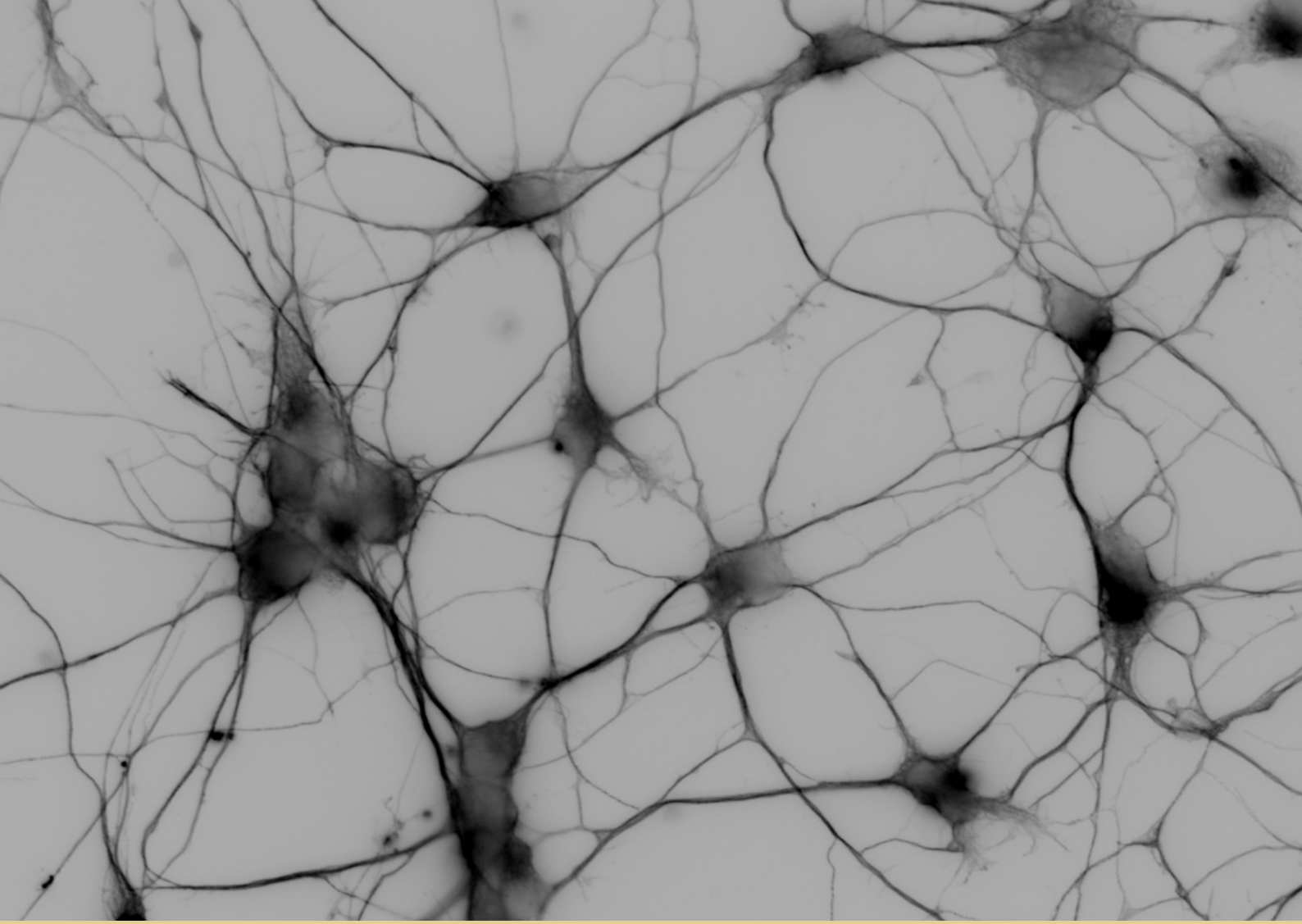

Miranda Mele

MODULATION OF $GABA_A$ RECEPTORS IN CEREBRAL ISCHEMIA:
ALTERATIONS IN RECEPTOR TRAFFICKING COUPLED TO NEURONAL DEATH AFTER OXYGEN/GLUCOSE DEPRIVATION

Tese de Doutoramento em Biociências na especialidade de Neurociências, orientada pelo Professor Carlos B. Duarte, apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra

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Universidade de Coimbra

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alterations in receptor trafficking coupled to neuronal death after oxygen/glucose deprivation

Miranda Mele

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Dissertação apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra para prestação de provas de Doutoramento em Biociências, na especialidade de Neurociências.

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Cover note:

The image presented in the cover of this thesis represents cultured hippocampal neurons labeled with an antibody against tubulin.

Dedico questa tesi ai miei genitori

e a tutte le persone che mi vogliono bene.

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The present thesis is mostly based on the work that has been submitted for publication in an international peer-reviewed journal

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Caldeira MV, Curcio M, Leal G, Salazar IL, **Mele M**, Santos AR, Melo CV, Pereira P, Canzoniero LM, Duarte CB (2013) Excitotoxic stimulation downregulates the ubiquitin-proteasome system through activation of NMDA receptors in cultured hippocampal neurons. Biochim Biophys Acta. 1832(1): 263-74.

Lobo AC, Gomes JR*, Catarino T*, **Mele M***, Fernandez P, Inácio AR, Bahr BA, Santos AE, Wieloch T, Carvalho AL, Duarte CB. (2011) Cleavage of the vesicular glutamate transporters under excitotoxic conditions. Neurobiol Dis. 44(3): 292-303.

* Equal contribution

INDEX

ABBREVIATIONS

AIF, apoptosis inducing factor

ALLN, N-acetyl-leu-leu-norleucinal

AMP, adenosine monophosphate

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPAR, AMPA receptor

ANOVA, analysis of variance

AP2, adaptor protein 2

APV, (2)-amino-5-phosphonovaleric acid; (2)-amino-5-phosphonopentanoate

ATP, adenosine-5'-triphosphate

BCA, bicinchoninic acid

Bcl-2, B-cell lymphoma 2 protein

BIG2, brefeldin A inhibited GDP/GTP exchange factor 2

Bim, Bcl2-interacting mediator of cell death

BZ, benzodiazepines

CA1, cornu ammonis 1 region of the hippocampus

$[Ca^{2+}]_i$, cytosolic calcium concentration

CaM, Ca^{2+}/calmodulin

CBP, CREB binding protein

CCVs, clathrin-coated vesicles

CD95, cluster of differentiation 95

cDNA, complementary DNA

ClC-2, voltage-gated Cl^- channel

CNS, central nervous system

CREB, cyclic-AMP response element binding protein

Ct, threshold cycle

DAPK, death-associated protein kinase

DG, dentate gyrus

DIV, days in vitro

DNA, deoxyribonucleic acid

dNTP, deoxyribonucleotide triphosphate

DOC, sodium deoxycholate

DTT, dithiothreitol

E, embryonic

ECF, enhanced chemifluorescence

E_{Cl^-}, chloride equilibrium potential

EDTA, ethylenediaminetetraacetic acid

EGTA, ethylene glycol tetraacetic acid

ELISA, enzyme-linked immunosorbent assay

EPSP, excitatory postsynaptic potentials

ER, endoplasmic reticulum

ERK, extracellular signal-regulated kinase

ERM, (ezrin, radixin, moesin) proteins

F-actin, filamentous actin

FasL, Fas ligand

FBS, FOXO-binding site

FOXO, forkhead box protein O

FRAP, fluorescence recovery after photobleaching

GABA, γ-aminobutyric acid

$GABA_AR$, GABA type A receptors

$GABA_BR$, GABA type B receptors

GABARAP, $GABA_AR$-associated protein

GAD, glutamic acid decarboxylase

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

GDP, guanosine diphosphate

GODZ, Golgi-specific DHHC zinc finger protein

GTP, guanosine triphosphate

HAP1, huntingtin-associated protein 1

HBSS, Hank's balanced salt solution

HEPES, hydroxyethyl piperazineethanesulfonic acid

HIF-1, hypoxia-inducible factor-1

i.e., *id est* (that is)

i.v., intravenous

IC, infarct core

ICD, intracytoplasmic domain

IL-1, interleukin 1

InsP3, inositol 1,4,5-trisphosphate

IP, immunoprecipitation

IPSPs, inhibitory postsynaptic potentials

IκB, inhibitor of NF-κB

Jacob, juxtasynaptic attractor of caldendrin on dendritic boutons protein

Lys, lysine

MAP2, microtubule-associated protein 2

MCA, middle cerebral artery

MCAO, MCA occlusion

MDL28170, N-[(1S)-1-[[(1-formyl-2-phenylethyl)amino]carbonyl]-2-methylpropyl]-carbamic acid, phenylmethyl ester

mGluR, metabotropic glutamate receptors

mRNA, messenger RNA

N.S., not significant

NBQX, 1,2,3,4-tetrahydro-6-nitro-2, 3-dioxo[f]quinoxaline-7-sulfonamide disodium

NF-kB, nuclear factor-kappa B

NMDA, N-methyl-D-aspartate

NMDAR, NMDA receptor

nNOS, neuronal NO synthase

NO, nitric oxide

NRSF, neuron-restrictive silencer factor

NSF, N-ethylmaleimide-sensitive factor

O^{2-}, superoxide anion

OGD, oxygen and glucose deprivation

p53, protein 53

PBS, phosphate buffered saline

PCR, polymerase chain reaction

PGG2, prostaglandin G2

PGH, prostaglandin H

Pi, inorganic phosphate

PI3K, phosphoinositide 3-kinase

PKA, cAMP-dependent protein kinase

PKC, calcium/phospholipid-dependent protein kinase C

PLIC, proteins linking integrin-assocated protein with cytoskeleton

PMSF, phenylmethylsulfonyl fluoride

PP1α, protein phosphatase 1α

PP2A, protein phosphatase 2A

PP2B, protein phosphatase 2B

PRIP, phospholipase C-related but catalytically inactive protein

PSD, postsynaptic density

PTEN, phosphatase and tensin homolog on chromosome ten

PVDF, polyvinylidene difluoride

qPCR, quantitative PCR

rCBF, regional cerebral blood flow

REST, RE1-silencing transcription factor

RIPA, radioimmunoprecipitation assay lysis buffer

RNA, ribonucleic acidB

RT, room temperature

SDS, sodium dodecyl sulfate

SEM, standard error of the mean

Ser, serine

SPT, single particle tracking

STATs, signal transducers and activators of transcription

STEP, striatal enriched tyrosine phosphatase

TE, tris-EDTA

Thr, threonine

TM, transmembrane domains

TNF, tumor necrosis factor

TORC, transducer of regulated CREB activity

TTC, triphenyltetrazolium chloride

tVGAT, truncated VGAT

Txnip, thioredoxin-interacting protein

Tyr, tyrosine

Uba, ubiquitin-associated

Ubl, ubiquitin-like

VGAT, vesicular GABA transporter

WT, wild type

Ψm, mitochondrial membrane potential

KEY WORDS

$GABA_A$ receptors

Cerebral ischemia

Oxygen/glucose deprivation (OGD)

Cell death

Hippocampus

PALAVRAS CHAVE

Receptores de GABA do tipo $GABA_A$

Isquémia cerebral

Ausência de oxigénio e glucose (OGD)

Morte celular

Hipocampo

SUMÁRIO

A isquémia cerebral resulta de um fornecimento insuficiente de sangue ao cérebro, levando a uma desregulação no equilíbrio entre a neurotransmissão excitatória/inibitória e consequente morte celular por excitotoxicidade. A actividade das redes neuronais no sistema nervoso central (SNC) é determinada maioritariamente pelo balanço entre a neurotransmissão glutamatérgica e GABAérgica, que se encontra aumentada e reduzida, respectivamente, nas lesões isquémicas. O papel desempenhado pelo glutamato nos danos isquémicos está largamente documentado, ao contrário das alterações na neurotransmissão inibitória que permanecem pouco estudadas.

Estudos in vivo e in vitro mostraram uma desregulação da neurotransmissão GABAérgica em cérebro isquémicos, ao nível pré- e pós-sináptico. A incubação de fatias de hipocampo na ausência de oxigénio e glucose (OGD) induz uma libertação rápida de GABA por exocitose, seguida de uma fase tardia em que ocorre a libertação do neurotransmissor mediada por reversão do transportador da membrana plasmática. A diminuição dos transportadores vesiculares do GABA na sinapse e a perda de ATP são dois mecanimos que podem estar na origem da redução da libertação excitotóxica de GABA. Os receptores de GABA do tipo $GABA_A$ ($GABA_AR$) são os principais intervenientes na inibição sináptica rápida no SNC e a diminuição da sua expressão superficial foi demonstrada em modelos de isquémia in vivo e in vitro. A estabilização da expressão superficial dos $GABA_AR$ foi recentemente correlacionada com a protecção de neurónios do hipocampo e do córtex cerebral sujeitos a OGD, e o bloqueio da internalização dos $GABA_AR$ dependente da interacção AP2/clatrina reduz também a morte neuronal causada pela OGD. Estas evidências indicam que o número de $GABA_AR$ na superfície celular e a internalização deste receptor desempenham um papel modulador da morte celular causada pela isquémia. Porém, os

mecanismos moleculares envolvidos na internalização dos $GABA_AR$ não foram ainda desvendados.

Neste trabalho investigámos os mecanismos moleculares envolvidos na diminuição dos $GABA_AR$ em culturas de hipocampo submetidas OGD, um modelo in vitro de isquémia cerebral. A exposição transitória de neurónios de hipocampo a OGD fez diminuir os níveis totais das subunidades dos receptores $GABA_A$ características de receptores sinápticos (α1, α2, β3, γ2), por um mecanismo dependente da activação de calpaínas, mas não afectou os níveis da subunidade δ, tipicamente encontrada em receptores extra-sinápticos. Resultados semelhantes foram observados na região de enfarte em murganhos sujeitos à oclusão da artéria cerebral média (MCAO). Experiências de PCR quantitativo mostraram uma diminuição da expressão das subunidades dos $GABA_AR$ do tipo α1, α2, β2, β3 e γ2 mediada pela activação dos receptores do glutamato. Contudo, a inibição da transcrição não contribuiu para a diminuição dos níveis de proteína total das subunidades dos $GABA_AR$.

A maioria dos $GABA_AR$ presentes no cérebro contêm as subunidades 2α, 2β, e 1γ2, e apresentam uma grande mobilidade entre a localização sináptica e extra-sináptica. A acumulação do receptor nas sinapses inibitórias é regulada pela gefirina, uma proteína estrutural que permite a estabilização dos $GABA_AR$ na sinapse. A população de $GABA_AR$ da superfície neuronal é reciclada continuamente entre a membrana plasmática e os compartimentos intracelulares. Os mecanismos de regulação da expressão superficial dos $GABA_AR$ desempenham um papel fundamental no controlo dos níveis de receptor na sinapse e, consequentemente, da actividade sináptica inibitória. A internalização dos $GABA_AR$ é regulada negativamente pela fosforilação das subunidades β3 ou γ2 numa sequência intracelular. Em condições fisiológicas normais a fosforilação destas subunidades é controlada pela calcineurina, uma fosfatase activada pela entrada de Ca^{2+} através dos receptores NMDA. Contudo, não foram ainda identificadas as alterações no controlo do tráfego dos receptores $GABA_A$ durante a isquémia cerebral. Neste estudo,

combinámos abordagens bioquímicas e de imagiologia celular para investigar os mecanismos que regulam a internalização dos $GABA_AR$ após um estímulo de OGD transitório. Verificámos que a OGD diminui a interação $GABA_AR$/Gefirina e induz a internalização dos $GABA_AR$ pela via endocítica dependente de clatrina. A redução da interação $GABA_AR$/Gefirina e o aumento da internalização dos $GABA_AR$ é regulada por fosforilação, como demonstrámos pelos ensaios de co-imunoprecipitação de proteínas com os receptores de superfície e pelo ensaio de "antibody-feeding", respectivamente. Seguidamente, mostrámos que a OGD induz a desfosforilação e a internalização das subunidades β3 dos receptores $GABA_A$, expressos em grande quantidade no hipocampo e no córtex cerebral, duas regiões particularmente vulneráveis à excitotoxicidade. Os dados obtidos usando fosfo-mutantes da subunidade β3 dos $GABA_AR$ permitiram-nos concluir que a desfosforilação do receptor causada pela OGD e a sua consequente internalização contribuem para a morte neuronal.

Após a internalização, os $GABA_AR$ são rapidamente reciclados e voltam para a membrana plasmática ou são encaminhados para os lisossomas a fim de serem degradados. O rumo que os $GABA_AR$ endocitados tomam depende da interacção das subunidades β1-3 com a proteína associada à huntingtina 1 (HAP1). Verificámos que a OGD reduz também a reciclagem dos $GABA_AR$ de volta para a membrana plasmática e diminui a sua interacção com a proteína HAP1.

Em resumo, neste trabalho propomos um novo modelo no qual a dissociação do complexo $GABA_AR$/Gefirina e a desfosforilação do receptor são passos fulcrais na diminuição da actividade GABAérgica durante a isquémia cerebral, com consequente morte neuronal.

SUMMARY

Cerebral ischemia is a pathological condition caused by insufficient blood supply to the brain, which causes an imbalance between excitatory/inhibitory neurotransmission and excitotoxic neuronal death. The activity of neuronal networks in the CNS is mainly determined by the balance between glutamatergic and GABAergic neurotransmission, which is up- and down-regulated, respectively, during ischemic insults. In contrast with the role of glutamate in ischemic damage, which is largely documented, the alterations in inhibitory neurotransmission remain poorly understood.

In vivo and in vitro studies have shown a downreregulation of GABAergic neurotransmission in the ischemic brain, both at the pre- and post-synaptic levels. Exposure of hippocampal slices to oxygen and glucose-deprivation induces an early release of GABA by exocytosis, followed by a delayed phase of neurotransmitter release mediated by reversal of the plasma membrane transporter. The downregulation of vesicular GABA transporters and the loss of ATP is likely to cause a delayed inhibition of exocytotic release of GABA. $GABA_A$ receptors ($GABA_AR$) are the major players in fast synaptic inhibition in the CNS, and a downregulation of the surface expression of $GABA_ARs$ has been shown in in vivo and in vitro models of ischemia. Furthermore, it was recently shown that stabilization of $GABA_AR$ surface expression correlates with neuroprotection in hippocampal and cerebrocortical neurons subjected to Oxygen Glucose Deprivation (OGD), and blockade of AP2/clathrin dependent internalization of $GABA_AR$ also reduces OGD induced cell death. Together, these evidence indicates that the number of $GABA_AR$ at the cell surface and receptor internalization play a key modulatory role in the induction of ischemic cell death, but the molecular mechanisms involved in receptor internalization have not been elucidated.

In the present work we investigated the molecular mechanisms underlying $GABA_AR$ downregulation in cultured hippocampal neurons

subjected to OGD, an in vitro model of ischemia. Transient exposure of hippocampal neurons to OGD downregulated the total protein levels of $GABA_AR$ subunits characteristic of synaptic receptors (α1, α2, β3, γ2), but was without effect on the δ subunit that is typically found in extrasynaptic recepors. Similar results were observed in the infarct core of mice subjected to middle cerebral artery occlusion (MCAO). The downregulation of $GABA_AR$ subunits in cultured hippocampal neurons subjected to OGD was mediated by calpains. Quantitative PCR experiment showed a decrease in the expression levels of α1, α2, β2, β3 and γ2 $GABA_AR$ subunits that was mediated by activation of glutamate receptor, but inhibition of transcription activity did not account for the downregulation of $GABA_AR$ subunit protein levels.

The majority of $GABA_AR$ in the brain are assembled from at least 2 α-, 2 β-, and 1 γ2-subunits. $GABA_AR$ present also a dynamic mobility between synaptic and extrasynaptic localization, being the accumulation of the receptor at the inhibitory synapses regulated by its scaffold protein gephyrin. Furthermore, the neuronal surface $GABA_AR$ are in a continue cycle between the plasma membrane and intracellular compartments, and the regulation of the total receptor surface expression plays a key role in the control of the postsynaptic pool size and the strength of synaptic inhibition. The $GABA_AR$ internalization rate is negatively regulated by phosphorylation of β3 or γ2 $GABA_AR$ subunits on their intracellular loop. Thus, NMDAR signaling is known to control the stability of synaptic $GABA_AR$ via calcineurin and $GABA_AR$ dephosphorylation. However, so far the alterations in the regulation of $GABA_AR$ trafficking that occur during pathological conditions, such as brain ischemia, remain completely unexplored. In this work we combined biochemical approaches and cell imaging to investigate the mechanisms regulating the internalization of $GABA_AR$ following transient OGD. We found that OGD decreases $GABA_AR$/Gephyrin interaction and induces the internalization of $GABA_AR$ via clathrin dependent endocytosis. Both reduction of $GABA_AR$/Gephyrin interaction and the increase in $GABA_AR$

internalization were found to be regulated by phosphorylation as assessed by surface co-immunoprecipitation assay and antibody-feeding, respectively. Moreover, we demonstrated that OGD-induced dephosphorylation and internalization of β3 $GABA_AR$ subunits, which are present in a large proportion of receptor subtypes in the hippocampus and cortex, regions that are particularly vulnerable to excitotoxicity. Furthermore, our data showed that the OGD-induced receptor dephosphorylation and consequent internalization contributes to neuronal cell death, as demonstrated using a phospho-mutant of the β3 $GABA_AR$ subunit.

Following internalization, $GABA_ARs$ are rapidly recycled back to the neuronal plasma membrane or targeted for lysosomal degradation. The decision regarding the sorting of endocytosed $GABA_ARs$ depends on the interaction of $GABA_AR$ β1-3 subunits with huntingtin-associated protein 1 (HAP1). We found that OGD also reduced the recycling of $GABA_AR$ back to the plasma membrane and decrease their interaction with the HAP1 protein. Overall, we propose a new model in which $GABA_AR$/Gephyrin dissociation and receptor dephosphorylation are key steps for GABAergic down modulation during cerebral ischemia and consequent neuronal cell death.

CHAPTER 1 – Introduction

1.1. CEREBRAL ISCHEMIA

Stroke is the second most common cause of death worldwide and a leading global cause of disability (Lopez et al., 2006). From the clinical point of view the stroke may be classified as ischemic, intracerebral hemorrhagic and sub-arachnoid hemorrhagic (Warlow et al., 2003).

Cerebral ischemia is the pathological condition in which the brain is subjected to hypoxia, normally resulting from an arterial obstruction that reduces the blood supply to the affected area. Brain ischemia is usually classified into two main groups, global and focal ischemia. During global ischemia the blood flow is transiently blocked to the entire brain, resulting in delayed and selective neuronal death. Focal ischemia is a consequence of a temporary or permanent obstruction of local blood supply injuring a specific area of the brain.

In humans global ischemia occurs mostly as a consequence of cardiac arrest, open-heart surgery, profuse bleeding, or carbon monoxide poisoning. Only selected neuronal populations degenerate and die during a brief transient global ischemic insult, both in humans (Brillman, 1993; Petito et al., 1987; Roach et al., 1996; Swain et al., 1993) and in animal models (Schmidt-Kastner and Freund, 1991). The most vulnerable cells are pyramidal neurons in the cornu ammonis 1 (CA1) region of the hippocampus, hilar neurons of the dentate gyrus (DG), medium aspiny neurons of the striatum, pyramidal neurons in neocortical layers II, V, and VI, and Purkinje neurons of the cerebellum (Crain et al., 1988; Kirino, 1982). The molecular mechanisms underlying the cell-specific pattern of global ischemia–induced neuronal death are not well understood.

Focal ischemia in humans occurs mainly as a consequence of stroke, cerebral hemorrhage, or traumatic brain injury. Stroke is mainly caused by a clot that occludes a cerebral artery, while in the other cases the ischemic injury is caused by a bursting of a weakened blood vessel in the brain and bleeding into the surrounding tissue (in cerebral hemorrhage or traumatic brain injury).

Tissues in risk of damage due to the cerebral artery occlusion are the core and penumbra. The core corresponds to the center of the stroke and receives essentially no blood supply; this area contains cells that are dependent on the affected blood vessel to obtain oxygen and nutrients required for their metabolism. The penumbra is the region surrounding the core and contains cells that receive a supply of oxygen and nutrients from nearby blood vessels, although it is not sufficient to keep the normal metabolic activity. The duration of the ischemic episode determines the extent or grade of damage (Memezawa et al., 1992). Although the infarct starts in the core, at its maximum it encompasses both core and penumbra, generally after 6 to 24 hours of permanent ischemia (Garcia et al., 1993).

To improve the understanding of the etiology, prevention and treatment strategies for the different subtypes of stroke, it is very important to choose the most appropriate animal model according to the question to be addressed. Different animal models that have been developed to study specific aspects of this pathological condition are described in the next section.

1.1.1. Animal models of brain ischemia

Various models of stroke have been developed in the past decade, (Ginsberg and Busto, 1989; James et al., 2008), most of them performed in rodents (Bailey et al., 2009). These include models of global and focal ischemia, and in vitro and in vivo models are available.

1.1.1.1. In vivo models

1.1.1.2. Global ischemia models

Global ischemia models mimic the cerebral damage that occurs after cardiac arrest. To study acute global ischemic damage in rodents, the four-vessel occlusion model (Pulsinelli and Brierley, 1979; Xu and Pulsinelli, 1994) and the two-vessel occlusion model (Smith et al., 1984; Wellons et al., 2000) are commonly used. Both methods cause extensive

bilateral forebrain injury. The first model consists in a permanent occlusion of both vertebral arteries and temporary ligation of the two common carotid arteries, while the second is obtained by temporary occlusion of the common carotid arteries combined with induced systemic hypotension. Chronic global hypoperfusion models in rodents include ligation of both common carotid arteries (Wakita et al., 1998) and bilateral common carotid artery stenosis using external microcoils (Wakita et al., 1998). Both models have been shown to produce mainly white matter lesions.

1.1.1.3. Focal ischemia models

Animal models of focal ischemia mimic the pathologic condition of stroke or cerebral infarction in humans (DeGirolami et al., 1984; Longa et al., 1989; Nagasawa and Kogure, 1989). Since ischemic stroke in humans occurs mainly in the vascular territory of the middle cerebral artery (MCA) (del Zoppo et al., 1992), the models of MCA occlusion (MCAO) were developed to study the consequences of this clinical condition. In rodents MCAO induces long term sensorimotor deficits, cognitive deficits and impairment of postural and sensory reflexes (Bouet et al., 2007; Freret et al., 2009; Gerlai et al., 2000). Permanent or transient vessel occlusion is performed using endovascular or surgical approaches (Kuge et al., 1995; Robinson et al., 1975; Tamura et al., 1981; Tureyen et al., 2005), and may be either proximal or distal. In proximal occlusion, the MCA is occluded close to its branching from the internal carotid, before the origin of the lenticulostriate arteries (Ginsberg and Busto, 1989; McAuley, 1995). After MCAO, blood flow is reduced to less than 15% in the center of the stroke, or core. The region in which blood flow is reduced to less than 40% is defined as penumbra. In the case of distal MCAO, blood flow to the basal ganglia is not interrupted; consequently the damage is restricted to the neocortex. This type of occlusion can be induced surgically by means of a clip (Buchan et al., 1992) or by inducing thrombotic clots (Kilic et al., 1998; Markgraf et al., 1993), in combination with transient unilateral occlusion of the common carotid

arteries (Brint et al., 1988; Chen et al., 1986; Lipton, 1999). The reduction of blood flow achieved in the core and penumbra with distal MCAO is similar to that achieved in the proximal model.

1.1.1.4. Oxygen and glucose deprivation (OGD) - In Vitro model

Oxygen and glucose deprivation (OGD) is considered an in vitro model of global ischemia (Dawson et al., 1996; Goldberg and Choi, 1993; Martin et al., 1994). OGD is commonly performed in primary cultures of neurons or glia from different brain regions, such as the neocortex, hippocampus, cerebellum and hypothalamus of embryonic or early postnatal rats or mice. The effect of OGD on organotypic hippocampal slice cultures from perinatal rats, which keep the cellular organization of the hippocampus, has also been tested (Newell et al., 1995; Rimvall et al., 1987; Strasser and Fischer, 1995a; Strasser and Fischer, 1995b). Cultures of dissociated neurons and organotypic hippocampal slice cultures are usually incubated in a deoxygenated and glucose-free medium (OGD) to mimic the interruption of the oxygen and nutrient supply to the brain. Following the induction of in vitro ischemia, the cultures are often incubated in fresh or conditioned culture medium, in an oxygen-containing atmosphere environment, to simulate the in vivo blood flow reperfusion period. The absence of blood vessels and blood flow makes OGD a simple model system to analyze but at the same time a less complete model. In the last years this model has been increasingly used to better understand the molecular injury pathways of brain ischemia.

No animal model reproduces exactly the complexity of ischemic stroke. Therefore, the right model to choose depends on the research question being addressed. This is very important in order to prevent ambiguous interpretation of the results.

1.1.2. Ischemia-induced neuronal cell death

Brain ischemia mediates neuronal death through a series of events that involve multiple interdependent molecular pathways. These pathways are thought to be activated following the extracellular accumulation of excitatory amino acids, especially glutamate (Faden et al., 1989). Global and focal ischemia induce neuronal death with hallmarks of both necrosis and apoptosis (Choi, 1996; Ginsberg and Busto, 1989). From a morphological point of view, necrotic cell death is generally characterized by early mitochondrial swelling and loss of integrity of the plasma membrane, with preservation of the nuclear membrane. In necrotic cell death two main states can be distinguished, edematous death and ischemic death. The former state is characterized by cytoplasm swelling, absence of plasma membrane blebbing and absence of microtubules. Furthermore, the endoplasmic reticulum, Golgi apparatus and polysomes appear as incomplete structures, and although the nucleus appears almost normal there is irregular chromatin condensation (Kalimo et al., 1977; Kalimo et al., 1982). CA1 neurons undergoing delayed death in the rat and gerbil models of global ischemia show the characteristics of edematous death (Kirino and Sano, 1984; Petito and Pulsinelli, 1984). These edematous changes are typically observed upon global ischemia in the end stages of degeneration. The ischemic death is characterized by darkening and shrinkage of the nucleus and cytoplasm (Brown, 1977; Brown and Brierley, 1972; Inamura et al., 1987); the nuclear and plasma membranes become highly irregular, and therefore the cell shape changes.

Unlike necrotic cells, apoptotic neurons in the ischemic brain exhibit characteristic morphologic features such as cytoplasmic shrinkage, chromatin condensation, dynamic membrane blebbing and apoptotic bodies. Moreover, in vitro experiments apoptotic cells do not exhibit membrane damage until the last stages of death, when the membranes become permeable to normally retained solutes (Martin et al., 1995; Matylevitch et al., 1998). A number of specific apoptotic death cascades involving different signaling molecules have now been identified.

Molecular hallmarks of apoptosis include phosphatidylserine exposure (translocation from the inner leaflet to the outer surface of the plasma membrane), activation of the cell surface receptors such as Fas/CD95, a member of the tumor necrosis factor (TNF) family of death receptors (Martin-Villalba et al., 1999), mitochondrial release of cytochrome c (Fujimura et al., 1998), activation of the caspases, notably caspase-3, (Namura et al., 1998) and DNA fragmentation (Benveniste et al., 1984; Cardell et al., 1989; Tominaga et al., 1993). The classical positive definition of necrotic cell death is based on morphological criteria, including early plasma membrane rupture and dilatation of cytoplasmic organelles, in particular mitochondria (Edinger and Thompson, 2004; Kroemer et al., 2005). However, this mode of cell death is also characterized by molecular signaling, including generation of ROS, ATP depletion (Tiwari et al., 2002) and changes in the actin cytoskeleton (Thomas et al., 2006b).

1.1.2.1. Excitotoxic neuronal death

Neuronal death in brain ischemia has been shown to involve multiple molecular pathways, largely triggered by the increase in extracellular glutamate (Faden et al., 1989). The massive release of synaptic glutamate following anoxia, during the ischemic episode (Choi, 1988), the release of the neurotransmitter by reversal of the plasma membrane transporters, and the inhibition of the glutamate reuptake mechanisms (Danbolt, 1994; Kanner and Schuldiner, 1987; Nicholls and Attwell, 1990), contribute to the increase in the extracellular glutamate concentration, with consequent overactivation of the ionotropic glutamate receptors (N-methyl-D-aspartate [NMDA] receptors [NMDAR], AMPA [α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid] receptors [AMPAR] and kainate receptors). Among the mechanisms involved in glutamate-mediated excitotoxicity there are alterations in the intracellular ion concentration, especially Ca^{2+} and Na^{+}, induced by excessive activation of glutamate receptors. The signaling by NMDAR and their intracellular

binding partners, in addition to the glutamate-mediated generation of free-radicals, play a key role in the activation of the cell death machinery.

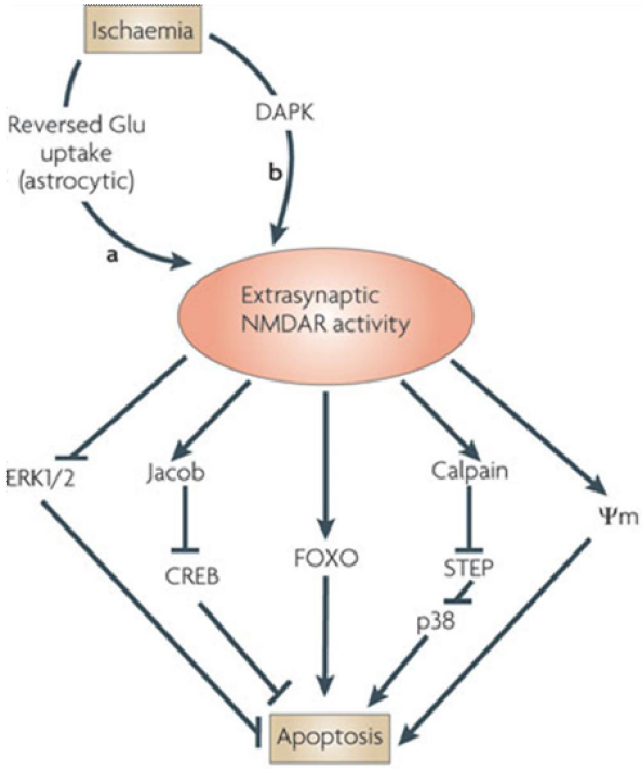

Figure 1.1. Activators and effectors of extrasynaptic NMDAR activity in brain ischemia.
Brain ischaemia results in activation of extrasynaptic NMDAR through the reversal of the glutamate uptake system from astrocytes (a). Extrasynaptic NMDA receptor currents are also preferentially enhanced by ischaemia-induced activation of death-associated protein kinase (DAPK) (b). Increased extrasynaptic (but not synaptic) NMDAR activity in turn preferentially activates a number of pro-death pathways. Mitochondrial membrane potential (Ψm) is disrupted by extrasynaptic NMDAR activity. CREB, cyclic-AMP response element binding protein ; ERK, extracellular signal-regulated kinase; FOXO, forkhead box protein O; Jacob, juxtasynaptic attractor of caldendrin on dendritic boutons protein, STEP, striatal enriched tyrosine phosphatase. From (Hardingham and Bading, 2010)

NMDARs and GluA2-lacking AMPAR allow the influx of Ca^{2+} and Na^{+} into postsynaptic cells (Gorter et al., 1997; Tsubokawa et al., 1994; Tsubokawa et al., 1996), while activation of AMPA receptors containing GluA2 subunits, as well as kainate receptors, further contributes to the increase in Na^{+} permeability, thereby depolarizing the postsynaptic

membrane. The massive rise in cytosolic Ca^{2+} levels is also due to activation of metabotropic glutamate receptors (mGluR), mGluR1 and mGluR5, that trigger the release of Ca^{2+} from inositol 1,4,5-triphosphate (InsP3)-sensitive intracellular stores via stimulation of phospholipase C (Oguro et al., 1995). Moreover, the excessive rise in extracellular glutamate concentration allows the activation of extrasynaptic NMDA receptors, with a consequent influx of toxic amounts of Ca^{2+} that promote the shutoff of the CREB-initiated program of gene expression which promotes cell survival. This response induced by extrasynaptic NMDAR contrasts with the role of synaptic NMDAR which are coupled to the activation of CREB, thereby promoting cell survival (Hardingham and Bading, 2003; Lonze and Ginty, 2002). This evidence indicates that the site of NMDAR mediated Ca^{2+} entry into cells critically influences the fate of neurons (Hardingham and Bading, 2010; Hardingham et al., 2002). Interestingly, contemporaneous activation of synaptic and extrasynaptic NMDARs also shuts off CREB (Hardingham et al., 2002).

The excessive release and spillover of glutamate in brain ischemia allows the stimulation of both populations of NMDAR, ultimately leading to cell death. It has been proposed that the breakdown of regular synaptic transmission and the overactivation of extrasynaptic GluN2B-containing NMDAR are responsible for neuronal death in brain ischemia (Benveniste et al., 1984; Rossi et al., 2000) (Fig. 1.2). However, at this point the relative role of synaptic and extrasynaptic NMDAR activation in excitotoxicity is still controversial (Sattler et al., 2000). The observations supporting a preferential neuroprotective role of synaptic GluN2A-containing NMDAR (Hardingham et al., 2002; Leveille et al., 2008) contrast with those pointing to a role in excitotoxicity (Papouin et al., 2012). Evidence suggested that the subunit composition of NMDAR plays a more important role in determining the downstream pathways activated than the cellular localization of the receptors (Liu et al., 2007). For example, during the early period of development only GluN2B-containing receptors are expressed and, therefore, at this stage the cells are more vulnerable to excitotoxic events. During development, with the

appearance of GluN2A-containing receptors, the cells become more resistant to these harmful factors (Thomas et al., 2006a; Zhou and Baudry, 2006).

The role of AMPAR in excitotoxic cell death has been related to the expression of GluA2 subunits. The relative expression of GluA2 in neurons is dynamic, being regulated in a cell-specific manner during development and remodeled by activity and in pathological conditions (Friedman et al., 1994; Prince et al., 1995), such as ischemia (Tanaka et al., 2000). This subunit governs the biophysical properties of AMPAR, including their Ca^{2+} permeability (Hollmann et al., 1991; Verdoorn et al., 1991). GluA2-lacking AMPAR are an important route of Ca^{2+} and Zn^{2+} entry into insulted neurons (Weiss and Sensi, 2000). In the adult brain, hippocampal neurons express high levels of GluA2 and exhibit relatively low Ca^{2+} influx via AMPAR. However, injurious stimuli, such as ischemia, induce the suppression of GluA2 mRNA, with a consequent downregulation in the expression of the protein in vulnerable CA1 neurons. This effect is subunit-specific and is observed in a cell-specific manner before the onset of cell death (Garthwaite and Garthwaite, 1989; Paschen et al., 1996; Pellegrini-Giampietro et al., 1997; Takuma et al., 1999).

The strength of the ischemic insult influences the cytosolic Ca^{2+} concentration and determines the mode of cell death. In fact, stronger insults induce a massive increase in cytosolic Ca^{2+} that results in necrotic cell death (Choi, 1995), while less severe insults cause a smaller elevations in Ca^{2+} and may trigger apoptosis (Bonfoco et al., 1995; Yu et al., 2001).

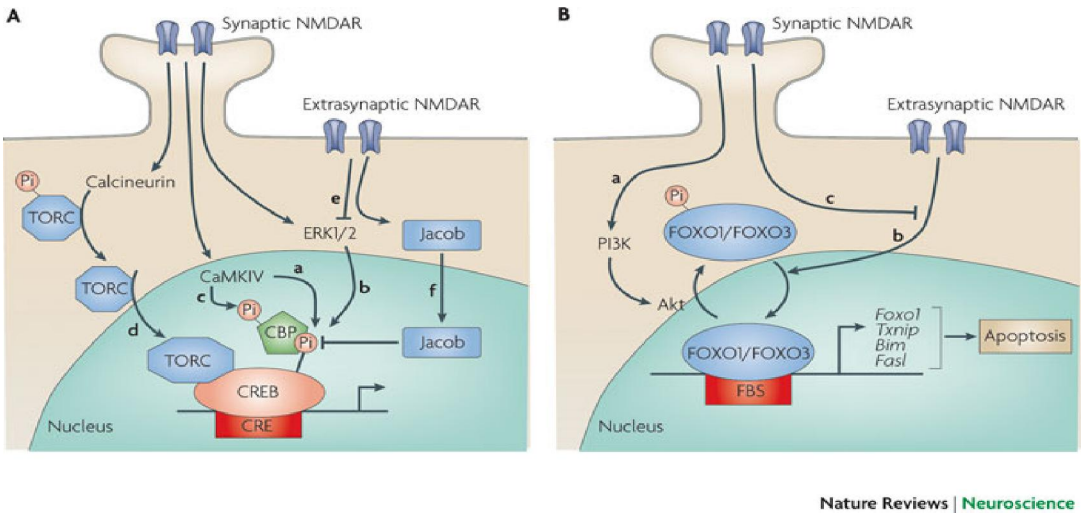

FIGURE 1.2. Opposing effects of synaptic and extrasynaptic NMDAR signalling on gene expression.
(A) Phosphorylated CREB at serine 133 recruits its co-activator CREB binding protein (CBP). This phosphorylation is mediated by the fast-acting nuclear Ca^{2+}/calmodulin-dependent protein (CaM) kinase pathway (Aa) and by the slower acting (but longer lasting) Ras–extracellular signal-regulated kinase 1/2 (ERK1/2) pathway (Ab), both of which promoted by activation of synaptic NMDAR. CBP is subject to Ca^{2+}-mediated transactivation by nuclear Ca^{2+} dependent CaM kinase IV which phosphorylates CBP (Ac). Synaptic NMDAR-induced Ca^{2+} signals promote TORC import into the nucleus through calcineurin-dependent dephosphorylation (Ad). TORC acts by assisting in the recruitment of CBP to CREB. In contrast, extrasynaptic NMDAR suppress CREB activity through inactivation of the Ras–ERK1/2 pathway 41 (Ae) and by inducing the nuclear translocation of juxtasynaptic attractor of caldendrin on dendritic boutons protein (Jacob), which promotes CREB dephosphorylation (Af). (B) Opposing effects of synaptic and extrasynaptic NMDAR signalling on forkhead box protein O (FOXO)-dependent gene expression. Synaptic NMDAR activity suppresses FOXO activity by promoting the Akt-mediated phosphorylation and nuclear export of FOXOs (Ba), of which FOXO1 and FOXO3 are the predominant neuronal subtypes. FOXO1 is also regulated transcriptionally by FOXOs and thus signals that cause FOXO export also result in the suppression of FOXO1 transcription. In contrast, bath activation of NMDAR, which also triggers extrasynaptic NMDAR activity, stimulates FOXO nuclear import (Bb), an event that contributes to excitotoxic cell death by promoting the transcription of pro-death genes. Synaptic NMDAR activity can exert a long-lasting block on this import signal (Bc), but the mechanism involved remains unclear. Bim, Bcl2-interacting mediator of cell death; Fasl, Fas ligand; FBS, FOXO binding site; Pi, inorganic phosphate; PI3K, phosphoinositide 3 kinase; Txnip, thioredoxin-interacting protein. From (Hardingham and Bading, 2010)

1.1.2.2. Intracellular mediators of excitotoxic cell death

Under physiological condition the calcium ions are intracellular messengers involved in the regulation of important functions, including synaptic activity, membrane excitability, exocytosis and enzyme activation (Lee et al., 2005; Yadavalli et al., 2004). A dysregulation of the $[Ca^{2+}]_i$ homeostases, with a dramatic increase in the cytoplasmatic calcium levels, is one of the first indicators of neuronal cell death (Banay-Schwartz et al., 1994; Bouet et al., 2007; Nixon, 2003; Polster et al., 2005). The $[Ca^{2+}]_i$ overload under excitotoxic conditions contributes to neuronal injury through activation of different classes of enzymes, including calpains (Araujo et al., 2004; Bano et al., 2005; Lee et al., 2005; Lob et al., 1975). Calpain activation was initially implicated in the necrotic process, but it is now accepted that these cysteine proteases play a prominent role in the apoptotic process (Liou et al., 2005).

The excessive activation of calpains contributes to neuronal death by cleaving proteins with different functions. Calpain overactivation leads to cytoskeletal protein breakdown, with a consequent loss of structural integrity and disturbance of axonal transport, and finally inducing neuronal death (Yamashima, 2004). The disruption of the cytoskeleton is mediated by the cleavage of several essential cytoskeletal proteins of the axons (Kieran and Greensmith, 2004), including tau, microtubule-associated protein 2 (MAP2), neurofilaments, and spectrin (Goll et al., 2003; Liu et al., 2008). For example, calpains were shown to be involved in the proteolysis of tau during retinal cell death (Benuck et al., 1996). In cerebellar granule neurons, excitotoxic stimulation with glutamate also induces the cleavage of myosin Va by calpains, while calpain inhibitors improved neuronal viability by preventing myosin Va proteolysis (Alavez et al., 2004). The excessive activation of calpains under excitotoxic conditions also leads to the abnormal cleavage of mitochondrial proteins. The cleavage of apoptosis inducing factor (AIF), a protein associated with the inner mitochondrial membrane, releases this protein to the cytosol (Pike et al., 2001). AIF is then translocated to the nucleus, activating caspase-independent apoptosis (Daugas et al., 2000; Polster et al., 2005).

Calpains are also implicated in the degradation of apoptotic proteins such as Bid (Li et al., 1998; O'Donovan et al., 2001). In addition to the direct effects of Ca^{2+}, it was suggested that calpains may be activated by DNA damage (Sedarous et al., 2003). In particular, calpains regulate the activation of p53 (Saulle et al., 2004) and calpain inhibitors were shown to reduce the p53 activity induced by DNA damage, most likely by preventing the release of cytochrome c and the activation of caspases (Sedarous et al., 2003). These data suggest that calpains are modulators of apoptosis stemming from DNA damage upstream of p53.

Calpains may also target plasma membrane receptors and ion transporters, thereby affecting neuronal death under excitotoxic conditions. Thus, calpains were shown to cleave NMDA receptors in hippocampal neurons exposed to toxic concentrations of glutamate (Adamec et al., 1998). Overactivation of NMDA also induces the cleavage of mGluR1a through a calpain-dependent mechanism, thereby altering the mGluR1a signaling and contributing to excitotoxic neuronal damage (Xu et al., 2007). It was also shown that calpains cleave the plasma membrane Na^{+}/Ca^{2+} exchanger during brain ischemia in neurons undergoing excitotoxicity (Bano et al., 2005). The proteolytic inactivation Na^{+}/Ca^{2+} exchanger is responsible for the delayed excitotoxic upregulation of Ca^{2+} and the consequent neuronal death. In this model, the overexpression of calpastatin (an endogenous calpain inhibitor) protects neurons from excitotoxic death by decreasing secondary Ca^{2+} overload (Bano et al., 2005).

NO is also considered an important downstream mediator of NMDA-induced excitotoxicity. The high cytosolic Ca^{2+} concentration resulting from the excessive activation of NMDAR stimulates the neuronal isoform of nitric oxide synthase (nNOS), which binds Ca^{2+}-calmodulin complexes and forms NO and citrulline from arginine (Bredt et al., 1992; Garthwaite, 1991; Kumura et al., 1996). Several studies implicate the free radical form of NO and the superoxide anion (O^{2-}) in the oxidative damage of cellular DNA, lipid peroxidation, and excitotoxic cell death (Choi, 1990; Choi, 1995; Liu et al., 2001; Tsubokawa et al., 1992). In this

pathway NO reacts with the superoxide anion to form peroxynitrite, a cytotoxic oxidant that induces DNA damage, thereby triggering apoptotic cell death (Choi, 1995; Takei and Endo, 1994).

NMDAR-mediated influx of Ca^{2+} also activates phospholipase A2 which releases arachidonic acid, an unsaturated fatty acid, and promotes the production of free radicals via activation of the lipoxygenase and cyclooxygenase pathways (Aronowski et al., 1996). Cyclooxygenase catalyzes the addition of two molecules of O_2 to arachidonic acid to produce prostaglandin PGG2, which is rapidly peroxidized to PGH2 with concomitant release of superoxide anion (Aguilar et al., 1996). The metabolism of free arachidonic acid is thought to be a major source of superoxide anion. Free radicals damage proteins by oxidation of side chains and modification of disulfide bonds. Moreover, they inactivate and damage nucleic acids. The oxidative damage caused by free radicals results from single- and double-stranded breaks in DNA, chemical modification of nucleic acid bases, breaking the glycosylic bond between ribose and individual bases, and by crosslinking proteins to DNA strands (Liu et al., 2001).

Overall Ca^{2+} and Zn^{2+} are critical players in ischemic cell death (Choi and Koh, 1998). In addition to the mechanisms mentioned above, high cytosolic Ca^{2+} contributes to neuronal death by depleting the energy stores of the cell due to activation of Ca^{2+}-ATPases and uncoupling of mitochondrial oxidative phosphorylation, leading to acute swelling of dendrites and cell bodies. Moreover, high $[Ca^{2+}]_i$ levels activate Ca^{2+}-sensitive transcription factors, phospholipases, endonucleases, and proteases (Choi and Koh, 1998). The dysregulation of the proteolytic activity not only affects intracellular proteins, including cytoskeletal proteins such as actin and spectrin (Furukawa et al., 1997) (see above), but also downregulate extracellular proteins (e.g. extracellular matrix proteins like laminin) (Chen and Strickland, 1997). Similar to the role of Ca^{2+}, the neurotoxic effects of Zn^{2+} in brain ischemia have been attributed to disruption of mitochondrial function (impairment of glycolysis and energy production, and inhibition of respiration) and

potentiation of AMPAR-mediated currents (Weiss and Sensi, 2000). Furthermore, Zn^{2+} influx via GluA2-lacking AMPAR also induces the production of free radicals, such as mitochondrial superoxide, in injured neurons (Bonfoco et al., 1995; Sensi et al., 1999).

The metabolic stress caused by energy depletion also contributes to neuronal cell death in cerebral ischemia. Neurons have a relatively high consumption of oxygen and glucose, and depend almost exclusively on oxidative phosphorylation for energy production. During the ischemic episode, impairment of cerebral blood flow restricts the delivery of substrates, particularly oxygen and glucose, and impairs the energetics required to maintain ionic gradients (Martin et al., 1994). The rapid decrease in ATP levels induces neuronal depolarization, promoting cell death by necrosis in the core region. Energetic impairment also reverses the operation of glutamate transporters in astrocytes and neurons, leading to an extracellular accumulation of the neurotransmitter. The resulting overactivation of ionotropic glutamate receptors contributes to cells swelling (edema) and consequent rupture of the plasma membrane (Meldrum and Garthwaite, 1990). Apoptotic and necrotic stimuli also compromise mitochondria integrity by the disruption of the mitochondrial membrane. The apoptotic cascade can be initiated by the release of cytochrome c into the cytoplasm, allowing the formation of the apoptosome, the signaling complex required for activation of caspase-9 (Broughton et al., 2009). The precise mechanisms by which the integrity of mitochondrial membrane breaks down are unknown, but Bcl-2 family members are known to play a critical role (Hengartner, 2000; Kroemer and Reed, 2000).

Other intracellular mechanisms triggered by ischemia are related with transcriptional pathways. The transcription factors that are thought to contribute to the changes in gene expression after global ischemia include CREB and nuclear factor kappa B (NF- kB), which control pro-survival programs, and the forkhead family of transcription factors and REST/NRSF, which direct pro-death pathways in adult neurons. As previously described, although the influx of Ca^{2+} via synaptic NMDAR

induces the activation of CREB and promote neuronal survival, the massive glutamate release during cerebral ischemia induces the influx of Ca^{2+} via extrasynaptic NMDAR eliciting CREB shutoff (Hardingham and Bading, 2003; Lonze and Ginty, 2002; Riccio and Ginty, 2002).

Under physiologic conditions the transcription factor NF-κB exists in the inactive form, composed by the transcription factor dimer bound to the IκB (inhibitor of NF-κB) protein, which maintains NF-κB inactive. Upon focal ischemia NF-κB is activated due to the phosphorylation and proteasomal degradation of IκB. Activated NF-κB translocates to the nucleus, where it binds to upstream regulatory elements in NF-κB-responsive genes (Schneider et al., 1999). Upon activation, NF-κB plays an important role in regulating neuronal survival. Accordingly, targeted deletion of NF-κB significantly reduces ischemic damage, suggesting a cell death–promoting role of NF-κB in focal ischemia (Schneider et al., 1999).

Dysregulation of REST and its target genes is also implicated in global ischemia (Calderone et al., 2003), which triggers a pronounced upregulation of REST mRNA and protein in selectively vulnerable CA1 neurons.

Finally, inflammatory responses are also involved in the pathogenesis of ischemia-induced neuronal death (Dirnagl et al., 1999). Ischemia-hypoxia triggers the activation of transcription factors such as NF-κB, hypoxia-inducible factor-1 (HIF-1), interferon regulatory factor-1 and signal transducers and activators of transcription (STATs). In particular STAT3 induces the expression of a group of proinflammatory target genes, such as platelet-activating factor and the cytokines TNFα and IL-1β (Ishibashi et al., 2002). Cytokines play a mutifacted response in the immune response following stroke. For example IL-1 can inhibit, exacerbate, or induce neuronal cell damage and death, while TNF-α induces apoptosis in a variety of cells, and can stimulate a proadhesive and pro-inflammatory state, in addition to the production of reactive oxygen species (ROS) in the endothelium, further exasperating the immune response (Tuttolomondo et al., 2008).

1.2. $GABA_A$ RECEPTOR-MEDIATED NEUROTRANSMISSION

1.2.1. $GABA_AR$ structure and trafficking

Inhibitory neurotransmission in the Central Nervous System (CNS) is largely mediated by γ-aminobutyric acid (GABA). GABA exerts its inhibitory control by acting on two classes of receptors with distinct electrophysiological and pharmacological properties. GABA type A receptors ($GABA_AR$) are ionotropic fast-acting ligand-gated chloride channels (Sieghart, 2006), while GABA type B receptors ($GABA_BR$) belong to the metabotropic G protein-coupled receptor superfamily and produce slow and prolonged inhibitory responses (Bettler and Tiao, 2006).

Under normal physiological conditions $GABA_AR$ respond to the binding of GABA by opening an integral chloride channel and allowing chloride to enter the neuron. The result is a membrane hyperpolarization and neuronal inhibition. Deficits in $GABA_AR$ function have been associated with both psychiatric diseases and neurological disorders (Benarroch, 2007; D'Hulst and Kooy, 2007; Lewis and Gonzalez-Burgos, 2006; Rudolph and Mohler, 2004; Thompson-Vest et al., 2003).

Many distinct but homologous $GABA_AR$ subunits (α 1–6, β1–3, γ1–3, δ, ε, θ, π and ρ1–3) have been cloned and sequenced from the mammalian CNS. These receptor subunits share a common ancestral structure that includes an extracellular N-terminal domain, four transmembrane domains (TM1-4) and an extended cytoplasmic loop region between TM3 and TM4. The latter sequence is subject to posttranslational modifications and interacts with various regulatory, chaperone, and scaffolding proteins (Fig. 1.4). The various $GABA_AR$ subunits are preferentially assembled to form heteropentameric receptors. Despite the vast theoretically possible number of heteropentameric assemblies, only a limited number of receptor subtypes are expressed physiologically (Sieghart and Sperk, 2002). The majority of $GABA_AR$ subtypes in the brain are composed of α1β2γ2, followed by α2β3γ2 and α3β3γ2 (Chang et al., 1996; Knight et al., 2000; Massaria et al., 1976; Tretter et al., 1997). $GABA_AR$ with different subunit compositions have different physiological

and pharmacological properties, are differentially expressed throughout the brain and are targeted to different subcellular regions. Receptors composed of α1, α2 or α3 subunits together with β and γ subunits are benzodiazepine - sensitive, and largely synaptically located, mediating most phasic inhibition in the brain (Rudolph and Mohler, 2004). Instead, $GABA_AR$ composed of α4 or α6 subunits, together with β and δ subunits, are predominantly extrasynaptic, mediate tonic inhibition and are insensitive to benzodiazepine modulation (Brunig et al., 2002). $GABA_AR$ are also present at presynaptic sites (Draguhn et al., 2008) (Fig. 1.3).

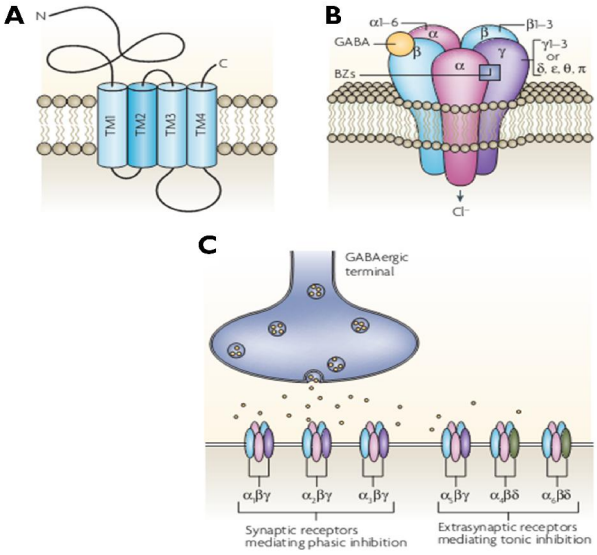

FIGURE 1.3. $GABA_AR$ structure and neuronal localization.
A) $GABA_AR$ are ligand-gated ion-channels formed by oligomerization of 5 subunits. $GABA_AR$ subunits consist of four hydrophobic transmembrane domains (TM1–4), with TM2 believed to line the pore of the channel. The large extracellular amino terminus is the site of GABA binding, and also contains binding sites for psychoactive drugs, such as benzodiazepines (BZ). Each receptor subunit also contains a large intracellular domain between TM3 and TM4 that is the site for interaction with various proteins, as well as for various post-translational modifications that modulate receptor activity. B) Five subunits belonging to seven subunit subfamilies (α, β, γ, δ, ε, θ and π) assemble to form a heteropentameric Cl^--permeable channel. Most $GABA_AR$ expressed in the brain consist of two α subunits, two β subunits and one γ subunit; the γ subunit can be replaced by δ, ε, θ or π subunits. Binding of the neurotransmitter GABA occurs at the interface between the α and β subunits and triggers the opening of the channel, allowing the rapid influx of Cl^- into the cell. BZ binding occurs at the interface between the α (1, 2, 3 or 5) and γ subunits, and potentiates GABA-induced Cl^- flux. C) $GABA_AR$ composed of α (1–3) subunits together with β and γ subunits are thought to be primarily synaptically localized, whereas α5βγ receptors are located largely at extrasynaptic sites. Both types of $GABA_AR$ are BZ sensitive. In contrast, receptors composed of α (4 or 6) βδ subunits are BZ insensitive and localized at extrasynaptic sites. From (Jacob et al., 2008)

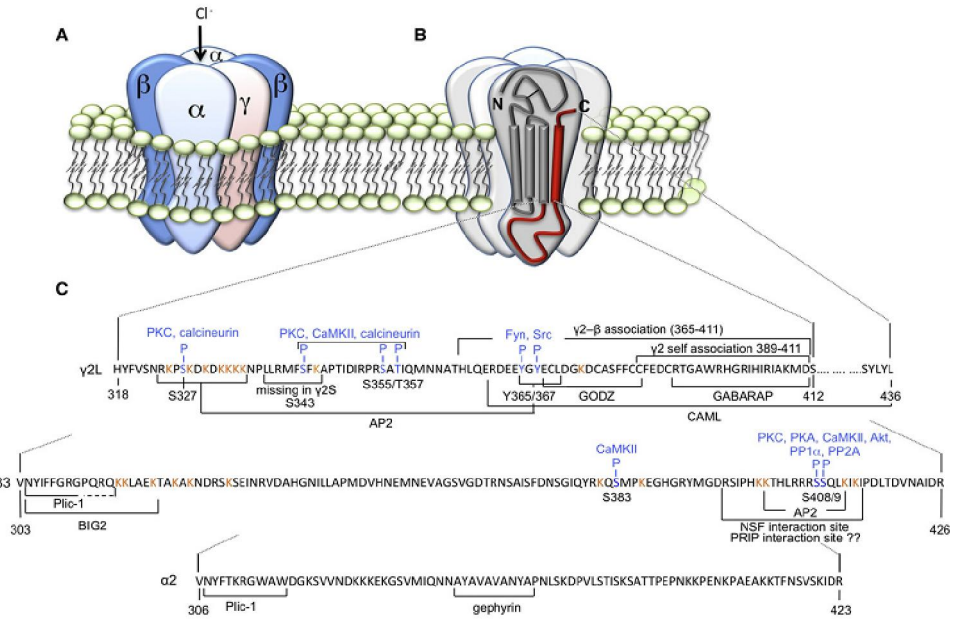

FIGURE 1.4. GABA$_A$R subunit structure and intracellular loop sequences
A) Schematic representation of GABA$_A$R heteropentamers consisting of two α, two β, and a single γ2 subunit. B) Every subunit includes an extracellular N-terminal domain, four transmembrane domains (TM1-4) separated by an extended cytoplasmic loop region between TM3 and TM4, and a short extracellular C terminus. The cytoplasmic loop and the TM4 regions of the γ2 subunit are essential for postsynaptic clustering of GABA$_A$R (see also Figure 1.3). C) Sequences of the cytoplasmic loop regions of representative subunits (γ2, β3, α2) with amino acid numbers referring to mature polypeptides from the mouse. Interaction sites for binding partners are marked by brackets beneath the sequence, along with amino acid numbers of known Ser/Thr and Tyr phosphorylation sites. Phosphorylation sites are shown in blue; Lys residues representing putative ubiquitination sites are in orange. From (Luscher et al., 2011)

GABA$_A$R are assembled from their component subunits in the endoplasmic reticulum (ER). The assembly process plays a critical role in determining the diversity of receptor subtypes expressed on the neuronal plasma membrane. Proteins only exit the ER if they have achieved their correctly folded conformation, and misfolded or unassembled proteins are retrotranslocated from ER for degradation in the proteasome, restricting the number of subunit combinations that can access the cell surface (Kittler et al., 2002) (Fig. 1.5). Following assembly in the endoplasmic reticulum, the receptors are trafficked to the cell surface where there is

dynamic regulation of their surface expression. Such regulation profoundly affects the efficacy of GABAergic transmission and the overall excitability of the central nervous system. The entry of $GABA_AR$ into the secretory pathway is regulated by interaction of α and β subunits with PLIC-1 (the protein that links integrin-associated protein with the cytoskeleton-1) (Bedford et al., 2001). PLIC-1 contains an ubiquitin-like (ubl) proteasome binding domain and ubiquitin-associated (uba) domain. The interaction with these two domains interferes with ubiquitin-mediated proteolysis of diverse substrates (Kleijnen et al., 2003; Kleijnen et al., 2000; Walters et al., 2002; Wu et al., 1999). PLIC-1 promotes the surface expression of $GABA_AR$ in neurons (Bedford et al., 2001), presumably by inhibiting ubiquitination and proteasomal degradation of α and β subunits.

Along the secretory pathway the newly synthesized and assembled receptors are palmitoylated by the Golgi apparatus-specific protein with the DHHC zinc finger domain (GODZ), and interact with the brefeldin-A-inhibited GDP/GTP exchange factor 2 (BIG2) and with the microtubule-associated protein $GABA_AR$ associated protein (GABARAP) to reach the cell surface by mechanisms that are not completely understood. More specifically, GODZ interacts with the $GABA_AR$ γ2 subunit recognizing a 14-amino acid cysteine-rich domain conserved in the intracellular domain of γ1–3 subunits, NH_2-terminal to the GABARAP binding site (Rathenberg et al., 2004). The γ2 subunit is palmitoylated at all four cysteines within the GODZ binding domain (Rathenberg et al., 2004). Mutation of these cysteine residues resulted in a loss of $GABA_AR$ clusters at the cell surface (Rathenberg et al., 2004). Therefore, GODZ controls $GABA_AR$ trafficking in the secretory pathway and the delivery of these receptors to the plasma membrane (Keller et al., 2004).

BIG2 has an important role in the vesicular trafficking of $GABA_AR$ to the plasma membrane. This protein can bind to the intracellular domain of the β3 subunit, and has a high binding affinity for the intracellular loops of all β subunits (Charych et al., 2004). BIG2 is largely localized to the trans-Golgi network (Charych et al., 2004) and has a known role in

membrane budding and vesicular transport from the Golgi apparatus (Moss and Vaughan, 1995). These data suggest that the main function of BIG2 is in the intracellular trafficking of $GABA_AR$ to the plasma membrane.

GABARAP is a 13.9 kDa microtubule-associated protein that interacts with the γ subunit cytoplasmic loop through its N-terminal domain (Wang et al., 1999; Wang and Olsen, 2000). The same protein also binds tubulin C-terminal region, suggesting that it may link the receptor to microtubule networks (Wang and Olsen, 2000). Several evidence indicate a role for GABARAP in the transport of $GABA_AR$ to the plasma membrane, but GABARAP is not essential for receptor surface expression since GABARAP knockout mice do not display alterations in either the total number of $GABA_AR$ or in their synaptic localization (O'Sullivan et al., 2005). In addition, GABARAP promotes clustering of receptors (Chen et al., 2000; Everitt et al., 2004) by a mechanism that requires polymerized microtubules and both the γ2 subunit and tubulin binding regions of GABARAP (Chen et al., 2000).

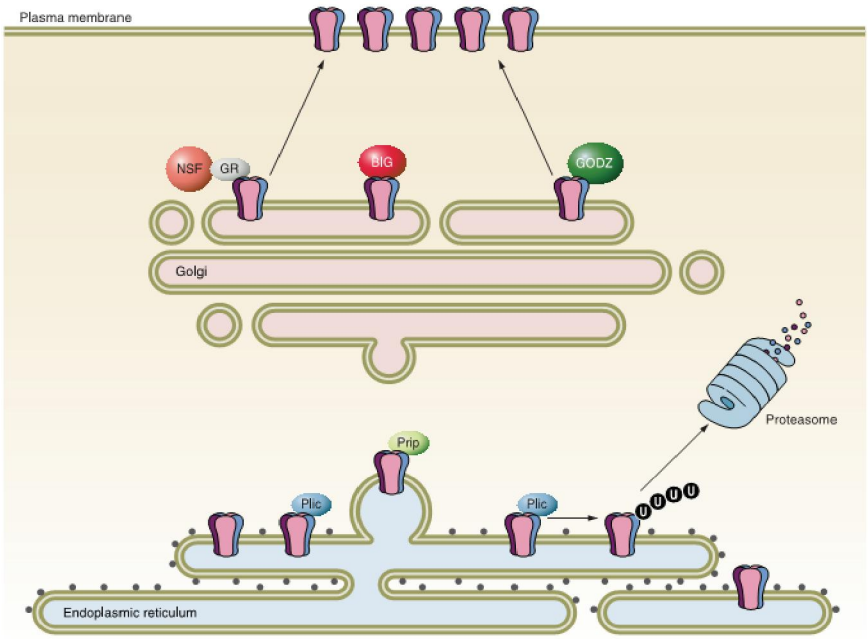

FIGURE 1.5. $GABA_AR$ trafficking in the secretory pathway is regulated by multiple receptor-associated proteins.

$GABA_AR$ are assembled within the ER and transported to the Golgi. Within the ER, unassembled receptor subunits are subjected to polyubiquitination that targets them for proteasomal degradation, a phenomenon that is dependent on the level of neuronal

activity. This process is negatively regulated by Plic-1, which binds directly to the receptor α- and β-subunits, prolonging their ER residence times. Within the Golgi, $GABA_AR$ receptors bind to complexes of GABARAP/NSF, facilitating their transport to the plasma membrane. BIG2 is also found within the Golgi and modulates receptor forward trafficking. GODZ is a Golgi resident palmitoyltransferase that regulates palmitoylation of γ subunits, a critical step in the delivery of $GABA_AR$ to the plasma membrane. Finally, PRIP proteins also play essential roles in the trafficking of $GABA_AR$ and in modulating their phosphorylation state. From (Vithlani et al., 2011)

1.2.2. Regulation of $GABA_AR$ cell surface expression

$GABA_AR$ can be delivered to the cell surface either as newly assembled channel complexes, via de novo secretory pathway, or reinserted following internalization. They can access inhibitory postsynaptic specializations or extrasynaptic sites, depending on their subunit composition. Once on the neuronal surface $GABA_AR$ are not static but are in a continue cycle between the plasma membrane and intracellular compartments. The regulation of receptor exo- and endocytosis plays a key role in the control of the postsynaptic pool size and the strength of synaptic inhibition. Furthermore, $GABA_AR$ were shown to be inserted into and removed from the plasma membrane exclusively at extrasynaptic sites (Bogdanov et al., 2006; Thomas et al., 2005). This aspect corroborates the importance of lateral diffusion for their postsynaptic specialization.

The membrane localization of $GABA_AR$ is highly selective in terms of receptors subtypes and the subunit composition is determinant for the postsynaptic targeting and clustering of these receptors. Although the molecular mechanisms that control $GABA_AR$ accumulation at inhibitory synapses are not fully understood, a number of receptor-associated proteins and cytoskeletal elements present at GABAergic postsynaptic densities (PSD) are involved in this process.

1.2.2.1. Postsynaptic $GABA_A$ receptors

The most important protein for the stabilization of $GABA_AR$ at synapses is gephyrin, considered the principal subsynaptic scaffold protein of both GABAergic and glycinergic synapses (Fritschy et al., 2008). Gephyrin, a 93 KDa polypeptide (Pfeiffer et al., 1982), is a largely expressed multi-functional protein, and also plays an essential in the postsynaptic clustering of glycine receptors (Feng et al., 1998; Kirsch et al., 1993; Prior et al., 1992; Sola et al., 2004). The role of gephyrin in the clustering of $GABA_A$ and glycine receptors results from its interaction both with microtubules (Kirsch et al., 1995) and with several regulators of microfilament dynamics, including profilin I and II (Mammoto et al., 1998). The direct interaction between $GABA_AR$ and gephyrin was firstly observed for the α2 subunit (Saiepour et al., 2010). Additional studies allowed the identification of gephyrin interaction motifs in the homologous region of α1 and α3 (Mukherjee et al., 2011; Tretter et al., 2011). Moreover recently a novel gephyrin-binding motif was identified in the $GABA_AR$ β2 and β3 large cytoplasmic loops (Kowalczyk et al., 2013).
At postsynaptic sites gephyrin is known to oligomerize and forms clusters (Saiyed et al., 2007) through the N-terminal gephyrin domain (G-gephyrin), that assumes a trimeric structure (Schwarz et al., 2001; Sola et al., 2001), and the C-terminal domain (E- gephyrin) that forms a dimer (Schwarz et al., 2001; Sola et al., 2001; Xiang et al., 2001). The linker region between the E and G domains is thought to interact with microtubules (Ramming et al., 2000).
The gephyrin structure allows the organization of a microtubule and microfilament-associated hexagonal protein lattice that may facilitate the spatial distribution of receptors in the postsynaptic membrane. However, it is not yet clear how structural changes affect the postsynaptic scaffold organized by gephyrin and the relative role played by $GABA_AR$ versus gephyrin phosphorylation. Recent studies showed that the dynamics of gephyrin clustering is regulated by neuronal activity (van Versendaal et al., 2012; Vlachos et al., 2012). Considering that phosphorylation and intracellular Ca^{2+} rises make gephyrin susceptible to proteolysis by

calpain (Tyagarajan et al., 2011), it is reasonable to hypothesize that neuronal gephyrin dynamics may be phosphorylation-dependent. However, few data are available on the functional characterization of the diverse phosphorylation sites that have been identified on gephyrin (Kuhse et al., 2012; Specht et al., 2011; Tyagarajan and Fritschy, 2010; Tyagarajan et al., 2013b; Zita et al., 2007). Gephyrin phosphorylation status directly impacts on GABAergic synaptic function, presumably by allowing formation of synapses (Tyagarajan et al., 2011) and recruitment, or stabilization, of $GABA_AR$ to the postsynaptic density. Furthermore, recent work demonstrated that calpain activation is a general mechanism to confine gephyrin to the postsynaptic cluster, in a phosphorylation-dependent manner (Tyagarajan et al., 2013b). Overall, multiple signaling cascades converge onto gephyrin to modify its scaffolding properties at the GABAergic postsynaptic density and to influence synaptic function in the CNS.

1.2.2.2. Extrasynaptic $GABA_A$ receptors

The clustering of $GABA_AR$ at the extrasynaptic site is mediated by radixin. This protein belongs to the family of ERM (ezrin, radixin, moesin) proteins, which are known to link transmembrane proteins to the actin cytoskeleton. Radixin is an α5 $GABA_AR$ subunit-interacting protein and is essential for extrasynaptic clustering of α5βγ2 receptors (Loebrich et al., 2006). In fact, among different γ2-containing $GABA_AR$ only those composed by α5βγ2 subunits showed an extrasynaptic distribution. The extrasynaptic clustering of α5-containing receptors was abolished when neurons were transfected with a dominant-negative radixin, but no no effect was observed on α5-containing $GABA_AR$ surface expression under the same conditions (Loebrich et al., 2006), suggesting that the synaptic accumulation of $GABA_AR$ containing α5 subunits is prevented by a radixin-independent mechanisms. Nevertheless, the functional relevance of α5βγ2 receptor clustering at the extrasynaptic region is not known.

1.2.2.3. Lateral diffusion of $GABA_A$ receptors

It is well established that synaptic strength is influenced by the number of postsynaptic receptors. In addition to the classical machinery of receptor endocytosis or membrane insertion/recycling, the lateral diffusion of receptors from and into the synaptic regions also plays an important role in the regulation of $GABA_AR$ density at the synapse (Fig. 1.6) (Dahan et al., 2003; Groc et al., 2004; Tardin et al., 2003). Both single particle tracking (SPT) and electrophysiological studies have demonstrated that $GABA_AR$ are rapidly exchanged between synaptic and extrasynaptic domains by lateral diffusion (Bogdanov et al., 2006; Jacob et al., 2005; Thomas et al., 2005). SPT has shown a rapid exchange between the extrasynaptic and synaptic populations of $GABA_AR$ and this process was found to be modulated by the activity of protein phosphatase 2B (PP2B). This calcium dependent mechanism activated via NMDA receptors leads to an increase in the lateral mobility of $GABA_AR$ and reduces the size of inhibitory synapses, a process that favors neuronal depolarization (Bannai et al., 2009).

$GABA_AR$ clusters are stabilized by gephyrin in the postsynaptic areas. In fact, fluorescence recovery after photobleaching (FRAP) experiments showed significantly higher fluorescence recovery rates at extrasynaptic sites than at postsynaptic membrane domains, indicating a greater mobility of extrasynaptic $GABA_AR$ when compared with the postsynaptic population of receptors (Jacob et al., 2005). Gephyrin knock-down significantly increased FRAP recovery rates at the synapse, indicating that the $GABA_AR$ mobility at postsynaptic sites is controlled by direct or indirect interactions with the scaffold protein (Jacob et al., 2005).

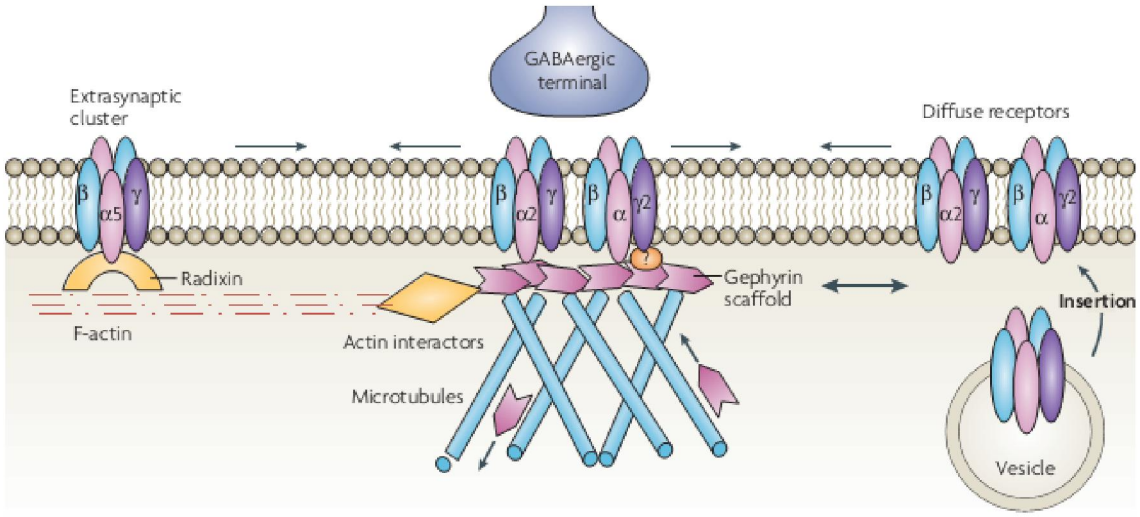

FIGURE 1.6. Dynamic regulation of receptor lateral mobility at the GABAergic synapse. $GABA_AR$ are inserted into the plasma membrane at extrasynaptic sites from where they can then diffuse into synaptic sites. Lateral diffusion (indicated by the horizontal single-headed arrows) in the plasma membrane allows continual exchange between diffuse receptor populations and synaptic or extrasynaptic receptor clusters, with anchoring molecules tethering or corralling moving receptors. The synaptic localization of α2-containing $GABA_AR$ is maintained by direct binding to gephyrin, which binds to microtubules and actin interactors. Gephyrin also displays local lateral movements (indicated by the double-headed arrow) and removal or addition by microtubule-dependent trafficking. This traffic of gephyrin further contributes to the regulation of GABAergic synaptic transmission. The extrasynaptic localization of α5-containing $GABA_AR$ is controlled by the binding of the α5 subunit to activated radixin, which directly binds F-actin. From (Jacob et al., 2008)

1.2.2.4. Endocytosis of $GABA_AR$ from the plasma membrane

The process of $GABA_AR$ endocytosis occurs mainly via clathrin- and dynamin-dependent mechanisms upon interaction of the $GABA_AR$ β and γ subunits with the AP2 clathrin adaptor protein complex (Kittler et al., 2005; Kittler et al., 2008; Kittler et al., 2000). In one-week-old cultures $GABA_AR$ endocytosis occurs within 30 min for 25% of the receptors present in the membrane, and 70% of these receptors are recycled back to the cell surface within one hour. Six hours after internalization about 30% of $GABA_AR$ are subjected to lysosomal degradation (Kittler et al., 2004).

$GABA_AR$ are intimately associated with AP2 in the brain through a direct binding of the β1–3 and γ2 $GABA_AR$ subunits (Kittler et al., 2000). The first sequence motifs important for AP2/clathrin/dynamin-mediated endocytosis of $GABA_AR$ was identified in an heterologous system and correspond to a dileucine motif present in β subunits (Herring et al., 2005; Herring et al., 2003). Additional studies performed in neurons identified a ten amino acid sequence motif (KTHLRRRSSQLK in the β3 subunit) that includes a major phosphorylation site conserved in the cytoplasmic loop region of β1-3 subunits (S408, S409 in β3) as an important motif for AP2/clathrin/dynamin-mediated $GABA_AR$ internalization (Kittler et al., 2005; Kittler et al., 2008). This motif also contains the major sites of phosphorylation by cAMP-dependent protein kinase (PKA) and calcium/phospholipid-dependent protein kinase (PKC) within this class of receptor subunits: S409 in β1, S410 in β2, and S408/9 in β3 (Moss et al., 1995). The interaction of the AP2 μ2 subunit with $GABA_AR$ is negatively regulated by phosphorylation of $GABA_AR$ β subunits. In fact, AP2 binds $GABA_AR$ when this site is dephosphorylated triggering their internalization. More recently, a tyrosine-based AP2-μ2 adaptin-binding motif ($Y^{365}GY^{367}ECL$) was indentified in the $GABA_AR$ γ2 subunit, which is also conserved in the γ1 and γ3 subunits (Kittler et al., 2008). These tyrosine residues are the major sites for phosphorylation by Fyn and Src kinases (Bogdanov et al., 2006; Jacob et al., 2005; Nishikawa et al., 2002). (Tab. 1.1)

TABLE 1. $GABA_AR$ phosphorylation sites. Adapted from (Vithlani et al., 2011)

		Protein Kinase		
Subunit	Phophorylation Site	In vitro	Heterologous cell lines	Primary neurons
β1	S384	CaMKII		
	S409	PKA, PKC, CaMKII, PKG	PKA, PKC	
β2	S410	PKA, PKC, Akt, CaMKII, PKG	PKc, Akt	Akt
β3	S383	CaMKII		
	S408	PKC	PKA, PKC	PKA, PKC
	S409	PKA, PKC, CaMKII, PKG	PKA, PKC	PKA, PKC
γ2	S327	PKC		
	S343	PKC, CaMKII		
	S348	CaMKII		
	T350	CaMKII		
	Y365	Src	Src	
	Y367	Src	Src	

1.2.3. Post-endocytic $GABA_AR$ sorting

Following internalization, $GABA_AR$ are rapidly recycled back to the neuronal plasma membrane or targeted for lysosomal degradation (Fig. 1.7). The destiny of internalized receptors is determinant for surface receptor levels.

1.2.4. Recycling of $GABA_AR$

The decision regarding the sorting of endocytosed $GABA_AR$ depends on the interaction of $GABA_AR$ β1-3 subunits with huntingtin-associated protein 1 (HAP1) (Fig. 1.7) (Kittler et al., 2004). HAP1 is a $GABA_AR$-associated protein that binds the intracellular loop of β subunits in vitro and in vivo (Kittler et al., 2004). This protein is localized in the cytoplasm and contains several central coil-coiled domains that are likely to regulate protein–protein interactions. Overexpression of HAP1 in neurons inhibits $GABA_AR$ degradation and consequently increases receptor recycling (Kittler et al., 2004). Furthermore, HAP1 overexpression was shown to increase surface levels of $GABA_AR$ and mIPSC amplitude (Kittler et al., 2004). An unanswered question is whether HAP1 promotes recycling of $GABA_ARs$ or prevents their lysosomal degradation.

1.2.5. Degradation of $GABA_AR$

Endocytosed $GABA_AR$ that fail to be recycled are targeted for lysosomal degradation (Kittler et al., 2004). This process is regulated by ubiquitination of a series of lysine residues within the intracellular domain of the γ2 subunit. Accordingly, an increase of $GABA_AR$ accumulation at the synapses is observed when lysosomal activity is blocked or the trafficking of ubiquitinated cargo to lysosomes is disrupted (Arancibia-Carcamo et al., 2009). Studies performed in primary neuronal cultures showed a biphasic degradation of $GABA_AR$, with about 42 % of the receptors displaying a short half-live of 3.8 hours, while the remaining 58% of the receptors show a half-life of 32 h (Borden and Farb, 1988). The stability of the former pool of receptors is not affected by lysosomal inhibitors, indicating that they are degraded by a non-lysosomal pathway. A surface biotinylation–degradation assay using cortical neuronal cultures, to assess the degradation of surface receptors, revealed that approximately 25 % of previously biotinylated surface receptors were degraded in 6 h and this effect was shown to be mediated by lysosomes (Kittler et al., 2004). In addition to the lysosomal system, a major mechanism for protein degradation involves the 26S proteasome, which promotes the degradation of polyubiquitinated substrates, a system largely recognized to play a role in the degradation of short-lived cytoplasmic proteins. Singly expressed to oligomeric structures formed by 3 $GABA_AR$ subunits may be degraded quickly by the proteasome (Bedford et al., 2001), but it is unknown whether receptor subunits are polyubiquitinated.

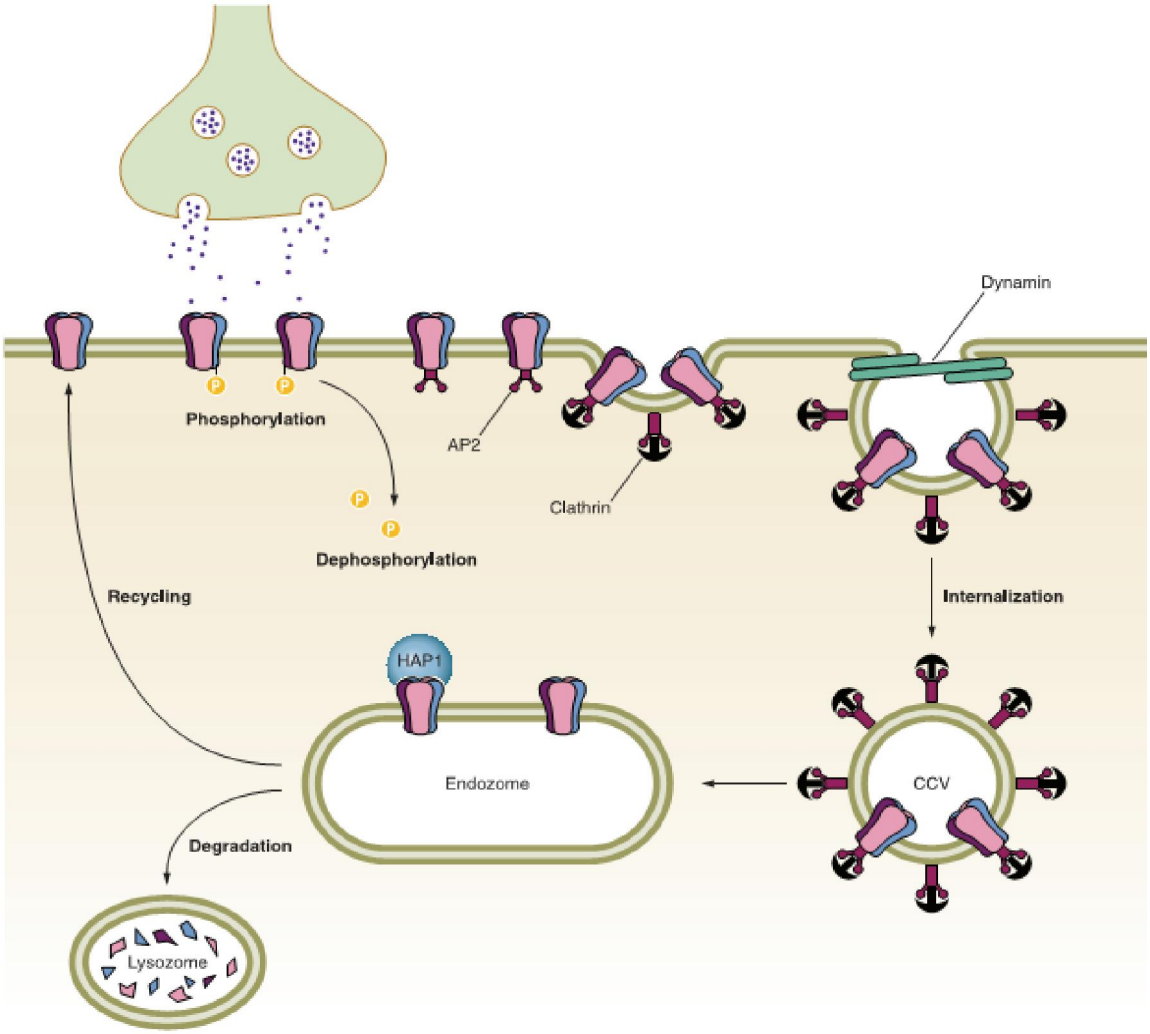

FIGURE 1.7. GABA$_A$R clathrin-mediated endocytosis.
The receptors cluster in specialized sites at the plasma membrane known as clathrin-coated pits, which invaginate and pinch off to form clathrin-coated vesicles (CCVs), a process that is dependent on dynamin. The clathrin adaptor protein (AP)-2 is a central component in the formation of these vesicles, forging a link between membrane proteins and clathrin that forms the outer layer of the coat. The vesicles subsequently lose their coat and fuse together to form an early endosome. Internalized receptors are then either subjected to rapid recycling or targeted for lysosomal degradation, an endocytic sorting decision that is regulated by the Huntingtin-associated protein (HAP)-1. From (Vithlani et al., 2011)

1.2.6. Pharmacology of $GABA_AR$

$GABA_AR$ are the site of action of diverse pharmacologically and clinically important drugs such as benzodiazepines, barbiturates, neuroactive steroids, anesthetics and convulsants, which allosterically modulate GABA-induced currents (Sieghart, 1995). The study of the activity of these drugs also contribute to elucidate the role of $GABA_AR$ in the modulation of anxiety, excitability of the brain, muscle tonus, vigilance, circadian rhythms, learning and memory (Ramerstorfer et al., 2011; Sieghart, 1995).

The binding sites for GABA and for some allosteric modulators of $GABA_AR$ were already identified (Olsen and Sieghart, 2008). Two GABA binding sites are located at the two β^+-α^- interfaces in the extracellular region of $GABA_AR$ composed of 2α, 2β and one γ subunit (Smith and Olsen, 1995). The benzodiazepines bind to a site located at the α^+ γ^- interface (Ernst et al., 2003; Sigel and Buhr, 1997) but in contrast to GABA or GABA agonists they do not activate directly $GABA_AR$. The high-affinity benzodiazepine binding site modulates allosterically GABA-induced currents. In fact, the transduction of benzodiazepine-induced conformational changes to the channel is less efficient as compared with GABA, in addition only a single high-affinity benzodiazepine binding site at the α^+ γ^- interface is present, which alone is not able to directly activate the channel in the absence of GABA. In contrast to the benzodiazepines allosteric modulation, steroids, inhalation anesthetics, i.v. anesthetics or barbiturates exhibit two different actions depending on the concentration. At low concentrations, they enhance GABA-induced currents, and at higher concentrations, they are able to directly provoke $GABA_AR$-mediated currents in the absence of GABA (Sieghart, 1995). These compounds, thus, presumably interact with at least two binding sites at $GABA_AR$.

As mentioned before $GABA_ARs$ exhibit an heterogeneous subunits composition with distinct but overlapping regional distribution in the brain. At the single cell level, there are cells expressing only a few $GABA_AR$ subunits, and others expressing most of these subunits (Pirker

et al., 2000; Wisden et al., 1992); giving rise to a multiplicity of these receptors. Moreover individual receptor subtypes often have a quite specific regional, cellular and subcellular distribution. (Kasugai et al., 2010; Nusser et al., 1998b) (Brunig et al., 2002; Crestani et al., 2002; Farrant and Nusser, 2005). A distinct subunits composition and distribution of receptor subtypes also suggests a distinct function. From a pharmacological point of view studies performed in transgenic mice, indicates that $GABA_AR$s containing α1 subunits seem to be involved in the sedative, anticonvulsant and anterograde amnestic actions of diazepam (McKernan et al., 2000; Rudolph et al., 1999). Similar experiments indicate that receptors containing α2 subunits primarily mediate the anxiolytic effects of diazepam (Low et al., 2000), and the analgesic action of local diazepam in the spinal cord (Knabl et al., 2008). Steroids seem to preferentially modulate receptors containing the δ subunit (Hosie et al., 2009; McKernan et al., 2000; Stell et al., 2003); these and other studies for the first time indicated a possible function of specific $GABA_AR$ subtypes in the rodent brain.

1.3. EFFECTS OF ISCHEMIA ON GABA NEUROTRANSMISSION

The insufficient blood supply to the brain during cerebral ischemia leads to excitotoxic neuronal death. This pathological condition is characterized by an unbalance between excitatory/inhibitory neurotransmission which contributes to neuronal damage (Choi, 1992; Lipton, 1999). In the CNS this balance is mainly regulated by glutamatergic and GABAergic neurotransmission, which are respectively up- and downregulated during ischemic insults. While the role of glutamate in neuronal death in brain ischemia is well documented, the alterations in GABAergic neurotransmission have received little attention and are not as well characterized. The experimental evidence presently available, using in vivo and in vitro models, point to alterations in GABAergic synaptic transmission in brain ischemia, both at the pre- and post-synaptic levels. Brain ischemia has been shown to induce an extracellular accumulation of GABA, which may be due to: i) an increase in Ca^{2+}-dependent release of the neurotransmitter before depletion of ATP, which is required for exocytosis; ii) reversal of GABA transporters induced by plasma membrane depolarization and changes in the Na^{+} electrochemical gradient; and iii) the leakage of GABA from injured, permeable terminals (Hutchinson et al., 2002; Phillis et al., 1994). However, in the case of transient cerebral ischemia, the extracellular levels of GABA return to normal within one hour of the reperfusion onset (Globus et al., 1991; Inglefield et al., 1995; Phillis et al., 1994; Schwartz et al., 1995). Interestingly previous results from our lab shown that excitotoxic conditions lead to the cleavage of glutamic acid decarboxylase (GAD) in cultured hippocampal neurons in a UPS-dependent manner (Baptista et al., 2010). GAD is the key enzyme in the synthesis of GABA (Martin and Rimvall, 1993) and was already known to be cleaved in cerebrocortical neurons subjected to excitotoxic conditions by a mechanism that is sensitive to inhibitors of calpain (Sha et al., 2008) (Monnerie and Le Roux, 2007). Cleavage of GAD diminished the activity of the enzyme and changed the its subcellular distribution (Baptista et al., 2010), which

should decrease GABA production and may affect the accumulation of the neurotransmitter in synaptic vesicles. Moreover under excitotoxic conditions also the GABA vesicular transporter VGAT is cleaved in a calpain dependent manner gives rise to a truncated form of the transporter (tVGAT) (Gomes et al., 2011). These aspects are expected to decrease the release of GABA by exocytosis under excitotoxicity Therefore the extracellular accumulation of the neurotransmitter likely does not depend by an increased of the GABA exocytose

This large accumulation of extracellular GABA can have several functional consequences despite being transient. For example, extracellular accumulation of GABA down-regulates GABA synthesis transiently, as shown in the mouse neocortex following a permanent middle cerebral artery occlusion (Green et al., 1992), and can induce adaptations in $GABA_AR$ and changes in the Cl^- gradient. In fact, sustained exposure of receptors to high concentrations of agonists usually leads to receptor down-regulation and there is evidence that this may happen in vivo, after transient cerebral ischemia. In gerbils subjected to transient global ischemia, $GABA_AR$ are down-regulated in the hippocampus and cerebral cortex within 30 min of the onset of reperfusion, when GABA levels have started to normalize (Alicke and Schwartz-Bloom, 1995).

The effects of in vivo and in vitro ischemia on $GABA_AR$ function have been assessed mainly by electrophysiology, optical imaging of intracellular Cl^- changes and Cl^--flux assays. Electrophysiological studies showed that GABA-induced inhibitory postsynaptic potentials (IPSPs) disappear earlier than excitatory postsynaptic potentials (EPSPs) (Xu and Pulsinelli, 1994). Similar findings were reported in hippocampal slice preparations exposed to anoxia in vitro (Congar et al., 1995). Also, during reperfusion $GABA_AR$ response results are attenuated. In forebrain synaptoneurosomes, a subcellular fraction containing the pre- and post-synaptic regions, GABA-gated Cl^--flux is reduced during the first 2 h after cerebral ischemia (Verheul et al., 1993). Optical imaging of the hippocampal slice also showed that $GABA_AR$ responses in area CA1

pyramidal neurons are reduced early after the onset of reoxygenation (Inglefield and Schwartz-Bloom, 1998). A reduction in $GABA_A$ currents was also observed in cultured hippocampal neurons subjected to OGD, and this effect was attributed to the depletion of ATP and to an increase in intracellular Ca^{2+} (Harata et al., 1997).

The ischemia-induced alterations that decrease $GABA_AR$ activity comprise two major types of postsynaptic cellular events: the reduction in the transmembrane Cl^- gradient and the production of cellular mediators that alter $GABA_AR$ and their functional responses. There are indeed evidences pointing to an increase in the intracellular Cl^- concentration in adult neurons following oxygen-glucose deprivation as well as in in vitro cerebral ischemia. Thus, in hippocampal slices deprived of oxygen and glucose, an increase in intracellular Cl^- was observed in cell bodies at the CA1 area (Taylor et al., 1995). Furthermore, intracellular Cl^- was shown to increase in CA1 pyramidal neurons and in interneurons early after the onset of reoxygenation (Inglefield and Schwartz-Bloom, 1998). In accordance with these results, reduced $GABA_A$ responses in CA1 pyramidal neurons are observed following the rise in intracellular Cl^- induced by oxygen-glucose deprivation (Inglefield and Schwartz-Bloom, 1998). These in vitro results are supported by in vivo studies showing that focal cerebral ischemia reduces the GABA-mediated inhibition through a depolarizing shift in the reversal potential for $GABA_A$-mediated IPSPs in the primary somatosensory cortex (Mittmann et al., 1998). Anoxia was also found to suppress GABA-mediated IPSCs in hippocampal slices due to a positive shift in E_{Cl-} (Mittmann et al., 1998).

There are several possible mechanisms by which cerebral ischemia increases the intracellular Cl^- concentration, including the passive influx together the influx of Na^+, influx through GABA-gated Cl^- channels, inhibition of the voltage-gated Cl^- channel (ClC-2), hypofunction or reversal of outward Cl^- cotransporters and activation of inward Cl^- cotransporters. Studies demonstrate that oxygen-glucose deprivation causes an ATP-dependent rundown of $GABA_A$ currents in hippocampal neurons (Harata et al., 1997), suggesting that the Cl^- ATPase fails to

transport Cl^- into the extracellular space early during reperfusion, when ATP levels are still very low.

$GABA_AR$ function, similarly to glutamate receptors, may also be modulated by cellular signals generated during cerebral ischemia. Firstly, an increased intracellular Ca^{2+} concentration decreases GABA-gated Cl^- conductance (Inoue et al., 1986; Stelzer et al., 1988) and GABA-induced currents in neuronal cultures (Llano et al., 1991; Martina et al., 1994). One of the Ca^{2+}-dependent enzymes activated by ischemia is phospholipase A2, which generates arachidonic acid from the hydrolysis of membrane phospholipids. It was shown that phospholipase A2, arachidonic acid and its metabolites (i.e. prostaglandins and thromboxanes) decrease $GABA_A$ responses in cerebral cortical synaptoneurosomes (Schwartz-Bloom et al., 1996; Schwartz et al., 1988; Schwartz and Yu, 1992). In addition, there are several studies demonstrating the sensitivity of $GABA_A$ neurotransmission to oxidative stress. Generation of superoxide radicals inhibits $GABA_A$ responses in cerebral cortical synaptoneurosomes in a Ca^{2+}-dependent manner (Schwartz et al., 1988). In addition, the generation of superoxide radicals and H_2O_2 have direct effects on $GABA_AR$, decreasing the maximal density of Cl^- channel sites in brain homogenates (Sah et al., 2002). Exposure of hippocampal (Pellmar, 1995) and thalamocortical slices (Frantseva et al., 1998) to H_2O_2 also reduced significantly the inhibitory postsynaptic potentials (IPSPs).

The down-regulation of GABAergic synapses in brain ischemia may also result from the reduction of $GABA_AR$ phosphorylation which leads to receptor desensitization (Gyenes et al., 1994) and a reduction of cell surface density of $GABA_AR$ (Nusser et al., 1997; Nusser et al., 1998a). These receptors, similarly to most plasma membrane proteins, are very dynamic at neuronal cell surface, not only for their cycle between the plasma membrane and intracellular compartments, but also for lateral diffusion (see section 1.2.2.3). The lateral diffusion of $GABA_AR$ has recently gained increased importance following the observations showing that the receptors are inserted into and removed from the plasma

membrane exclusively at extrasynaptic sites (Bogdanov et al., 2006; Thomas et al., 2005). Therefore, the key mechanism controlling the size of the $GABA_AR$ postsynaptic pool, thereby accounting for the strength of inhibitory synapses, is the receptor exo- and endocytosis. Several studies have reported a decrease in the surface and synaptic $GABA_AR$ expression during ischemia, suggesting an increase in receptor endocytosis (Arancibia-Carcamo and Kittler, 2009; Liu et al., 2010; Mielke and Wang, 2005; Zhan et al., 2006). In vitro studies, using cell culture ELISA as a cell surface receptor assay, showed that OGD decreases cell surface $GABA_AR$ in cultured cortical neurons without altering the total amount of receptors. Inhibition of receptor endocytosis with hypertonic sucrose treatment prevented receptor internalization and similar results were obtained in cells treated with insulin. Under the latter conditions the cells were protected from OGD-induced cell death, and the authors suggested that $GABA_AR$ internalization contributes to neuronal death (Mielke and Wang, 2005). This hypothesis was later supported in studies using the same technique to follow receptor internalization in addition to the biotinylation assay. In this set of experiments the activation of phosphatidylinositol 3-kinase/Akt–dependent signaling pathway, through PTEN downregulation, was shown to protect neurons from the toxic effects of OGD by preventing the reduction in the surface expression of $GABA_AR$ (Liu et al., 2010). More recently, it was shown that the downmodulation of $GABA_ARs$ from dendritic clusters during OGD is dependent on the AP2 pathway for cell surface removal of the receptors. Moreover, blockade of this pathway reduced the neuronal death induced by OGD (Smith et al., 2012). Although these findings point to a key role of $GABA_AR$ endocytosis in OGD-induced downmodulation of GABAergic neurotransmission and cell death the strategy employed may also interfere with the internalization of other proteins mediate by the AP2 pathway. The hypothesis that the reduction of surface $GABA_AR$ is accompanied by alteration of the $GABA_AR$ subunits was not much investigated however as shown that in vivo ischemia there is a loss of

$GABA_AR$ subunit mRNA expression in hippocampal neurons before the degeneration of CA1 pyramidal cells (Li et al., 1993).

1.3.1. Neuroprotection by GABAergic drugs after cerebral ischemia

Considering the evidence for the alteration in GABAergic neurotransmission in ischemia, and its role in neuronal death, the GABAergic system is an obvious target for neuroprotection studies. The upregulation of the GABAergic system as a neuroprotective strategy can be achieved by acting at different levels, using GABA agonists, GABA modulators, GABA transporter inhibitors and GABA transaminase inhibitors.

Injection of the benzodiazepine diazepam directly into area CA1 of the hippocampus was shown to be neuroprotective (Schwartz et al., 1995). Also, the GABA modulator chlomethiazole reduces cerebral cortical and striatal infarct size in rats and marmosets when administered 1 h after occlusion of the middle cerebral artery (Green et al., 2000; Marshall et al., 2000; Sydserff et al., 1995).

In general the therapeutic window of GABAergic drugs is relatively short (Cross et al., 1991; Hall et al., 1997; Inglefield et al., 1995; Schwartz-Bloom et al., 1998; Schwartz-Bloom et al., 2000; Schwartz et al., 1994; Schwartz et al., 1995; Shuaib et al., 1995). For example, the benzodiazepine partial agonist imidazenil and the GABA uptake inhibitor tiagabine were shown to be neuroprotective in CA1 hippocampal neurons when the effects were evaluated 4-7 days after transient global ischemia, but not 21-35 days after ischemia (Inglefield et al., 1995; Schwartz-Bloom et al., 1998). Independently of the strategy used, the current model postulates that to be effective neuroprotective drugs need to be administered early, within a few hours of a stroke (De Keyser et al., 1999).

Despite the neuroprotective effects of $GABA_AR$ agonists (e.g. clomethiazole) in both global and focal ischemia models, as shown by various outcome measures such as histopathology, excitatory amino acid

release in vivo, and edema formation (Green, 1998), clinical trials failed to confirm its benefit. (Lyden et al., 2001). Diazepam was also investigated in clinical trials, in the search for a potential neuroprotective effect in acute stroke, with the treatment initiated within 12 h from onset, but no significant effects were obtained (Lodder et al., 2006). The negative outcomes of GABAergic drugs in the treatment of cerebral ischemia suggest that the activation of $GABA_AR$ may not be the better strategy to upregulate GABAergic transmission in this pathologic condition.

OBJECTIVES

$GABA_A$ receptors ($GABA_AR$) are the main mediators of inhibitory neurotransmission in the CNS and play an essential role in maintaining the excitatory/inhibitory balance required for the correct function of neuronal networks (Smith and Kittler, 2010). Modulation of $GABA_AR$ expression at the synapse plays a key role in determining the strength of synaptic inhibition (Arancibia-Carcamo and Kittler, 2009). In brain ischemia the surface downmodulation of $GABA_AR$ contributes to compromise neuronal inhibition thereby altering neuronal excitability, but the molecular mechanisms underlying the changes in the $GABA_AR$ surface expression under pathological conditions remain poorly understood.

The present work was aimed at investigating the molecular mechanisms underlying $GABA_AR$ downregulation in cultured hippocampal neurons subjected to Oxygen Glucose Deprivation (OGD), an in vitro model of ischemia. More specifically we investigated the effect of transient exposure of hippocampal neurons to OGD on:

- the total protein levels of $GABA_AR$ subunits characteristic of synaptic (α1, α2, β3, γ2) and extrasynaptic (δ subunit) receptors. The role of calpains in the alterations of $GABA_AR$ total protein levels was also investigated;

- the expression levels of α1, α2, β2, β3 and γ2 $GABA_AR$ subunits, and the contribution of glutamate receptor activation, using quantitative PCR experiments;

- the internalization of $GABA_AR$. In particular we studied the effect of OGD on i) $GABA_AR$/Gephyrin interaction, using a surface co-immunoprecipitation assay, and on ii) the internalization of $GABA_AR$ via clathrin dependent endocytosis, using a antibody-feeding assay;

- the dephosphorylation and internalization of β3 $GABA_AR$ subunits, and the contribution of dephosphorylation and consequent internalization of the receptors to neuronal cell death. The role of receptor phosphorylation in OGD-induced neuronal death was investigated using a phospho-mutant form of the β3 $GABA_AR$ subunit;

- the recycling of $GABA_AR$ back to the plasma membrane and their interaction with the HAP1, the protein that determines the sorting of endocytosed $GABA_AR$.

CHAPTER 2 – Material and Methods

2.1. Hippocampal cultures

Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin (0.06%, 15 min, 37°C; GIBCO Invitrogen) in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS; 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red). The hippocampi were then washed with HBSS containing 10% fetal bovine serum (GIBCO Invitrogen), to stop trypsin activity, and transferred to Neurobasal medium (GIBCO Invitrogen) supplemented with B27 supplement (1:50 dilution; GIBCO Invitrogen), 25 µM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin. The cells were dissociated in this solution and were then plated on 6 well plates ($90.0x10^3$ cells/cm^2), previously coated with poly-D-lysine (0.1 mg/mL), or on poly-D-lysine coated glass coverslips, at a density of $80.0x10^3$ cells/cm^2. The cultures were maintained in a humidified incubator with 5% CO_2/95% air, at 37°C, for 15 days.

2.2. Oxygen-glucose deprivation

Hippocampal neurons (15 DIV) were incubated in a glucose-free saline buffer (116 mM NaCl, 25 mM sucrose, 10 mM HEPES, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 25 mM $NaHCO_3$) in an anaerobic chamber with 10% H_2, 85% N_2, 5% CO_2 (Forma Anaerobic System, Thermo Fisher Scientific), at 37°C, for the indicated period of time. The OGD buffer was then replaced by conditioned medium and the cultures were returned to the humidified 95% air/5% CO_2 incubator for the indicated post-incubation time period. Under control conditions (Sham) the cells were incubated in the saline buffer described above, supplemented with 25 mM glucose instead of sucrose, and kept in the humidified 95% air/5% CO_2 incubator at 37°C. When appropriate the cells were pre-incubated with glutamate receptor or calpain inhibitors (20

μM NBQX [Tocris] and 100 μM APV [Tocris] were added 30 min before OGD; 50 μM ALLN [Calbiochem] or 50 μM MDL28170 [Calbiochem], 1 h before OGD), and the drugs were also present during and after the insult.

2.3. Nuclear morphology analysis

After OGD followed by incubation in culture conditioned medium, neurons were fixed in 4% sucrose/paraformaldehyde and incubated with the fluorescent dye Hoechst 33342 (1 μg/ml) for 10 min. The coverslips were then mounted on a slide with a fluorescence mounting medium (DAKO), and imaging was performed on a Zeiss Axiovert 200 fluorescence microscope coupled to an Axiocam HRm digital camera. For each experimental condition three coverslips were analyzed (at least 200 cells per coverslip were counted), and at least three independent experiments were performed, using distinct preparations.

2.4. Western blotting

Total cell extracts were prepared after washing the cells twice with ice-cold PBS buffer. The cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS, at a final pH 7.5) supplemented with 1 mM DTT and a cocktail of protease inhibitors (0.1 mM PMSF, 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin; Sigma-Aldrich Química). For phosphorylation studies the lysis buffer was contained 10 mM HEPES, 150 mM NaCl, 10 mM EDTA and 1% Triton (pH 7.4), and was supplemented with phosphatase inhibitors (50 mM NaF and 1.5 mM sodium orthovanadate). After centrifugation at 16,100x g for 10 min, protein levels present in the supernatants were quantified using the BCA method (Thermo Scientific). Samples were then diluted with a 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). Protein samples were separated by SDS-PAGE, in 10% polyacrylamide gels, transferred to PVDF membranes (Millipore) and immunoblotted. Membranes were incubated with primary antibodies (overnight at 4°C), washed and exposed to alkaline phosphatase-conjugated secondary antibodies (1:20,000 dilution; 1h at room temperature) (GE Healthcare or Jackson ImmunoResearch). Alkaline phosphatase activity was visualized using ECF on the Storm 860 Gel and Blot Imaging System (GE Healthcare). The following primary antibodies were used: anti-Alpha1 $GABA_A$ receptor (1:1000, NeuroMab), anti-Alpha2 $GABA_A$ receptor (1:1000, Synaptic System), anti-Beta 3 $GABA_A$ receptor (1:1000, NeuroMab), anti-Phospho-$Ser^{408/409}$ Beta 3 $GABA_A$ receptor (1:1000, Symansis), anti-Gama 2 $GABA_A$ receptor (1:1000, Synaptic Systems) and anti-Gephyrin (1:1000, Synaptic Systems). Anti-Synaptophysin (1:10000, Abcam) and anti-β-tubulin (1:300000, Sigma) antibodies were used as loading controls.

Dephosphorylation of the lysate proteins was performed by incubating 30 µg of protein with 1 µl of λ protein phosphatase (Final concentrations: ~20 U/ml; New England BioLabs), in 1x NEBuffer supplemented with 1 mM $MnCl_2$, for 1 h at 30°C. Samples were then diluted with a 2x concentrated denaturing buffer and the proteins were separated by SDS-PAGE as described above.

2.5. q-PCR Analyses

2.5.1. Total RNA isolation, RNA quality and RNA concentration

Total RNA extraction from cultured hippocampal neurons was performed with TRIzol (Invitrogen). Briefly, 1mL of TRIzol was added to each well (density of $90.0x10^3$ cells/cm^2) of a 6-well cluster plate and the content of each experimental condition (two wells) was collected. Chloroform was then added for phase separation and the RNA was precipitated by isopropanol addition. The precipitated RNA was washed with 75% ethanol, centrifuged, air-dried and resuspended in 20 µl of RNase-free water (GIBCO Invitrogen). RNA quality and integrity was evaluated using

the Experion automated gel-electrophoresis system (Bio-Rad). RNA concentration was determined using a NanoDrop 2000c/2000 UV-Vis Spectrophotomer (Thermo scientific). The samples were stored at -80°C until further use.

2.5.2. Reverse transcription reaction

First strand cDNA was synthesized from 1 µg of total RNA using iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's specifications.

2.5.3. Primer design

Primers for real-time PCR were designed using the "Beacon Designer 7" software (Premier Biosoft International), with the following specification: (1) GC content about 50%; (2) Annealing temperature (Ta) between 55 ± 5°C; (3) Secondary structures and primer-dimers were avoided; (4) Primer length between 18-24 bp; (5) Final product length between 100-200 bp.

2.5.4. Real-Time PCR

Gene expression analysis was performed using SsoFast™ SuperMix (Bio-Rad). Briefly, 2 µl of 1:10 diluted cDNA were added to 10 µl of 2x EvaGreen and to specific primers (final concentration of each was 250 nM in 20 µl total volume). The thermocycling reaction was composed of the following steps: 1) activation of the Sso7d fusion DNA polymerase (95°C for 30 s), 2) denaturation (45 cycles of a 10s step at 95°C), 3) annealing (30 s at the optimal annealing temperature for each set of primers) and 4) elongation (30s at 72°C). At the end of the thermocycling reaction a melting step was performed (starting at 55°C with a rate of 0.5°C per 10 s, up to 95°C). The fluorescence was measured after the extension step, using the iQ5 Multicolor Real-Time PCR Detection System (BioRad). To calculate the efficiency of each set of primers all assay included a non-template control and a standard curve of cDNA. The reactions were run in duplicate. The value used for the quantification was the threshold cycle (Ct; the detectable fluorescence signal above background resulting from the accumulation of amplified product), a

value that is a proportional measure of the starting concentration of the target sequence. The threshold base line was always set at the beginning of the exponential phase. Data analysis was performed using the GenEx (MultiD Analyses) software for Real-Time PCR expression profiling.

2.6. Fluorescence assay of receptor internalization

Cultured living hippocampal neurons (15 DIV) were incubated at RT for 10 min in the presence of a high concentration (1:100) of an anti-Alpha1 $GABA_A$ receptor antibody (Millipore), directed against the N-terminus of the α1 subunits, or an anti-myc antibody (1:300, Cell Signaling). The cells were then washed with PBS at 37°C, to remove the unbound antibody, and were further incubated in an antibody free conditioned medium at the same temperature (for different periods) to allow the internalization of antibody-bound receptors. After this incubation neurons were fixed for 15 min in 4% sucrose/paraformaldehyde. Next, neurons were exposed to a super-saturating concentration (1:300) of the first of two secondary antibodies (Alexa Fluor 488 goat anti-rabbit; Invitrogen) for 1h at RT. After permeabilization (0.25% Triton X-100 for 5 min) the cells were incubated with the second secondary antibody (Alexa Fluor 568 goat anti-rabbit, 1:500 Invitrogen) for 1 h at RT. This strategy allows distinguishing the surface receptors from those receptors that have been internalized before fixation (Goodkin et al., 2005). The coverslips were then mounted on slides with a fluorescence mounting medium (DAKO). Images were acquired on Axio Observer 2.1 fluorescence microscope (Zeiss) coupled to an Axiocam HRm digital camera, using a 63x oil obective and were quantified using the ImageJ image analysis software. For each experiment analyzed the cells were stained and imaged using identical settings. The ratio of internalization was calculated using the internalized antibody signal/total antibody signal ratio (Fig. 2.1).

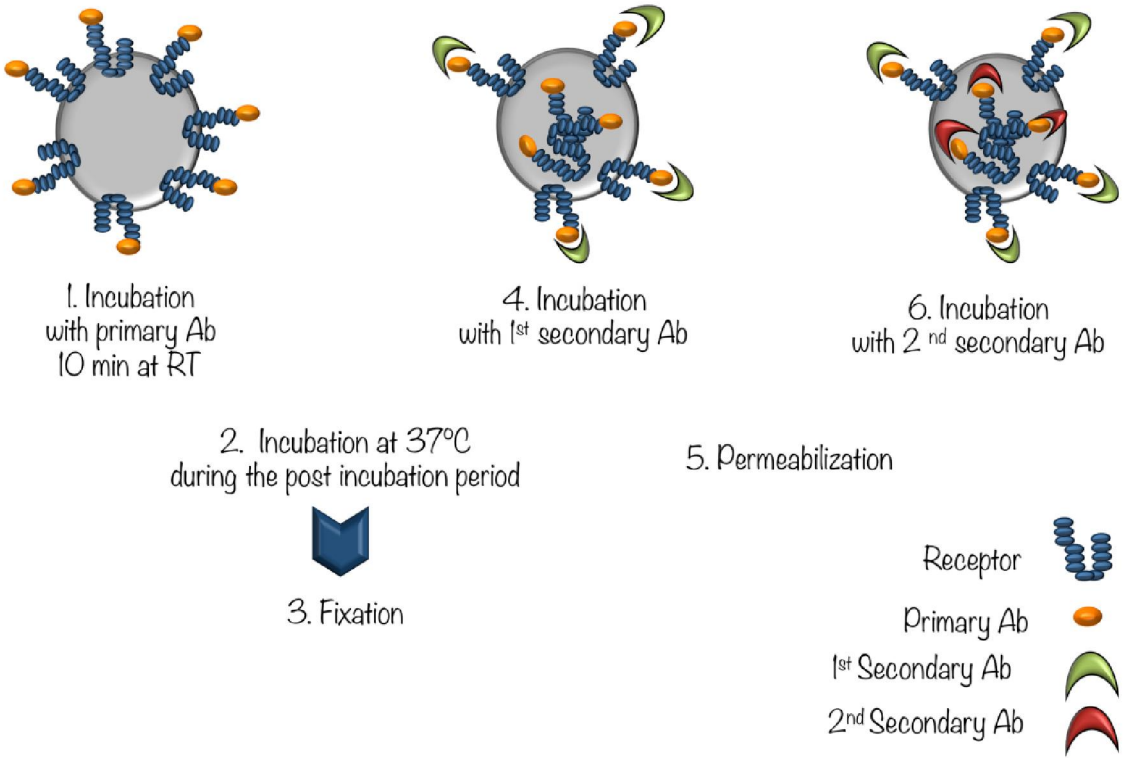

FIGURE 2.1. Schematic representation of the fluorescence assay used to assess receptor internalization.

2.7. Immunocytochemistry

Hippocampal neurons were fixed in 4% sucrose/paraformaldehyde (in PBS) and permeabilized with 0.3% Triton X-100 in PBS. The neurons were than incubated with 10% BSA in PBS for 30 min at 37°C, and incubated with the primary antibody anti-myc (1:500, Cell Signaling) diluted in 3% BSA in PBS, overnight at 4°C. The cells were washed with PBS and incubated with the secondary antibody (anti-mouse IgG) conjugated with Alexa Fluor 488 (Invitrogen), for 1 h at RT. The coverslips were mounted in a fluorescence mounting medium (DAKO, Denmark). Imaging was performed in an Axio Observer 2.1 fluorescence microscope, coupled to an Axiocam HRm digital camera, using a 63x oil objective. The cells to count were chosen by the myc (green) channel to check for the presence of transfected neurons. Measurements were performed in three independent preparations, and at least 50 cells were counted per experimental condition for each preparation.

2.8. Surface co-immunoprecipitation assay

After stimulation using the indicated experimental conditions hippocampal neurons were washed twice with ice-cold PBS and incubated with Sulfo-NHS–SS–biotin (0.25 mg/ml in PBS; Thermo Scientific) for 15 min on ice. Cells were then washed twice with 50 mM NH_4Cl and two times more with PBS. After biotinylation, the cells were lysed with RIPA buffer and α1-containing $GABA_AR$s were immunoprecipitated.

Protein G Plus-Agarose beads (50 µl; Santa Cruz Biotechnology) were added to Lysis buffer (1 ml) containing 5 µg of an anti-$GABA_AR$ alpha 1 subunit (NeuroMab) monoclonal antibody and incubated for 2 h on a head-over-head shaker at 4°C. Antibody excess was removed by two rinses with lysis buffer. Lysed samples (400 µg) were added to the beads and incubated for 6 h on a head-over-head shaker at 4°C. Beads were centrifuged at 800× g to remove the antibody, and the samples were then washed three times with lysis buffer. The residual buffer was removed and bead–IP–$GABA_AR$s were incubated with 50 µL of 1% SDS (80 min at 37°C) to disrupt the interaction between the beads and IP-$GABA_AR$s. Finally, beads were centrifuged at 800× g, and the supernatants were mixed with 150 µl of lysis buffer before being used in NeutAvidin pull-downs.

NeutAvidin® Plus UltraLink Resin beads (40 µL; Thermo scientific) were added to the samples and mixed on a head-over-head shaker for 4 h. Beads were then centrifuged at 800× g and washed three times with lysis buffer. The residual lysis buffer was removed and then 60 µL of 2× loading buffer was added. Samples were heated at 90°C for 5 min and beads were centrifuged at 800× g. The bead supernatants were used for western blot analysis (Fig. 2.2).

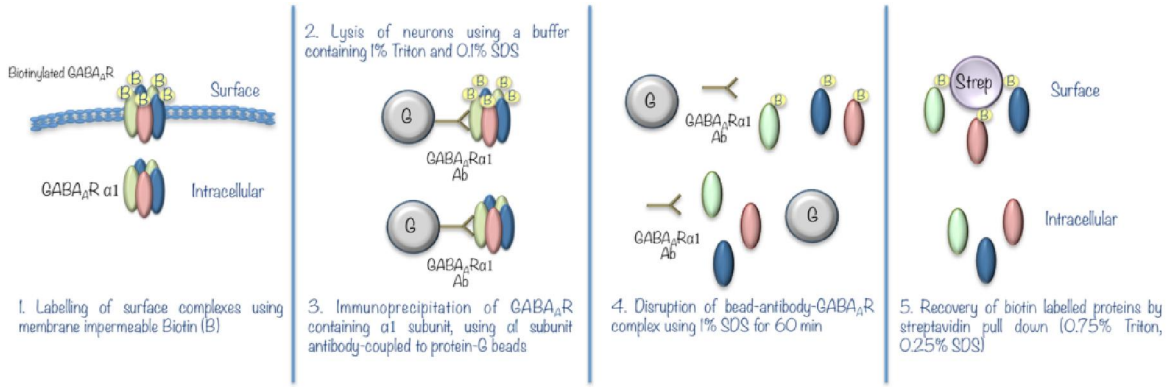

FIGURE 2.2. Schematic representation of the surface co-immunoprecipitation assay.

2.9. Neuron transfection with calcium phosphate

Transfection of cultured hippocampal neurons with myc-huGABA$_A$R β3 (WT), myc-huGABA$_A$R β3 (p-mimetic) or myc-huGABA$_A$R β3 (p-null) constructs was performed by the calcium phosphate coprecipitation method. Briefly, 2 µg of plasmid DNA were diluted in Tris-EDTA (TE) pH 7.3 and mixed with 2.5 M $CaCl_2$. This DNA/TE/calcium mix was added to 10 mM HEPES-buffered saline solution (270 mM NaCl, 10 mM KCl, 1.4 mM Na_2 HPO_4, 11 mM dextrose, 42 mM HEPES, pH 7.2). The precipitates were allowed to form for 30 min at room temperature, protected from light, with vortex mixing every 5 min, to ensure that the precipitates had similar small sizes. Meanwhile, cultured hippocampal neurons were incubated with cultured-conditioned medium with 2 mM kynurenic acid (Sigma). The precipitates were added drop-wise to each well and incubated for 2 h at 37°C, in an incubator with 95% air/ 5% CO_2. The cells were then washed with acidic 10% CO_2 equilibrated culture medium containing 2 mM kynurenic acid and returned to the 95% air /5% CO_2 incubator for 20 min at 37°C. Finally, the medium was replaced with the initial culture-conditioned medium, and the cells were further incubated in a 95% air /5% CO_2 incubator for 48 h at 37°C to allow protein expression. Cell cultures were then subjected to OGD for 90 min, and 8 h after the insult the cells were fixed to proceed with the cell

death assay. In the case of the fluorescence internalization assay cells were subjected to 70 min of OGD.

2.10. Mutagenesis

The plasmid containing the human WT $GABA_AR$ β3 subunit sequence was a kind offer of Doctor Martin Wallner (Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, UCLA). In the same vector, two myc-Tag sequences (GAGCAGAAGCTGATCTCAGAGGAGGATCTGGAGC AGAAGCTGATCTCAGAGGAGGATCTG) were added between the 4th and 5th codon of human $GABA_AR$ β3 cDNA that code for the amino acids belonging to the N-terminus of the protein (NZYTech, Lda). To obtain the phospho-mimetic and a phospho-null mutants of the human $GABA_AR$ β3 subunit, we performed a site directed mutagenesis of the serine residues 432/433 (homologous of mouse 408/409), using QuikChange® II XL Site–Directed Mutagenesis Kit (Agilent Technology). Briefly, specific primers were designed to mutate the two serine residues to two aspartate residues (5' gcacaagaagacccatctacggaggagggatgatcagctcaaaattaaaatacctgatctaac3'), in the case of the phospho-mimetic mutant, and to two alanine residues (5' gacccatctacggaggagggctgcacagctcaaaattaaaat 3') in the case of the phospho-null mutant (primers were synthesized by Sigma Aldrich). For each mutagenesis the reaction contained 13.5 ng of dsDNA template (myc-huGABA$_A$R β3), 5 µl of 10x reaction buffer, 125 ng of each oligonucleotide primer, 1 µl of dNTP mix, 3 µl of QuikSolution, ddH_2O to final volume of 50 µl and 1 µl of *PfuUltra* HF DNA polymerase (2.5 U/µl). The following thermal cycling was then performed: 95°C for 1 min, 18 cycles (95°C for 50 s, 60°C for 50 s, 68°C for 6 min 30 s) and 68°C for 7 min. The parental methylated dsDNA was then digested using 1 µl of *Dpn* I enzyme (New England BioLabs) at 37°C for 1 h. *Dpn* I digested dsDNA was used to transform E. coli Top 10 cells to be then amplified. The

obtained plasmid DNA was extracted using Plasmid Mini Kit (Quiagen) and sequenced to confirm the mutagenesis (STABvida).

2.11. Middle cerebral artery occlusion

Focal cerebral ischemia was induced by the transient occlusion of the right middle cerebral artery (MCA), using the intraluminal filament placement technique as described previously (Nygren and Wieloch, 2005). Briefly, adult male mice were anesthetized by inhalation of 2.5% isoflurane (IsobaVet, Schering-Plough Animal Health) in O_2:N_2O (30:70). Anesthesia was subsequently reduced to 1.5–1.8% isoflurane and sustained throughout the occlusion period. Body temperature was kept at ~37°C throughout the surgery period. To monitor regional cerebral blood flow (rCBF), an optical fiber probe (Probe 318-I, Perimed) was fixed to the skull at 2 mm posterior and 4 mm lateral to bregma and connected to a laser Doppler flow meter (Periflux System 5000, Perimed). A filament composed of 6 – 0 polydioxanone suture (PSD II, Ethicon) with a silicone tip (diameter of 225–275 µm) was inserted into the external carotid artery and advanced into the common carotid artery. The filament was retracted, moved into the internal carotid artery, and advanced until the origin of the MCA, given by the sudden drop in rCBF (~70% of baseline). After 45 min, the filament was withdrawn and reperfusion observed. The animals were placed in a heating box at 37°C for the first 2 h after surgery and thereafter transferred into a heating box at 35°C, to avoid postsurgical hypothermia. Thirty minutes and 24 h after the onset of reperfusion, 0.5 ml of 5% glucose was administered subcutaneously. Temperature and sensorimotor deficits were assessed at 1, 2 h and 24 h after the surgery. Body weight was controlled daily. In sham surgeries, the filament was advanced up to the internal carotid artery, and withdrawn before reaching the MCA. The Ethics Committee for Animal Research at Lund University approved animal housing conditions, handling, and surgical procedures. Eleven to 36 weeks old C57BL/6J

male mice (weight: 23.0 g to 37.9 g; Lund University breeding facility) were housed under diurnal conditions with ad libitum access to water and food before and after surgery.

Mice were anesthetized 48 h after MCA occlusion (MCAO) or sham surgery, by inhalation of 2.5 % isoflurane and were then perfused transcardially with 0.9 % NaCl for 2 min before decapitation. Upon removal of meninges, brains were rapidly isolated and frozen by immersion in isopentane at -40°C, further cooled down to -70°C and stored at -80°C. The infarct core and remaining ipsilateral tissue (designated as penumbra for simplification) were dissected, as well as the contralateral cortex, from coronal brain sections covering the majority of damage. More specifically, consecutive 2 mm, 1 mm and 2 mm thick brain sections were made, starting at 2 mm from the olfactory bulb. Dissections were performed at -15 °C, a temperature that allows an easy detachment of the infarct core and penumbra. The cortical-striatal infarcts obtained were illustrated in (Inacio et al., 2011). Equivalent brain regions were dissected from sham-operated mice, which were also designated as infarct core and penumbra, and contralateral cortex. For each animal, corresponding regions from each of 3 consecutive brain sections were pulled together. Samples were then homogenized and processed for Western blotting as previously described (Inacio et al., 2011). Cellular protein extraction was performed by mechanical homogenization of the tissue and incubation in lysis buffer: 20 mM Tris (pH 7.5), 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1% Triton-X100, 2.5 mM sodium pyrophosphate, 1mM β-glycerolphosphate, 1mM orthovanadate and 1 mM PMSF, supplemented with a protease inhibitor cocktail (P8340, Sigma-Aldrich). Following 30 min incubation at 4°C, samples were centrifuged at 18000x g, for 15 min. Total protein concentration in lysates was determined by the Bradford assay, using bovine albumin (Sigma) as standard.

2.12. Receptor recycling assay

Cultured living hippocampal neurons (15 DIV), transfected with myc-huGABA$_A$R β3 (WT), were incubated at RT for 10 min in the presence of a high concentration of an anti-myc antibody (Cell Signaling). The myc-tag was located at the N-terminus of the β3 GABA$_A$R subunits. The cells were then washed with PBS at 37°C to remove the unbound antibody and further incubated in an antibody free conditioned medium at 37°C for 20 min allowing the internalization of antibody-bound receptors. The antibodies that remained on the cell surface were then stripped away by incubation in stripping buffer (0.5 M NaCl and 0.2 M acetic acid) on ice for 4 min (Passafaro et al., 2001). Neurons were then washed extensively with ice-cold PBS and returned back to culture medium at 37°C (for different periods of time) for recycling. After recycling, neurons were fixed, and Myc-antibody complexes recycling back to the surface were detected by incubation of the cells with a secondary antibody (anti-mouse IgG conjugated with Alexa Fluor 488). Neurons were then permeabilized, and intracellular Myc-antibody complexes were detected with a different secondary antibody (anti-mouse IgG conjugated with Alexa Fluor 568). The coverslips were then mounted on slides with a fluorescence mounting medium (DAKO). Images were acquired on Axio Observer 2.1 fluorescence microscope (Zeiss) coupled to an Axiocam HRm digital camera and were quantified using the ImageJ image analysis software. For each experiment analyzed, the cells were stained and imaged using identical settings. The recycling of the receptors was calculated using a recycled antibody signal/total antibody signal ratio (Fig. 2.3).

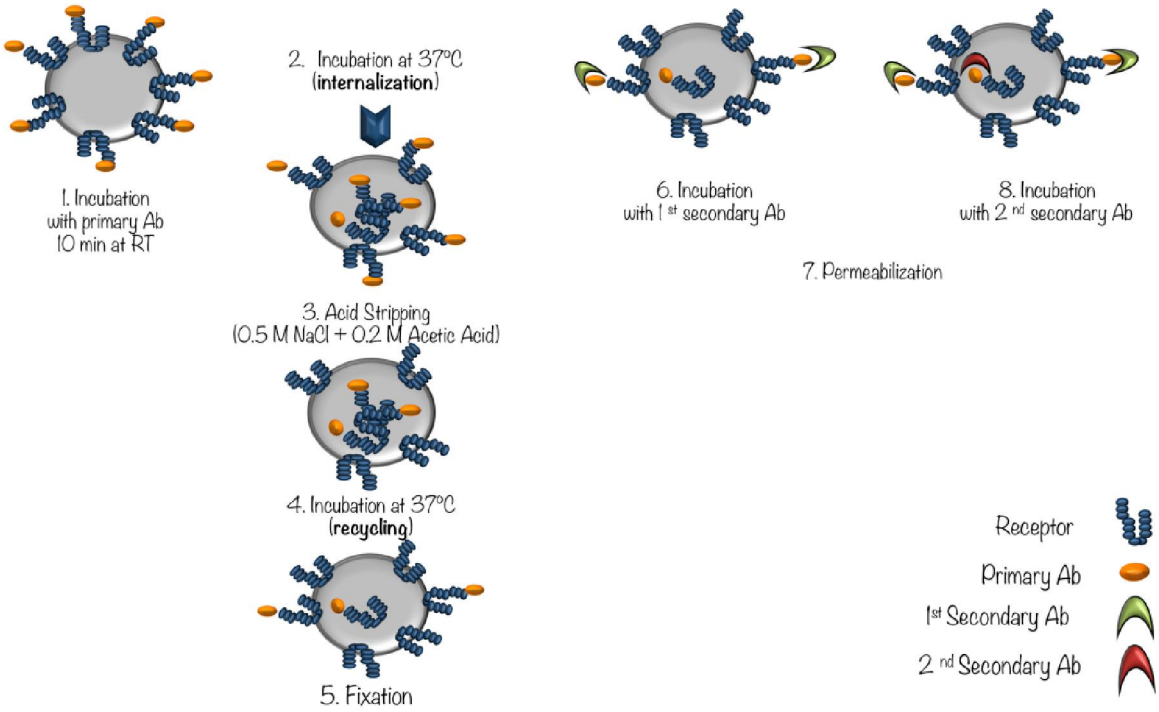

FIGURE 2.3. Schematic representation of the receptor recycling assay.

2.13. Co-immunoprecipitation assay

After protein extraction and quantification, 5 µg of an anti-$GABA_AR$ β3 subunit (NeuroMab) monoclonal antibody or anti-HAP1 (Santa Cruz Biotechnology) antibody were added to lysed samples (400 µg) and incubated overnight on a head-over-head shaker at 4°C in 1 ml of RIPA. Protein G Plus-Agarose beads (40 µl; Santa Cruz Biotechnology) were then added to lysis buffer and incubated for 2 h on a head-over-head shaker at 4°C. Beads were centrifuged at 800× g to remove the antibody, and the samples were then washed three times with lysis buffer and once with urea 1 M. Finally, beads were centrifuged at 800× g, the residual lysis buffer was removed and 50 µL of 2× loading buffer was added. Samples were heated at 90°C for 5 min and beads were centrifuged at 800× g. The bead supernatants were used for western blot analysis.

2.14. Statistical analysis

Statistical analysis was performed using one-way ANOVA analysis of variance, followed by the Dunnett's or Bonferroni post-hoc test, or using the Student's *t* test, as indicated in the figure captions.

CHAPTER 3 – Results

3.1. OGD induces cell death and downregulates $GABA_AR$ subunit total protein levels by a calpain-dependent mechanism

OGD is a well-established in vitro model of global cerebral ischemia (Dawson et al., 1996; Goldberg and Choi, 1993; Martin et al., 1994). Exposure of cultured hippocampal neurons to OGD for 60 min-120 min induced a time-dependent cell death, as determined by analysis of nuclear morphology 7 h or 12 h after the insult (Fig. 3.1). The short periods of OGD tested, 60 min or 75 min, induced ~20% cell death, while 90 min or 120 min of OGD induced ~30% and ~40% cell death, respectively. We did not observe significant differences in cell death between the two post-incubation times used, 7 h and 12 h ($p>0.05$).

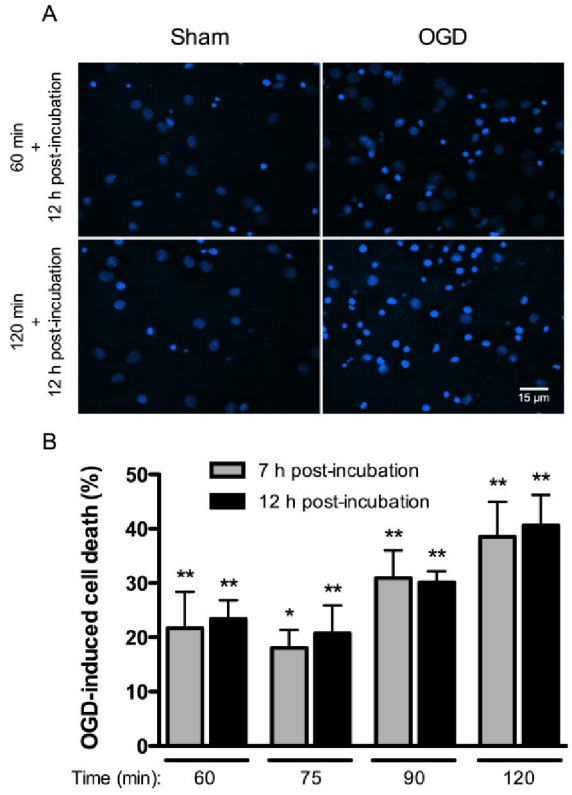

FIGURE 3.1. OGD-induced neuronal death.
Cultured hippocampal neurons (15 DIV) were subjected to OGD for the indicated periods of time (60 min, 75 min, 90 min and 120 min), and further incubated in culture-conditioned medium for 7 h or 12 h (post-incubation). Cell death was analyzed

after nuclei staining with Hoechst 33342. Representative results are shown in panel (A). Panel (B) shows the OGD-induced neuronal death, as calculated after subtracting neuronal death determined in preparations incubated under sham conditions for the same period of time. The results are average ± SEM of 3-5 different experiments performed in triplicate and in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's test. *p<0.05, **p<0.01 - significantly different when compared to control conditions.

To assess the effect of OGD on $GABA_AR$ subunit total protein levels, cultured hippocampal neurons were subjected to 90 min of OGD, and further incubated in culture conditioned medium for 8 h. $GABA_AR$ subunit protein levels were analyzed by western blot using specific antibodies against α1, α2, β3, γ2 and δ subunits. α1, α2, β3 and γ2 subunits are localized preferentially at the synapse (Alldred et al., 2005; Nusser et al., 1998b), mediating phasic inhibition (Brickley et al., 1996), in contrast with δ subunits which are extrasynaptic (Nusser et al., 1998b). The results show a downregulation of all the synaptic $GABA_AR$ subunits, of ~40% for α1 subunits, ~20% for α2 subunits, and ~35% for β3 and γ2 subunits (Fig. 3.2A-D), but no effect was observed for the δ subunit (Fig. 3.2E). Shorter periods of OGD (60 min) did not affect $GABA_AR$ total protein levels (not shown). Similarly to the results obtained in hippocampal neurons subjected to OGD, a downregulation of α1, β3 and γ2 subunits was observed in the infarct core of mice subjected to transient MCAO, a model of focal brain ischemia, but no effect was observed for the δ subunit. No significant changes in $GABA_AR$ subunit protein levels were observed in the penumbra (Fig.3.3A).

The OGD-induced $[Ca^{2+}]_i$ overload activates calpains (Brorson et al., 1995; Saido et al., 1994; Vanderklish and Bahr, 2000) which cleave numerous intracellular proteins in the ischemic brain (Bevers and Neumar, 2008). To investigate whether calpains are involved in the OGD-induced downregulation of $GABA_AR$ subunits, hippocampal neurons were subjected to OGD in the presence or in the absence of the chemical inhibitors ALLN or MDL28170. Western blot analysis performed 8 h after injury showed that MDL28170 fully abrogated the effect of OGD on α1,

β3, α2 and γ2 $GABA_AR$ subunits (p>0.05) (Fig. 3.2A-E). Furthermore, ALLN clearly prevented the reduction of β3 and α2 subunits in hippocampal neurons subjected to OGD (Fig. 3.2B, C).

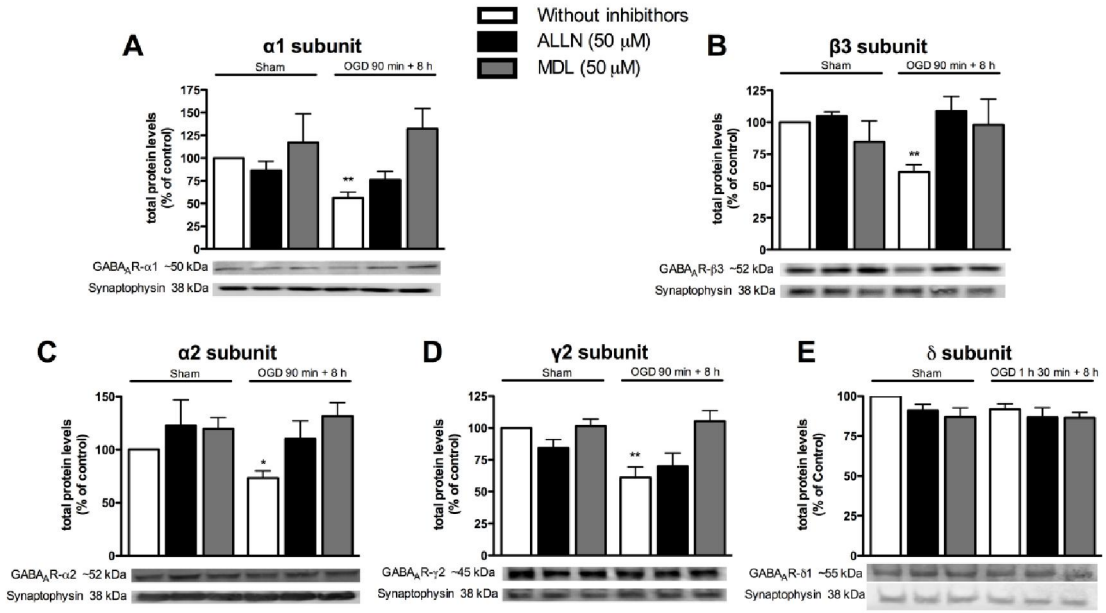

FIGURE 3.2. α1, α2, β3 and γ2 $GABA_AR$ subunit protein levels are downregulated in in vitro ischemia (OGD) by a calpain-dependent mechanism.
Cultured hippocampal neurons (15 DIV) were exposed to OGD for 90 min in the presence or in the absence of 50 μM ALLN and 50 μM MDL28170. α1 (A), β3 (B), α2 (C), and γ2 (D) $GABA_AR$ subunit total protein levels was determined by Western Blot analysis, 8 h after the insult, and the results were normalized with the loading control Synaptophysin. Results are the mean ± SEM of at least 3 independent experiments performed in different preparations, and are expressed as percentage of the control. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. *p<0.05, **p <0.01- significantly different when compared to control conditions, as depicted in the figure.

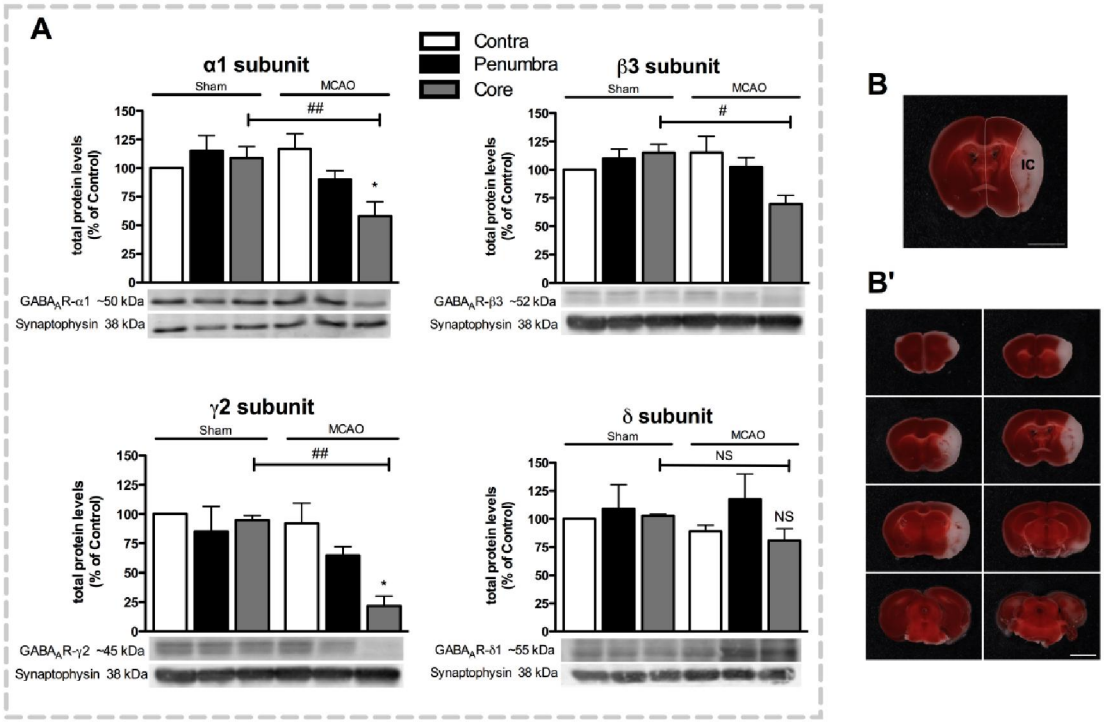

FIGURE 3.3. α1, β3 and γ2 GABA$_A$R subunit protein levels are downregulated in transient brain ischemia in vivo (MCAO).

(A) Effect of transient in vivo ischemia (MCAO) on α1, β3, γ2 and δ GABA$_A$R subunit total protein levels, as determined 48 h after the lesion in the infarct core, penumbra and contralateral cortex. GABA$_A$R subunit protein levels was determined by Western blot as indicated above. (B) Representative images of the regions dissected from the ipsilateral brain hemisphere of C57BL/6 mice subjected to sham surgery or MCAO, considered as infarct core (IC) and penumbra (delineated). Scale bar: 3mm. (B') Representative image of the cerebral infarct core following a transient (45 min) occlusion of the MCA in C57BL/6 mice, as given by lack of TTC staining in contiguous 1 mm tick coronal slices (white). Scale bar, 3 mm. Results are the mean ± SEM of at least 3 independent animals, and are expressed as percentage of the control. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. *p<0.05, **p <0.01 - significantly different from the contralateral region in sham operated animals; $^{\#}$p<0.05, $^{\#\#}$p<0.01 - significantly different when compared to the corresponded region in sham operated animals, as depicted in the figure. N.S. - not significant

3.2. OGD downregulates the $GABA_AR$ subunit mRNA through activation of glutamate receptors

The pool of protein in the cells is maintained by the balance between newly synthesized proteins and protein degradation. Considering the effect of OGD on the protein levels of $GABA_A$ receptor subunits (Figs. 3.2, 3.3), we hypothesized that in vitro ischemia could also downregulate the mRNA for the various subunits causing a long-term effect on the synthesis of new receptors. Quantitative PCR experiments showed a decrease in the expression levels of α1, α2, β2, β3 and γ $GABA_AR$ subunits in hippocampal neurons subjected to OGD for 90 min, and further incubated in culture conditioned medium for 5 h, but no effect was observed for shorter periods of in vitro ischemia (75 min), even when determined 7 h after the insult (Fig. 3.4 A-E). The former experimental conditions led to a 70% reduction in mRNA levels of α1, 50% in α2 and β2, 25% in β3 and 40% in γ2 subunits, which are typically found in synaptic receptors. In contrast, no changes in the mRNA levels for the extrasynaptic $GABA_AR$ δ subunits was observed, even for 90 min of OGD (Fig. 3.4F). The OGD-induced downregulation of mRNA levels for α1, α2, β2, β3 and γ2 subunits was prevented by incubation with the NMDA and non-NMDA glutamate receptors inhibitors APV (100 μM) and NBQX (20 μM) (Fig. 3.5).

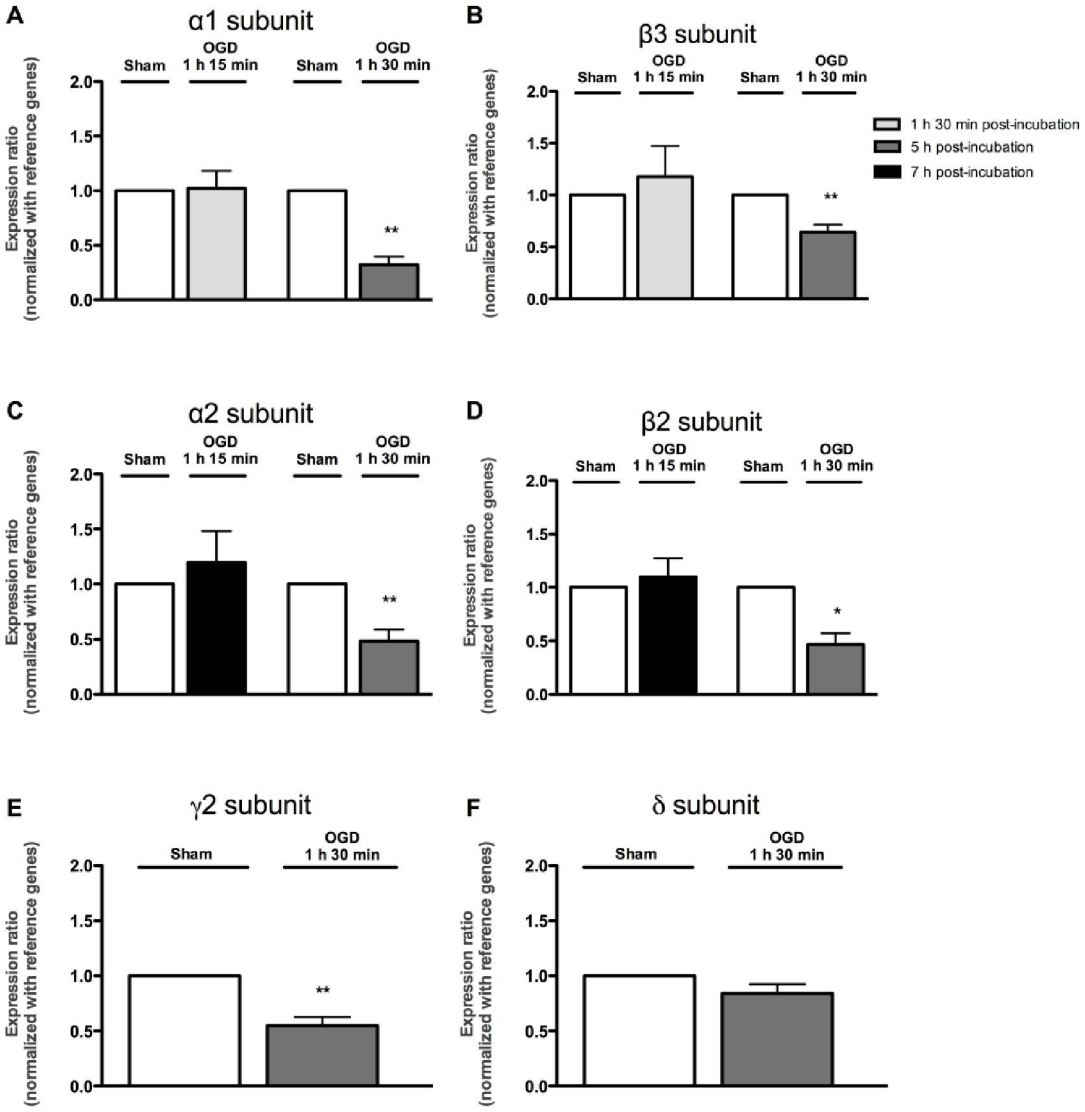

FIGURE 3.4. OGD downregulates the mRNA levels of α1, α2, β3 and γ2 $GABA_AR$s subunits.

Cultured hippocampal neurons (15 DIV) were exposed to OGD for 75 min or 90 min and further incubated for the indicated periods of time. The mRNA for α1 (A), α2 (C), β2 (D), β3 (B), γ2 (D) and δ (F) $GABA_AR$ subunits was determined by qPCR analysis. GAPDH and 18S ribosomal RNA were used as reference genes. Results are means ± SEM of at least 3 independent experiments, and expressed as percentage of control (sham). Statistical analysis was performed by one-way ANOVA, followed by Dunnett's test. *p<0.05, **p <0.01 - significantly different when compared to control conditions.

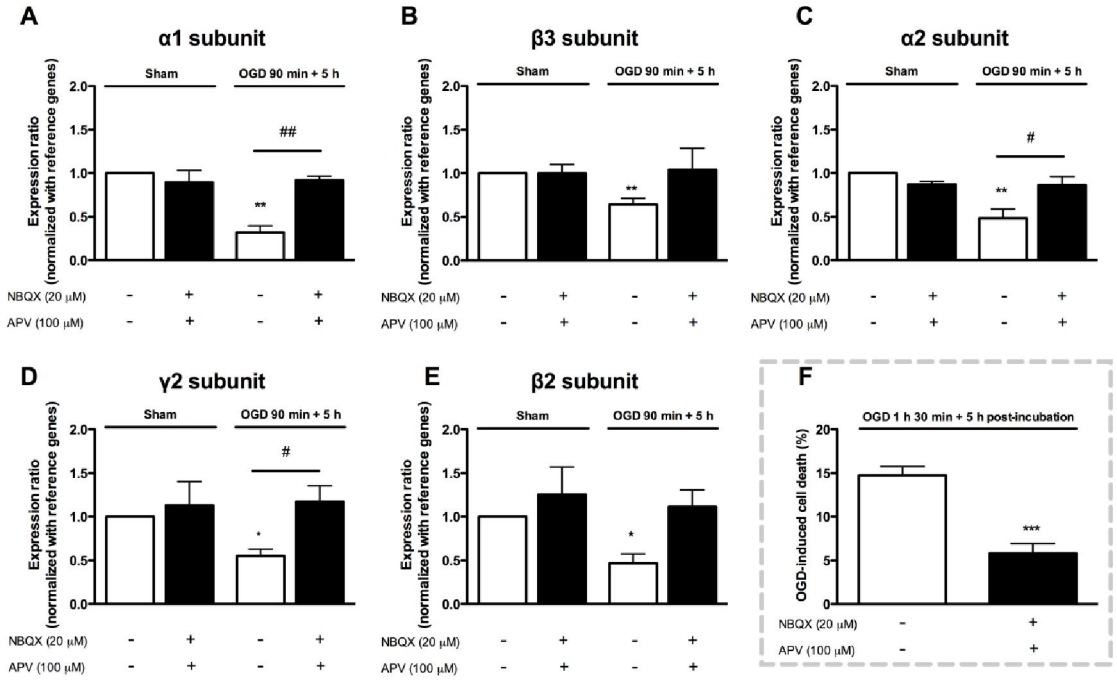

FIGURE 3.5. The effect of OGD on GABA$_A$R mRNA levels is mediated by activation of glutamate receptors.
Cultured hippocampal neurons were subjected to OGD (for 90 min) or incubated under control conditions (sham), in the presence or in the absence of the glutamate receptor inhibitors NBQX (20 μM) and APV (100 μM), as indicated, and further incubated in culture conditioned medium for 5 h. When the effect of glutamate receptor inhibitors was tested, the cells were pre-incubated with the drugs for 30 min, and were present during the period of OGD as well as during the subsequent incubation in culture conditioned medium. The mRNA levels for the α1 (A), α2 (C), β3 (B), β2 (D) and γ2 (E) GABA$_A$R subunits was determined by qPCR. GAPDH and 18S ribosomal RNA were used as reference genes. (F) Neurons were subjected to OGD for the 90 min or incubated under control conditions (sham), in the presence or in the absence of the glutamate receptor inhibitors NBQX (20 μM) and APV (100 μM), as indicated, and further incubated in culture-conditioned medium for 5 h (post-incubation). Cell death was analyzed after nuclei staining with Hoechst 33342. Results are means ± SEM of at least 3 independent experiments, and expressed as percentage of control (sham). Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. *p<0.05, **p <0.01, #p<0.05, ##p<0.01 - significantly different when compared to control conditions or for the indicated comparisons.

The results in Fig. 3.5F show a role for glutamate receptors in hippocampal neuronal death following OGD, as determined by nuclear morphology analysis. Although OGD decreased the mRNA levels for α1, α2, β2 and β3 subunits, inhibition of transcription is unlikely to contribute to the observed downregulation of GABA$_A$R protein levels since

transcription blockage with actinomycin D for 9.5 h (the maximal duration of the OGD experiments) did not affect the abundance of α1, α2, β3 and γ2 protein levels (Fig. 3.6).

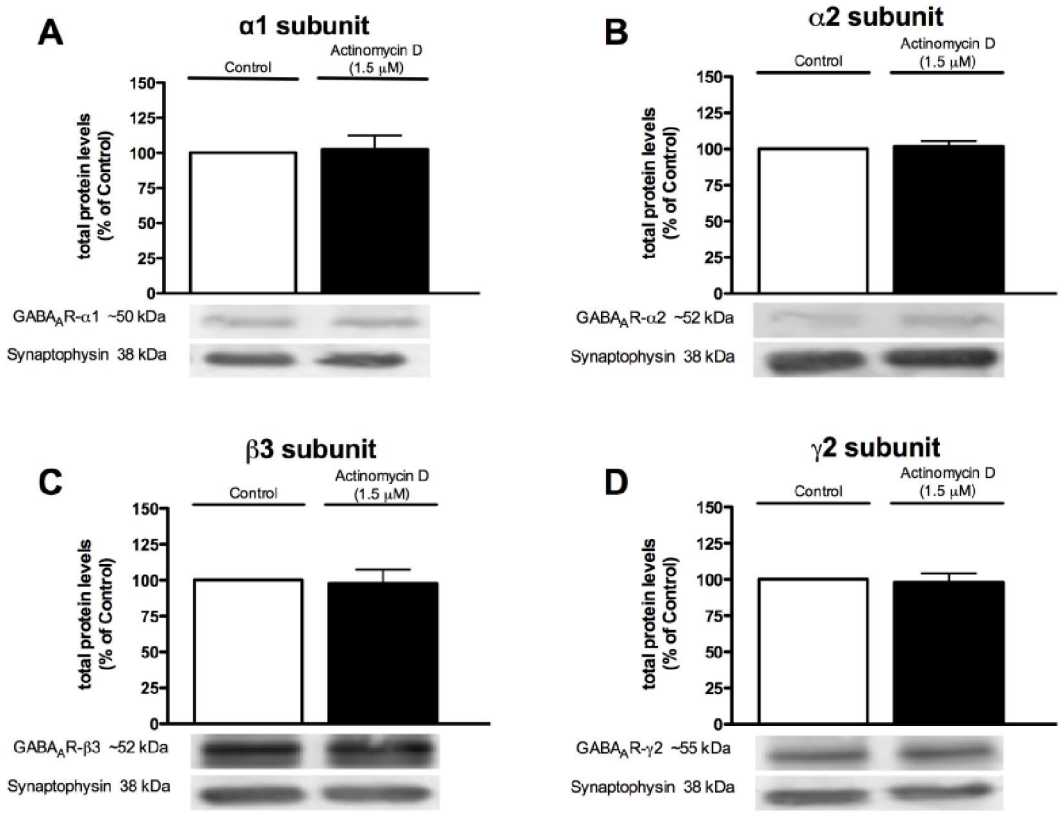

FIGURE 3.6. Inhibition of transcription does not affect total protein levels of α1, α2, β3 and γ2 GABA$_A$R subunits.

Cultured hippocampal neurons (15 DIV) were incubated with Actinomycin D (1.5 μm) for 9.5 h, and α1 (A), α2 (C), β3 (B) and γ2 (D) GABA$_A$R subunit total protein levels was determined by Western Blot analysis. The results were expressed as a percentage of the control, normalized with the loading control synaptophysin, and are the mean ± SEM of at least 3 independent experiments performed in different preparations. The differences obtained were not statistically significant, as determined by the Student's *t* test

3.3. Downregulation of GABA$_A$R α1 subunit/gephyrin interaction during OGD

The number of GABA$_A$R at the synapse determines the strength of inhibitory signaling. These receptors are very dynamic structures in the cell membrane, moving between synaptic and extrasynaptic sites

(Thomas et al., 2005), and their accumulation at the synapse is regulated by interaction with the scaffold protein gephyrin (Jacob et al., 2005). To evaluate if $GABA_AR$/gephyrin interaction is altered in ischemic conditions, a surface co-immunoprecipitation protocol was used. Exposure of hippocampal neurons to OGD for 70 min, which does not affect total $GABA_AR$ α1 subunit protein levels (Fig. 3.7D), reduced by about 50% the interaction between surface-expressed receptor subunit and gephyrin (Fig. 3.7A, C), as demonstrated by immunoprecipitation of surface subunits followed by Western blot with an anti-gephyrin antibody (Fig. 3.7C). This effect was completely inhibited by cyclosporin A (1 μM), a calcineurin inhibitor, but was insensitive to okadaic acid (0.5 μM), an inhibitor of PP1α and PP2A phosphatases.

Although okadaic acid did not affect the interaction between gephyrin and $GABA_AR$ α1 subunits, western blot experiments showed a shift of the band corresponding to gephyrin in extracts prepared from neurons incubated with okadaic acid (Fig. 3.7F). This shift did not correspond to an increase of total gephyrin protein levels, as confirmed by western blot quantification (Fig. 3.7E). To investigate whether the shift in the gephyrin band was due to phoshorylation of the scaffold protein, we performed the λ-phosphatase assay. Indeed, the observed shift in the gephyrin band was not observed when cell lysates were incubated with λ-phosphatase (Fig. 3.7F). Furthermore, no shift in gephyrin immunoreactivity was observed in extracts prepared from hippocampal neurons subjected to OGD, further suggesting that the phosphorylation sites regulated by protein phosphatases sensitive to okadaic acid are not involved in the regulation of the interaction between gephyrin and $GABA_AR$ α1 subunits.

In contrast with the effect of phosphatase inhibitors on the OGD-induced downregulation of gephyrin/$GABA_AR$ α1 subunit interaction, the decrease in total surface expression of $GABA_AR$ α1 subunits under the same conditions was completely abrogated in the presence of okadaic acid (Fig. 3.7B and 3.7C), but was insensitive to cyclosporin A (1 μM). These results indicate that different protein phosphatases mediate the effect of OGD on the dissociation of $GABA_AR$ α1 subunits from gephyrin

and the decrease in surface expression $GABA_AR$s, presumably due an increase in the rate of internalization.

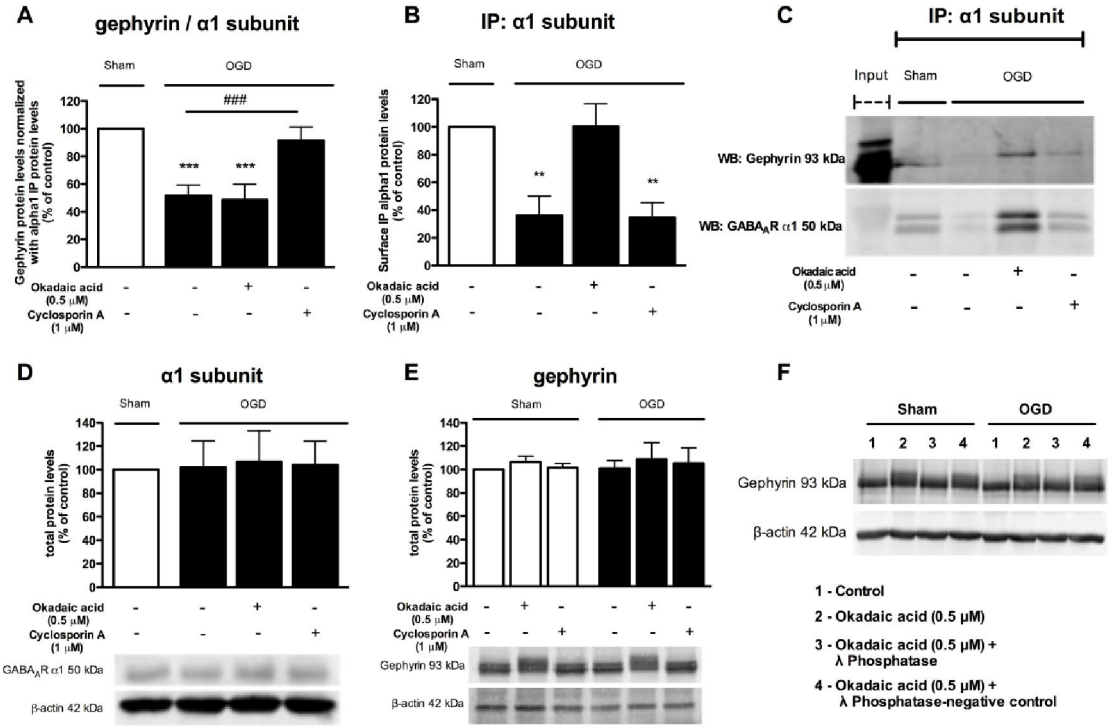

FIGURE 3.7. OGD reduces the interaction of surface α1 $GABA_AR$ subunits with gephyrin.

(A-C) A surface co-immunoprecipitation protocol was used to investigate the $GABA_AR$/gephyrin interaction. Cultured hippocampal neurons were exposed to OGD for 70 min and biotinylated as described in the methods section. Where indicated the cells were incubated with okadaic acid (0.5 µM) or with cyclosporin A (1 µM) during the OGD period. The surface $GABA_AR$ α1 subunits were analyzed by western blot with a specific antibody after purification with a surface co-immunoprecipitation assay (B). The co-immunoprecipitation of gephyrin with the surface $GABA_AR$ α1 subunits was also analyzed by western blot with a specific antibody, and the ratio between gephyrin associated with surface $GABA_AR$ α1 and the plasma membrane associated $GABA_AR$ α1 subunit is expressed in panel (A). A representative image is shown in panel (C). The effects of OGD on the total $GABA_AR$ α1 subunit and gephyrin protein levels were determined under the same experimental conditions (70 min of OGD) and the results are shown in panels (D) and (E) respectively. (F) Extracts prepared from cells treated under the indicated experimental conditions were incubated with 1 µl of λ-phosphatase (~20U/µl) for 60 min at 30°C before western blot analysis with an anti-gephyrin antibody. Results are means ± SEM of at least 3 independent experiments performed in different preparations, and expressed as percentage of the control. β-actin or $GABA_AR$ α1 were used as loading controls. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. *$p<0.05$, **$p<0.01$, #$p<0.05$ -

significantly different when compared to control conditions or for the indicated comparisons.

3.4. OGD increases α1 $GABA_AR$ subunit internalization

From the functional point of view, it is the population of $GABA_AR$ associated with the plasma membrane that is expected to play a role in the modulation of the demise process after OGD. To determine the rate at which the cell-surface $GABA_ARs$ are internalized, we used an antibody feeding technique (Connolly et al., 1999; Lin et al., 2000) that allows distinguishing the cell-surface and the internalized pools of native $GABA_ARs$. Cell-surface $GABA_ARs$ on living cultured hippocampal neurons were labeled with an anti-$GABA_AR$-α1 (N-terminus) antibody.

Under resting conditions the $GABA_AR$ α1 subunit presented a constant rate of internalization for 30 min, when about 80% of the surface receptors were internalized. This rate of internalization, of about 10% of the surface receptors/10 min, was calculated both in the soma and neurites. In both compartments there was a pool of $GABA_AR$ α1 subunit, corresponding to about 20% of the labeled proteins, which was stable and did not undergo internalization during 60 min (Fig. 3.8A). Therefore, in all other experiments the internalization of $GABA_AR$ α1 subunits was followed for 10-20 min.

The effect of OGD on $GABA_AR$ α1 subunit internalization was tested in hippocampal neurons subjected to the ischemic injury for 70 min, which does not affect the total protein levels of the receptor subunit (Fig. 3.7D). The experimental conditions used induce about 20% cell death as measured 7 h - 12 h after the insult (Fig. 3.1). Labeling of surface receptors was performed immediately after OGD, to capture the initial alterations in the mechanisms regulating receptor trafficking, and receptor internalization was measured for different periods of time (0-20 min). Immunocytochemistry analysis revealed ~25% increase in the ratio of α1 subunit internalization compared to the corresponding sham condition, when tested for 20 min (Figs. 3.8B and 3.8C), both in the soma

and neurite compartments. This effect was abolished when internalization was blocked by a hyperosmolar concentration of sucrose (350 mM) (Figs. 3.8D and 3.8F), and with the specific dynamin inhibitor dynasor (125 μM) (Fig. 3.8E and 3.8G). Furthermore, the OGD-induced increase in the ratio of α1 subunit internalization was prevented by incubation with the NMDA and non-NMDA glutamate receptors inhibitors APV (100 μM) and NBQX (20 μM) (Fig. 3.9).

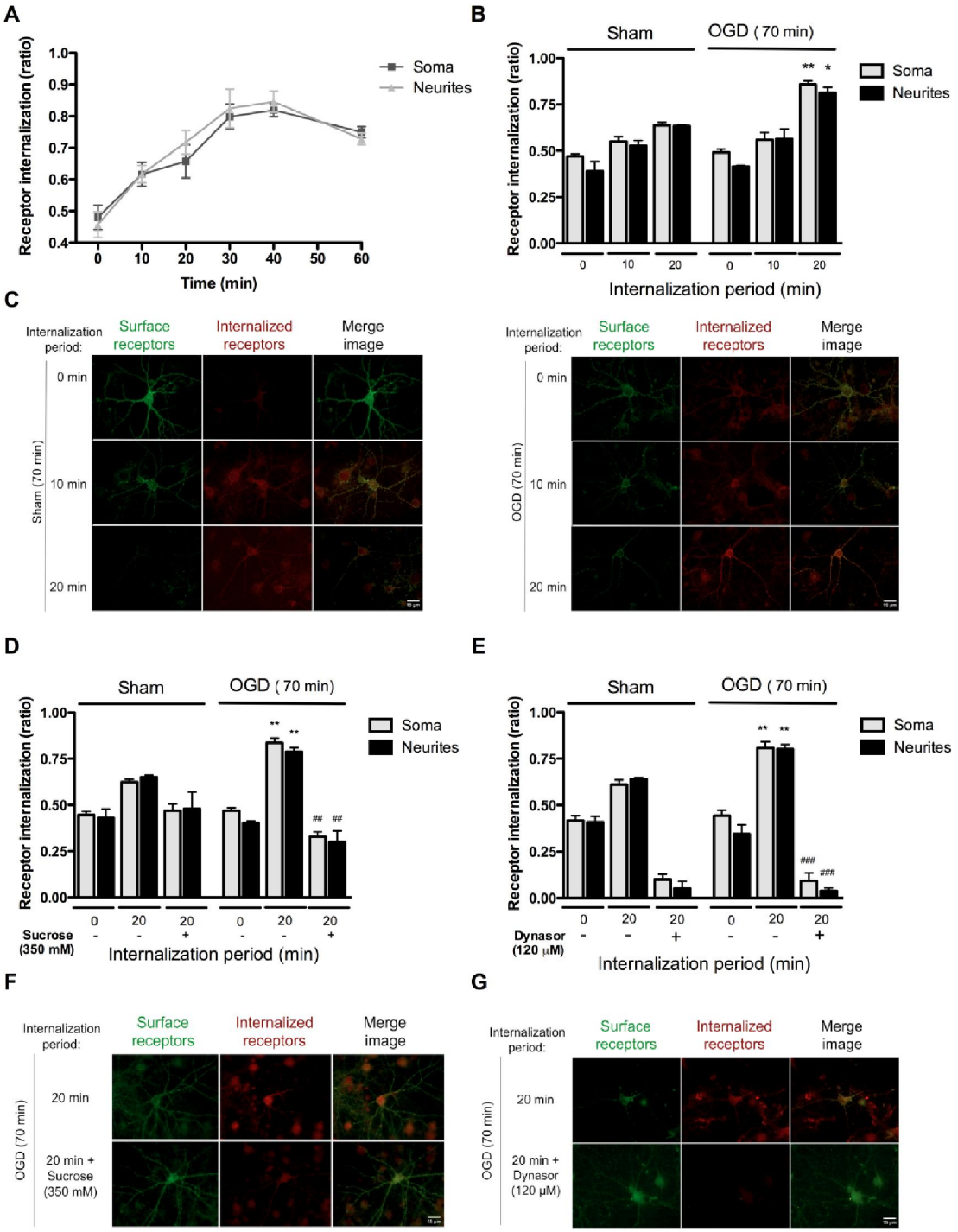

FIGURE 3.8. OGD increases α1 $GABA_AR$ subunit internalization by clathrin-mediated endocytosis.

Receptor internalization was assessed through an antibody-feeding assay and analyzed by fluorescence microscopy in cells labelled with an anti-$GABA_AR$-α1 (N-terminus) antibody. A time-course analysis of receptor internalization was performed in basal conditions (in culture conditioned medium) to validate the method (A). After quantification of the images at the soma and dendritic compartments, the results were expressed as a ratio of internalized receptors/total receptor immunoreactivity. Different

internalization periods were also tested in cells subjected to OGD (70 min) or maintained under control conditions (sham) before incubation with the anti-α1 $GABA_AR$ subunit antibody (B, C). Panels (D-G) show the effect of an hyperosmolar concentration of sucrose (350 mM) (D, F) and treatment with the dynamin inhibitor Dynasor (125 μM) (E, G) on the internalization of the α1 $GABA_AR$ subunit. Internalization of α1 $GABA_AR$ subunits was allowed for 20 min. When the effect of an hyperosmolar treatment was tested, the cells were incubated with 350 mM sucrose during the incubation period of surface receptor live staining and during the internalization period. The same strategy was adopted in the experiments with dynasor. Results are means ± SEM of at least 3 independent experiments, performed in different preparations. At least 10 cells were analysed for each experimental condition/experiment. Internalization ratio was calculated by the ratio internalized antibody signal/total antibody signal. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. *p<0.05, **p<0.01, ***p<0.001, ##p<0.01, ###p<0.001 - significantly different when compared to control conditions or for the indicated comparisons.

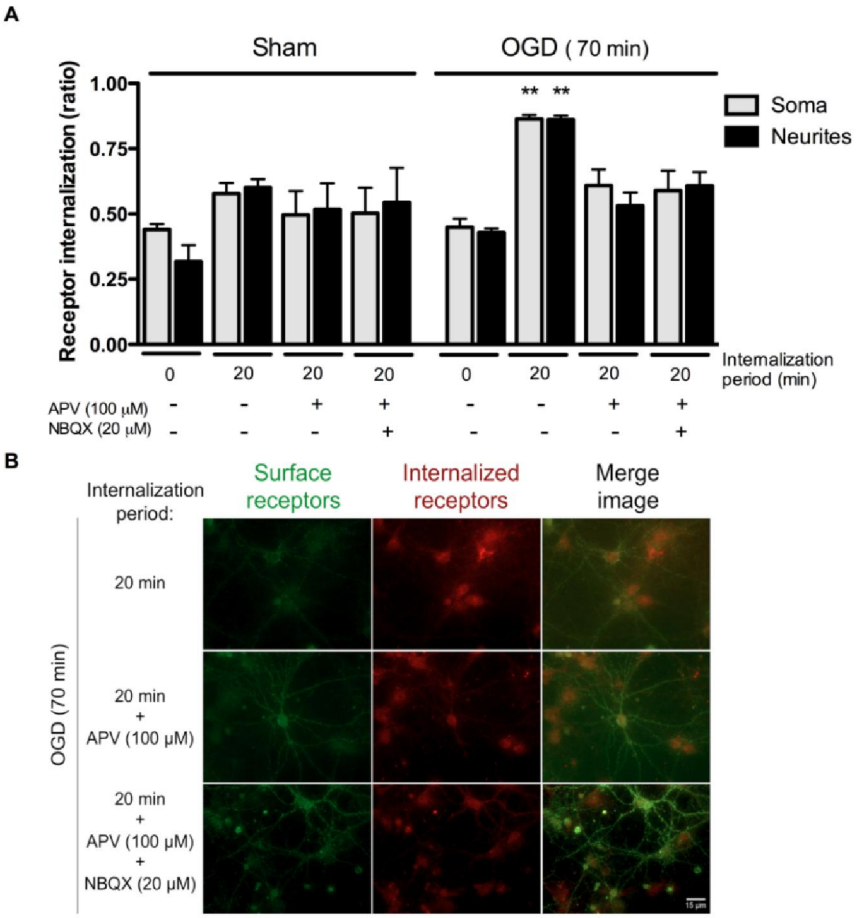

FIGURE 3.9. Effect of OGD on $GABA_AR$ α1 subunit internalization is mediated by activation of glutamate receptors.

Cultured hippocampal neurons were subjected to OGD (70 min) or maintained under control conditions (sham), and the internalization of $GABA_AR$ (20 min) was assessed through an antibody-feeding assay. When the effect of glutamate receptor antagonists

was tested, the cells were pre-incubated (or not) with NBQX (20 μM) and AP-5 (100 μM) for 30 min before OGD, and the inhibitors were also present during the whole experimental period. Representative fluorescence images are shown in panel (B) and the results in (A) are means ± SEM of at least 3 different experiments performed in independent preparations. At least 10 cells were analysed in each condition per experiment. Ratio of internalization was calculated by internalized antibody signal/total antibody immunoreactivity. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. **p<0.01 - significantly different when compared to the sham condition.

3.5. OGD-induced dephosphorylation of $GABA_AR$ β3 subunits leads to receptor internalization and mediates cell death

The internalization of $GABA_AR$ is a process negatively regulated by phosphorylation of β3 or γ2 $GABA_AR$ subunit intracellular loop (Kittler et al., 2005; Kittler et al., 2008). The $GABA_AR$ β3 subunits are present in a large proportion of receptor subtypes in the hippocampus and cerebral cortex, regions that are particularly vulnerable to excitotoxicity (Lo et al., 2003). To evaluate if the observed increase of $GABA_AR$ internalization (Fig. 3.8) is mediated by receptor dephosphorylation, the levels of $GABA_AR$ β3 subunit phosphorylation were evaluated by western blot analysis using a phospho-specific antibody against the β3 subunit serine residues 408/409 (mouse sequence) (Fig. 3.10). After 70 min of OGD, $GABA_AR$ β3 subunit phosphorylation was reduced by 60% (Fig. 3.10A, B), and a decrease in β3 subunit phosphorylation was also observed in the infarct core after transient MCAO (Fig. 3.10C, D). The effect of OGD on $GABA_AR$ β3 subunit phosphorylation level was reduced when the NMDA receptor inhibitor AP-5 (100 μM) was used (Fig. 3.10A). These observations are correlated with the role of NMDA receptors in OGD-induced internalization of $GABA_AR$ α1 subunits (Fig. 3.9). Furthermore, the effect of OGD on β3 subunit dephosphorylation was prevented when neurons were incubated with 0.5 μM of okadaic acid (PP1/PP2A phosphatase inhibitor) but not in the presence of 1μM of cyclosporin A (calcineurin inhibitor) (Fig. 3.10B).

In vitro studies showed that phosphorylation of $GABA_AR$ β3 subunit on serine residues 408/409 negatively regulates receptor endocytosis

(Terunuma et al., 2008). These two serine residues are located in an AP2-binding motif conserved within the ICD of all $GABA_AR$ subunit isoforms (KTHLRRRSSQLK) (Kittler et al., 2005). To evaluate the role of β3 subunit dephosphorylation in the increase of $GABA_AR$ internalization during OGD, and its contribution to the excitotoxicity-induced neuronal death, we made phosphomutants of the $GABA_AR$ β3 subunit. Cultured hippocampal neurons (13 DIV) were transfected with the myc-tagged wild-type or the phospho-mimetic form (SS432/433DD) (homologous of mouse 408/409) of the hu$GABA_AR$β3 subunit, subjected to OGD for 90 min and further incubated in culture conditioned medium for 12 h. The transfected cells were identified by immunocytochemistry with an anti-myc antibody (as shown in Fig. 3.11B), and nuclear morphology analysis of transfected cells (Fig. 3.11A-B) showed a protective effect of the phospho-mimetic form of the receptor that reduced OGD-induced cell death by about 50% when compared with the wild-type β3 subunit. In contrast, non-transfected cells in the two types of cultures exhibited a similar rate of OGD-induced neuronal death (Fig. 3.11A'), showing the specificity of the effects resulting from the expression of the phospho-mimetic form (SS432/433DD) of the hu$GABA_AR$ β3 subunit.

The surface expression of the mutant myc-tagged $GABA_AR$ β3 subunits was evaluated by immunocytochemistry with an anti-myc antibody under non-permeabilizing conditions (see Fig. 3.12A). The SS432/433DD mutant of the $GABA_AR$ β3 subunits presented an increased surface expression in transfected hippocampal neurons, both in control condition and after OGD (70 min), when compared to the WT $GABA_AR$ β3 subunits (Fig. 3.12B and B'). In contrast, transfection with the phospho-null mutant of $GABA_AR$ β3 subunits reduced the surface expression of the receptor, both in the somal (Fig. 3.12B) and neuritic compartments (Fig. 3.12B'). The total expression of the myc-tagged wild-type, phospho-mimetic and phospho-null (SS432/433AA) forms of the $GABA_AR$ β3 subunit was evaluated by immunocytochemistry with an anti-myc antibody after permeabilization and showed a similar expression level of the three proteins (Fig. 3.12C-D'). The internalization rate of myc-tagged

wild type, phospho-mimetic and phospho-null $GABA_AR$ β3 (SS432/433) subunits, in cultured hippocampal neurons maintained under control conditions or subjected to OGD, was assessed using the antibody feeding technique (see Fig. 13A-B). A decrease in internalization ratio was observed for the phospho-mimetic mutant when compared with the WT $GABA_AR$ β3, in contrast with the phospho-null mutant which showed no alteration in internalization. Differential analysis of soma (Fig. 3.13A) and neurites (Fig. 3.13A') showed similar results for the two cellular compartments.

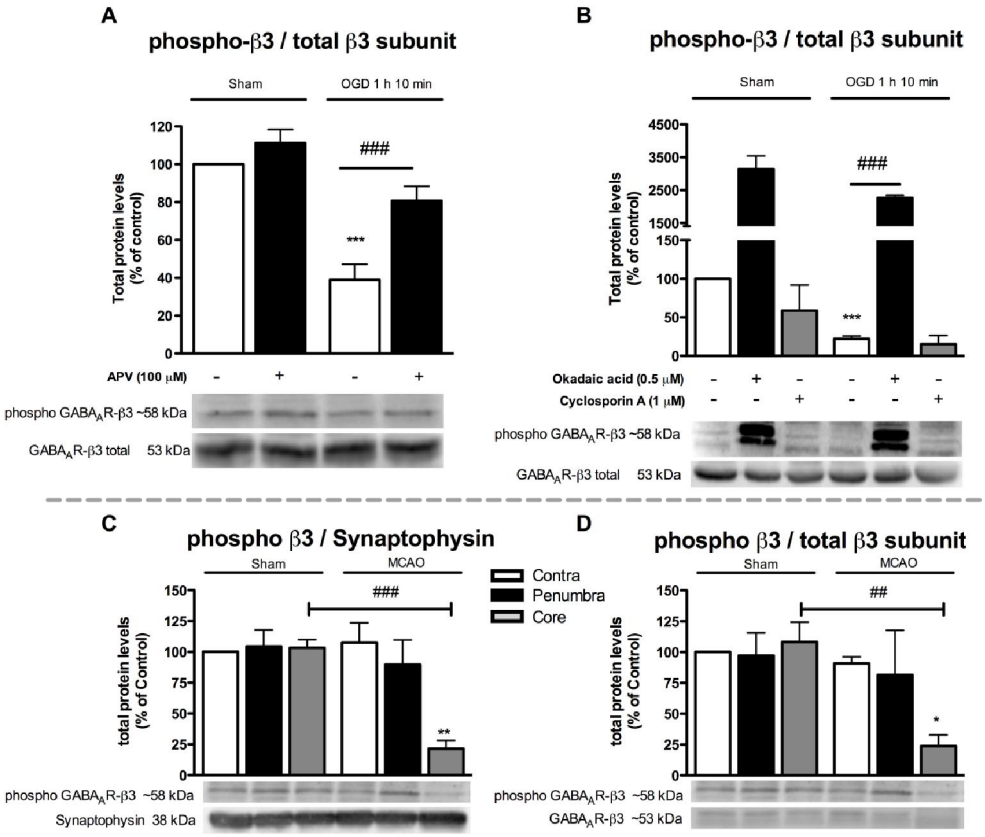

FIGURE 3.10. OGD decreases $GABA_AR$ β3 subunit dephosphorylation by a mechanism dependent on the activity of NMDAR and PP1/PP2A phosphatases.
$GABA_AR$ β3 subunit phosphorylation was evaluated by western blot analysis using a phospho-specific antibody against the β3 subunit serine 408/409 (A, B). The cells were subjected to OGD (70 min) or maintained under control conditions (sham), and the following inhibitors were tested: APV (100 μM) (A), okadaic acid (0.5 μM) and cyclosporin A (1 μM) (B). When the effect of glutamate receptor antagonists was tested, the cells were pre-incubated with the drugs for 30 min and they were also present during the entire experiment. $GABA_AR$ β3 subunit phosphorylation was determined with

a specific antibody, which binds to the phosphorylated serine 408/409. (C-D) Effect of in vivo ischemia (MCAO) on β3 $GABA_AR$ subunit phosphorylation levels, as determined 48 h after the lesion, in the infarct core, penumbra and contralateral cortex. $GABA_AR$ β3 subunit phosphorylation was evaluated by Western blot, as indicated above. Results were normalized to the total protein levels of $GABA_AR$ β3 subunit or synaptophysin, and were expressed as percentage of control. The bars represent the means ± SEM of at least 3 independent experiments performed in different preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. **p<0.01, ***p <0.001, ###p<0.01 - significantly different when compared to control conditions.

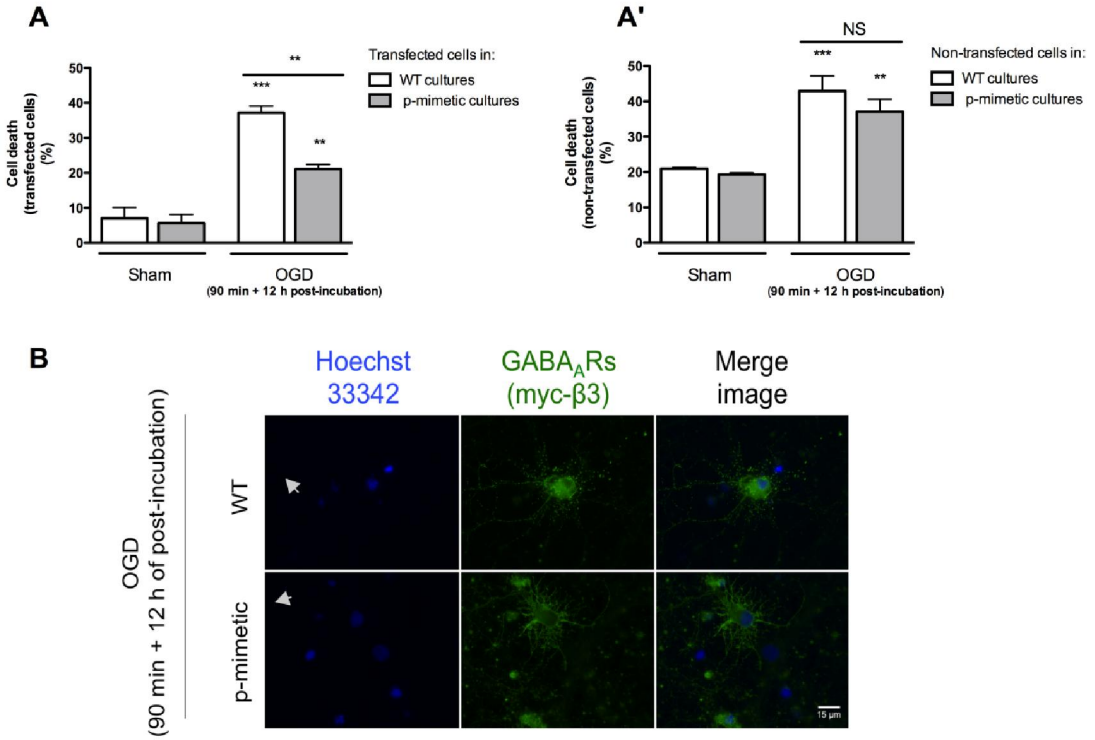

FIGURE 3.11. $GABA_AR$ β3 subunit dephosphorylation mediates OGD-induced cell death.

(A-B) Cultured hippocampal neurons were transfected with the myc-tagged wild-type or the phospho-mimetic form (Ser408/409) of the $GABA_AR$ β3 subunit and subjected to OGD for 90 min before incubation in culture conditioned medium for 12 h. Where indicated (sham) the cells were treated under control conditions. The transfected cells were identified by immunocytochemistry with an anti-myc antibody, and the viability of transfected (A) and non-transfected (A') cells was evaluated with Hoechst 33342. Representative images are shown in panel (B). The arrows point to the nuclei of hippocampal neurons transfected with the wild type or the phospho-mimetic forms of $GABA_AR$ β3 subunit. Under the same conditions, hippocampal neurons transfected with phospho-mimetic form of the $GABA_AR$ β3 subunit show a decrease in cell death (the arrow in the panel B points to the nuclei of transfected cells). For each experimental condition two coverslips were analyzed and at least 40 cells were counted per coverslip.

Results are means ± SEM of at least 3 independent experiments, performed in different preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. **p<0.01; ***p<0.001 - significantly different when compared to Sham condition. N.S. – not significant.

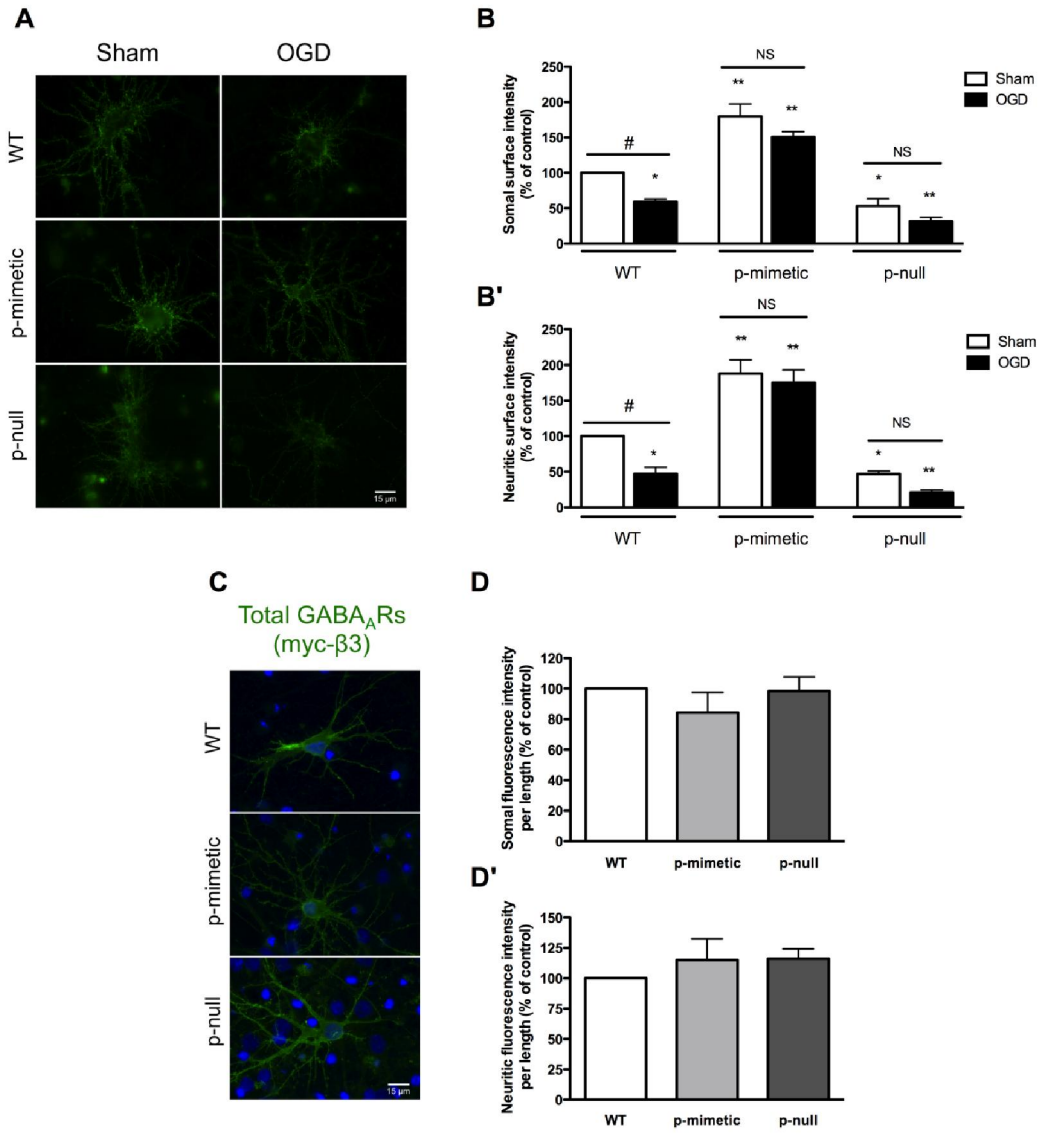

FIGURE 3.12. OGD-induced GABA$_A$R β3 subunit dephosphorylation decreases surface receptor protein levels.

(A-B) Cultured hippocampal neurons were transfected with the myc-tagged wild-type, the phospho-mimetic form (Ser408/409) or the phospho-null GABA$_A$R β3 (Ser432/433) form of the GABA$_A$R β3 subunit and subjected to OGD for 70 min. Where indicated (sham) the cells were treated under control conditions. The effect of OGD on the surface expression of the myc-tagged GABA$_A$R β3 subunits (phospho-mimetic and phospho-null forms) was evaluated by immunocytochemistry, in the somal (B) and neuritic compartments (B'), with an anti-myc antibody under non-permeabilizing conditions.

Representative images are shown in panel (C). The total expression of the myc-tagged wild-type, phospho-mimetic (SS432/433DD) and phospho-null (SS432/433AA) forms of the $GABA_AR$ β3 subunit was evaluated by immunocytochemistry with an anti-myc antibody after permeabilizing the cells (D and D'), and representative images are shown in panel (C). At least 10 cells were analysed in each condition per experiment. Results are means ± SEM of at least 3 independent experiments, performed in different preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. *p<0.05; **p<0.01; #p<0.05 - significantly different when compared to control conditions or for the indicated comparisons. N.S. – not significant.

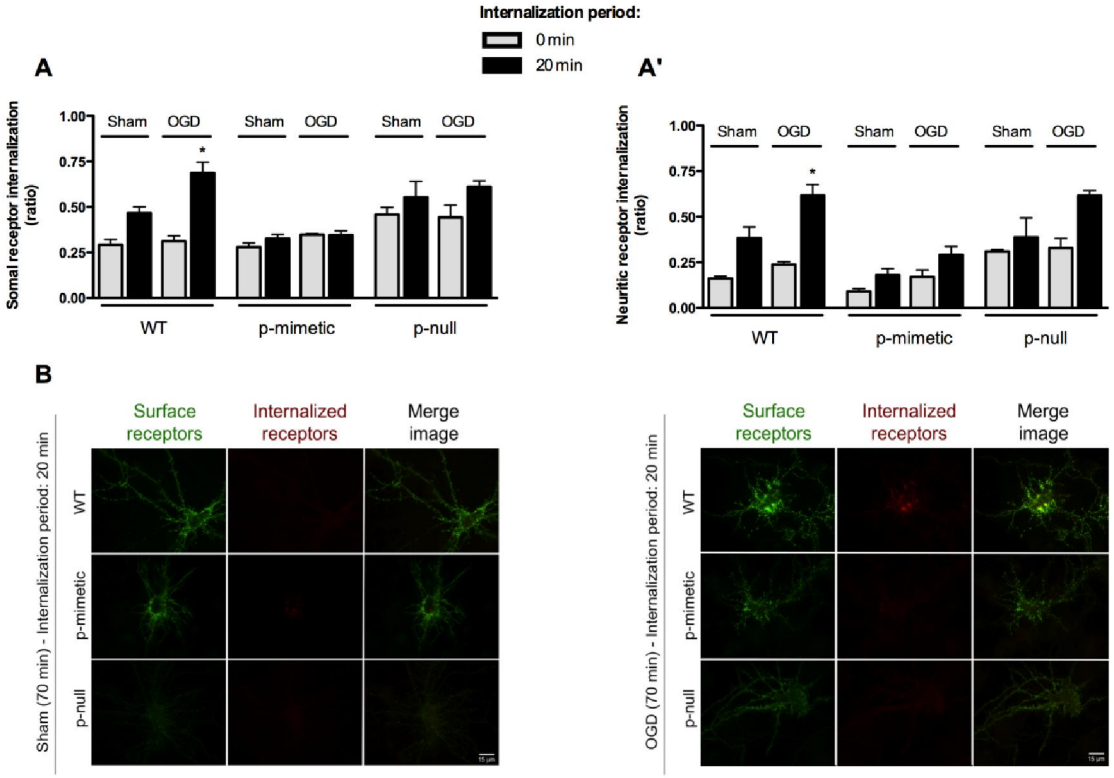

FIGURE 3.13. OGD-induced $GABA_AR$ β3 subunit dephosphorylation leads to receptor internalization.

(A-C') Cultured hippocampal neurons were transfected with the myc-tagged wild-type, the phospho-mimetic (Ser408/409) or the phospho-null $GABA_AR$ β3 (Ser432/433) form of the $GABA_AR$ β3 subunit and subjected to OGD for 70 min. Where indicated (sham) the cells were treated under control conditions. The rate of internalization of myc-tagged wild type, phospho-mimetic and phospho-null $GABA_AR$ β3 (Ser432/433) (homologous of mouse 408/409) subunits in cultured hippocampal neurons maintained under control conditions (sham) or subjected to OGD is shown in panels (A) and (A'). The internalization ratio, obtained by the antibody feeding assay, was calculated based on the immunoreactivity of the internalized antibody/total antibody signal. At least 10 cells were analysed in each condition per experiment. Results are means ± SEM of at least 3 independent experiments, performed in different preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's or Bonferroni test. *p<0.05 - significantly different when compared to sham condition.

3.6. OGD reduces $GABA_AR$ β3 subunits recycling and affects its interaction with HAP1

Following internalization $GABA_AR$ are recycled back to the membrane or targeted to lysosomes for degradation (Kittler et al., 2004). To evaluate if the observed increase in $GABA_AR$ internalization (Figs. 3.8 and 3.13) is accompanied by an alteration in receptor recycling, the rate of $GABA_AR$ β3 subunit recycling was evaluated with a receptor recycling assay (Fig. 3.14A-B). Cultured hippocampal neurons (13 DIV) were transfected with the myc-tagged wild-type $GABA_AR$ β3 subunit and the recycling rate was evaluated under control or OGD conditions. Labelling of surface receptors was performed immediately after OGD, and receptor internalization was allowed for 20 min (this incubation period allows the detection of the OGD-induced increase in the internalization of $GABA_AR$ α1 and β3 subunits [Figs. 3.8C-E and 3.13]) and the receptor recycling was measured at different periods of time (0-30 min). Immunocytochemistry analysis showed a ~15% decrease in the ratio of beta 3 subunit recycling compared to the correspondent sham condition when tested for 15 min and 30 min in the soma compartment (Figs. 3.14B). In neurites this reduction corresponds to ~20% after 15 min (Figs. 3.14B) and ~25% when tested for 30 min.

The sorting of $GABA_AR$ after the internalization is determined by the interaction of $GABA_AR$ with the HAP1 protein (Kittler et al., 2004). To evaluate if the $GABA_AR$/HAP1 interaction is altered in ischemic conditions, a co-immunoprecipitation protocol was used. Exposure of hippocampal neurons to OGD for 70 min reduced by at least 30% the interaction between the surface-expressed $GABA_AR$ β3 subunit and HAP1 (Fig. 3.14C, D), as demonstrated by immunoprecipitation of HAP1 followed by western blot with a $GABA_AR$ β3 subunit antibody (Fig. 3.14C). The same result was obtained by immunoprecipitation of $GABA_AR$ β3 subunits followed by western blot for HAP1 (Fig. 3.14D). These results indicate that OGD reduces $GABA_AR$ recycling possibly due to a decrease in the $GABA_AR$/HAP1 interaction.

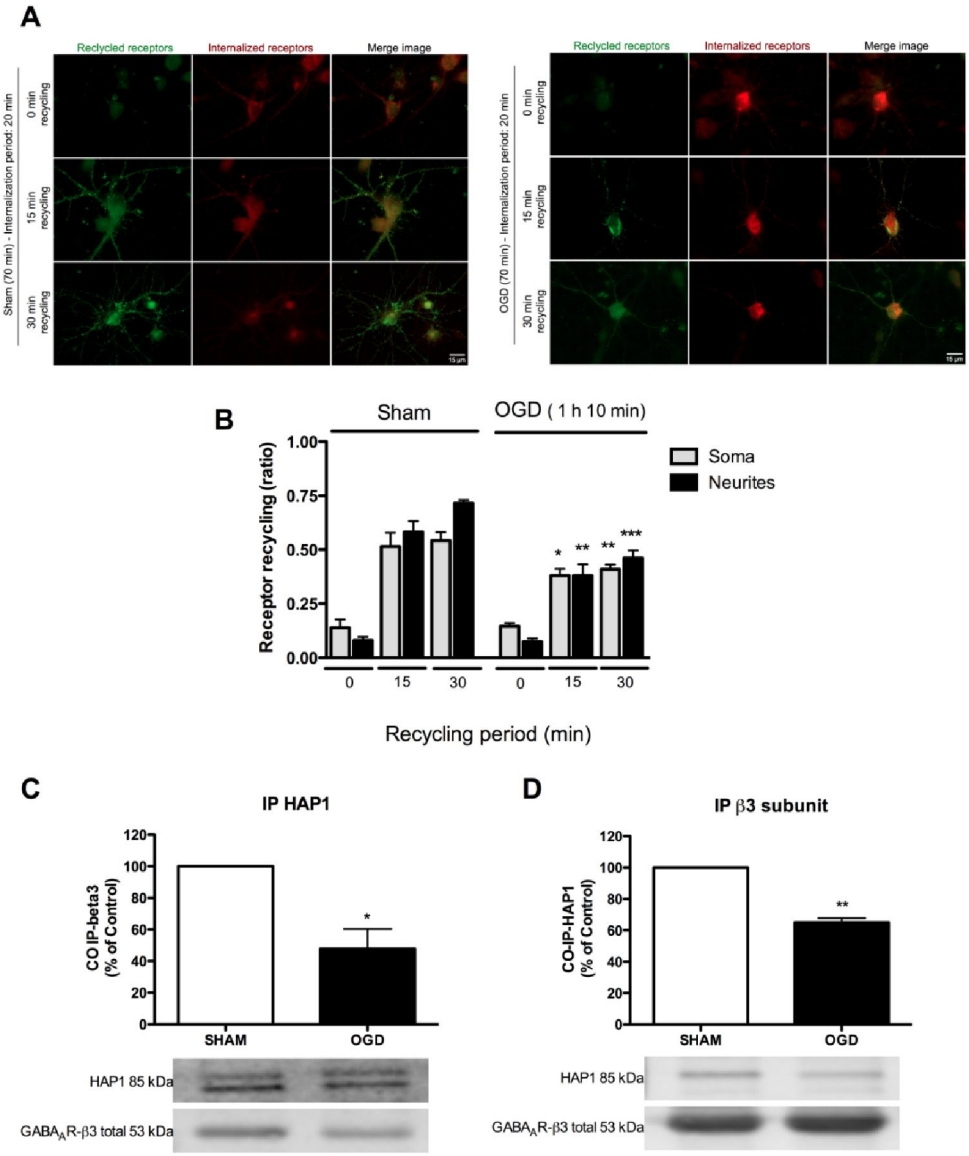

FIGURE 3.14. OGD decreases β3 $GABA_AR$ subunit interaction with HAP1 and $GABA_AR$ recycling.

(A) Cultured hippocampal neurons were transfected with the myc-tagged wild-type β3 $GABA_AR$ subunits. Receptor recycling was assessed through an antibody-feeding assay and analyzed by fluorescence microscopy in cells labelled with an anti-myc (N-terminus) antibody. Internalization of β3 $GABA_AR$ subunits was allowed for 20 min and recycling was measured for different periods of time (0, 15 and 30 min). (B) After quantification of the images somal and neuritic compartments, the results were expressed as a ratio of recycled receptors/total receptor immunoreactivity. (C-D) The co-immunoprecipitation protocol was used to determine $GABA_AR$/HAP1 interaction. Cultured hippocampal neurons were exposed to OGD for 70 min and the immuneprecipitated HAP1 (C) or $GABA_AR$ β3 subunits (D) were analyzed by western blot with a specific antibody; the co-immunoprecipitation of $GABA_AR$ or HAP1, respectively, was also analyzed by western blot with a specific antibody. The ratio between the co-immunoprecipitated protein levels and the immunoprecipitated protein was used for the quantification showed in

panels (C) and (D). Results are means ± SEM of at least 3 independent experiments, performed in different preparations. At least 10 cells were analysed for each experimental condition/experiment. Recycling ratio was calculated by the ratio recycled antibody signal/total antibody signal. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni test or Student's *t* test when appropriated. *p<0.05, **p<0.01, ***p<0.001 - significantly different when compared to control conditions.

CHAPTER 4 – Discussion

Stroke, or cerebral ischemia, is characterized by an early disruption of GABAergic neurotransmission due to alterations at both pre- and post-synaptic sides of the GABAergic synapse (Schwartz-Bloom and Sah, 2001), but the molecular mechanisms involved are not fully understood. In this work we show a key role for protein phosphatases in the regulation of $GABA_AR$ α1 subunit interaction with gephyrin, an anchoring protein responsible for the receptor clustering at the synapse, and in the internalization of $GABA_AR$ in hippocampal neurons subjected to OGD, an in vitro model of global ischemia. In particular, the dephosphorylation of β3 $GABA_ARs$ subunits was found to play a key role in receptor internalization following OGD and the resulting loss of inhibitory activity contributes to neuronal death. Moreover, we demonstrate that OGD reduces $GABA_AR$ recycling, probably by interfering with their interaction with the HAP1 protein.

Using the antibody feeding assay we observed an increased internalization of $GABA_AR$ α1 and β3 subunits in hippocampal neurons subjected to OGD, in agreement with the evidence indicating that the majority of the receptors contain 2 α-, 2 β-, and 1 γ2-subunits (Rudolph and Mohler, 2004). The α1 $GABA_AR$ subunit is greatly expressed in the hippocampus (Hortnagl et al., 2013; Laurie et al., 1992; Wisden et al., 1992), a brain region that is highly vulnerable to ischemic conditions (Kirino and Sano, 1984; Schmidt-Kastner and Freund, 1991; Sugawara et al., 1999). The OGD-induced internalization of $GABA_AR$ α1 subunits was mediated by clathrin-dependent endocytosis, sensitive to an hyperosmolar concentration of sucrose and to dynasor, and required glutamate receptor activation. A similar mechanism was shown to contribute to the internalization of $GABA_AR$ in an in vitro model of epilepsy, a condition also characterized by excitation/inhibition imbalance (Goodkin et al., 2005).

$GABA_ARs$ are clustered at the synapse through interaction with gephyrin (Tyagarajan and Fritschy, 2010), and the $GABA_AR$ α1 subunits were shown to interact directly with the scaffold protein (Mukherjee et al., 2011; Tretter et al., 2011). This is in agreement with the results obtained

in the present study showing that surface $GABA_AR$ α1 subunits co-immunoprecipitate with gephyrin. The interaction between the α1 $GABA_AR$ subunit and gephyrin promotes the receptor accumulation at inhibitory synapses by limiting its lateral diffusion, and this interaction depends on residues 360–375 of the α1 subunit that bind directly to gephyrin (Mukherjee et al., 2011). Modulating this interaction via covalent modifications, such as phosphorylation, may be a potent mechanism to control the strength of fast GABAergic signaling. Accordingly, we observed that OGD significantly decreases the co-immunoprecipitation of surface $GABA_AR$ α1 subunits with gephyrin by a mechanism sensitive to calcineurin inhibition. Since calcineurin had no effect on the apparent mobility of gephyrin in SDS-PAGE, the results suggest that the phosphatase may dephosphorylate $GABA_ARs$ (Kapur and Lothman, 1990). There are indeed evidences showing that calcineurin activation mediates the effect of NMDA receptors in the reduction of GABA-mediated inhibition (Chen and Wong, 1995; Lu et al., 2000; Stelzer and Shi, 1994), but various mechanisms may be involved. Thus, the induction of long-term depression at CA1 inhibitory synapses resulted in a reduction in the synaptic $GABA_ARs$ number by a calcineurin-dependent mechanism (Wang et al., 2003), while a direct effect of calcineurin on the functional properties of $GABA_ARs$ was proposed in a different study (Jones and Westbrook, 1997). The effect of calcineurin on the dissociation of gephyrin-$GABA_AR$ α1 complexes induced by OGD clearly favors the former mechanism of action. However, at this point it is not possible to rule out an effect of OGD on the activity of protein kinase(s) responsible for the phosphorylation of the amino acid residues targeted by calcineurin (Chapell et al., 1998; Connolly et al., 1999; Filippova et al., 2000; Jovanovic et al., 2004).

The possibility that gephyrin phosphorylation might regulate $GABA_AR$ binding to gephyrin, and their post-synaptic localization or trafficking, has not been investigated. Evidence available for glycine receptors (GlyR) demonstrate that proline-directed phosphorylation of gephyrin may induce a conformational change favoring GlyR binding (Tyagarajan and

Fritschy, 2010; Zita et al., 2007). Several studies identified gephyrin as a target for serine/threonine directed phosphorylation (Beausoleil et al., 2006; Lundby et al., 2012), and a recent study detailed 18 different phosphorylation residues on gephyrin (Herweg and Schwarz, 2012), suggesting a key role for phosphorylation in the regulation of gephyrin function. Moreover, given that phosphorylation and intracellular Ca^{2+} rise make gephyrin susceptible to proteolysis by calpain (Tyagarajan et al., 2013a) the neuronal activity-driven gephyrin dynamics could very likely be phosphorylation-dependent. Accordingly, gephyrin phosphorylation on Ser268 was recently shown to be important for scaling (up or down) GABAergic transmission (Tyagarajan et al., 2013a).

In contrast with the role of calcineurin in OGD-induced dissociation of gephyrin – $GABA_AR$ α1 complexes, the dephosphorylation of $GABA_AR$ β3 subunits under the same conditions is mediated by okadaic acid-sensitive protein phosphatases (PP1 or PP2A). Recruitment of $GABA_AR$ into the endocytic pathway is facilitated via the interaction of the intracellular domains of β1–3 and γ2 subunits with μ2-AP2 (Kittler et al., 2005; Kittler et al., 2008). This motif incorporates the major sites of phosphorylation by PKC and protein kinase A (PKA), corresponding to serine residues S408 and S409 in the case of the $GABA_AR$ β3 subunit (mouse sequence) (Brandon et al., 2002; McDonald and Moss, 1997). Phosphorylation of these sites has been shown to impair $GABA_AR$ endocytosis by preventing the interaction of the β3 subunit with AP2 (Jacob et al., 2009; Kittler et al., 2005). The role of $GABA_AR$ β3 dephosphorylation in receptor internalization and neuronal death in hippocampal neurons subjected to OGD is supported by the following evidences: i) OGD reduced the phosphorylation of $GABA_AR$ β3 subunit by a mechanism sensitive to okadaic acid, as determined by western blot with a phosphospecific antibody against serine residues 408/409; ii) the phospho-mimetic mutant of $GABA_AR$ β3 subunit (SS432/433AA) (homologous of mouse 408/409) was accumulated at cell surface and showed no OGD-induced internalization, and iii) the same mutation reduced significantly OGD-induced cell death. The neuroprotection

provided by the phospho-mimetic mutant of $GABA_AR$ β3 subunit, resulting from receptor activation by endogenous GABA, is highly remarkable considering that less than 10% of the cells present in the culture are GABAergic (Baptista et al., 2010).

The triple arginine motif of the β3 $GABA_AR$ subunit that mediates direct binding of the receptor to the clathrin adaptor protein AP2 plays a key role in regulating the synaptic distribution of the receptor (Smith et al., 2012). Furthermore, a peptide overlapping with the AP2 binding region in the β3 subunit, to compete the β3/AP2 interaction, was shown to decrease OGD-induced cell death, in agreement with the results obtained in this work using a distinct experimental approach. However, the use of a peptide overlapping with the AP2 binding region in the β3 subunit does not rule out non-specific effects on the internalization of other plasma membrane proteins, which may also interact with AP2 on the same binding motif.

$GABA_AR$ cell surface stability is determined not only by their internalization ratio but also by the post-internalization sorting mechanisms. Upon internalization $GABA_ARs$ are rapidly recycled back to the cell surface, whereas over longer periods of internalization the receptors are also targeted for lysosomal degradation (Kittler et al., 2004). HAP1 plays a key role in these processes since it directly binds to $GABA_AR$, thereby preventing their degradation and enhancing receptor recycling to the plasma membrane (Kittler et al., 2004). We showed that OGD downregulates $GABA_AR$ recycling, possibly due to a reduction in the interaction of the receptor with HAP1, as suggested by the co-immunoprecipitation assay. These observations are relevant to understand the down-modulation of $GABA_AR$ surface expression not only in brain ischemia but also in pathological conditions such as epilepsy, in which an acute reduction in receptor surface expression and loss of synaptic $GABA_AR$ leads to a compromised neuronal inhibition and altered excitability states (Mielke and Wang, 2005; Naylor et al., 2005; Tan et al., 2007). However, the mechanisms involved in the downregulation of $GABA_AR$ under the latter conditions remain unclear.

In addition to the effect on the surface expression of $GABA_A$Rs, OGD also downregulated the total protein levels (α1, α2, β3 and γ2 subunits) and mRNA (α1, α2, β2, β3 and γ2 subunits) for $GABA_A$R subunits, which is likely to have a delayed and long-lasting effect on GABAergic synaptic transmission. At least some of the effects of OGD on $GABA_A$R subunits (e.g. α2 and β3) are mediated by calpains. The upregulation of calpain activity under excitotoxic conditions and in brain ischemia is also coupled to an abnormal cleavage and/or degradation of several other proteins (Gomes et al., 2012; Gomes et al., 2011; Lobo et al., 2011), thereby contributing to neural death. The OGD-induced downregulation of the mRNA levels for α1, α2, β2, β3 and γ2 $GABA_A$ receptor subunits was mediated by activation of glutamate receptors and would prevent the replenishment of the $GABA_A$ receptor pool degraded in response to the injury. Interestingly, under the OGD conditions used the mRNA levels for the $GABA_A$R δ subunit were not significantly altered. Considering that this subunit is found at extrasynaptic regions (Nusser et al., 1998b), the results suggest that OGD has differential effects on the synaptic and extrasynaptic pools of $GABA_A$Rs.

In conclusion, we showed that (de)phosphorylation of $GABA_A$R β3 subunits on serines 408/409 (mouse sequence) is a master regulator of $GABA_A$R surface localization in ischemic conditions, and receptor internalization, together with a reduction in the rate of recycling, contributes to the death of hippocampal neurons after transient OGD. Recruitment of $GABA_A$R for internalization is induced by glutamate receptor activation and follows the impairment in their interaction with the scaffold protein gephyrin, by a mechanism that is also regulated by protein phosphatases (Fig. 4.1). The degradation of $GABA_A$Rs and the downregulation of their mRNAs may further reduce GABAergic synaptic transmission. Taken together, these results suggest that modulation of $GABA_A$R phosphorylation might be a therapeutic target to preserve synaptic inhibition in brain ischemia.

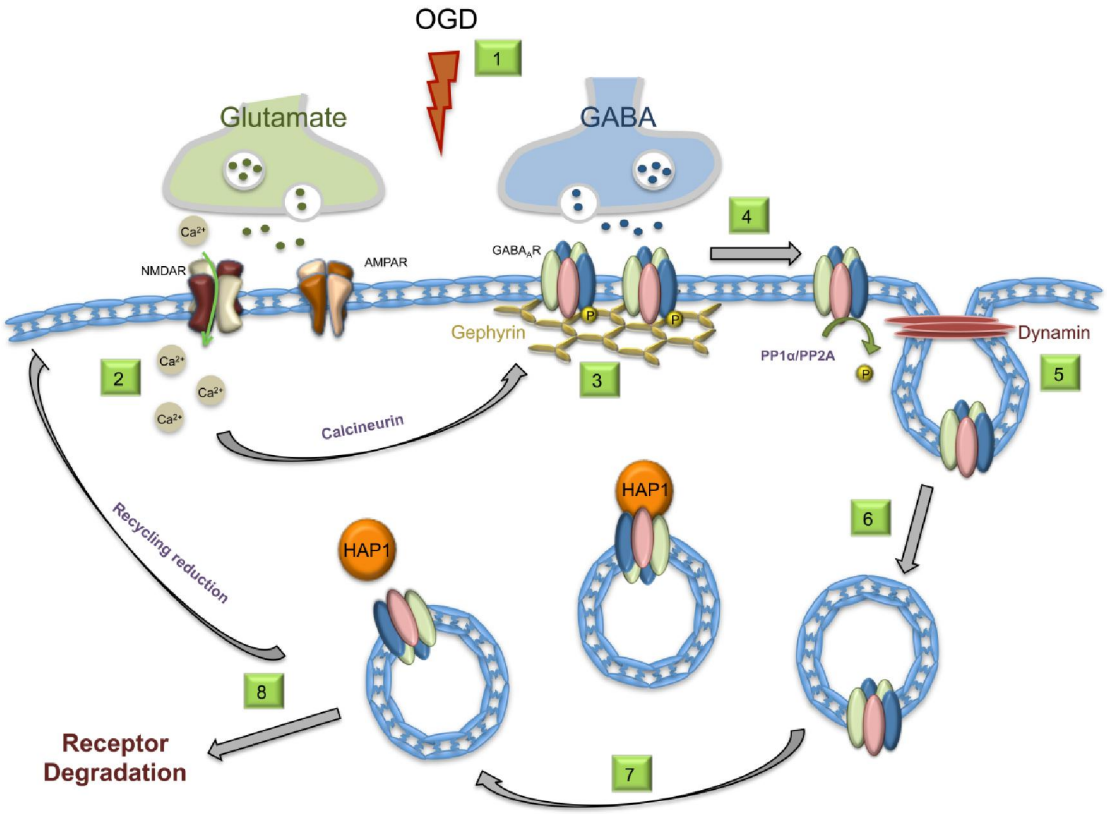

FIGURE 4.1. **Model of GABA$_A$R internalization during cerebral ischemia.**
Ischemic insult (1) overactivates NMDAR signalling (2) and the resulting activation of calcineurin decreases GABA$_A$R/Gephyrin interaction (3). In parallel, OGD reduces phosphorylation of GABA$_A$R β3 subunit by a mechanism sensitive to okadaic acid (4), inducing the internalization of GABA$_A$R via clathrin dependent endocytosis (5, 6). OGD also reduce GABA$_A$R/HAP1 interaction and GABA$_A$R recycling rate (7, 8), driving GABA$_A$R to degradation.

CHAPTER 5 – References

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