regulator homolog (human)	NM_031506	0.44	0.013
		Ache	Acetylcholinesterase	NM_172009	0.44	0.015
		Htr2c	5-hydroxytryptamine (serotonin) receptor 2C	NM_012765	0.44	0.041

		Fut7	Fucosyltransferase 7 (alpha (1,3) fucosyltransferase)	NM_199491	0.44	0.024
		Gal3st2	Galactose-3-O-sulfotransferase 2	XM_001063652	0.44	0.036
		Adra2a	Adrenergic, alpha-2A-, receptor	NM_012739	0.44	0.040
		Acot5	Acyl-coa thioesterase 5	NM_001079709	0.44	0.024
		Socs7	Suppressor of cytokine signaling 7	XM_213443	0.45	0.004
		Nppa	Natriuretic peptide precursor A	NM_012612	0.45	0.014
		Snca	Synuclein, alpha (non A4 component of amyloid precursor)	S73008	0.45	0.030
		F12	Coagulation factor XII (Hageman factor)	NM_001014006	0.45	0.011
		Loc680273	Similar to Forkhead box protein L1 (Forkhead-related protein FKHL11) (Forkhead-related transcription factor 7) (FREAC-7)	XM_001056413	0.45	0.036
		Akr1c13	Aldo-keto reductase family 1, member C13	NM_001014240	0.45	0.029
		Lrrn3	Leucine rich repeat neuronal 3	NM_030856	0.45	0.043
		Gucy2g	Guanylate cyclase 2G	NM_139042	0.45	0.001
		Adcy3	Adenylate cyclase 3	NM_130779	0.45	0.014
		Gstm3	Glutathione S-transferase mu 3	NM_020540	0.46	0.026
		Rdh5	Retinol dehydrogenase 5	ENSRNOT00000010217	0.46	0.028
		Atp6v0a4	Atpase, H+ transporting, lysosomal V0 subunit A4	NM_001106591	0.46	0.028
		Ckmt2	Creatine kinase, mitochondrial 2, sarcomeric	NM_001127652	0.46	0.048
		Bmx	BMX non-receptor tyrosine kinase	NM_001109016	0.46	0.020
		Madd	MAP-kinase activating death domain	NM_053585	0.46	0.019
		Tceanc	Transcription elongation factor A (SII) N-terminal and central domain containing	NM_001109015	0.46	0.007
		Dpp4	Dipeptidylpeptidase 4	NM_012789	0.46	0.044
		Zfp112	Zinc finger protein 112	NM_001107487	0.47	0.005
		Pcsk4	Proprotein convertase subtilisin/kexin type 4	NM_133559	0.47	0.006
		Apbb1	Amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65)	NM_080478	0.47	0.017
		Mc4r	Melanocortin 4 receptor	NM_013099	0.47	0.033
		Dpyd	Dihydropyrimidine dehydrogenase	NM_031027	0.32	0.031
		Ugt2b36	Dihydropyrimidine dehydrogenase	NM_031027	0.32	0.031

		Plekhg5	Pleckstrin homology domain containing, family G (with rhogef domain) member 5	NM_201272	0.48	0.003
		Zfp324	Zinc finger protein 324	ENSRNOT00000036874	0.48	0.009
		Prlr	Prolactin receptor	NM_012630	0.48	0.013
		Sult4a1	Sulfotransferase family 4A, member 1	NM_031641	0.48	0.013
		Rpusd3	RNA pseudouridylate synthase domain containing 3	NM_001108641	0.48	0.010
		Tex15	Testis expressed 15	NM_001106087	0.48	0.046
		Pick1	Protein interacting with PRKCA 1	NM_053460	0.48	0.018
		Prlhr	Prolactin releasing hormone receptor	NM_139193	0.48	0.002
		Zcchc12	Zinc finger, CCHC domain containing 12	NM_001014065	0.48	0.021
		Asphd2	Aspartate beta-hydroxylase domain containing 2	NM_001009716	0.48	0.011
		Itgb2	Integrin, beta 2	NM_001037780	0.49	0.035
		Tnfrsf11a	Tumor necrosis factor receptor superfamily, member 11a	XM_001063501	0.49	0.005
		Ece2	Endothelin-converting enzyme 2	NM_001002815	0.49	0.004
		Ampd2	Adenosine monophosphate deaminase 2 (isoform L)	NM_001101681	0.49	0.025
		Mgc109340	Similar to Microsomal signal peptidase 23 kda subunit (spase 22 kda subunit) (SPC22/23)	NM_001024267	0.49	0.023
		Insig2	Insulin induced gene 2	NM_178091	0.49	0.013
		Rgd1308116	Similar to hypothetical protein MGC42105	XM_001076547	0.50	0.022
		Afp	Alpha-fetoprotein	NM_012493	0.50	0.039
		Zbtb24	Zinc finger and BTB domain containing 24	NM_001098667	0.50	0.013
		Mstn	Myostatin	NM_019151	0.50	0.021
		Lor	Loricrin	ENSRNOT00000056500	0.50	0.004

	Up-regulated	**Gene Symbol**	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
		Unc13c	Unc-13 homolog C (C. Elegans)	NM_173146	2.51	0.039

Neurotransmitter Secretion		Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value
	Down-regulated	**Unc13b**	Unc-13 homolog B (C. Elegans)	NM_001042579	0.33	0.017
		Htr6	5-hydroxytryptamine (serotonin) receptor 6	NM_024365	0.39	0.046
		Snap25	Synaptosomal-associated protein 25	NM_030991	0.43	0.027
		Htr2c	5-hydroxytryptamine (serotonin) receptor 2C	NM_012765	0.44	0.041
		Snca	Synuclein, alpha (non A4 component of amyloid precursor)	S73008	0.45	0.030
		Syt3	Synaptotagmin III	NM_019122	0.48	0.014

Signaling Pathways	Up-regulated	Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value
		Mcc	Mutated in colorectal cancers	NM_001170534	0.04	0.036
		Tcf7l2	Transcription factor 7-like 2 (T-cell specific, HMG-box)	NM_001191052	0.05	0.050
		Il6	Interleukin 6	NM_012589	0.02	0.022
		Ereg	Epiregulin	NM_021689	0.00	0.002
		Fst	Follistatin	NM_012561	0.02	0.020
		Adamts1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	NM_024400	0.00	0.001
		Bmp2	Bone morphogenetic protein 2	NM_017178	0.00	0.003
		Itgb6	Integrin, beta 6	NM_001004263	0.00	0.003
		Olr374	Olfactory receptor 374	NM_001001289	0.01	0.011
		Tlr2	Toll-like receptor 2	NM_198769	0.01	0.005
		Areg	Amphiregulin	NM_017123	0.02	0.017
		Sgsm1	Small G protein signaling modulator 1	ENSRNOT00000056824	0.04	0.038
		Rgd1564791	Similar to hypothetical protein 4930474N05	XM_574052	0.04	0.041
		Adm	Adrenomedullin	NM_012715	0.03	0.029
		Hbegf	Heparin-binding EGF-like growth factor	NM_012945	0.01	0.010
		Fgf18	Fibroblast growth factor 18	NM_019199	0.00	0.000
		Erc1	ELKS/RAB6-interacting/CAST family member 1	NM_170788	0.03	0.033

Taok3	TAO kinase 3	NM_001024254	0.04	0.036
F2rl1	Coagulation factor II (thrombin) receptor-like 1	NM_053897	0.01	0.007
Wnt6	Wingless-type MMTV integration site family, member 6	NM_001108226	0.05	0.048
Wwox	WW domain-containing oxidoreductase	NM_001106188	0.04	0.038
Olr1694	Olfactory receptor 1694	NM_001001110	0.00	0.001
Sox7	SRY (sex determining region Y)-box 7	NM_001106045	0.04	0.044
Rab20	RAB20, member RAS oncogene family	NM_001109535	0.01	0.006
Olr1673	Olfactory receptor 1673	NM_001000268	0.01	0.014
Cd44	Cd44 molecule	NM_012924	0.04	0.041
Tgfb1	Transforming growth factor, beta 1	NM_021578	0.05	0.047
Rnd3	Rho family gtpase 3	NM_001007641	0.04	0.039
Shc4	SHC (Src homology 2 domain containing) family, member 4	NM_001191065	0.00	0.005
Glra2	Glycine receptor, alpha 2	NM_012568	0.03	0.028
Bst2	Bone marrow stromal cell antigen 2	NM_198134	0.03	0.031
Rxfp3	Relaxin/insulin-like family peptide receptor 3	NM_001008310	0.02	0.018
Fzd6	Frizzled homolog 6 (Drosophila)	NM_001130536	0.01	0.005
Pdgfc	Platelet derived growth factor C	NM_031317	0.04	0.044
Rspo3	R-spondin 3 homolog (Xenopus laevis)	NM_001100990	0.02	0.025
Olr803	Olfactory receptor 803	NM_001000853	0.01	0.009
Gadd45g	Growth arrest and DNA-damage-inducible, gamma	NM_001077640	0.01	0.014
Cx3cr1	Chemokine (C-X3-C motif) receptor 1	NM_133534	0.04	0.041
Shoc2	Soc-2 (suppressor of clear) homolog (C. Elegans)	NM_001013155	0.00	0.003
Ppp1r15a	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	NM_133546	0.03	0.035

Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
Blnk	B-cell linker	NM_001025767	0.13	0.003
Hes5	Hairy and enhancer of split 5 (Drosophila)	NM_024383	0.18	0.033

	Down-regulated	**Olr282**	Olfactory receptor 282	NM_001000224	0.19	0.038
		Agtr1b	Angiotensin II receptor, type 1b	NM_031009	0.21	0.002
		Cdkn1c	Cyclin-dependent kinase inhibitor 1C	NM_182735	0.25	0.005
		Tp63	Tumor protein p63	NM_019221	0.25	0.000
		Ptafr	Platelet-activating factor receptor	NM_053321	0.30	0.025
		Gpr6	G protein-coupled receptor 6	NM_031806	0.31	0.003
		Htr4	5-hydroxytryptamine (serotonin) receptor 4	NM_012853	0.32	0.002
		Unc13b	Unc-13 homolog B (C. Elegans)	NM_001042579	0.33	0.017
		Olr428	Olfactory receptor 428	NM_001000394	0.34	0.010
		Il4	Interleukin 4	NM_201270	0.34	0.002
		Esr1	Estrogen receptor 1	NM_012689	0.34	0.002
		Plek2	Pleckstrin 2	NM_001114180	0.34	0.006
		Sstr1	Somatostatin receptor 1	NM_012719	0.35	0.010
		Fgf8	Fibroblast growth factor 8	NM_133286	0.35	0.004
		Olr1378	Olfactory receptor 1378	NM_214828	0.36	0.044
		Afap1l2	Actin filament associated protein 1-like 2	XM_001064140	0.36	0.002
		Prkcz	Protein kinase C, zeta	NM_022507	0.37	0.012
		Rasal3	RAS protein activator like 3	NM_001134562	0.37	0.028
		Htr6	5-hydroxytryptamine (serotonin) receptor 6	NM_024365	0.39	0.046
		Rasgrp2	RAS guanyl releasing protein 2 (calcium and DAG-regulated)	NM_001082977	0.39	0.010
		Adcyap1	Adenylate cyclase activating polypeptide 1	NM_016989	0.40	0.002
		Casp8	Caspase 8	NM_022277	0.40	0.028
		Gna15	Guanine nucleotide binding protein, alpha 15	NM_053542	0.40	0.001
		Oprl1	Opiate receptor-like 1	NM_031569	0.40	0.028
		Dclk3	Doublecortin-like kinase 3	NM_001191800	0.41	0.000
		Lck	Lymphocyte-specific protein tyrosine kinase	NM_001100709	0.41	0.006
		Olr1331	Olfactory receptor 1331	NM_001000790	0.41	0.040
		Mas1	MAS1 oncogene	NM_012757	0.42	0.030

		Calcr	Calcitonin receptor	NM_053816	0.42	0.040
		Grem2	Gremlin 2, cysteine knot superfamily, homolog (Xenopus laevis)	NM_001105974	0.42	0.028
		Fgf3	Fibroblast growth factor 3	NM_130817	0.43	0.015
		Wnt3	Wingless-type MMTV integration site family, member 3	NM_001105715	0.43	0.003
		Rerg	RAS-like, estrogen-regulated, growth-inhibitor	TC629838	0.43	0.017
		Htr2c	5-hydroxytryptamine (serotonin) receptor 2C	NM_012765	0.44	0.041
		Slc2a8	Solute carrier family 2, (facilitated glucose transporter) member 8	NM_053494	0.44	0.005
		Adra2a	Adrenergic, alpha-2A-, receptor	NM_012739	0.44	0.040
		Socs7	Suppressor of cytokine signaling 7	XM_213443	0.45	0.004
		Nppa	Natriuretic peptide precursor A	NM_012612	0.45	0.014
		Chn1	Chimerin (chimaerin) 1	NM_032083	0.45	0.010
		Snca	Synuclein, alpha (non A4 component of amyloid precursor)	S73008	0.45	0.030
		Gucy2g	Guanylate cyclase 2G	NM_139042	0.45	0.001
		Adcy3	Adenylate cyclase 3	NM_130779	0.45	0.014
		Olr1217	Olfactory receptor 1217	NM_001000439	0.46	0.047
		Bmx	BMX non-receptor tyrosine kinase	NM_001109016	0.46	0.020
		Lingo1	Leucine rich repeat and Ig domain containing 1	NM_001100722	0.46	0.031
		Rab40b	Rab40b, member RAS oncogene family	NM_001107076	0.46	0.023
		Madd	MAP-kinase activating death domain	NM_053585	0.46	0.019
		Sgef	Src homology 3 domain-containing guanine nucleotide exchange factor	ENSRNOT00000019553	0.47	0.049
		Olr750	Olfactory receptor 750	NM_001000366	0.47	0.001
		Mc4r	Melanocortin 4 receptor	NM_013099	0.47	0.033
		Olr40	Olfactory receptor 40	NM_001000127	0.47	0.039
		Rtn4rl1	Reticulon 4 receptor-like 1	NM_181377	0.47	0.020
		Plekhg5	Pleckstrin homology domain containing, family G (with rhogef domain) member 5	NM_201272	0.48	0.003
		Prlr	Prolactin receptor	NM_012630	0.48	0.013
		Gpr64	G protein-coupled receptor 64	NM_181366	0.48	0.000

		Pick1	Protein interacting with PRKCA 1	NM_053460	0.48	0.018
		Mapk8ip2	Mitogen-activated protein kinase 8 interacting protein 2	ENSRNOT00000055792	0.48	0.017
		Prlhr	Prolactin releasing hormone receptor	NM_139193	0.48	0.002
		Olr777	Olfactory receptor 777	NM_001000579	0.49	0.017
		Tac1	Tachykinin 1	NM_012666	0.49	0.010
		Olr1697	Olfactory receptor 1697	NM_001001111	0.49	0.024
		Asb2	Ankyrin repeat and SOCS box-containing 2	NM_001011984	0.49	0.005
		Itgb2	Integrin, beta 2	NM_001037780	0.49	0.035
		Tnfrsf11a	Tumor necrosis factor receptor superfamily, member 11a	XM_001063501	0.49	0.005
		Insig2	Insulin induced gene 2	NM_178091	0.49	0.013
		Afp	Alpha-fetoprotein	NM_012493	0.50	0.039
		Mstn	Myostatin	NM_019151	0.50	0.021

Synapse	**Up-regulated**	**Gene Symbol**	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
		Unc13c	Unc-13 homolog C (C. Elegans)	NM_173146	2.51	0.039
		Fmr1	Fragile X mental retardation 1	NM_052804	2.34	0.027
		Glra2	Glycine receptor, alpha 2	NM_012568	2.24	0.028
	Down-regulated	**Gene Symbol**	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
		Unc13b	Unc-13 homolog B (C. Elegans)	NM_001042579	0.33	0.017
		Chrng	Cholinergic receptor, nicotinic, gamma	NM_019145	0.38	0.035
		Rasgrp2	RAS guanyl releasing protein 2 (calcium and DAG-regulated)	NM_001082977	0.39	0.010
		Snap25	Synaptosomal-associated protein 25	NM_030991	0.43	0.027
		Chrna4	Cholinergic receptor, nicotinic, alpha 4	NM_024354	0.43	0.025
		Ache	Acetylcholinesterase	NM_172009	0.44	0.015
		Slc2a8	Solute carrier family 2, (facilitated glucose transporter) member 8	NM_053494	0.44	0.005

		Svop	SV2 related protein	NM_134404	0.44	0.033
		Adra2a	Adrenergic, alpha-2A-, receptor	NM_012739	0.44	0.040
		Slc17a7	Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7	NM_053859	0.45	0.022
		Sema4f	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4F	NM_019272	0.45	0.031
		Snca	Synuclein, alpha (non A4 component of amyloid precursor)	S73008	0.45	0.030
		Slc17a6	Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6	NM_053427	0.46	0.047
		Apbb1	Amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65)	NM_080478	0.47	0.017
		Syt3	Synaptotagmin III	NM_019122	0.48	0.014
		Pick1	Protein interacting with PRKCA 1	NM_053460	0.48	0.018
		Synpr	Synaptoporin	NM_023974	0.48	0.025
		Syt17	Synaptotagmin XVII	NM_138849	0.49	0.013

		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
Transcription	**Up-regulated**	**Pax3**	Paired box 3	NM_053710	10.65	0.044
		Mkx	Mohawk homeobox	ENSRNOT00000025623	9.17	0.040
		Tcf7l2	Transcription factor 7-like 2 (T-cell specific, HMG-box)	NM_001191052	7.39	0.050
		Il6	Interleukin 6	NM_012589	7.19	0.022
		Ereg	Epiregulin	NM_021689	6.11	0.002
		Fst	Follistatin	NM_012561	4.89	0.020
		Nr4a3	Nuclear receptor subfamily 4, group A, member 3	NM_017352	4.54	0.003
		Bmp2	Bone morphogenetic protein 2	NM_017178	4.00	0.003
		Runx1	Runt-related transcription factor 1	NM_017325	3.90	0.000
		Tlr2	Toll-like receptor 2	NM_198769	3.58	0.005
		Batf3	Basic leucine zipper transcription factor, ATF-like 3	NM_021865	2.95	0.005
		Uncx	UNC homeobox	NM_017179	2.90	0.001

		Foxe3	Forkhead box E3	ENSRNOT00000010208	2.85	0.017
		Hlx	H2.0-like homeobox	NM_001077674	2.83	0.000
		Hmga1	High mobility group AT-hook 1	NM_139327	2.82	0.011
		Pax1	Paired box 1	NM_001107787	2.59	0.030
		Wnt6	Wingless-type MMTV integration site family, member 6	NM_001108226	2.39	0.048
		Myc	Myelocytomatosis oncogene	NM_012603	2.38	0.014
		Sox7	SRY (sex determining region Y)-box 7	NM_001106045	2.37	0.044
		Eid3	EP300 interacting inhibitor of differentiation 3	NM_001044304	2.37	0.027
		Npm3	Nucleophosmin/nucleoplasmin, 3	ENSRNOT00000023963	2.29	0.012
		Tgfb1	Transforming growth factor, beta 1	NM_021578	2.27	0.047
		Junb	Jun B proto-oncogene	NM_021836	2.26	0.001
		Rgd1561672	Similar to novel protein	XM_001065540	2.24	0.014
		Klf5	Kruppel-like factor 5	NM_053394	2.23	0.012
		Atf3	Activating transcription factor 3	NM_012912	2.20	0.021
		Fzd6	Frizzled homolog 6 (Drosophila)	NM_001130536	2.15	0.005
		Zfp217	Zinc finger protein 217	NM_001107813	2.14	0.003
		Sin3a	SIN3 homolog A, transcription regulator (yeast)	NM_001108761	2.03	0.004
		Hmgb1	High mobility group box 1	NM_012963	2.00	0.009
		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
	Down-regulated	**Hes5**	Hairy and enhancer of split 5 (Drosophila)	NM_024383	0.18	0.033
		Nostrin	Nitric oxide synthase trafficker	NM_001024260	0.24	0.029
		Cdkn1c	Cyclin-dependent kinase inhibitor 1C	NM_182735	0.25	0.005
		Tp63	Tumor protein p63	NM_019221	0.25	0.000
		Pou4f3	POU class 4 homeobox 3	NM_001108889	0.26	0.013
		Tbx1	T-box 1	NM_001108322	0.33	0.037
		Il4	Interleukin 4	NM_201270	0.34	0.002

		Esr1	Estrogen receptor 1	NM_012689	0.34	0.002
		Rgd1304879	Similar to Zinc finger protein 398 (Zinc finger DNA binding protein p52/p71)	NM_001014056	0.35	0.004
		Afap1l2	Actin filament associated protein 1-like 2	XM_001064140	0.36	0.002
		Hoxc6	Homeo box C6	XM_001069410	0.40	0.015
		Dmrtc1a	DMRT-like family c1a	NM_001025288	0.40	0.008
		Plagl1	Pleiomorphic adenoma gene-like 1	NM_012760	0.41	0.015
		Hcfc1	Host cell factor C1	NM_001139507	0.43	0.005
		Foxl2	Forkhead box L2	ENSRNOT00000023091	0.44	0.004
		Egr4	Early growth response 4	NM_019137	0.44	0.008
		Loc680273	Similar to Forkhead box protein L1 (Forkhead-related protein FKHL11) (Forkhead-related transcription factor 7) (FREAC-7)	XM_001056413	0.45	0.036
		Tceanc	Transcription elongation factor A (SII) N-terminal and central domain containing	NM_001109015	0.46	0.007
		Zfp112	Zinc finger protein 112	NM_001107487	0.47	0.005
		Apbb1	Amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65)	NM_080478	0.47	0.017
		Zfp324	Zinc finger protein 324	ENSRNOT00000036874	0.48	0.009
		Zcchc12	Zinc finger, CCHC domain containing 12	NM_001014065	0.48	0.021
		Itgb2	Integrin, beta 2	NM_001037780	0.49	0.035
		Tnfrsf11a	Tumor necrosis factor receptor superfamily, member 11a	XM_001063501	0.49	0.005
		Zbtb24	Zinc finger and BTB domain containing 24	NM_001098667	0.50	0.013
		Mstn	Myostatin	NM_019151	0.50	0.021

		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
Receptor Activity	**Up-regulated**	**Thbd**	Thrombomodulin	NM_031771	7.03	0.012
		Nr4a3	Nuclear receptor subfamily 4, group A, member 3	NM_017352	4.54	0.003
		Vom2r75	Vomeronasal 2 receptor, 75	NM_173320	3.83	0.028
		Itgb6	Integrin, beta 6	NM_001004263	3.75	0.003

		Olr374	Olfactory receptor 374	NM_001001289	3.65	0.011
		Tlr2	Toll-like receptor 2	NM_198769	3.58	0.005
		Plaur	Plasminogen activator, urokinase receptor	NM_134352	3.13	0.006
		F2rl1	Coagulation factor II (thrombin) receptor-like 1	NM_053897	2.46	0.007
		Extl3	Exostoses (multiple)-like 3	NM_020097	2.38	0.029
		Olr1694	Olfactory receptor 1694	NM_001001110	2.37	0.001
		Pvr	Poliovirus receptor	NM_017076	2.33	0.021
		Olr1673	Olfactory receptor 1673	NM_001000268	2.29	0.014
		Cd44	Cd44 molecule	NM_012924	2.28	0.041
		Glra2	Glycine receptor, alpha 2	NM_012568	2.24	0.028
		Rxfp3	Relaxin/insulin-like family peptide receptor 3	NM_001008310	2.15	0.018
		Fzd6	Frizzled homolog 6 (Drosophila)	NM_001130536	2.15	0.005
		Olr803	Olfactory receptor 803	NM_001000853	2.08	0.009
		Cx3cr1	Chemokine (C-X3-C motif) receptor 1	NM_133534	2.06	0.041
		Vom2r56	Vomeronasal 2 receptor, 56	NM_001099484	2.02	0.049

	Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
Down-regulated	**Olr282**	Olfactory receptor 282	NM_001000224	0.19	0.038
	Agtr1b	Angiotensin II receptor, type 1b	NM_031009	0.21	0.002
	Ptafr	Platelet-activating factor receptor	NM_053321	0.30	0.025
	Klrb1a	Killer cell lectin-like receptor subfamily B, member 1A	NM_001010964	0.31	0.001
	Gpr6	G protein-coupled receptor 6	NM_031806	0.31	0.003
	Htr4	5-hydroxytryptamine (serotonin) receptor 4	NM_012853	0.32	0.002
	Sstr3	Somatostatin receptor 3	NM_133522	0.32	0.003
	Cd244	Cd244 molecule, natural killer cell receptor 2B4	NM_022259	0.33	0.007
	Gpr160	G protein-coupled receptor 160	NM_001025147	0.34	0.008
	Olr428	Olfactory receptor 428	NM_001000394	0.34	0.010

		Gfra4	GDNF family receptor alpha 4	NM_023967	0.34	0.010
		Trpc7	Transient receptor potential cation channel, subfamily C, member 7	NM_001191691	0.34	0.031
		Esr1	Estrogen receptor 1	NM_012689	0.34	0.002
		Sstr1	Somatostatin receptor 1	NM_012719	0.35	0.010
		Sorcs3	Sortilin-related VPS10 domain containing receptor 3	NM_001106367	0.35	0.013
		Olr1378	Olfactory receptor 1378	NM_214828	0.36	0.044
		Trpc6	Transient receptor potential cation channel, subfamily C, member 6	NM_053559	0.37	0.009
		Gpr68	G protein-coupled receptor 68	NM_001108049	0.38	0.000
		Il8ra	Interleukin 8 receptor, alpha	NM_019310	0.38	0.015
		Chrng	Cholinergic receptor, nicotinic, gamma	NM_019145	0.38	0.035
		Htr6	5-hydroxytryptamine (serotonin) receptor 6	NM_024365	0.39	0.046
		Pth2r	Parathyroid hormone 2 receptor	NM_031089	0.40	0.038
		Oprl1	Opiate receptor-like 1	NM_031569	0.40	0.028
		Olr1331	Olfactory receptor 1331	NM_001000790	0.41	0.040
		Mas1	MAS1 oncogene	NM_012757	0.42	0.030
		Calcr	Calcitonin receptor	NM_053816	0.42	0.040
		Rtn4r	Reticulon 4 receptor	NM_053613	0.43	0.006
		Chrna4	Cholinergic receptor, nicotinic, alpha 4	NM_024354	0.43	0.025
		Htr2c	5-hydroxytryptamine (serotonin) receptor 2C	NM_012765	0.44	0.041
		Tnfrsf17	Tumor necrosis factor receptor superfamily, member 17	NM_001105761	0.44	0.026
		Adra2a	Adrenergic, alpha-2A-, receptor	NM_012739	0.44	0.040
		Lpar4	Lysophosphatidic acid receptor 4	NM_001106940	0.45	0.021
		Chn1	Chimerin (chimaerin) 1	NM_032083	0.45	0.010
		Sema4f	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4F	NM_019272	0.45	0.031
		Gucy2g	Guanylate cyclase 2G	NM_139042	0.45	0.001
		Olr1217	Olfactory receptor 1217	NM_001000439	0.46	0.047
		Prom2	Prominin 2	NM_138857	0.46	0.031

		Olr750	Olfactory receptor 750	NM_001000366	0.47	0.001
		Mc4r	Melanocortin 4 receptor	NM_013099	0.47	0.033
		Olr40	Olfactory receptor 40	NM_001000127	0.47	0.039
		Rtn4rl1	Reticulon 4 receptor-like 1	NM_181377	0.47	0.020
		Prlr	Prolactin receptor	NM_012630	0.48	0.013
		Gpr176	G protein-coupled receptor 176	ENSRNOT00000007882	0.48	0.014
		Gpr64	G protein-coupled receptor 64	NM_181366	0.48	0.000
		Prlhr	Prolactin releasing hormone receptor	NM_139193	0.48	0.002
		Olr777	Olfactory receptor 777	NM_001000579	0.49	0.017
		Olr1697	Olfactory receptor 1697	NM_001001111	0.49	0.024
		Itgb2	Integrin, beta 2	NM_001037780	0.49	0.035
		Trpm2	Transient receptor potential cation channel, subfamily M, member 2	NM_001011559	0.49	0.002
		Tnfrsf11a	Tumor necrosis factor receptor superfamily, member 11a	XM_001063501	0.49	0.005

		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
Glutamate Secretion	**Down-regulated**	**Htr6**	5-hydroxytryptamine (serotonin) receptor 6	NM_024365	0.39	0.046
		Snca	Synuclein, alpha (non A4 component of amyloid precursor)	S73008	0.45	0.030

		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
RNA Biosynthetic Process	**Up-regulation**	**Pax3**	Paired box 3	NM_053710	10.65	0.044
		Mkx	Mohawk homeobox	ENSRNOT00000025623	9.17	0.040
		Tcf7l2	Transcription factor 7-like 2 (T-cell specific, HMG-box)	NM_001191052	7.39	0.050
		Il6	Interleukin 6	NM_012589	7.19	0.022
		Ereg	Epiregulin	NM_021689	6.11	0.002
		Fst	Follistatin	NM_012561	4.89	0.020

Nr4a3	Nuclear receptor subfamily 4, group A, member 3	NM_017352	4.54	0.003
Bmp2	Bone morphogenetic protein 2	NM_017178	4.00	0.003
Runx1	Runt-related transcription factor 1	NM_017325	3.90	0.000
Tlr2	Toll-like receptor 2	NM_198769	3.58	0.005
Batf3	Basic leucine zipper transcription factor, ATF-like 3	NM_021865	2.95	0.005
Uncx	UNC homeobox	NM_017179	2.90	0.001
Foxe3	Forkhead box E3	ENSRNOT00000010208	2.85	0.017
Hlx	H2.0-like homeobox	NM_001077674	2.83	0.000
Hmga1	High mobility group AT-hook 1	NM_139327	2.82	0.011
Pax1	Paired box 1	NM_001107787	2.59	0.030
Wnt6	Wingless-type MMTV integration site family, member 6	NM_001108226	2.39	0.048
Myc	Myelocytomatosis oncogene	NM_012603	2.38	0.014
Sox7	SRY (sex determining region Y)-box 7	NM_001106045	2.37	0.044
Npm3	Nucleophosmin/nucleoplasmin, 3	ENSRNOT00000023963	2.29	0.012
Tgfb1	Transforming growth factor, beta 1	NM_021578	2.27	0.047
Junb	Jun B proto-oncogene	NM_021836	2.26	0.001
Rgd1561672	Similar to novel protein	XM_001065540	2.24	0.014
Klf5	Kruppel-like factor 5	NM_053394	2.23	0.012
Atf3	Activating transcription factor 3	NM_012912	2.20	0.021
Fzd6	Frizzled homolog 6 (Drosophila)	NM_001130536	2.15	0.005
Sin3a	SIN3 homolog A, transcription regulator (yeast)	NM_001108761	2.03	0.004
Hmgb1	High mobility group box 1	NM_012963	2.00	0.009

Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
Hes5	Hairy and enhancer of split 5 (Drosophila)	NM_024383	0.18	0.033
Nostrin	Nitric oxide synthase trafficker	NM_001024260	0.24	0.029
Cdkn1c	Cyclin-dependent kinase inhibitor 1C	NM_182735	0.25	0.005

	Down-regulation	**Tp63**	Tumor protein p63	NM_019221	0.25	0.000
		Pou4f3	POU class 4 homeobox 3	NM_001108889	0.26	0.013
		Tbx1	T-box 1	NM_001108322	0.33	0.037
		Il4	Interleukin 4	NM_201270	0.34	0.002
		Esr1	Estrogen receptor 1	NM_012689	0.34	0.002
		Rgd1304879	Similar to Zinc finger protein 398 (Zinc finger DNA binding protein p52/p71)	NM_001014056	0.35	0.004
		Afap1l2	Actin filament associated protein 1-like 2	XM_001064140	0.36	0.002
		Hoxc6	Homeo box C6	XM_001069410	0.40	0.015
		Plagl1	Pleiomorphic adenoma gene-like 1	NM_012760	0.41	0.015
		Foxl2	Forkhead box L2	ENSRNOT00000023091	0.44	0.004
		Loc680273	Similar to Forkhead box protein L1 (Forkhead-related protein FKHL11) (Forkhead-related transcription factor 7) (FREAC-7)	XM_001056413	0.45	0.036
		Tceanc	Transcription elongation factor A (SII) N-terminal and central domain containing	NM_001109015	0.46	0.007
		Zfp112	Zinc finger protein 112	NM_001107487	0.47	0.005
		Apbb1	Amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65)	NM_080478	0.47	0.017
		Zfp324	Zinc finger protein 324	ENSRNOT00000036874	0.48	0.009
		Itgb2	Integrin, beta 2	NM_001037780	0.49	0.035
		Tnfrsf11a	Tumor necrosis factor receptor superfamily, member 11a	XM_001063501	0.49	0.005
		Zbtb24	Zinc finger and BTB domain containing 24	NM_001098667	0.50	0.013

		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
Response to Oxidative Stress	**Up-regulation**	**Areg**	Amphiregulin	NM_017123	3.07	0.017
		Sin3a	SIN3 homolog A, transcription regulator (yeast)	NM_001108761	2.03	0.004
		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value**
		Mmp9	Matrix metallopeptidase 9	NM_031055	0.23	0.027

	Down-regulation	**Trpc7**	Transient receptor potential cation channel, subfamily C, member 7	NM_001191691	0.34	0.031
		Fgf8	Fibroblast growth factor 8	NM_133286	0.35	0.004
		Lck	Lymphocyte-specific protein tyrosine kinase	NM_001100709	0.41	0.006
		Nox1	NADPH oxidase 1	NM_053683	0.43	0.002
		Chrna4	Cholinergic receptor, nicotinic, alpha 4	NM_024354	0.43	0.025
		Snca	Synuclein, alpha (non A4 component of amyloid precursor)	S73008	0.45	0.030
		Trpm2	Transient receptor potential cation channel, subfamily M, member 2	NM_001011559	0.49	0.002

Supplemental Table 2. List of genes up-regulated and down-regulated at 24h after OGD for different ontological classes. Gene ontology analyses included genes that had a *p*-value <0.05 and a fold change of 2.0 and were performed using GoMiner™. Classes were selected manually. Note that some genes are included in more than one class.

		Gene Symbol	Gene name	Accession Number	Fold Change 24h	*p*-value
Apoptosis	**Up-regulated**	**Ctgf**	Connective tissue growth factor	NM_022266	9.84	0.012
		Angptl4	Angiopoietin-like 4	NM_199115	4.91	0.008
		Anxa1	Annexin A1	NM_012904	4.62	0.018
		Casp1	Caspase 1	NM_012762	4.56	0.026
		Mycs	Myc-like oncogene, s-myc protein	NM_021837	4.25	0.042
		Cd44	Cd44 molecule	NM_012924	3.07	0.032
		Nqo1	NAD(P)H dehydrogenase, quinone 1	NM_017000	2.76	0.037
		Ccr5	Chemokine (C-C motif) receptor 5	NM_053960	2.75	0.006
		Hoxa13	Homeo box A13	XM_575481	2.72	0.047
		Pdgfrb	Platelet derived growth factor receptor, beta polypeptide	NM_031525	2.54	0.04
		Six1	SIX homeobox 1	NM_053759	2.48	0.025
		B4galt1	UDP-Gal:betaglcnac beta 1,4- galactosyltransferase, polypeptide 1	NM_053287	2.35	0.009
		Cdkn2b	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	NM_130812	2.31	0.034
		Birc3	Baculoviral IAP repeat-containing 3	NM_023987	2.3	0.012
		Gata6	GATA binding protein 6	NM_019185	2.27	0.035
		Prkcd	Protein kinase C, delta	NM_133307	2.2	0.013
		Id1	Inhibitor of DNA binding 1	NM_012797	2.17	0.032
		Pparg	Peroxisome proliferator-activated receptor gamma	NM_001145367	2.17	0.024
		Mitf	Microphthalmia-associated transcription factor	NM_001191089	2.12	0.019
		Casp14	Caspase 14	NM_001191776	2.06	0.019
		Hand2	Heart and neural crest derivatives expressed 2	NM_022696	2.03	0.038
		F3	Coagulation factor III (thromboplastin, tissue factor)	NM_013057	2,00	0.04

		Gene Symbol	Gene name	Accession Number	Fold Change 24h	*p*-value
	Down-regulated	**Cd27**	CD27 molecule	NM_001024335	0.31	0.035
		Grin1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	NM_017010	0.39	0.038
		Dhcr24	24-dehydrocholesterol reductase	NM_001080148	0.43	0.045
		Grin2a	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A	NM_012573	0.44	0.038
		Ptgs2	Prostaglandin-endoperoxide synthase 2	NM_017232	0.48	0.008

		Gene Symbol	Gene name	Accession Number	Fold Change 24h	*p*-value
Inflammatory Response	Up-regulated	**Cxcl2**	Chemokine (C-X-C motif) ligand 2	NM_053647	8.61	0.049
		Cxcl10	Chemokine (C-X-C motif) ligand 10	NM_139089	7.57	0.015
		Reg3g	Regenerating islet-derived 3 gamma	NM_173097	6.57	0.005
		Calca	Calcitonin-related polypeptide alpha	NM_017338	5.11	0.032
		Anxa1	Annexin A1	NM_012904	4.62	0.018
		Il5	Interleukin 5	NM_021834	4.57	0.026
		Olr1	Oxidized low density lipoprotein (lectin-like) receptor 1	NM_133306	4.1	0.016
		Fgg	Fibrinogen gamma chain	NM_012559	3.36	0.039
		Ccl3	Chemokine (C-C motif) ligand 3	NM_013025	3.27	0.018
		Cd44	Cd44 molecule	NM_012924	3.07	0.032
		Ccr5	Chemokine (C-C motif) receptor 5	NM_053960	2.75	0.006
		S100a9	S100 calcium binding protein A9	NM_053587	2.49	0.029
		Il1rl1	Interleukin 1 receptor-like 1	NM_001127689	2.39	0.022
		B4galt1	UDP-Gal:betaglcnac beta 1,4- galactosyltransferase, polypeptide 1	NM_053287]	2.35	0.009
		Pparg	Peroxisome proliferator-activated receptor gamma	NM_001145367	2.17	0.024
		F3	Coagulation factor III (thromboplastin, tissue factor)	NM_013057	2,00	0.04

		Gene Symbol	Gene name	Accession Number	Fold Change 24h	*p*-value
	Down-regulated	F2	Coagulation factor II (thrombin)	NM_022924	0.3	0.009
		Ptgs2	Prostaglandin-endoperoxide synthase 2	NM_017232	0.48	0.008

		Gene Symbol	Gene name	Accession Number	Fold Change 24h	*p*-value
Ion Transmembrane Transporter Activity	Up-regulated	S100a6	S100 calcium binding protein A6	NM_053485	7.79	0.027
		Clca2	Chloride channel calcium activated 2	NM_001013202	4.67	0.038
		Ucp3	Uncoupling protein 3 (mitochondrial, proton carrier)	NM_013167	4.16	0.012
		Slc17a1	Solute carrier family 17 (sodium phosphate), member 1	NM_133554	3.4	0.012
		Clic1	Chloride intracellular channel 1	NM_001002807	3.04	0.017
		Clic5	Chloride intracellular channel 5	NM_053603	2.26	0.026
		Slco2b1	Solute carrier organic anion transporter family, member 2b1	NM_080786	2.17	0.048
		Kcna10	Potassium voltage-gated channel, shaker-related subfamily, member 10	NM_001191713	2.1	0.026
		Atp1b4	Atpase, (Na+)/K+ transporting, beta 4 polypeptide	NM_053381	2.07	0.026
		Slc12a7	Solute carrier family 12 (potassium/chloride transporters), member 7	NM_001013144	2.07	0.045
		Atp1a4	Atpase, Na+/K+ transporting, alpha 4 polypeptide	NM_022848	2.02	0.018

		Gene Symbol	Gene name	Accession Number	Fold Change 24h	*p*-value
	Down-regulated	Grin1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	NM_017010	0.39	0.038
		Trpc5	Transient receptor potential cation channel, subfamily C, member 5	NM_080898	0.41	0.048
		Grin2a	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A	NM_012573	0.44	0.038
		Grin2b	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	NM_012574	0.44	0.022
		Kcnq3	Potassium voltage-gated channel, KQT-like subfamily, member 3	NM_031597	0.46	0.036
		Gabra3	Gamma-aminobutyric acid (GABA) A receptor, alpha 3	NM_017069	0.47	0.004
		Gabrb3	Gamma-aminobutyric acid (GABA) A receptor, beta 3	NM_017065	0.48	0.015
		Rgd156249	Similar to Orphan sodium- and chloride-dependent neurotransmitter	NM_001109146	0.5	0.038

		2	transporter NTT5 (Solute carrier family 6 member 16)			
		Slc8a1	Solute carrier family 8 (sodium/calcium exchanger), member 1	NM_019268	0.5	0.022

		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 24h**	***p*-value**
Metabolic Process	**Up-regulated**	**Eif4g2**	Eukaryotic translation initiation factor 4, gamma 2	AB256044	12.23	0.005
		Loc293989	Connective tissue growth factor	NM_022266	9.84	0.012
		Ctgf	Connective tissue growth factor	NM_022266	9.84	0.012
		Ptgr1	Prostaglandin reductase 1	NM_138863	7.03	0.028
		Hmga2	High mobility group AT-hook 2	NM_032070	6.99	0.023
		Cd80	Cd80 molecule	NM_012926	6.69	0.049
		Lhx1	LIM homeobox 1	NM_145880	6.02	0.022
		Loc687736	Similar to double homeobox, 4	XM_001079968	5.48	0.05
		Akr1b8	Aldo-keto reductase family 1, member B8	NM_173136	5.34	0.031
		Agpat2	1-acylglycerol-3-phosphate O-acyltransferase 2 (lysophosphatidic acid acyltransferase, beta)	NM_001107821	5.26	0.031
		Srpx2	Sushi-repeat-containing protein, X-linked 2	NM_001108243	5.22	0.034
		Calca	Calcitonin-related polypeptide alpha	NM_017338	5.11	0.032
		Loc300308	Similar to hypothetical protein 4930509O22	XM_001079328	5.08	0.016
		Rgd1565071	Similar to hypothetical protein 4930509O22	XM_001079328	5.08	0.016
		Angptl4	Angiopoietin-like 4	NM_199115	4.91	0.008
		Rgd1563833	Similar to putative homeobox protein	ENSRNOT00000066263	4.82	0.029
		Rgd1564033	Similar to Homeobox protein OTX1	XM_001059672	4.73	0.041
		Anxa1	Annexin A1	NM_012904	4.62	0.018
		Il5	Interleukin 5	NM_021834	4.57	0.026
		Casp1	Caspase 1	NM_012762	4.56	0.026
		Mycs	Myc-like oncogene, s-myc protein	NM_021837	4.25	0.042

		Pla1a	Phospholipase A1 member A	NM_138882	4.24	0.031
		Ucp3	Uncoupling protein 3 (mitochondrial, proton carrier)	NM_013167	4.16	0.012
		Olr1	Oxidized low density lipoprotein (lectin-like) receptor 1	NM_133306	4.1	0.016
		Rgd1563334	Similar to novel protein similar to esterases	XM_578509	4.1	0.023
		Loc503327	Similar to serine/threonine kinase	XM_578861	3.96	0.023
		Akr1c18	Aldo-keto reductase family 1, member C18	NM_138510	3.91	0.016
		Loc100365510	Homeobox protein-like	XM_002727408	3.7	0.033
		Adamts14	ADAM metallopeptidase with thrombospondin type 1 motif, 14	NM_001107636	3.68	0.004
		Loc100363448	Double homeobox, 1-like	XM_002728977	3.62	0.042
		Map3k6	Mitogen-activated protein kinase kinase kinase 6	NM_001107909	3.48	0.044
		Rgd1561620	Similar to isopentenyl diphosphate delta-isomerase type 2	XM_225507	3.42	0.034
		Hoxb7	Homeo box B7	NM_001017480	3.41	0.038
		Loc686581	Similar to transmembrane protease, serine 11A	XM_001074837	3.4	0.017
		Rbms1	RNA binding motif, single stranded interacting protein 1	NM_001012184	3.32	0.042
		Itgb3	Integrin, beta 3	NM_153720	3.28	0.039
		Pcolce	Procollagen C-endopeptidase enhancer	NM_019237	3.25	0.045
		Loc687536	Similar to Forkhead box protein F1 (Forkhead-related protein FKHL5) (Forkhead-related transcription factor 1) (FREAC-1) (Hepatocyte nuclear factor 3 forkhead homolog 8) (HFH-8)	XM_001079002	3.17	0.026
		Mgst2	Microsomal glutathione S-transferase 2	NM_001106430	3.14	0.014
		Cd44	Cd44 molecule	NM_012924	3.07	0.032
		Loc291480	Similar to ribosomal protein L19	XM_225800	3.01	0.02
		Plcb2	Phospholipase C, beta 2	NM_053478	2.94	0.034
		Mmp3	Matrix metallopeptidase 3	NM_133523	2.94	0.041
		Ptgs1	Prostaglandin-endoperoxide synthase 1	NM_017043	2.92	0.004
		Icam1	Intercellular adhesion molecule 1	NM_012967	2.91	0.033
		Nr6a1	Nuclear receptor subfamily 6, group A, member 1	XM_001060553	2.87	0.038

		Lyve1	Lymphatic vessel endothelial hyaluronan receptor 1	NM_001106286	2.8	0.038
		Nqo1	NAD(P)H dehydrogenase, quinone 1	NM_017000	2.76	0.037
		Ccr5	Chemokine (C-C motif) receptor 5	NM_053960	2.75	0.006
		Hoxa13	Homeo box A13	XM_575481	2.72	0.047
		Rgd1565321	Similar to MAP/microtubule affinity-regulating kinase 4 (MAP/microtubule affinity-regulating kinase like 1)	XM_344642	2.71	0.014
		Rac2	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	NM_001008384	2.64	0.008
		Gcm1	Glial cells missing homolog 1 (Drosophila)	NM_017186	2.63	0.002
		Foxs1	Forkhead box S1	NM_001012091	2.61	0.038
		Pfkfb1	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1	ENSRNOT00000033656	2.56	0.004
		Pdgfrb	Platelet derived growth factor receptor, beta polypeptide	NM_031525	2.54	0.04
		S100a9	S100 calcium binding protein A9	NM_053587	2.49	0.029
		Six1	SIX homeobox 1	NM_053759	2.48	0.025
		Cyp2d2	Cytochrome P450, family 2, subfamily d, polypeptide 2	NM_012730	2.48	0.021
		G6pc	Glucose-6-phosphatase, catalytic subunit	NM_013098	2.47	0.015
		Sult2al1	Sulfotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring-like 1	NM_012695	2.45	0.003
		Loxl4	Lysyl oxidase-like 4	NM_001107592	2.43	0.011
		Rgd1565390	Similar to putative protein kinase	XM_344843	2.39	0.037
		Cyp2c23	Cytochrome P450, family 2, subfamily c, polypeptide 23	NM_031839	2.37	0.011
		B4galt1	UDP-Gal:betaglcnac beta 1,4- galactosyltransferase, polypeptide 1	NM_053287	2.35	0.009
		P4ha3	Prolyl 4-hydroxylase, alpha polypeptide III	NM_198775	2.35	0.037
		Cdkn2b	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	NM_130812	2.31	0.034
		Gadd45a	Growth arrest and DNA-damage-inducible, alpha	NM_024127	2.29	0.03
		Rdh7	Retinol dehydrogenase 7	NM_133543	2.29	0.023
		Mmp25	Matrix metalloproteinase 25	XM_002742434	2.27	0.005
		Gata6	GATA binding protein 6	NM_019185	2.27	0.035
		Iyd	Iodotyrosine deiodinase	NM_001025000	2.25	0.031

		Etv2	Ets variant 2	ENSRNOT00000031873	2.24	0.027
		Adamts5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	NM_198761	2.24	0.005
		Dpep1	Dipeptidase 1 (renal)	NM_053591	2.23	0.035
		Ferd3l	Fer3-like (Drosophila)	NM_001108980	2.22	0.005
		Dse	Dermatan sulfate epimerase	NM_001108933	2.21	0.028
		Prkcd	Protein kinase C, delta	NM_133307	2.2	0.013
		Cpn1	Carboxypeptidase N, polypeptide 1	NM_053526	2.18	0.045
		Vegfc	Vascular endothelial growth factor C	NM_053653	2.17	0.011
		Samd4a	Sterile alpha motif domain containing 4A	NM_001107254	2.17	0.017
		Sdr16c5	Short chain dehydrogenase/reductase family 16C, member 5	NM_001106634	2.17	0.004
		Id1	Inhibitor of DNA binding 1	NM_012797	2.17	0.032
		Pparg	Peroxisome proliferator-activated receptor gamma	NM_001145367	2.17	0.024
		Mixl1	Mix1 homeobox-like 1 (Xenopus laevis)	NM_001105979	2.15	0.005
		Tgm6	Transglutaminase 6	XM_001079039	2.14	0.004
		Cpm	Carboxypeptidase M	NM_001108098	2.12	0.045
		Mitf	Microphthalmia-associated transcription factor	NM_001191089	2.12	0.019
		Nme4	Non-metastatic cells 4, protein expressed in	NM_001109478	2.11	0.044
		Atp1b4	Atpase, (Na+)/K+ transporting, beta 4 polypeptide	NM_053381	2.07	0.026
		Casp14	Caspase 14	NM_001191776	2.06	0.019
		Rgd1564878	Similar to natural killer cell protease 7	XM_001058604	2.06	0.013
		Enpp3	Ectonucleotide pyrophosphatase/phosphodiesterase 3	NM_019370	2.06	0.043
		Ugt2b37	UDP-glucuronosyltransferase 2 family, member 37	NM_001007264	2.04	0.017
		Hand2	Heart and neural crest derivatives expressed 2	NM_022696	2.03	0.038
		Vrk2	Vaccinia related kinase 2	NM_001108366	2.03	0.025
		Atp1a4	Atpase, Na+/K+ transporting, alpha 4 polypeptide	NM_022848	2.02	0.018
		Cfb	Complement factor B	NM_212466	2.02	0.027
		Sqrdl	Sulfide quinone reductase-like (yeast)	NM_001047913	2.01	0.032

		Loc682652	Similar to 60S ribosomal protein L29 (P23)	XM_001062490	2,00	0.011
		Hoxc4	Homeo box C4	NM_001109884	2,00	0.018
		F3	Coagulation factor III (thromboplastin, tissue factor)	NM_013057	2,00	0.04
		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 24h**	***p*-value**
	Down-regulated	**Agbl1**	ATP/GTP binding protein-like 1	XM_218798	0.27	0.015
		F2	Coagulation factor II (thrombin)	NM_022924	0.3	0.009
		Grin1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	NM_017010	0.39	0.038
		Gal3st3	Galactose-3-O-sulfotransferase 3	NM_001024290	0.4	0.042
		Tesc	Tescalcin	ENSRNOT00000001490	0.41	0.02
		Prss35	Protease, serine, 35	NM_001008560	0.41	0.049
		Dhcr24	24-dehydrocholesterol reductase	NM_001080148	0.43	0.045
		Grin2a	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A	NM_012573	0.44	0.038
		Grin2b	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	NM_012574	0.44	0.022
		Nfil3	Nuclear factor, interleukin 3 regulated	NM_053727	0.45	0.048
		Fam65b	Family with sequence similarity 65, member B	NM_001014009	0.46	0.002
		Prkce	Protein kinase C, epsilon	NM_017171	0.46	0.022
		Kndc1	Kinase non-catalytic C-lobe domain (KIND) containing 1	ENSRNOT00000034426	0.47	0.03
		Ptgs2	Prostaglandin-endoperoxide synthase 2	NM_017232	0.48	0.008
		Ambp	Alpha-1-microglobulin/bikunin precursor	NM_012901	0.48	0.005
		Adra1b	Adrenergic, alpha-1B-, receptor	NM_016991	0.48	0.022
		Mapk10	Mitogen activated protein kinase 10	NM_012806	0.49	0.025

Neurotransmitter secretion	**Down-regulated**	**Gene Symbol**	**Gene name**	**Accession Number**	**Fold Change 24h**	***p*-value**
		Syt1	Synaptotagmin I	NM_001033680	0.44	0.031

		Gene Symbol	Gene name	Accession Number	Fold Change 24h	*p*-value
Signaling Pathways	**Up-regulated**	**Ctgf**	Connective tissue growth factor	NM_022266	9.84	0.012
		Hmga2	High mobility group AT-hook 2	NM_032070	6.99	0.023
		Olr1297	Olfactory receptor 1297	NM_001000461	5.93	0.045
		Calca	Calcitonin-related polypeptide alpha	NM_017338	5.11	0.032
		Anxa1	Annexin A1	NM_012904	4.62	0.018
		Il5	Interleukin 5	NM_021834	4.57	0.026
		Casp1	Caspase 1	NM_012762	4.56	0.026
		Npvf	Neuropeptide VF precursor	NM_023952	3.99	0.021
		Olr1595	Olfactory receptor 1595	NM_001000500	3.93	0.011
		Olr60	Olfactory receptor 60	NM_001000748	3.88	0.048
		Rab32	RAB32, member RAS oncogene family	NM_001108902	3.77	0.031
		Olr907	Olfactory receptor 907	NM_001001357	3.75	0.041
		Olr1443	Olfactory receptor 1443	NM_001000018	3.69	0.009
		Rgd1564327	Similar to integrin alpha 8	NM_001173972	3.52	0.027
		Olr1666	Olfactory receptor 1666	NM_001000108	3.31	0.026
		Itgb3	Integrin, beta 3	NM_153720	3.28	0.039
		Olr1185	Olfactory receptor 1185	NM_001000982	3.27	0.009
		Gpr39	G protein-coupled receptor 39	NM_001100943	3.25	0.021
		Olr1261	Olfactory receptor 1261	NM_001000804	3.1	0.04
		Cd44	Cd44 molecule	NM_012924	3.07	0.032
		Plcb2	Phospholipase C, beta 2	NM_053478	2.94	0.034
		Olr1700	Olfactory receptor 1700	NM_001001113	2.88	0.028
		Olr834	Olfactory receptor 834	NM_001000407	2.82	0.022
		Ccr5	Chemokine (C-C motif) receptor 5	NM_053960	2.75	0.006

		Olr1453	Olfactory receptor 1453	NM_001000772	2.74	0.043
		Olr1012	Olfactory receptor 1012	NM_001000071	2.73	0.002
		Hoxa13	Homeo box A13	XM_575481	2.72	0.047
		Rac2	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	NM_001008384	2.64	0.008
		Pfkfb1	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1	ENSRNOT00000033656	2.56	0.004
		Pdgfrb	Platelet derived growth factor receptor, beta polypeptide	NM_031525	2.54	0.04
		Olr1361	Olfactory receptor 1361	NM_173333	2.5	0.008
		Il1rl1	Interleukin 1 receptor-like 1	NM_001127689	2.39	0.022
		Olr326	Olfactory receptor 326	NM_001000248	2.34	0.025
		Olr1061	Olfactory receptor 1061	NM_001000065	2.32	0.032
		Cdkn2b	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	NM_130812	2.31	0.034
		Gadd45a	Growth arrest and DNA-damage-inducible, alpha	NM_024127	2.29	0.03
		Olr1368	Olfactory receptor 1368	NM_214825	2.28	0.026
		Olr1369	Olfactory receptor 1369	NM_001000494	2.28	0.023
		Gata6	GATA binding protein 6	NM_019185	2.27	0.035
		Olr1512	Olfactory receptor 1512	NM_001001350	2.26	0.014
		Olr1174	Olfactory receptor 1174	NM_001001016	2.25	0.023
		Asb15	Ankyrin repeat and SOCS box-containing protein 15	ENSRNOT00000008917	2.23	0.004
		Prkcd	Protein kinase C, delta	NM_133307	2.2	0.013
		Olr718	Olfactory receptor 718	NM_001000361	2.2	0.024
		Vegfc	Vascular endothelial growth factor C	NM_053653	2.17	0.011
		Olr185	Olfactory receptor 185	NM_001000183	2.17	0.004
		Id1	Inhibitor of DNA binding 1	NM_012797	2.17	0.032
		Rgd1563046	Similar to cerberus-like	NM_001115031	2.16	0.031
		Vom1r52	Vomeronasal 1 receptor 52	NM_001008930	2.14	0.018
		Olr1022	Olfactory receptor 1022	NM_001001075	2.14	0.043

		Olr1440	Olfactory receptor 1440	NM_001000017	2.13	0.031
		Loc685368	Similar to Ral guanine nucleotide dissociation stimulator (ralgef) (ralgds)	XM_001063509	2.12	0.019
		Mitf	Microphthalmia-associated transcription factor	NM_001191089	2.12	0.019
		Olr464	Olfactory receptor 464	NM_001000296	2.12	0.018
		Olr713	Olfactory receptor 713	NM_001000360	2.08	0.022
		Olr768	Olfactory receptor 768	NM_001000919	2.08	0.007
		Olr306	Olfactory receptor 306	NM_001000766	2.07	0.003
		Olr233	Olfactory receptor 233	NM_001000207	2.07	0.036
		Olr866	Olfactory receptor 866	NM_001000411	2.04	0.031
		Chrm2	Cholinergic receptor, muscarinic 2	NM_031016	2.01	0.012
		Olr127	Olfactory receptor 127	NM_001001026	2.01	0.006

	Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 24h**	***p*-value**
Down-regulated	**Olr1256**	Olfactory receptor 1256	NM_001001086	0.17	0.011
	Gpr83	G protein-coupled receptor 83	NM_080411	0.3	0.031
	F2	Coagulation factor II (thrombin)	NM_022924	0.3	0.009
	Cd27	CD27 molecule	NM_001024335	0.31	0.035
	Grin1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	NM_017010	0.39	0.038
	Dhcr24	24-dehydrocholesterol reductase	NM_001080148	0.43	0.045
	Grin2a	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A	NM_012573	0.44	0.038
	Grin2b	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	NM_012574	0.44	0.022
	Prkce	Protein kinase C, epsilon	NM_017171	0.46	0.022
	Gabra3	Gamma-aminobutyric acid (GABA) A receptor, alpha 3	NM_017069	0.47	0.004
	Kndc1	Kinase non-catalytic C-lobe domain (KIND) containing 1	ENSRNOT00000034426	0.47	0.03
	Adra1b	Adrenergic, alpha-1B-, receptor	NM_016991	0.48	0.022
	Mapk10	Mitogen activated protein kinase 10	NM_012806	0.49	0.025
	Rab6b	RAB6B, member RAS oncogene family	NM_001108775	0.5	0.039

		Gene Symbol	Gene name	Accession Number	Fold Change 24h	*p*-value
Synapse	**Up-regulated**	**Cald1**	Caldesmon 1	NM_013146	2.55	0.011
		Samd4a	Sterile alpha motif domain containing 4A	NM_001107254	2.17	0.017
		Chrm2	Cholinergic receptor, muscarinic 2	NM_031016	2.01	0.012
		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 24h**	***p*-value**
	Down-regulated	**Grin1**	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	NM_017010	0.39	0.038
		Grin2a	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A	NM_012573	0.44	0.038
		Syt1	Synaptotagmin I	NM_001033680	0.44	0.031
		Grin2b	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	NM_012574	0.44	0.022
		Clstn2	Calsyntenin 2	NM_134377	0.44	0.018
		Cabp1	Calcium binding protein 1	NM_001033676	0.47	0.041
		Gabra3	Gamma-aminobutyric acid (GABA) A receptor, alpha 3	NM_017069	0.47	0.004
		Gabrb3	Gamma-aminobutyric acid (GABA) A receptor, beta 3	NM_017065	0.48	0.015

		Gene Symbol	Gene name	Accession Number	Fold Change 24h	*p*-value
Transcription	**Up-regulated**	**Ferd3l**	Connective tissue growth factor	NM_022266	9.84	0.012
		Hmga2	High mobility group AT-hook 2	NM_032070	6.99	0.023
		Lhx1	LIM homeobox 1	NM_145880	6.02	0.022
		Loc687736	Similar to double homeobox, 4	XM_001079968	5.48	0.05
		Calca	Calcitonin-related polypeptide alpha	NM_017338	5.11	0.032
		Rgd1563833	Similar to putative homeobox protein	ENSRNOT00000066263	4.82	0.029
		Rgd1564033	Similar to Homeobox protein OTX1	XM_001059672	4.73	0.041

		Mycs	Myc-like oncogene, s-myc protein	NM_021837	4.25	0.042
		Loc100365510	Homeobox protein-like	XM_002727408	3.7	0.033
		Loc100363448	Double homeobox, 1-like	XM_002728977	3.62	0.042
		Hoxb7	Homeo box B7	NM_001017480	3.41	0.038
		Loc687536	Similar to Forkhead box protein F1 (Forkhead-related protein FKHL5) (Forkhead-related transcription factor 1) (FREAC-1) (Hepatocyte nuclear factor 3 forkhead homolog 8) (HFH-8)	XM_001079002	3.17	0.026
		Icam1	Intercellular adhesion molecule 1	NM_012967	2.91	0.033
		Nr6a1	Nuclear receptor subfamily 6, group A, member 1	XM_001060553	2.87	0.038
		Hoxa13	Homeo box A13	XM_575481	2.72	0.047
		Gcm1	Glial cells missing homolog 1 (Drosophila)	NM_017186	2.63	0.002
		Foxs1	Forkhead box S1	NM_001012091	2.61	0.038
		Six1	SIX homeobox 1	NM_053759	2.48	0.025
		Cdkn2b	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	NM_130812	2.31	0.034
		Gata6	GATA binding protein 6	NM_019185	2.27	0.035
		Etv2	Ets variant 2	ENSRNOT00000031873	2.24	0.027
		Id1	Inhibitor of DNA binding 1	NM_012797	2.17	0.032
		Pparg	Peroxisome proliferator-activated receptor gamma	NM_001145367	2.17	0.024
		Mixl1	Mix1 homeobox-like 1 (Xenopus laevis)	NM_001105979	2.15	0.005
		Mitf	Microphthalmia-associated transcription factor	NM_001191089	2.12	0.019
		Atp1b4	Atpase, (Na+)/K+ transporting, beta 4 polypeptide	NM_053381	2.07	0.026
		Hand2	Heart and neural crest derivatives expressed 2	NM_022696	2.03	0.038
		Hoxc4	Homeo box C4	NM_001109884	2,00	0.018

	Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 24h**	***p*-value**
Down-regulated	**Grin1**	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	NM_017010	0.39	0.038
	Tesc	Tescalcin	ENSRNOT00000001490	0.41	0.02

		Nfil3	Nuclear factor, interleukin 3 regulated	NM_053727	0.45	0.048

		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 24h**	***p*-value**
Receptor Activity	**Up-regulated**	**Olr1012**	Connective tissue growth factor	NM_022266	9.84	0.012
		Mrgprf	MAS-related GPR, member F	NM_153722	6.88	0,000
		Vom2r73	Vomeronasal 2 receptor, 73	NM_001099486	6.16	0.03
		Olr1297	Olfactory receptor 1297	NM_001000461	5.93	0.045
		Loc680894	Similar to putative pheromone receptor (Go-VN4)	XM_001059364	5.11	0.025
		Olr1	Oxidized low density lipoprotein (lectin-like) receptor 1	NM_133306	4.1	0.016
		Olr1595	Olfactory receptor 1595	NM_001000500	3.93	0.011
		Ccr6	Chemokine (C-C motif) receptor 6	NM_001013145	3.88	0.013
		Olr60	Olfactory receptor 60	NM_001000748	3.88	0.048
		Olr907	Olfactory receptor 907	NM_001001357	3.75	0.041
		Olr1443	Olfactory receptor 1443	NM_001000018	3.69	0.009
		Rgd1564327	Similar to integrin alpha 8	NM_001173972	3.52	0.027
		Vom2r3	Vomeronasal 2 receptor, 3	NM_001099460	3.38	0.009
		Olr1666	Olfactory receptor 1666	NM_001000108	3.31	0.026
		Itgb3	Integrin, beta 3	NM_153720	3.28	0.039
		Olr1185	Olfactory receptor 1185	NM_001000982	3.27	0.009
		Gpr39	G protein-coupled receptor 39	NM_001100943	3.25	0.021
		Olr1261	Olfactory receptor 1261	NM_001000804	3.1	0.04
		Cd44	Cd44 molecule	NM_012924	3.07	0.032
		Ly49i4	Ly49 inhibitory receptor 4	NM_001009495	3.06	0.021
		Olr1700	Olfactory receptor 1700	NM_001001113	2.88	0.028
		Nr6a1	Nuclear receptor subfamily 6, group A, member 1	XM_001060553	2.87	0.038
		Olr834	Olfactory receptor 834	NM_001000407	2.82	0.022

		Lyve1	Lymphatic vessel endothelial hyaluronan receptor 1	NM_001106286	2.8	0.038
		Ccr5	Chemokine (C-C motif) receptor 5	NM_053960	2.75	0.006
		Olr1453	Olfactory receptor 1453	NM_001000772	2.74	0.043
		Pdgfrb	Platelet derived growth factor receptor, beta polypeptide	NM_031525	2.54	0.04
		Olr1361	Olfactory receptor 1361	NM_173333	2.5	0.008
		Vom2r49	Vomeronasal 2 receptor, 49	NM_001099515	2.48	0.007
		Loxl4	Lysyl oxidase-like 4	NM_001107592	2.43	0.011
		Il1rl1	Interleukin 1 receptor-like 1	NM_001127689	2.39	0.022
		Olr326	Olfactory receptor 326	NM_001000248	2.34	0.025
		Clec4b2	C-type lectin domain family 4, member b2	NM_001005896	2.34	0.045
		Olr1061	Olfactory receptor 1061	NM_001000065	2.32	0.032
		Olr1368	Olfactory receptor 1368	NM_214825	2.28	0.026
		Olr1369	Olfactory receptor 1369	NM_001000494	2.28	0.023
		Cxcl16	Chemokine (C-X-C motif) ligand 16	NM_001017478	2.28	0.015
		Olr1512	Olfactory receptor 1512	NM_001001350	2.26	0.014
		Olr1174	Olfactory receptor 1174	NM_001001016	2.25	0.023
		Olr718	Olfactory receptor 718	NM_001000361	2.2	0.024
		Olr185	Olfactory receptor 185	NM_001000183	2.17	0.004
		Pparg	Peroxisome proliferator-activated receptor gamma	NM_001145367	2.17	0.024
		Vom1r52	Vomeronasal 1 receptor 52	NM_001008930	2.14	0.018
		Olr1022	Olfactory receptor 1022	NM_001001075	2.14	0.043
		Olr1440	Olfactory receptor 1440	NM_001000017	2.13	0.031
		Olr464	Olfactory receptor 464	NM_001000296	2.12	0.018
		Olr713	Olfactory receptor 713	NM_001000360	2.08	0.022
		Olr768	Olfactory receptor 768	NM_001000919	2.08	0.007
		Olr306	Olfactory receptor 306	NM_001000766	2.07	0.003
		Olr233	Olfactory receptor 233	NM_001000207	2.07	0.036
		Enpp3	Ectonucleotide pyrophosphatase/phosphodiesterase 3	NM_019370	2.06	0.043

		Olr866	Olfactory receptor 866	NM_001000411	2.04	0.031
		Taar4	Trace amine-associated receptor 4	RatNM_175583	2.03	0.039
		Chrm2	Cholinergic receptor, muscarinic 2	NM_031016	2.01	0.012
		Olr127	Olfactory receptor 127	NM_001001026	2.01	0.006
		Vom2r71	Vomeronasal 2 receptor, 71	NM_001099516	2.01	0.029
	Down-regulated	**Gene Symbol**	**Gene name**	**Accession Number**	**Fold Change 24h**	***p*-value**
		Olr1256	Olfactory receptor 1256	NM_001001086	0.17	0.011
		Gpr83	G protein-coupled receptor 83	NM_080411	0.3	0.031
		F2	Coagulation factor II (thrombin)	NM_022924	0.3	0.009
		Cd27	CD27 molecule	NM_001024335	0.31	0.035
		Grin1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	NM_017010	0.39	0.038
		Trpc5	Transient receptor potential cation channel, subfamily C, member 5	NM_080898	0.41	0.048
		Grin2a	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A	NM_012573	0.44	0.038
		Grin2b	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	NM_012574	0.44	0.022
		Gabra3	Gamma-aminobutyric acid (GABA) A receptor, alpha 3	NM_017069	0.47	0.004
		Adra1b	Adrenergic, alpha-1B-, receptor	NM_016991	0.48	0.022
		Gabrb3	Gamma-aminobutyric acid (GABA) A receptor, beta 3	NM_017065	0.48	0.015
		Gpr123	G protein-coupled receptor 123	NM_001107559	0.49	0.028

Glutamate Secretion	**Down-regulated**	**Gene Symbol**	**Gene name**	**Accession Number**	**Fold Change 24h**	***p*-value**
		Grin2b	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	NM_012574	0.44	0.022

RNA Biosynthetic Process	**Up-regulated**	**Gene Symbol**	**Gene name**	**Accession Number**	**Fold Change 24h**	***p*-value**
		Cdkn2b	Connective tissue growth factor	NM_022266	9.84	0.012

Hmga2	High mobility group AT-hook 2	NM_032070	6.99	0.023
Lhx1	LIM homeobox 1	NM_145880	6.02	0.022
Loc687736	Similar to double homeobox, 4	XM_001079968	5.48	0.05
Rgd1563833	Similar to putative homeobox protein	ENSRNOT00000066263	4.82	0.029
Rgd1564033	Similar to Homeobox protein OTX1	XM_001059672	4.73	0.041
Mycs	Myc-like oncogene, s-myc protein	NM_021837	4.25	0.042
Loc100365510	Homeobox protein-like	XM_002727408	3.7	0.033
Loc100363448	Double homeobox, 1-like	XM_002728977	3.62	0.042
Hoxb7	Homeo box B7	NM_001017480	3.41	0.038
Loc687536	Similar to Forkhead box protein F1 (Forkhead-related protein FKHL5) (Forkhead-related transcription factor 1) (FREAC-1) (Hepatocyte nuclear factor 3 forkhead homolog 8) (HFH-8)	XM_001079002	3.17	0.026
Icam1	Intercellular adhesion molecule 1	NM_012967	2.91	0.033
Nr6a1	Nuclear receptor subfamily 6, group A, member 1	XM_001060553	2.87	0.038
Hoxa13	Homeo box A13	XM_575481	2.72	0.047
Gcm1	Glial cells missing homolog 1 (Drosophila)	NM_017186	2.63	0.002
Foxs1	Forkhead box S1	NM_001012091	2.61	0.038
Six1	SIX homeobox 1	NM_053759	2.48	0.025
Gata6	GATA binding protein 6	NM_019185	2.27	0.035
Etv2	Ets variant 2	ENSRNOT00000031873	2.24	0.027
Id1	Inhibitor of DNA binding 1	NM_012797	2.17	0.032
Pparg	Peroxisome proliferator-activated receptor gamma	NM_001145367	2.17	0.024
Mixl1	Mix1 homeobox-like 1 (Xenopus laevis)	NM_001105979	2.15	0.005
Mitf	Microphthalmia-associated transcription factor	NM_001191089	2.12	0.019
Hand2	Heart and neural crest derivatives expressed 2	NM_022696	2.03	0.038
Hoxc4	Homeo box C4	NM_001109884	2,00	0.018

		Gene Symbol	Gene name	Accession Number	Fold Change 24h	*p*-value
	Down-regulated	**Grin1**	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	NM_017010	0.39	0.038
		Tesc	Tescalcin	ENSRNOT00000001490	0.41	0.02
		Nfil3	Nuclear factor, interleukin 3 regulated	NM_053727	0.45	0.048

		Gene Symbol	Gene name	Accession Number	Fold Change 24h	*p*-value
Oxidative Stress	**Up-regulated**	**F3**	Connective tissue growth factor	NM_022266	9.84	0.012
		Anxa1	Annexin A1	NM_012904	4.62	0.018
		Ucp3	Uncoupling protein 3 (mitochondrial, proton carrier)	NM_013167	4.16	0.012
		Olr1	Oxidized low density lipoprotein (lectin-like) receptor 1	NM_133306	4.1	0.016
		Ptgs1	Prostaglandin-endoperoxide synthase 1	NM_017043	2.92	0.004
		Nqo1	NAD(P)H dehydrogenase, quinone 1	NM_017000	2.76	0.037
		Pdgfrb	Platelet derived growth factor receptor, beta polypeptide	NM_031525	2.54	0.04
		Prkcd	Protein kinase C, delta	NM_133307	2.2	0.013
		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 24h**	***p*-value**
	Down-regulated	**Dhcr24**	24-dehydrocholesterol reductase	NM_001080148	0.43	0.045
		Ptgs2	Prostaglandin-endoperoxide synthase 2	NM_017232	0.48	0.008
		Slc8a1	Solute carrier family 8 (sodium/calcium exchanger), member 1	NM_019268	0.5	0.022

Supplemental Table 3. List of genes up-regulated and down-regulated at both 7h and 24h after OGD for different ontological classes. Gene ontology analyses included genes that had a *p*-value <0.05 and a fold change of 2.0 and were performed using GoMiner™. Classes were selected manually. Note that some genes are included in more than one class.

		Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value 7h	Fold Change 24h	*p*-value 24h
Apoptosis	**Up-regulated**	Gal	Galanin prepropeptide	NM_033237	9.39	0.004	5.6	0.029
		Serpine1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	NM_012620	5.03	0.018	9.77	0.028
		Foxb1	Forkhead box B1	NM_001013248	5.62	0.048	6.39	0.035
		Epha2	Eph receptor A2	NM_001108977	2.91	0.023	6.39	0.026
		Ripk3	Receptor-interacting serine-threonine kinase 3	NM_139342	2.98	0.049	5.02	0.025
		Cd44	Cd44 molecule	NM_012924	2.2	0.006	3.32	0.036
		Il1rn	Interleukin 1 receptor antagonist	NM_022194	2.79	0.001	2.2	0.024
	Down-regulated	**Gene Symbol**	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value 7h**	**Fold Change 24h**	***p*-value 24h**
		Acvr1c	Activin A receptor, type IC	NM_139090	0.49	0.023	0.3	0.043
		Pml	Promyelocytic leukemia	XM_236296	0.43	0.008	0.44	0.036

		Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value 7h	Fold Change 24h	*p*-value 24h
Inflammatory response	**Up-regulated**	Il1rl1	Interleukin 1 receptor-like 1	NM_013037	13.01	0.03	47.02	0.001
		Reg3b	Regenerating islet-derived 3 beta	NM_053289	17.43	0.015	29,00	0.007
		Cxcl1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	NM_030845	8.35	0.002	11.99	0.011
		Gal	Galanin prepropeptide	NM_033237	9.39	0.004	5.6	0.029
		Serpine1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	NM_012620	5.03	0.018	9.77	0.028
		Gpx2	Glutathione peroxidase 2	NM_183403	5.91	0.008	8.85	0.004

		Cd44	Cd44 molecule	NM_012924	2.2	0.006	3.32	0.036
		Il1rn	Interleukin 1 receptor antagonist	NM_022194	2.79	0.001	2.2	0.024

Ion Transmembr. Transporter Activity	**Down-regulated**	**Gene Symbol**	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value 7h**	**Fold Change 24h**	***p*-value 24h**
		Atp13a2	Activin A receptor, type IC	NM_139090	0.49	0.023	0.3	0.043

		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value 7h**	**Fold Change 24h**	***p*-value 24h**
Metabolic Process	**Up-regulated**	Gal	Galanin prepropeptide	NM_033237	9.39	0.004	5.6	0.029
		Serpine1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	NM_012620	5.03	0.018	9.77	0.028
		Gpx2	Glutathione peroxidase 2	NM_183403	5.91	0.008	8.85	0.004
		Plau	Plasminogen activator, urokinase	NM_013085	4.85	0.003	9.07	0.03
		Epha2	Eph receptor A2	NM_001108977	2.91	0.023	6.39	0.026
		Ripk3	Receptor-interacting serine-threonine kinase 3	NM_139342	2.98	0.049	5.02	0.025
		Lipg	Lipase, endothelial	NM_001012741	3,00	0.037	4.08	0.012
		Runx2	Runt-related transcription factor 2	NM_053470	2.86	0,000	2.98	0.049
		Hopx	HOP homeobox	NM_133621	2.08	0.032	3.47	0.037
		Cd44	Cd44 molecule	NM_012924	2.2	0.006	3.32	0.036
		Il1rn	Interleukin 1 receptor antagonist	NM_022194	2.79	0.001	2.2	0.024
		Adamts7	ADAM metallopeptidase with thrombospondin type 1 motif, 7	NM_001047101	2.29	0.018	2.64	0.023
		Mst1	Macrophage stimulating 1 (hepatocyte growth factor-like)	NM_024352	2.24	0.044	2.61	0.004
		Tgif1	TGFB-induced factor homeobox 1	NM_001015020	2.11	0.041	2.73	0.04
		Lbxcor1	LBXCOR1 homolog (mouse)	XM_002727063	2.35	0.042	2.49	0.024

		Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value 7h	Fold Change 24h	*p*-value 24h
	Down-regulated	Mmp28	Matrix metallopeptidase 28	NM_001079888	0.2	0.019	0.24	0.01
		Ube2ql1	Ubiquitin-conjugating enzyme E2Q family-like 1	NM_001145163	0.31	0.007	0.36	0.038
		Far2	Fatty acyl coa reductase 2	ENSRNOT00000002528	0.33	0.001	0.35	0.027
		Acvr1c	Activin A receptor, type IC	NM_139090	0.49	0.023	0.3	0.043
		Gpr26	G protein-coupled receptor 26	NM_138841	0.48	0.013	0.33	0.03
		St8sia5	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 5	NM_213628	0.49	0.007	0.37	0.016
		Pml	Promyelocytic leukemia	XM_236296	0.43	0.008	0.44	0.036
		Lass4	LAG1 homolog, ceramide synthase 4	NM_001107117	0.42	0.008	0.47	0.029
		Actn2	Actinin alpha 2	NM_001170325	0.48	0.001	0.44	0.032
		Atp13a2	Atpase type 13A2	NM_001173432	0.44	0.013	0.5	0.043
		Eno2	Enolase 2, gamma, neuronal	NM_139325	0.48	0.006	0.48	0.041

		Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value 7h	Fold Change 24h	*p*-value 24h
Signaling Pathway	**Up-regulation**	Il1rl1	Interleukin 1 receptor-like 1	NM_013037	13.01	0.03	47.02	0.001
		Gal	Galanin prepropeptide	NM_033237	9.39	0.004	5.6	0.029
		Postn	Periostin, osteoblast specific factor	NM_001108550	2.31	0.04	7.35	0.013
		Epha2	Eph receptor A2	NM_001108977	2.91	0.023	6.39	0.026
		Clcf1	Cardiotrophin-like cytokine factor 1	NM_207615	2.13	0.007	6.53	0.028
		Ripk3	Receptor-interacting serine-threonine kinase 3	NM_139342	2.98	0.049	5.02	0.025
		Runx2	Runt-related transcription factor 2	NM_053470	2.86	0,000	2.98	0.049
		Cd44	Cd44 molecule	NM_012924	2.2	0.006	3.32	0.036
		Il1rn	Interleukin 1 receptor antagonist	NM_022194	2.79	0.001	2.2	0.024
		Tgif1	TGFB-induced factor homeobox 1	NM_001015020	2.11	0.041	2.73	0.04
		Lbxcor1	LBXCOR1 homolog (mouse)	XM_002727063	2.35	0.042	2.49	0.024

		Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value 7h	Fold Change 24h	*p*-value 24h
	Down-regulation	Rasgrp1	RAS guanyl releasing protein 1 (calcium and DAG-regulated)	NM_019211	0.25	0.003	0.3	0.034
		Loc100360071	Neuropeptide S-like	ENSRNOT00000054896	0.19	0,000	0.38	0.05
		Chrm1	Cholinergic receptor, muscarinic 1	NM_080773	0.43	0.016	0.36	0.04
		Acvr1c	Activin A receptor, type IC	NM_139090	0.49	0.023	0.3	0.043
		Gpr26	G protein-coupled receptor 26	NM_138841	0.48	0.013	0.33	0.03
		Pml	Promyelocytic leukemia	XM_236296	0.43	0.008	0.44	0.036

		Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value 7h	Fold Change 24h	*p*-value 24h
Synapse	**Up-regulation**	Sypl2	Synaptophysin-like 2	NM_013037	13.01	0.03	47.02	0.001
		Gene Symbol	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value 7h**	**Fold Change 24h**	***p*-value 24h**
	Down-regulation	Chrm1	Cholinergic receptor, muscarinic 1	NM_080773	0.43	0.016	0.36	0.04
		Clstn3	Calsyntenin 3	NM_134376	0.45	0.014	0.39	0.036
		Dlgap2	Discs, large (Drosophila) homolog-associated protein 2	NM_053901	0.48	0.011	0.4	0.036

		Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value 7h	Fold Change 24h	*p*-value 24h
Transcription	**Up-regulation**	Tgif1	TGFB-induced factor homeobox 1	NM_013037	13.01	0.03	47.02	0.001
		Runx2	Runt-related transcription factor 2	NM_053470	2.86	0,000	2.98	0.049
		Hopx	HOP homeobox	NM_133621	2.08	0.032	3.47	0.037
		Lbxcor1	LBXCOR1 homolog (mouse)	XM_002727063	2.35	0.042	2.49	0.024

		Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value 7h	Fold Change 24h	*p*-value 24h
	Down-regulation	Pml	Promyelocytic leukemia	XM_236296	0.43	0.008	0.44	0.036
		Lass4	LAG1 homolog, ceramide synthase 4	NM_001107117	0.42	0.008	0.47	0.029
		Actn2	Actinin alpha 2	NM_001170325	0.48	0.001	0.44	0.032

		Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value 7h	Fold Change 24h	*p*-value 24h
Receptor Activity	**Up-regulation**	Tas2r120	Taste receptor, type 2, member 120	NM_001080937	3.91	0.047	6.21	0.008
		Il1rl1	Interleukin 1 receptor-like 1	NM_013037	13.01	0.03	47.02	0.001
		Cd93	CD93 molecule	NM_053383	4.77	0,000	6.84	0.008
		Epha2	Eph receptor A2	NM_001108977	2.91	0.023	6.39	0.026
		Procr	Protein C receptor, endothelial	NM_001025733	3.66	0.027	5.49	0.039
		Gpr4	G protein-coupled receptor 4	NM_001025680	3.24	0.01	3.72	0.035
		Cd44	Cd44 molecule	NM_012924	2.2	0.006	3.32	0.036
		Il1rn	Interleukin 1 receptor antagonist	NM_022194	2.79	0.001	2.2	0.024

		Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value 7h	Fold Change 24h	*p*-value 24h
	Down-regulation	Chrm1	Cholinergic receptor, muscarinic 1	NM_080773	0.43	0.016	0.36	0.04
		Acvr1c	Activin A receptor, type IC	NM_139090	0.49	0.023	0.3	0.043
		Gpr26	G protein-coupled receptor 26	NM_138841	0.48	0.013	0.33	0.03

		Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value 7h	Fold Change 24h	*p*-value 24h
Glutamate Secretion	**Up-regulated**	Il1rn	Interleukin 1 receptor antagonist	NM_022194	2.79	0.001	2.2	0.024

		Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value 7h	Fold Change 24h	*p*-value 24h
RNA Biosynthetic Process	**Up-regulation**	Hopx	HOP homeobox	NM_013037	13.01	0.03	47.02	0.001
		Runx2	Runt-related transcription factor 2	NM_053470	2.86	0,000	2.98	0.049
		Tgif1	TGFB-induced factor homeobox 1	NM_001015020	2.11	0.041	2.73	0.04
	Down-regulation	**Gene Symbol**	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value 7h**	**Fold Change 24h**	***p*-value 24h**
		Lass4	LAG1 homolog, ceramide synthase 4	NM_001107117	0.42	0.008	0.47	0.029

		Gene Symbol	Gene name	Accession Number	Fold Change 7h	*p*-value 7h	Fold Change 24h	*p*-value 24h
Response to Oxidative Stress	**Up-regulation**	Gpx2	Glutathione peroxidase 2	NM_013037	13.01	0.03	47.02	0.001
		Serpine1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	NM_012620	5.03	0.018	9.77	0.028
	Down-regulation	**Gene Symbol**	**Gene name**	**Accession Number**	**Fold Change 7h**	***p*-value 7h**	**Fold Change 24h**	***p*-value 24h**
		Pml	Promyelocytic leukemia	XM_236296	0.43	0.008	0.44	0.036