DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Alteration of $GABA_AR$ trafficking during cerebral ischemia:

the role of Huntingtin-associated protein 1

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Doutor Professor Carlos Duarte (Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra) e da Doutora Miranda Mele (Centro de Neurociências e Biologia Celular)

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2014

This work was performed at the Center for Neuroscience and Cell Biology, University of Coimbra, Portugal, with support from the Portuguese Foundation for Science and Technology (FCT) and FEDER/COMPETE with FCT grants: PTDC/NEU-NMC/0198/2012 and PEst-C/SAU/LA0001/2013-2014.

Agradecimentos/Acknowledgmentes

Um agradecimento especial è para o Professor Carlos Duarte por me ter acolhido no seu grupo, oferecendo-me a oportunidade de crescer profissionalmente e também pela sua total disponibilidade e paciência.

Ringrazio la professoressa Lorella Canzoniero che mi ha spinta ed incentivata ad affrontare questo cambiamento con positività e determinazione e per avermi fatto capire l'importanza di una tale esperienza.

Il mio grazie più affettuoso è rivolto a Miranda, la mia "Cheffa", che ha reso questo anno memorabile non solo per la complicità laboratoriale creatasi ma soprattutto per il forte legame di amicizia che si è instaurato fin da subito. La sua esperienza lavorativa e la sua capacità di comunicarla agli altri hanno contributo ad accrescere le mie conoscenze.

Um agradecimento carinho è para o Rui com quem tive uma "relaçao complicada" mas tambem muito especial. Muitas vezes ele foi uma ajuda real e preciosa.

Um sincero agradecimento a todos os meninos de laboratório por me ter acolhido desde o principio com carinho e simpatia e ter tornado o trabalho de laboratorio produtivo, mas antes de mais muito agradável.

Para os "Fixinhos" da salinha, com seu afeto e sua sincera amizade me acolheram e me fizeram sentir em casa todos os dias. Obrigada para os dias inesquecível.

Muito obrigada a todas as pessoas do segundo piso, nao há um piso com as pessoas e bolinhos milhores.

Un grazie di cuore alla mia famiglia che mi ha sostenuta ogni giorno in questo anno così lontana da casa. Grazie per non avermi fatto mai sentire la vostra mancanza e avermi sempre spinta a dare il massimo.

Grazie a mia sorella Lina, costantemente presente nella mia vita anche in questo anno di lontananza e alla mia migliore amica Rossella, con la quale condivido da anni tutti i momenti della mia vita. Ci siete sempre state per me in ogni istante.

Agli italiani trapiantati a Coimbra, in particolare al mio compagno di avventura Fabio con il quale ho condiviso molti momenti memorabili in questo anno e a tutti miei colleghi Italiani, è stato davvero bello e divertente aver condiviso momenti portoghesi con voi ed aver colonizzato insieme il CNC.

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INDEX

ABBREVIATIONS

Ab, antibody

Ala, alanine

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

AP2, adaptor protein 2

Arg, arginine

Asp, aspartic acid

ATP, adenosine-5'-triphosphate

BSA, bovine serum albumine

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

CA1, cornu ammonis 1 region of the hippocampus

cDNA, complementary DNA

DG, dentate gyrus

DIV, days in vitro

DMEM, Dulbecco's modified eagle medium

dNTP, deoxyribonucleoside triphosphate

DOC, deoxycholic acid

DTT, dithiothreitol

E, embryonic

ECF, enhanced chemiofluorescence

EDTA, ethylenediaminetetraacetic acid

EGTA, ethylene glycol tetraacetic acid

ER, endoplasmic reticulum

FBS, fetal bovine serum

FDU, 5-Fluoro-2′-deoxyuridine

FSK, forskolin

GABA, γ-aminobutyric acid

$GABA_AR$, GABA type A receptor

$GABA_BR$, GABA type B receptor

GABARAP, $GABA_AR$-associated protein

GAD, glutamic acid decarboxylase

GAT, GABA transporter

GFAP, glial fribrillary acidic protein

GFP, green fluorescence protein

Gln, glutamine

HAP1, huntingtin-associated protein 1

HAP1-A, huntingtin-associated protein 1 A

HAP1-B, huntingtin-associated protein 1 B

HBSS, Hank's balanced salt solution

HD, Huntington's disease

HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid

htt, huntingtin

ICD, intracytoplasmic domain

IgG, immunoglobulin G

IP_3, inositol 1,4,5-trisphosphate

LB, lysogeny broth medium

MAP2, microtubule-associated protein 2

MAPKs, mitogen-activated protein kinases

MCA, middle cerebral artery

MCAO, middle cerebral artery occlusion

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

MEM, minimum essential medium

mIPSC, miniature inhibitory post-synaptic currents

mRNA, messenger RNA

MTP, mitochondrial transition pore

NMDA, N-methyl-D-aspartate

NMDAR, NMDA receptor

ns, not significant

NSF, N-ethylmaleimide-sensitive factor

OGD, oxygen and glucose deprivation

p50, protein 50

PBS, phosphate buffered saline

PCD, programmed cell death

PKA, protein kinase A

PKC, protein kinase C

PLC, phospholipase C

PLIC, proteins linking integrin-assocated protein with cytoskeleton

PMA, phorbol-12-myristate-13-acetate

PMSF, phenylmethylsulfonyl fluoride

PP1, protein phosphatase 1

PP2A, protein phosphatase 2A

PP2A-C, protein phosphatase 2 catalytic subunit C

PP2C, protein phosphatase 2C

PSD, postsynaptic density

PVDF, polyvinildene difluoride

qPCR, quantitative PCR

rCBF, regional cerebral blood flow

RIPA, radioimmunoprecipitation assay lysis buffer

RNA, ribonucleic acid

ROS, radical oxygen species

RT, room temperature

SDS, sodium dodecyl sulphate

SEM, standard error of the mean

Ser, serine

TE, tris-EDTA

TM, transmembrane domains

TS, thymidylate - synthase

VGAT, vesicular GABA transporter

KEY WORDS

Huntigtin - associated protein 1 (HAP1)

Cerebral ischemia

$GABA_A$ receptor

Oxygen/glucose deprivation (OGD)

Neurons

PALAVRAS CHAVE

Proteína associada a huntingtina de tipo 1 (HAP1)

Isquémia cerebral

Receptores de GABA do tipo $GABA_A$

Privação de oxigénio e glucose (OGD)

Neurónios

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. No sistema nervoso central (SNC) a regulação deste equilíbrio é determinada principalmente pelo balanço entre a neurotransmissão glutamatérgica e GABAérgica e diversos estudos têm mostrado que a neurotransmissão glutamatérgica e GABAérgica está aumentada e reduzida, respectivamente, nas lesões isquémicas. Ao contrário das alterações na neurotransmissão glutamatérgica na isquémia cerebral que têm sido amplamente investigadas, poucos estudos têm abordado os mecanismos moleculares que contribuem para as alterações na neurotransmissão GABAérgica. Resultados recentes do nosso laboratório, obtidos utilizando o modelo de isquémia cerebral baseado na privação de oxigénio e glicose (OGD), mostraram que o insulto isquémico induz a desfosforilação e consequente internalização dos receptores de GABA do tipo A ($GABA_AR$), contribuindo 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). Estudos anteriores do nosso laboratório mostraram que a OGD transitória também reduz a reciclagem e o regresso para a membrana plasmática dos $GABA_AR$, e diminui a interacção dos receptores com a proteína HAP1 em neurónios do hipocampo em cultura.

A proteína HAP1 existe em duas isoformas, HAP1-A e HAP1-B, que compartilham a mesma região central (aminoácidos 277-445). A HAP1 está associada a microtúbulos e a diversos tipos de organelos, incluindo as mitocôndrias, lisossomas e vesículas sinápticas. Tendo em consideração estas observações, no presente trabalho investigámos o papel da HAP1 na redução da expressão à superfície e reciclagem dos $GABA_AR$ em neurónios de hipocampo em cultura após OGD. Os resultados obtidos mostram que a exposição transitória de neurónios de hipocampo a OGD (90 min) reduz os níveis da proteína HAP1, quando testado por *western blot* duas horas após o insulto isquémico. Este efeito

dependente do tempo de incubação, não foi observado em neurónios incubados na presença do inibidor das calpaínas MDL28170. A inibição das fosfatases PP1/PP2A com ácido ocadáico também diminuiu a redução de HAP1 induzida pela OGD.

A diminuição dos níveis da proteína HAP1 foi também observada em neurónios corticais expostos a OGD, à semelhança dos resultados obtidos em neurónios do hipocampo em cultura. Porém, a oclusão transitória da artéria cerebral média (MCAO), um modelo *in vivo* de isquémia cerebral, teve o efeito oposto sobre os níveis da proteína HAP1 no núcleo isquémico, localizado na região cortical. Esta discrepância pode ser devida ao efeito do insulto isquémico sobre os níveis da proteína Hap1 em células da glia, presentes no tecido cerebral mas ausentes nas culturas neuronais. De acordo com essa hipótese, a análise por *western blot* realizada com extractos de células da glia em cultura expostas a 90 min de OGD seguido de 12 h de pós-incubação mostraram um aumento dos níveis de proteína HAP1.

Para investigar o papel modulador de HAP1 nas alterações do tráfego dos $GABA_AR$ induzidas pelo OGD, foram realizadas experiências em culturas de neurónios de hipocampo transfectados com as isoformas HAP1-A ou-1B. A sobre-expressão das duas isoformas de HAP1, em neurónios de hipocampo em cultura, preveniu a redução da expressão superficial da subunidade β3 do $GABA_AR$ induzida pela OGD. Este efeito foi devido ao aumento da reciclagem da subunidade β3 do receptor $GABA_AR$, como mostrado através do ensaio de reciclagem.

Em resumo, os nossos resultados sugerem que a proteína HAP1 desempenha um papel fundamental na redução da neurotransmissão GABAérgica durante a isquémia cerebral.

ABSTRACT

Cerebral ischemia is a pathological condition characterized by a reduction of blood flow to the brain leading to an imbalance between excitatory and inhibitory neurotransmission and consequent neuronal cell death. In the CNS this balance is mostly regulated by glutamate and GABA meurotransmitters. Several studies have shown that during an ischemic insult the glutamatergic and GABAergic neurotransmission is up- and down-regulated respectively. However, few studies have addressed the molecular mechanisms contributing to the alterations in GABAergic neurotransmission in brain ischemia. Recent data from our laboratory using the oxygen and glucose deprivation (OGD) model of brain ischemia showed that the ischemic insult induces the dephosphorylation and consequent internalization of $GABA_A$ receptors ($GABA_AR$), contributing to the death of cultured hippocampal neurons. Following internalization, $GABA_AR$ are rapidly recycled back to the plasma membrane or targeted for lysosomal degradation. The sorting of endocytosed $GABA_AR$ depends on the interaction of $GABA_AR$ β1-3 subunits with huntingtin-associated protein 1 (HAP1). Previous studies from our laboratory also showed that transient OGD reduces the recycling of $GABA_AR$ back to the plasma membrane and decrease the interaction of the receptors with the HAP1 protein in cultured hippocampal neurons.

HAP1 consists of two isoforms, HAP1-A and HAP1-B, which share the same middle part (amino acids 277-445). The protein is associated with microtubules and with various types of membranous organelles, including mitochondria, lysosomes and synaptic vesicles. Taking into consideration these observations, in the present work we investigated the putative role of HAP1 in the reduction of the surface expression and recycling of $GABA_AR$ in cultured hippocampal neurons subjected to OGD. Our results show that exposure of hippocampal neurons to OGD (90 min) downregulates HAP1 protein levels when tested 2 h after ischemic insult by western blot analysis. This effect was time dependent and was inhibited in the presence of the calpain inhibitor MDL28170. Inhibition of PP1/PP2A phosphatases with okadaic acid also reduced the OGD-induced downregulation of HAP1.

A decrease in HAP1 protein levels was also observed in cortical neurons exposed to OGD, but transient middle cerebral artery occlusion (MCAO), an *in vivo* model of cerebral ischemia, had the opposite effect on HAP1 protein levels in the ischemic core located in

cortical region. This discrepancy may be due to the effect of the ischemic insult in HAP1 protein levels in glial cells present in the brain tissue but not in neuronal cultures. Accordingly, western blot analysis performed with extracts of cultured glial cells exposed to 90 min of OGD followed by 12 h of post-incubation showed an increase of HAP1 protein levels.

To investigate the modulatory role of HAP1 in OGD-induced changes in the traffic of $GABA_AR$, experiments were performed in cultured hippocampal neurons transfected with HAP1-A or -1B isoforms. Overexpression of the two isoforms of HAP1 in cultured hippocampal neurons decreased the OGD-induced downregulation of the surface expression of $GABA_AR$ β3 subunits. This effect was due to the increased recycling of $GABA_AR$ β3 as shown with receptor recycling assay. Taken together, our results suggest that HAP1 protein has a key role in the down-modulation of GABAaergic neurotransmission during cerebral ischemia.

INTRODUCTION

1.1. CEREBRAL ISCHEMIA

Stroke is a pathological condition caused by blockage or rupture of a blood vessel. An ischemic stroke occurs when a blood vessel supplying blood to the brain becomes blocked, for example by a clot. An hemorrhagic stroke occurs when a blood vessel bursts, leaking blood into the brain (Figure 1.1). This condition may result from a number of factors defined as risk factors (Sacco 1997) that are considered markers for increased stroke risk. For example hypertension, smoking, diabetes, high cholesterol, and lack of physical activity are defined modifiable risk factors (Prabhakaran and Chong 2014); age, sex and race are considered unmodifiable risk factors (Willey et al. 2014).

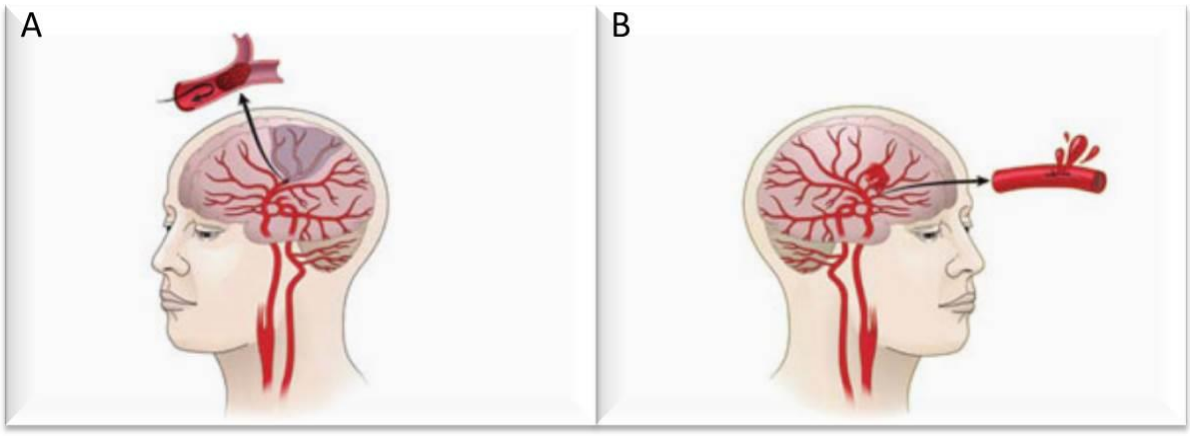

Figure 1.1. (A) Blockage of a blood vessel leads ischemic stroke; (B) Bleeding of a blood vessel of the brain induces an hemorrhagic stroke.

Cerebral ischemia, caused by insufficient blood supply to the brain, normally resulting from an arterial obstruction, leads to a cascade of damaging events in the brain. The severity and the extent of the damage depends on the degree and duration of the ischemic event (Aronowski et al. 1999; Back 1998; Fisher and Garcia 1996). In cerebral ischemia the blood flow can be transiently blocked to the entire brain, resulting in delayed and selective neuronal death (global ischemia) (Brillman 1993; Petito et al. 1987; Swain et al. 1993) or, alternatively, blood flow can be locally blocked in a temporary or permanent manner, injuring a specific area of the brain (focal ischemia) (Figure 1.2). Under the latter conditions it is possible to distinguish two different regions within the lesioned area: i) the core, corresponding to the center of the stroke that receives essentially no blood supply; this

region contains cells that are dependent on the affected blood vessel to obtain oxygen and nutrients required for their metabolism; ii) the penumbra, which is the region surrounding the core, containing cells that receive a supply of oxygen and nutrients from nearby non-affected blood vessels, although it is not sufficient to keep the normal metabolic activity (Hossmann 1994; Fisher and Garcia 1996). Focal ischemia in humans occurs mainly as a consequence of stroke, cerebral hemorrhage, or traumatic brain injury, whereas global ischemia is a consequence of cardiac arrest, open-heart surgery, profuse bleeding, or carbon monoxide poisoning.

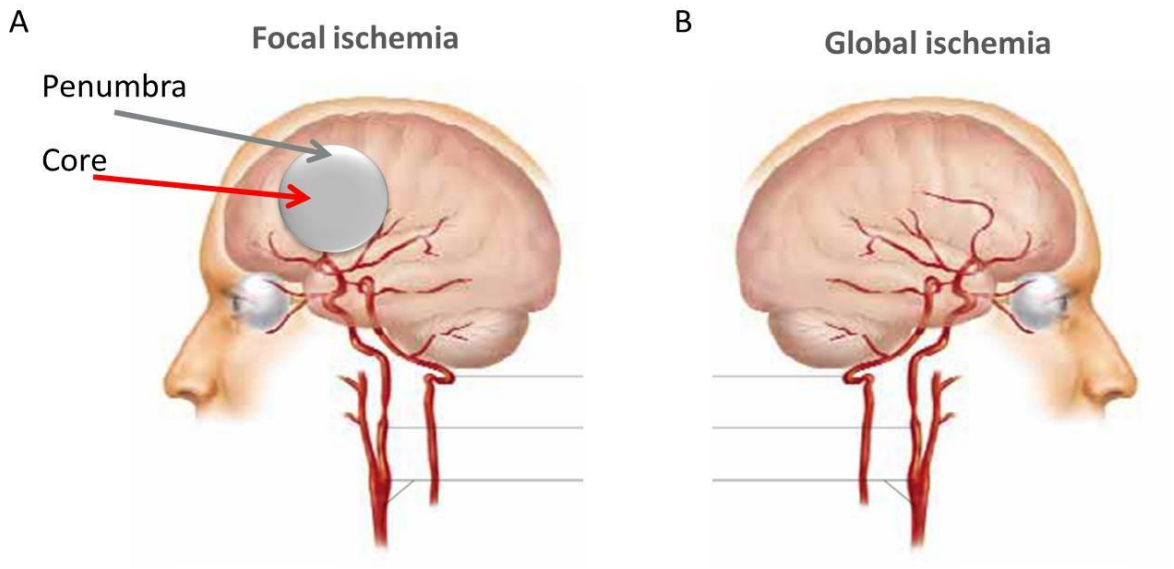

Figure 1.2. (A) Focal ischemia: temporary or permanent obstruction of local blood supply, injuring a specific area of the brain; (B) Global ischemia: blood flow is transiently blocked to the entire brain, resulting in delayed and selective neuronal death.

The brain regions that are more vulnerable to ischemic injury are the hippocampal CA1 area with pyramidal neurons, the dentate gyrus (DG), medium aspiny neurons of the striatum, pyramidal neurons in neocortical layers II, V, and VI, and cerebellar Purkinje neurons (Crain et al. 1988; Kirino 1982). The molecular mechanisms underlying the cell-specific pattern of global ischemia-induced neuronal death are not well understood. In order to address this question it is important to choose a model of brain ischemia that mimics the molecular and cellular mechanisms involved in neuronal damage following stroke in humans.

1.1.1. EXPERIMENTAL MODELS OF GLOBAL AND FOCAL ISCHEMIA

Different models of stroke have been used to study the mechanisms involved in focal and in global ischemia, including *in vivo* and *in vitro* models described below (Povlsen et al. 2012).

1.1.1.1. *IN VIVO* MODELS

MODELS OF GLOBAL ISCHEMIA

The most commonly used models of global ischemia are:

a) the four-vessel occlusion model in rats (4-VO) (Pulsinelli and Buchan 1988). This model consists in a permanent occlusion of both vertebral arteries and temporary ligation of the two common carotid arteries. In this model neuronal death is mainly restricted to pyramidal neurons of the hippocampal CA1 region.

b) the two-vessel occlusion (2-VO) also known as temporary bilateral common carotid occlusion, or BCCO in gerbils (Kirino 1982; Kitagawa et al. 1998) or (less commonly) mice (Kitagawa et al. 1998; Oguro et al. 2001). This model consists in a temporary occlusion of the common carotid arteries combined with induced systemic hypotension. These models induce extensive bilateral forebrain injury (Eklof and Siesjo 1972) and are clinically relevant to study global ischemia associated with cardiac arrest in humans.

MODELS OF FOCAL ISCHEMIA

The models of focal ischemia are the ones that better mimic stroke or cerebral infarction in humans (Oguro et al. 2001; Nagasawa and Kogure 1989). With permanent or temporary arterial occlusion (proximal or distal), it is possible to induce a necrotic cell death in the core region that leads to an irreversible damage.

The most commonly used model to study focal cerebral ischemia is the middle cerebral artery occlusion (MCAO), in which middle cerebral artery is occluded either transiently or permanently. The occlusion is induced by ligation of the common carotid and external carotid arteries, followed by insertion of a suture into the internal carotid artery at the bifurcation of the common carotid and external carotid arteries (Small and Buchan 2000; Ginsberg and Busto 1989; Longa et al. 1989). In the MCAO model the blood flow is lower

than 15% in the core region, and drops to less than 40% in the penumbra. Different experimental strategies can be used to evaluate the damage in the core region the stroke.

1.1.1.2. *IN VITRO* MODEL OF GLOBAL ISCHEMIA: OXYGEN AND GLUCOSE DEPRIVATION

The oxygen and glucose deprivation (OGD) is the most commonly used *in vitro* model to study global ischemia (Dawson et al. 1996; Goldberg and Choi 1993). OGD has been performed in primary cultures as well as in organotypic slices.

The most commonly used primary cultures in OGD studies are neurons or glia (Matute et al. 2002) isolated from different brain regions, such as the neocortex, hippocampus, cerebellum and hypothalamus of embryonic or early postnatal rats or mice (Gottron et al. 1997). Organotypic slices of the hippocampus are also frequently used in this type of studies (Newell et al. 1995; Rimvall et al. 1987; Strasser and Fischer 1995).

Primary cultures of neurons or organotypic 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 during the ischemic episode. Following the ischemic stimulus the cultures are normally incubated in fresh or conditioned culture medium, in an oxygen-containing atmosphere environment, to simulate the *in vivo* blood flow reperfusion period.

Similarly to what happens in *in vivo* models, OGD induces apoptotic as well as necrotic cell death. Despite being an *in vitro* model and considered a less complete model, the use of cell cultures in OGD experiments is a good system to analyze the molecular mechanisms of brain ischemia.

1.1.2. ISCHEMIA-INDUCED CELL DEATH: FEATURES AND MECHANISMS

In the ischemic brain cell death is induced by multiple factors, including a decrease in pH and ATP, free radical production by the mitochondrial respiratory chain, increased intracellular Na^+ concentration and membrane depolarization. These processes lead to secondary changes, resulting in the activation of damaging processes (Pulsinelli et al. 1982). Ischemic neuronal death is commonly considered a long and delayed process. In fact, between the insult and the manifestation of cell damage in some cases it may take days or

even weeks (Kirino et al. 1984; Du et al. 1996). However, this depends of the intensity and duration of the insult, as well as the brain region affected.

In addition to the multiple mechanisms that contribute to cell death, ischemic injury can also induces distinct modes of cell death. The most studied and accepted pathways of ischemic cell death are the necrotic and apoptotic cell death. In fact, both in global and focal ischemia it is possible to recognize hallmarks of necrotic as well as apoptotic events (Choi 1996; Ginsberg and Busto 1989). The hallmarks of necrotic cell death are the expansion of endoplasmic reticulum, disaggregation of polyribosomes, selective swelling of dendrites, dilation of organelles and intranuclear vacuoles (Kalimo et al. 1977; Kalimo et al. 1982). Apoptosis or programmed cell death (PCD) is a result of a series of events mediated by a dedicated set of gene products. Apoptotic neurons exhibit characteristic morphologic features that differentiate them from necrotic neurons, including cytoplasmic shrinkage, chromatin condensation and apoptotic bodies (Radi et al. 2014).

In general it is thought that in the initial periods of reperfusion there is the prevalence of necrotic markers due to immediate energy failure. Given that apoptotic cell death needs energy for cellular modifications such as cytoskeletal proteolysis and DNA alteration (Roy and Sapolsky 1999), its hallmarks appear later when mitochondrial physiology alterations occur.

The depletion of energy stores following an ischemic episode induces an ionic imbalance leading to increased neurotransmitter release and inhibition of the Na^+-dependent reuptake mechanisms. In particular the deregulation of glutamate and its receptors play an important role in the ischemic pathophysiology in different ways (Michaelis 1998). First, the increased binding of glutamate to ionotropic NMDA receptors and reduction of calcium impermeable subunit of AMPA receptors increase the influx of Ca^{2+} (Pellegrini-Giampietro et al. 1999) and the consequent $[Ca^{2+}]_i$ overload leads to the downstream activation of proteases that degrade membrane proteins, including phospholipases and proteases, inhibition of ATP production and also an increase of intracellular glutamate, thus, propagating the excitotoxic injury (Lo et al. 2003). In addition, ionotropic glutamate receptors promote an excessive influx of Na^+ with concomitant cell swelling and edema. Glutamate-induced neuronal cell death is associated with apoptosis, as evidenced by characteristic fragmentation of DNA, morphological changes, activation of calpains and

induction of caspase-dependent and -independent mechanisms (Strasser and Fischer 1995; Jover et al. 2002; MacManus et al. 1993). However, excessive stimulation with glutamate was shown to trigger necrotic cell death (Ankarcrona et al. 1995). In addition to the $[Ca^{2+}]_i$ dysregulation, the alterations in the homeostasis of other ions is also critical upon ischemia. For example, Zn^{2+} is an essential cofactor for many enzymes and transcription factors and its intracellular accumulation after cerebral ischemia is deleterious for the cells by affecting mitochondrial function and consequently inducing cell swelling and production of reactive oxygen species (ROS) by mitochondria (Weiss et al. 2000; Dineley et al. 2005; Lewen et al. 2000; Jiang et al. 2001).

ROS including the superoxide anion (O^{2-}), the hydroxyl radical (OH), hydrogen peroxide (H_2O_2), are particularly responsible for oxidative stress. After ischemia, particularly during the reperfusion period, the oxygen radical production and oxidative stress facilitate mitochondrial transition pore (MTP) formation. This process dissipates the proton gradient required for oxidative phosphorylation and ATP generation (Kroemer and Reed 2000). Oxidative stress has been shown to activate several intracellular signaling cascades that may have deleterious effects on the cellular homeostasis, such as activation of mitogen-activated protein kinases (MAPKs) (Cao et al. 2005). In addition to those already known, several other targets need to be identified and explored for the development of future therapeutic strategies in the brain ischemia field.

1.2. GABA MEDIATED NEUROTRANSMISSION

In the Central Nervous System (CNS), synaptic transmission is predominantly mediated by the neurotransmitters glutamate and γ-aminobutyric acid (GABA) (Bloom and Iversen 1971), which have an excitatory and inhibitory action, respectively. This work is focused on the neurotransmission by GABA which is considered the major inhibitory neurotransmitter of the CNS.

GABA is produced from glutamate by the cytosolic enzyme glutamic acid decarboxylase (GAD) and is stored inside small synaptic vesicles by a mechanism dependent on the activity of the vesicular inhibitory amino acid transporters. The neurotransmitter GABA is exocytosed into synaptic cleft and exerts its inhibitory control by acting on two classes of receptors with distinct electrophysiological and pharmacological properties: GABA type A

receptors ($GABA_AR$) (Macdonald and Olsen 1994; Wu and Sun 2014; Boue-Grabot et al. 1998) and GABA type B receptors ($GABA_BR$). $GABA_AR$ are ionotropic fast-acting ligand-gated chloride channels (Sieghart 2006) while $GABA_BR$ are metabotropic G protein-coupled receptors (Bettler and Tiao 2006) (Figure 1.3). Activation of $GABA_AR$ leads to the entrance of chloride into the cell according to the concentration gradient, causing membrane hyperpolarization.

$GABA_BR$ are responsible for the late and slower component of inhibitory synaptic transmission (Bettler and Tiao 2006). Their activation induces intracellular signal cascades that produce hyperpolarization in postsynaptic membrane and modulate neurotransmitter release in presynaptic neurons. The effects of $GABA_BR$ are typically mediated by activation of G-proteins coupled to the inhibition of adenylyl cyclase or voltage-gated Ca^{2+} channels, and activation of inward rectifying K^+ channels (Couve et al. 2000; Benke 2013). Inhibitory synapses exert a strong control on the neuronal response by modulating neuronal activity induced by excitatory neurotransmission.

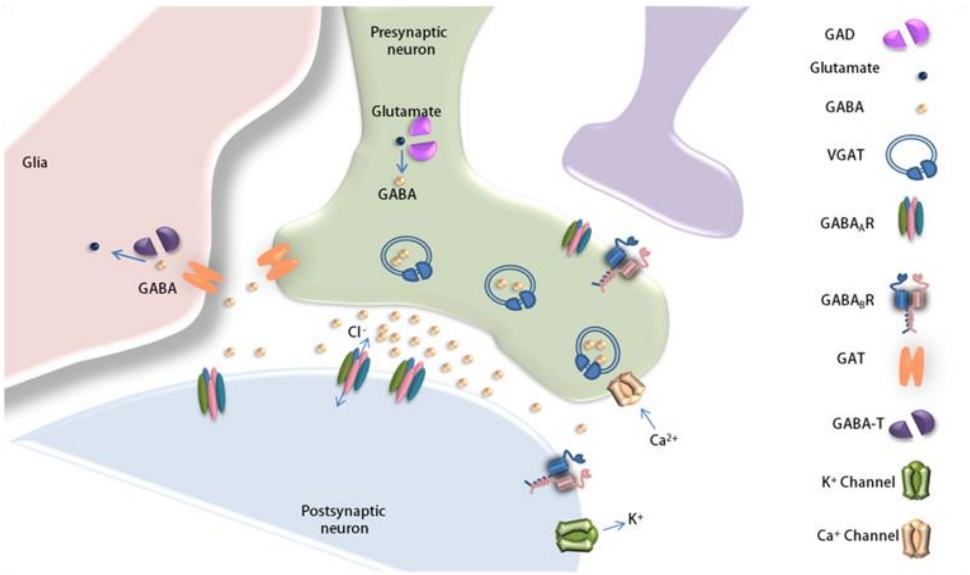

Figure 1.3. Schematic representation of a GABAergic synapse. GABA is synthesized in inhibitory neurons by glutamate decarboxylation performed by the enzyme glutamic acid decarboxylase (GAD), and is transported into synaptic vesicles by a vesicular neurotransmitter transporter (VGAT). GABA can be released both by exocytosis or by reversal of the plasma membrane transporters. GABA can acts through the activation of different classes of receptors: $GABA_A$ and $GABA_B$ receptors. $GABA_AR$ are pentameric receptors associated to chloride channel. $GABA_B$ receptors are metabotropic receptors that cause presynaptic inhibition by suppressing calcium influx. GABA reuptake by surrounding neurons and glia occurs through the activity of plasma membrane GABA transporters (GAT).

1.2.1. $GABA_AR$ STRUCTURE AND FUNCTION

As mentioned above GABA is the major inhibitory neurotransmitter in the CNS, and its fast inhibitory control is mediated by $GABA_AR$ which belong to the ionotropic family of neurotransmitter receptors (Sieghart 2006). Different gene products contribute to the formation of the $GABA_AR$ heteropentameric chloride channel, and the diversity of $GABA_AR$ is further increased by alternative splicing. The studies performed in mammals identified 19 genes encoding $GABA_AR$ subunits (α1–α6, β1–β3, γ1–γ3, δ, ε, τ, π, ρ1– ρ3), and most receptors belonging to this class are formed by the assembly of 2α and 2β subunits together with a single γ2 or δ subunit. In fact, the majority of $GABA_AR$ in brain are composed of α1β2γ2 subunits, followed by α2β3γ2 and α3β2γ2 (Knight et al. 2000; Massaria et al. 1976; Chang et al. 1996; Tretter et al. 1997; Luscher et al. 2011).

The different composition in subunits is the major determinant of the ligand binding and gating properties of the $GABA_AR$ channels. The precise subcellular localization of different $GABA_AR$ subtypes and the difference in subunit composition between synaptic and extrasynaptic receptors are reflected in a differential modulation of phasic and tonic signaling. Moreover depending on the subunit composition $GABA_AR$ have also different physiological and pharmacological properties.

Each $GABA_AR$ subunit present a structure characterized by an extracellular N-terminal domain that is also the site of action of various drugs, four hydrophobic transmembrane domains (TM1-4) and an extended cytoplasmic loop region (ICD) between TM3 and TM4 that mediates the interaction with trafficking and signaling factors and is subject to a number of posttranslational modifications. The C-terminal region of $GABA_AR$ subunits is extracellular (Sieghart 2006).

It is well established that $GABA_AR$ functions and synaptic strength are influenced by the number of postsynaptic receptors. The density of surface $GABA_AR$, and in particular the number of synaptic receptors, is determined by the balance between the rate of receptor exo- and endocytosis, to and from the membrane surface respectively (Belelli et al. 2009) (Brickley and Mody 2012), as well as by the lateral diffusion of receptors from and into the synaptic region.

1.2.2. TRAFFICKING OF $GABA_AR$

$GABA_AR$ are assembled upon oligomerization of the receptor subunits in the endoplasmic reticulum (ER). The receptors exit the ER only when the proteins are correctly folded, and the misfolded or unassembled receptor subunits are targeted for proteasomal degradation (Kittler et al. 2002). This process is negatively regulated by Plic-1, which binds to receptor α- and β-subunits and may also increase subunit maturation and production (Saliba et al. 2008). Following the assembly, $GABA_AR$ are transported to the Golgi apparatus where they bind the GABARAP/NSF complex. The receptors are then incorporated in vesicles and transported to the plasma membrane (Chen et al. 2000; Everitt et al. 2004).

$GABA_AR$ surface expression is very dynamic and regulated. Once in the membrane they can reach the postsynaptic region through lateral diffusion, and the synaptic receptors may be stabilized by interacting with its scaffold protein gephyrin. When localized in extra synaptic compartment the receptors can be removed from the plasma membrane by clathrin- and dynamin-dependent endocytosis. This process is facilitated by interactions of the $GABA_AR$ β and γ subunits intracellular domains with the clathrin adaptor protein AP2 (Kittler et al. 2000; Kittler et al. 2005; Kittler et al. 2008). This process is negatively regulated by phosphorylation, and therefore $GABA_AR$ are internalized when they are dephosphorylated (Kittler et al. 2005). The efficient endocytosis of $GABA_AR$ requires a di-leucin motif present in the intracellular loop region of the β subunit (Herring et al. 2003; Herring et al. 2005). Especially important for AP2/clathrin/dynamin-mediated $GABA_AR$ internalization in neurons is an amino acid sequence motif that includes a major phosphorylation site in the cytoplasmic loop region of β1-3 subunits (S408/409) and the γ2 subunit (Y365/367). The interaction between AP2 and β1-3/γ2 subunits can be regulated both by protein kinase A (PKA) and protein kinase C (PKC) while the same interaction with β2 subunit is regulated only by PKC (Kittler et al. 2005; Kittler et al. 2008).

1.3. HAP1 AND ITS ROLE IN $GABA_AR$ TRAFFICKING

1.3.1. HAP1 – STRUCTURE, FUNCTION AND LOCALIZATION

Huntingtin-associated protein-1 (HAP-1) was initially identified using the yeast two-hybrid system as an interacting partner for huntingtin (htt), the protein encoded by the Huntington disease (HD) gene (Li et al. 1995). Three transcripts that differ for their 3'-end (hap1A, 1B, and 1C) are expressed from the mouse hap1 gene due to alternative splicing but only two distinct protein isoforms (HAP-1A and HAP-1B) are encoded (Bertaux et al. 1998; Dragatsis et al. 2000). These two isoforms, HAP1-A (75 kDa) and HAP1-B (85 kDa), are different for their short C-terminal sequences (amino acids 579–599 of HAP1-A *versus* amino acids 579–629 of HAP1-B) and their localization in the brain (Gutekunst et al. 1998; Li et al. 1998a).

HAP1 contains coiled-coil domains in the middle region and multiple N-myristoylation sites. Coiled-coil domains and N-myristoylation sites are present in a large number of proteins that are associated with membrane-associated proteins and involved in vesicular trafficking. HAP1 does not contain transmembrane domains and nuclear localization signals, suggesting its cytoplasmic localization. It was also shown that the middle region of HAP1 (amino acids 277-445), which is present in both HAP1-A and HAP1-B isoforms, is responsible for the self-association (Figure 1.4). Moreover, this region also interacts with N-terminus of htt suggesting a role in the interaction of HAP1 with htt (Li et al. 1998a).

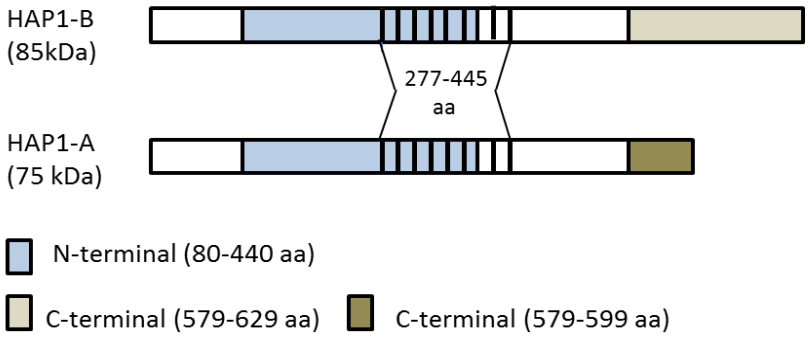

Figure 1.4. Schematic diagram of the two isoforms of HAP1: HAP1-B (85 kDa) and HAP1-A (75 kDa). rHAP1-A and rHAP1-B were isolated from rat brain cDNA libraries. The two isoforms have identical sequences in the overlapping regions, with different amino acids in the carboxy-terminus.

HAP1 is expressed in the olfactory bulb, hypothalamus, brain stem, striatum, cerebellum, hippocampus and colliculi of mouse and rat, as detected by northern blot and *in situ* hybridization analyses (Li et al. 1995; Bertaux et al. 1998; Dragatsis et al. 2000; Page et al. 1998). Expression of HAP1 has also been demonstrated in human different brain regions, including the amygdala, caudate nucleus, corpus callosum, substantia nigra, subthalamic nucleus and thalamus (Li et al. 1998c). So far hap1 transcripts have been associated with neurons and not glia (Li et al. 1996). Immunoblotting and immunohistochemistry studies have confirmed the expression of HAP1 protein in several regions of the central nervous system (CNS) (Li et al. 1995; Martin et al. 1999). During mouse development, hap1 transcripts are detected in the neuroepithelium, starting from the embryonic day 8.5 (E8.5), and are abundantly expressed in the hypothalamus, superior colliculus and cerebellum by E17.5 (Dragatsis et al. 2000). Furthermore, the two isoforms of HAP1 were found to be differently distributed. In developing hippocampal neurons in culture, HAP1-A immunoreactive puncta appear prevalently in neuronal processes, whereas HAP1-B staining remains diffuse (Li et al. 2000). In mature neurons (15 days), the majority of HAP1-A is concentrated in synaptic structures, in contrast with the majority of HAP1-B which remained diffuse in the cytoplasm and neurites (Li et al. 2000). In older neurons (>15 days) HAP1-A is highly expressed in axonal regions that might be in contact with dendrites, but the expression in dendrites is reduced when compared to younger neurons (Li et al. 2000). Studies performed in mice showed that HAP1 expression is not restricted to the brain, and the protein was found to be present at high levels in testis and at lower levels in lung and spleen. Furthermore, a differential expression of the two isoforms was also detected in these regions (Bertaux et al. 1998).

The molecular function of HAP1 is not completely understood. However, HAP1 has a crucial role in the regulation of rodent postnatal feeding behaviour. In fact, mice with homozygous disruption at the hap1 *locus* displayed normal brain and organ structure at birth, but showed decreased body weights. Interestingly, hap1$^{-/-}$ pups did not grow after birth and die around the 9th postnatal day due to decreased feeding (Chan et al. 2002). This phenotype may be caused by the degeneration of hypothalamic neurons that control feeding behaviour. Indeed, several lines of evidence suggest that HAP1 is involved in different cellular processes including vesicular transport, possibly along microtubules, and neuronal

transport of organelles and molecules. The role of HAP1 in intracellular trafficking was suggested by its interaction with proteins associated with microtubules. More specifically, HAP1 binds p150glued, a dynactin subunit that mediates the interaction of the motor protein dynein with microtubules (Li et al. 1998b; Engelender et al. 1997), thereby collaborating in the retrograde transport in neurons (McGuire et al. 2006). Furthermore, HAP1 also interacts with Duo (alternatively named P-CIP10 or Kalirin-7), a brain specific rac1 guanyl-nucleotide exchange factor that binds postsynaptic density-associated proteins (Colomer et al. 1997; Penzes et al. 2001).

The molecular functions of HAP1 are correlated with its subcellular localization. HAP1 has been observed to be associated with crude synaptic vesicles (Li et al. 1996; Engelender et al. 1997), and co-sediments with polymerized endogenous microtubules. Immunocytochemical studies indicated that HAP1 is primarily found in the cytoplasm of neurons within punctate structures (Li et al. 1996; Martin et al. 1999; Li et al. 1998b). This evidence was confirmed using electron microscopy, which showed HAP1 cytoplasmic localization and its association with axon terminals and post-synaptic dendritic spines (Gutekunst et al. 1998; Martin et al. 1999). Studies performed in PC12 cells transfected with HAP1-A also showed that this protein promotes neurite extension, suggesting an additional role in neuronal differentiation (Li et al. 2000). Nevertheless the precise function of HAP1 and its role in neuronal dysfunction remains to be elucidated, being this protein a possible contributor in different pathologies.

1.3.2. THE ROLE OF HAP1 IN $GABA_AR$ TRAFFICKING

After endocytosis $GABA_AR$ are targeted for lysosomal degradation or rapidly recycled back to the cell surface (Kittler and Moss 2003; Barnes 2000). It was shown that during short intervals the majority of internalized $GABA_AR$ are rapidly recycled back to the cell surface, but after longer periods of time the receptors are degraded. The degradation of $GABA_AR$ occur in lysosomes, and this was demonstrated in studies showing a reduction of receptor degradation in the presence of the lysosomal protease inhibitor leupeptin (Kittler et al. 2004). Blocking lysosomal activity or disrupting the trafficking of ubiquitinated cargo to lysosomes was shown to increase the accumulation of $GABA_AR$ at synapses with a consequent increase of GABAergic inhibition.

Previous studies provide evidence that a direct interaction of $GABA_AR$ with HAP1 might be involved in the endocytic receptor sorting under physiological condition (Kittler et al. 2004). HAP1 inhibits degradation of $GABA_AR$ and facilitates receptor recycling. Furthermore, HAP1 overexpression in cultured neurons showed an increase recycling of $GABA_ARs$ to the cell surface (Kittler et al. 2004). Y2H screen of a rat hippocampal library showed that rat HAP1 interacts specifically with β1-subunit intracellular domain of $GABA_AR$ (Bedford et al. 2001; Couve et al. 2000). This strong interaction was confirmed through pull-down assay and immunoprecipitation, identifying an intracellular domain of the protein HAP1 (residues 220-520) that interact with $GABA_AR$ (Kittler et al. 2004). Another piece of evidence suggesting a role for HAP1 in the intracellular fate of $GABA_AR$ was the co-localization of HAP1 with clathrin-coated vesicles (Li et al. 2002). Furthermore, HAP1 overexpression was shown to increase the surface levels of $GABA_AR$ and the miniature inhibitory post-synaptic currents (mIPSC) amplitude (Kittler et al. 2004).

1.4. EFFECTS OF ISCHEMIA ON GABA NEUROTRASMISSION

The reduction of blood flow that characterizes cerebral ischemia induces an imbalance between excitatory/inhibitory neurotransmission that is one of the major causes of excitotoxic neuronal death. The changes in metabolism associated to an insufficient energy supply to the brain induce the extracellular accumulation of glutamate (see section 1.1.2.) with the consequent overactivation of excitatory synapses. Under these conditions there are also pre- and post-synaptic alterations in GABAergic synapses. One of the early alterations occurring at inhibitory synapses upon an ischemic insult is the transient accumulation of GABA in the extracellular space which increases GABA-mediated neurotransmission (Hutchinson et al. 2002). This event is possibly caused by an increased Ca^{2+}-dependent release of GABA, as well by reversal of GABA transporters induced by plasma membrane depolarization with consequent changes in the Na^+ electrochemical gradient (Bazan 1970; Phillis et al. 1994).

Important also are the effects on $GABA_AR$ and the reduction of their activity. There are evidences *in vivo* that after transient cerebral ischemia $GABA_A$ receptors are down-regulated in regions such as the hippocampus and cerebral cortex, within 30 min of the reperfusion onset (Alicke and Schwartz-Bloom 1995). This down-regulation may result

from receptor internalization, but the cell surface density of $GABA_AR$ returns to normal within 2 h after ischemia. It was also shown that together with the down-regulation of $GABA_AR$, there is a very rapid decrease of the mRNA for α1 and β3 $GABA_AR$ subunits in the CA1 and CA3 hippocampal areas, as well as in the dentate gyrus (Li et al. 1993).

Exposure of cultured hippocampal neurons to oxygen and glucose deprivation (OGD), *in vitro* model of global ischemia, was used to examine molecular changes in GABAergic neurotrasmission. Previous results from our laboratory (Mele et al. 2014) established that in cultured hippocampal neurons subjected to 90 minutes of OGD there is a down-regulation of the $GABA_AR$ subunits α1, α2, β3, and γ2 when measured 8h after the stimulus. Moreover, western blot analysis using a phosphospecific antibody against serine residues 408/409 [which are the major sites of phosphorylation for PKA (protein kinase A)] showed that OGD reduces the phosphorylation of $GABA_AR$ β3 subunit by a mechanism sensitive to okadaic acid. Furthermore, transfection of hippocampal neurons with a phospho-mimetic mutant of the $GABA_AR$ β3 subunit (SS432/433AA) (homologous of mouse 408/409) showed an accumulation of the receptors at cell surface due to the reduction of the OGD-induced internalization. This mechanism also protected neurons from OGD-induced cell death (Mele et al. 2014).

1.5. OGD-INDUCED ALTEREATION OF $GABA_AR$ B3 RECYCLING AND ITS INTERACTION WITH HAP1

Previous results from our laboratory (Mele et al. 2014) uncovered the molecular mechanisms underlying $GABA_AR$ downregulation in cultured hippocampal neurons subjected to the OGD in vitro model of brain ischemia. Transient exposure of hippocampal neurons to OGD also down-regulated the total protein levels of $GABA_AR$ subunits (see previous section) by a mechanism dependent on the activity of calpains. Moreover, it was found that OGD i) decreases $GABA_AR$/Gephyrin interaction, ii) induces the internalization of $GABA_AR$ via clathrin-dependent endocytosis, iii) reduces the recycling of $GABA_AR$ back to the plasma membrane and iv) decreases their interaction with the HAP1 protein. Based on these observations a new model was proposed to explain the key steps in GABAergic down-modulation during cerebral ischemia (Figure 1.5). According to this

model, the increase in $GABA_AR$ internalization in brain ischemia is followed by a decrease in recycling, possibly due to a reduction in the interaction with the HAP1 protein.

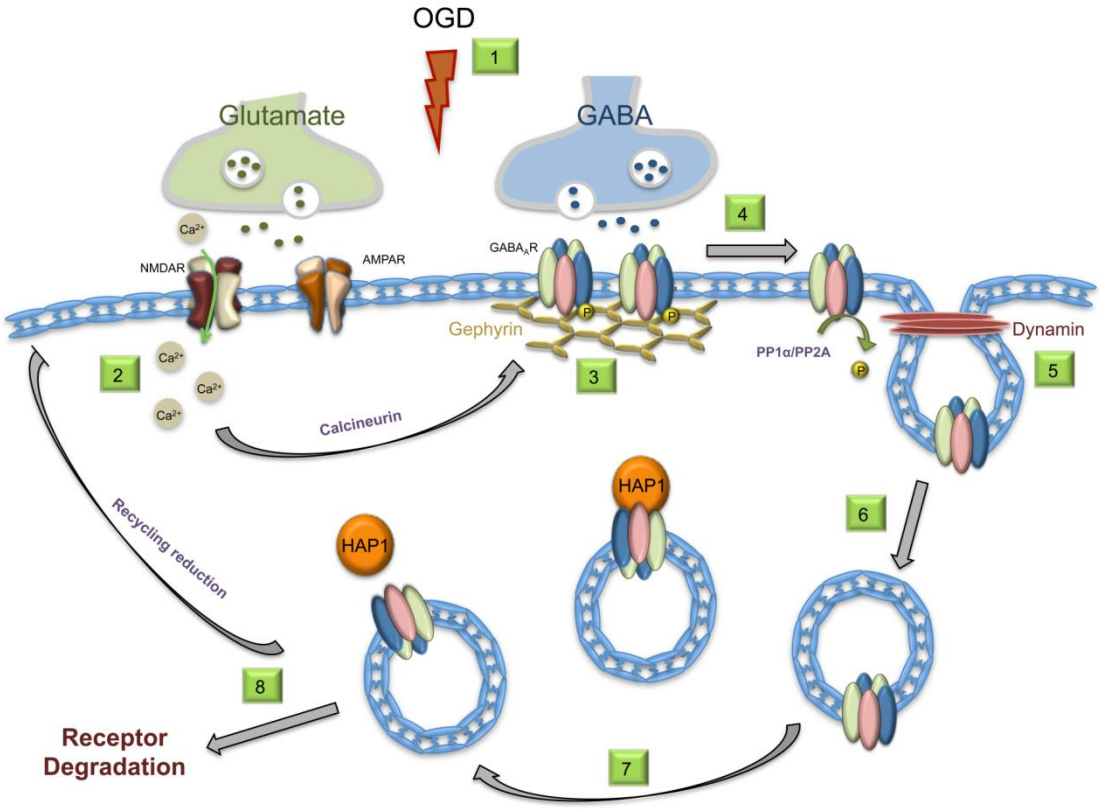

FIGURE 1.5. Model of the alteration in $GABA_AR$ trafficking during cerebral ischemia (Mele et al. 2014).
The strength of inhibitory synapses is determined by the postsynaptic $GABA_AR$ receptor pool size which is regulated by their stabilization on the membrane surface and their trafficking between the plasma membrane and intracellular compartment. Ischemic insult (1) overactivates NMDAR signalling (2) and the resulting stimulation of calcineurin decreases $GABA_AR$/Gephyrin interaction (3). In parallel, OGD reduces phosphorylation of $GABA_AR$ β3 subunit by a mechanism sensitive to okadaic acid (4), inducing the internalization of $GABA_AR$ via clathrin dependent endocytosis (5, 6). OGD also reduces $GABA_AR$/HAP1 interaction and $GABA_AR$ recycling rate (7, 8), driving $GABA_AR$ to degradation.

OBJECTIVES

The insufficient blood supply to the brain that characterize cerebral ischemia causes an imbalance between excitatory/inhibitory neurotransmission and excitotoxic neuronal death. $GABA_A$ receptors ($GABA_AR$) are the major mediators of inhibitory neurotransmission in the CNS and play an essential role in maintaining this balance, required for the correct function of neuronal networks (Smith and Kittler 2010).

The strength of inhibitory synapses is determined by the postsynaptic $GABA_AR$ receptor pool size which is regulated by their stabilization on the membrane surface and their trafficking between the plasma membrane and intracellular compartment. The number of plasma membrane receptors is mostly determined by the processes of internalization from plasma membrane (Kittler et al. 2000), and recycling to the surface. The $GABA_AR$ recycling is regulated by their interaction with the HAP1 cytoplasmic protein (Kittler et al. 2004).

The exposure of hippocampal neurons to OGD, an *in vitro* model of cerebral ischemia, was shown to increase the internalization of $GABA_AR$ via clathrin dependent endocytosis, and to reduce the recycling of $GABA_AR$ back to the plasma membrane, in addition to decreasing their interaction with the HAP1 protein (Mele et al. 2014). The major aim of this work was to investigate the role of the HAP1 protein on the alteration of $GABA_AR$ trafficking in cerebral ischemia. More specifically we investigated the effect of transient ischemia on:

- the total protein levels of HAP1 in hippocampal neurons, cortical neurons and glial cells subjected to OGD; The role of calpains and phosphatases in the alterations of HAP1 total protein levels was also investigated;

- the alterations in total HAP1 protein levels in the MCAO *in vivo* model of focal bran ischemia;

- the expression of HAP1 mRNA levels in glial cells exposed to OGD;

- the effects of HAP1 overexpression on the OGD-induced alteration of surface $GABA_AR$ and their recycling in cultured hippocampal neurons

MATERIALS AND METHODS

The antibodies and other reagents used in this work are listed in Tables 2.1 and 2.2.

Table 2.1. List of antibodies used in this work.

ANTIBODIES			
Name	**Used dilution**		**Supplier**
	Western Blot	Immunocytochemistry	
anti-Calpastatin	1:200		Santa Cruz Biotechnology
anti-Chicken IgG conjugated with AMCA Fluor 350		1:200	Invitrogen
anti-GFAP		1:1000	NeuroMab
anti-GFP (rabbit)	1: 500	1:1000	MBL
anti-HAP1	1:750	1:250	Santa Cruz Biotechnology
anti-MAP2		1:10000	Abcam
anti-mouse IgG conjugated with Alexa Flour 488		1:500	Invitrogen
anti-mouse IgG conjugated with Alexa Flour 568		1:500	Invitrogen
anti-Synaptophysin	1:20000		Abcam
anti-β-tubulin	1:300000		Sigma-Aldrich
goat Alexa Flour Far-red 647 anti-mouse		1:500	Invitrogen

Table 2.2. List of reagents used in this work.

REAGENTS	
Name	**Supplier**
FDU	Sigma-Aldrich
Actynomicin D	Calbiochem
Antipain	Sigma-Aldrich
Bromophenol blue	Merk
BSA	Enzytech or Sigma-Aldrich
CaCl	Panreac
Chloroform	Fisher-Scientific
Cyclosporin A	Santa Cruz Biotechnology
DAKO	Denmark
DOC	Sigma-Aldrich
DMSO	Sigma-Aldrich
DMEM	Sigma-Aldrich
DTT	Enzytech
ECF	GE Healthcare
EDTA	Sigma-Aldrich
EGTA	Sigma-Aldrich
Ethanol	Fisher-Scientific
Experion RNA StdSens Analysis Kit	Bio-Rad
Fetal bovine serum	Invitrogen
Forskolin	Tocris Bioscience

Gentamycin	Invitrogen
Glucose	VWR Chemicals Prolabo
Glutamate	Sigma-Aldirch
Glutamine	Sigma-Aldrich
Glycerol	Amresco
HEPES	Fisher-Scientific
Horse serum	Invitrogen
iScript cDNA synthesis kit	Bio-Rad
Isopropanol	Panreac
KCl	Panreac quimica S.A.U
KH_2PO_4	Panreac quimica S.A.U
Kynurenic acid	Sigma-Aldrich
Leupeptin	Sigma-Aldrich
MDL28170	Calbiochem
MEM	Sigma-Aldrich
$MgSO_4$	Merck
$Na_2HPO_4.2H_2O$	Merck
NaCl	Panreac quimica S.A.U
NaF	Sigma-Aldrich
$NaHCO_3$	Merck
Neurobasal medium	Invitrogen
Okadaic acid	Santa Cruz Biotechnology
PenStrep	Invitrogen
Pepstatin	Sigma-Aldrich
Phenol red	Sigma-Aldrich
PMA	Biomol, as part of Enzo Life Sciences
PMSF	Sigma-Aldrich
PVDF membranes	Millipore
Pyruvic acid	Sigma-Adlrich
RNAase free water	GIBCO Invitrogen
SM1 supplement	Stem Cell Technologies
SDS	Ficher-Scientific
Sodium orthovanadate	Sigma-Aldrich
SsoFast™ Eva Green SuperMix	Bio-Rad
Sucrose	VWR Chemicals Prolabo
TripleXtractor, reagent for RNA isolation	Grisp
Tris	Millipore
Triton X-100	Sigma-Aldrich
Trypsin	GIBCO Invitrogen
Tween	Fisher-Scientific
β-mercaptoethanol	Sigma-Aldrich

2.1. HIPPOCAMPAL CULTURES

Primary cultures of hippocampal neurons were prepared from the hippocampi of E18-E19 Wistar rat embryos. After dissection, hippocampi were treated with trypsin (0.06%, 15 min, 37°C) 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, to stop trypsin activity and transferred to Neurobasal medium supplemented with SM1 supplement (1:50 dilution), 25 µM glutamate, 0.5 mM glutamine and 50 µg/ml gentamycin. The cells were dissociated in this solution, and the homogenate was filtered (filter of 0.22 µm) to selected only the well dissociated cells. The cells were counted and plated on 6 well plates at the density of $90.0x10^3$cell/cm^2, previously coated with poly-D-lysine (0.1 mg/mL) or on poly-D-lysine coated glass coverslips at the density of $80x10^3$ cell/cm^2. The cells were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2, at 37°C for 15 days. At day 2 in vitro the cell division inhibitor 5-Fluoro-2′-deoxyuridine (FDU, 10µM, Sigma-Aldrich) was added to the cultures in order to prevent the proliferation of non-neuronal cells. After 7 days in vitro, one third of the culture medium was replaced with fresh medium without glutamate.

2.2. CORTICAL NEURON CULTURES

Primary cultures of cortical neurons were prepared from the brain cortex of E18-E19 Wistar rat embryos. After dissection, the cortices were washed with HBSS and were then treated with trypsin (0.25%, 15 min, 37°C). The trypsinized tissue was washed 6 times and the cells were dissociated in planting medium (MEM supplemented with 10% horse serum, 0.6% glucose and 1 mM pyruvic acid). The resulting suspension was filtered (filter of 0.22 µm) to isolate the well dissociated cells. The cells were plated at a density of $100x10^3$ cells/cm^2. After 2 hours the plating medium was replaced with Neurobasal medium supplemented with SM1 supplement (1:50 dilution), 0.5 mM glutamine and 50 µg/ml gentamycin. The cells were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2, at 37°C for 15 days. At day 2 in vitro the cell division inhibitor FDU

(10 µM) was added to the culture medium. One third of the culture medium was replaced with fresh medium twice a week.

2.3. GLIAL CELL CULTURES

Cultures of glial cells were prepared from the brain cortex of E18 Wistar rat embryos. After dissection, the brain cortices were washed with HBSS and mechanically dissociated in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 20% of fetal bovine serum (FBS) and 1% PenStrep. The cells were dissociated in this solution, and the resulting suspension was filtered with a filter of 0.22 µm to better separate the cells. Finally, the dissociated cells were plated in the same medium. The cells were kept in a humidified incubator at 37°C with an atmosphere of 95% air and 5% CO_2. The day after the culture medium was completely replaced with fresh medium, and additional changes of the medium were performed twice per week, with decreasing amount of FBS (20%, 15%, 10%) until the cells became confluent.

2.4. ASTROCYTE CULTURES

Astrocyte cultures were prepared from the brain cortex of E20 Wistar rat embryos. After dissection, the brain cortices were washed with HBSS and carefully homogenised. The resulting suspension was filtered with a filter of 0.22 µm to better separate the cells. The dissociated cells were then plated in the in 75 cm^2 flasks with glia medium (MEM supplemented with 10% of FBS, 6% glucose, 1% PenStrep) at the concentration of ~2-3x10^6 cells/flask. The cells were kept in a humidified incubator at 37°C with an atmosphere of 95% air and 5% CO_2. The day after the culture medium was completely replaced with fresh medium to remove dead cells. Twice a week, the flasks were vigorously shake and the medium was changed (this step allow the detachment of contaminating cells contributing for the purity of astrocyte cultures). Once confluent the astrocytes are ready to be used.

2.5. OXYGEN-GLUCOSE DEPRIVATION (OGD) OF HIPPOCAMPAL, CORTICAL NEURONS AND GLIAL CELLS

Hippocampal and cerebrocortical neurons (15 DIV) were incubated in a glucose-free saline medium (25 mM sucrose, 1.8 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES, 116 mM NaCl,

5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 0.0005% Phenol red), while glial cells were incubated in a glucose and sucrose-free saline medium (1.8 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES, 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 0.0005% Phenol red) in an anaerobic chamber with 10% H_2, 85% N_2, 5% CO_2 (Forma anaerobic System, Thermo Fisher Scientific), at 37°C for 1.5 h. The OGD medium was then replaced by conditioned medium or fresh medium in the case of glial cells, and the cultures were returned to the humidified 95% air/5% CO_2 incubator for the indicated periods of time (indicated as post-incubation period in the results section) (Chapter 3). Under control conditions (Sham) the cells were incubated in the saline buffer (25 mM glucose, 1.8 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES, 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 0.0005% Phenol red) and kept in the humidified 95% air/5% CO_2 incubator at 37°C.

When appropriate neurons were incubated with phosphatase inhibitors: Cyclosporin A 1µM (inhibitor of calcineurin) and Okadaic acid 1 µM (inhibitor of serine/threonine protein phosphatases PP2A-C), with the calpain inhibitor MDL28170 (50 µM) and with the phosphorylation activators: Forskolin (FSK, 1 µM) and Phorbol-12-Myristate-13-Acetate (PMA, 20 nM). Transcription inhibition was performed by incubation with Actynomicin D (1 µM). When tested, the calpain inhibitor and Actynomicin D were added 30 min before OGD and were also present during and after the insult.

To perform the experiments with calpastatin overexpression (endogenous calpain inhibitor), hippocampal neurons (10 DIV) were infected with adenoassociated virus serotype 1 (AAV type 1) expressing calpastatin or GFP. Five days after infection neurons were subjected to OGD.

2.6. 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 were 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 from the 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, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM 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 serum bovine albumin (Sigma) as standard.

2.7. WESTERN BLOTTING

Total cell extracts were prepared at different time period of post-incubation after OGD as indicated in the results section. After washing the cells twice with ice-cold PBS buffer (13.7 mM NaCl, 2.7 mM KCl, 1.8 mM K_2HPO_4, 10 mM $NaH_2PO_4.2H_20$), the cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris.HCl, 5 mM EGTA, 1% Triton X-100, 0.5% Deoxycholic acid, 0.1% Sodium dodecyl sulphate) 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 RIPA lysis buffer was also supplemented with 50 mM NaF and 1.5 mM sodium orthovanadate. The extracts were frozen (at -80°C), defrost and centrifuged at 16.000xg for 10 min. In the case of glial cells, before centrifugation, the extracts were sonicated for 5 min with ultrasonic sonicator bath at 4°C. The protein present in the supernatants was then quantified using the BCA method (Thermo Scientific). The samples were diluted with a 5x concentrated sample buffer (200 mM Tris.HCl, 8% glycerol, 1.6% SDS, 0.001% bromophenol blue and 5% β-mercaptoethanol). Protein samples were separated by SDS-

PAGE, in 10% polyacrylamide gels, and transferred to PVDF membranes. Membranes were blocked in 5% milk in TBS-T (20 nM Tris, 13.7 mM NaCl, 0.1% Tween, pH 7.6) and were then incubated with primary antibodies diluted in 0.5% milk TBS-T (overnight at 4°C). Finally, the membranes were washed and exposed to alkaline phosphatase-conjugated secondary antibodies (1:20.000 dilution; 1 h 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-HAP1 (1:750, Santa Cruz Biotechnology); anti-Synaptophysin (1:20000, Abcam), anti-β-tubulin (1:300000, Sigma-Aldrich), anti-GFP Rabbit (1:1000, MBL), anti-calpastatin (1:200, Santa Cruz Biotechnology).

2.8. IMMUNOCYTOCHEMISTRY

Hippocampal neurons (15 DIV) were fixed with 4% paraformaldehyde/sucrose in PBS and permeabilized with 0.3% Triton X-100 in PBS for 5 min. The cells were then incubated in PBS/BSA 10% for 60 min at RT, and further incubated with the primary antibodies diluted in PBS/BSA 3%, overnight at 4°C. Hippocampal neurons were washed 6 times with PBS and were incubated with the appropriate secondary antibodies, for 1 h at RT. The coverslips were mounted with 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 primary antibodies used were anti-HAP1 (1:250, Santa Cruz Biotechnology), anti-GFAP (1:1000, NeuroMab) and anti-MAP2 (1:10000, Abcam). The secondary antibodies used were: anti-Chicken IgG conjugated with AMCA Fluor 350 (1:200, Invitrogen), anti-mouse IgG conjugated with Alexa Flour 488 (1:500, Invitrogen) or with Alexa Flour 568 (1:500, Invitrogen).

2.9. q-PCR ANALYSES

2.9.1. TOTAL RNA EXTRACTION, RNA QUALITY AND RNA CONCENTRATION

RNA extraction from cultured glial cells was performed with TripleXtractor (Grisp). Briefly, 1 mL of TripleXtractor was added to each well of 6-well cluster plate. The extracts were diluted with chloroform and the samples were centrifuged for 15 minutes at 4°C to separate the different phases: inferior red phase containing soluble proteins and DNA, intermediate chloroform phase containing denatured proteins and top colorless phase containing total RNA. The top phase was removed and the RNA was precipitated with isopropanol followed by centrifugation. The resulting pellet containing RNA was washed with 75% ethanol, centrifuged and air-dried. RNA was resuspended with 20 µl of RNAase free water (GIBCO Invitrogen). RNA quality and integrity was evaluated using Experion electrophoresis for automated running RNA (Bio-Rad). RNA concentration was determined using NanoDrop 2000c/2000 UV –Vis spectrophotomer (Thermo scientific). The samples were kept at - 80°C.

2.9.2. 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. The thermocycler protocol includes four steps: 1) 5 min at 25°C; 2) 30 min at 42°C; 3) 5 min at 85°C; 4) Hold at 4°C.

2.9.3. PRIMER DESIGN

The primers for real-time PCR were designed using "Beacon Designer 7" software (Primer Biosoft Internationl), with the following specification: 1) GC content about 50%; 2) Anneling temperature (Ta) between 55 ± 5°C; 3) Secondary structures and primer-dimers were avoided; 4) Primer length 18-24 bp; 5) Final product length between 100-200 bp. The Primers were designed using a sequence common to the two HAP1 isoforms (HAP1-A and HAP1-B). The HAP1 Forward primer sequence was: CAGGAAGAAGATCACCGAAGA; the reverse primer sequence was: GTGTTCAGGTCCCGTTCT (Sigma).

2.9.4. REAL-TIME PCR

Real-time PCR experiments were performed as previously described (Mele et al. 2014). Gene expression analysis was performed using SsoFast^{TM} Eva Green SuperMix (BioRad). Briefly, 2 µl cDNA samples were diluted 1:10 and added to 10 µl of EvaGreen 2x and to the specific primers at the final concentration of 250 µM in a total volume of 20 µl. 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 10 s step at 95°C), 3) annealing (30 s at the optimal annealing temperature for each set of primers), 4) elongation (30 s at 72°C). At the end of 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 the assays included a nontemplate control and a standard curve of cDNA using serial dilutions (1:10, 1:100, 1:1000). All 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.

2.10. PLASMIDIC DNA AMPLIFICATION AND PURIFICATION

2.10.1. BACTERIA TRANSFORMATION

The following transformation protocol was used to obtain DNA from kanamycin resistant pEGFP-HAP1A and pEGFP-HAP1B vectors: 1µl of vector was added to competent E.Coli cells (DH5α) prepared with the Calcium Chloride protocol. After 30 min incubation on ice, the cells were submitted to an heat shock at 42°C for 45 s, followed by 5 min on ice. The cells were then pleated on Petri dishes containing LB-Agar and 50 µM kanamycin, and were then incubated overnight at 37°C.

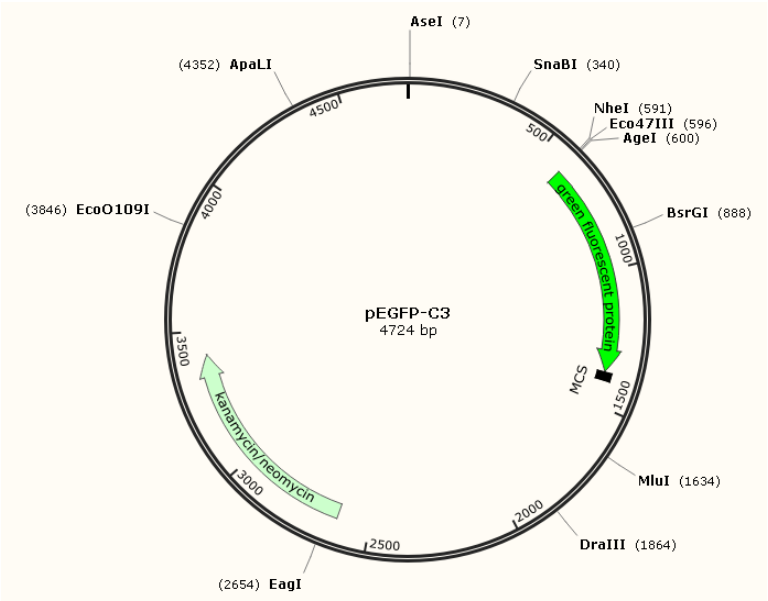

FIGURE 2.1. Map of the kanamycin resistant pEGFP (4724 bp) vector.

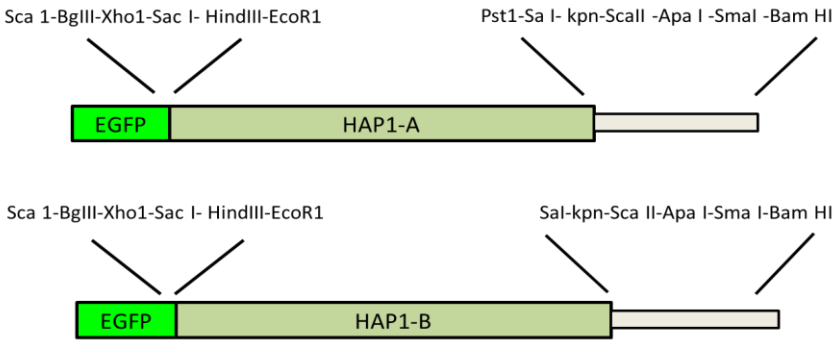

FIGURE 2.2. Schematic representation of the EGFP-HAP1-A and EGFP-HAP1-B fusion proteins.
These sequences were cloned in the kanamycin resistant pEGFP vector (4724 bp).

2.10.2. BACTERIA GROWTH

One single colony selected by kanamycin resistance after bacteria transformation was picked and incubated ON in LB medium supplemented with 50 µM kanamycin. After bacteria growth the DNA extraction was performed with HiPure Plasmid Filter Maxiprep Kit (Invitrogen) and NzyMiniprep (Nzytech). The concentration of DNA was determined

using NanoDrop 2000c/2000 UV–Vis spectrophotomer (Thermo scientific). The samples were kept at -20°C.

2.11. TRANSFECTION OF PRIMARY NEURONAL CULTURES

Hippocampal neurons were transfected with pEGFP, myc-huGABA$_A$R β3 (Mele et al. 2014), pEGFP-HAP1-A and pEGFP-HAP1-B (Gift from Ghislaine Poizat, Frédéric Saudou's laboratory, Institute Curie, Orsay, France), using the calcium phosphate co-precipitation method. 2 µg of DNA for each cover slip were used, diluted in Tris-EDTA (TE) pH 7.3 and mixed with 2.5 M of $CaCl_2$. The DNA/TE/$CaCl_2$ mix was added to 2x HEPES buffered saline solution pH 7.2 (250 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11 mM dextrose, 42 mM HEPES). The precipitates were allowed to develop at a room temperature for 30 min, protected from light, vortexing every 5 min. Neurons were prepared to receive the precipitates by adding 2 mM kynurenic acid (Sigma) in cultured-conditioned medium for 20 min. The precipitates were added to the cells drop-wise in the center of the coverslip and incubated for 2 h at 37°C, in an incubator with 95% air/5% CO_2. The cells were then washed with glutamate-free Neurobasal medium supplemented with 10 mM kynurenic acid and incubated for 20 min in an incubator with 95% air/5% CO_2. Finally, the medium was replaced with the initial culture-conditioned medium, and the cells were 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 different times of reoxygenation (0 h and 1 h). After the insult the cells were subjected to immunocytochemistry, using a fluorescence assay for receptor internalization and recycling.

2.12. IMMUNOCITOCHEMISTRY FOR MEMBRANE ASSOCIATED PROTEINS

A live staining protocol was used to study the effect of OGD on the levels of GABA$_A$R β3 subunit on the plasma membrane, in the absence of HAP1-A or HAP1-B. Cultured living hippocampal neurons (15 DIV), transfected with huGABA$_A$R β3, EGFP-HAP1 A or EGFP-HAP1 B, and subjected or not to OGD (90 min), were incubated with the primary antibody anti-myc GABA$_A$R β3 (1:300 in culture-conditioned medium; Cell Signaling) at RT for 10 min. The cells were washed with pre-warmed PBS and were then fixed with 4%

paraformaldehyde/sucrose in PBS and incubated with the secondary antibody (Alexa Flour 568 goat anti-mouse, 1:300 Invitrogen) for 1 h at RT. The preparations were washed six times with PBS and the cells were permeabilized with 0.3% Triton X-100 in PBS and blocked with 10% BSA in PBS for 60 min at RT. Then the cells were incubated ON at 4°C with an rabbit anti-GFP antibody (1:500, MBL), diluted in PBS/3% BSA. The cells were washed 6 times with PBS and incubated with the secondary antibody (Alexa Flour 488 goat anti-rabbit, 1:500 Invitrogen) for 1 h at RT. The coverslips were mounted in a florescence mounting medium and imaging was performed with Axio Observer 2.1 fluorescence microscope .

2.13. FLUORESCENCE ASSAY OF RECEPTOR INTERNALIZATION

The protocol used for fluorescence assay of receptor was previously described (Mele et al. 2014). After the OGD insult, cultured hippocampal neurons transfected with huGABA$_A$R β3, EGFP-HAP1 A or EGFP-HAP1 B, were incubated at RT for 10 min in the presence of a high concentration of the primary antibody (mouse) anti-myc (1:300, Cell Signaling) diluted in conditioned medium to label the receptors on the cell surface. The cells were then washed with PBS at 37°C to remove the unbound antibody and were again incubated in conditioned medium for 20 min at 37°C to allow receptor internalization. After this incubation the cells were fixed for 15 in 4% paraformaldehyde/sucrose and washed twice with PBS. Next, neurons were incubated with a super-saturating concentration (1:300) of the first secondary antibody (goat Alexa Flour 568 anti-mouse; Invitrogen) for 1 h at RT and washed 4 times with PBS. Then the cells were permeabilized (0.3% Triton X-100 for 5 min at 4°C) and washed for 5 min at RT before incubation with 10% BSA in PBS for 60 min at RT. To label the receptors that were internalized, the cells were incubated with the second secondary antibody (goat Alexa Flour Far-red 647 anti-mouse; 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). To identify the cells that were transfected with EGFP-HAP1 A or EGFP-HAP1 plasmids, the cells were then incubated with the primary antibody (rabbit) anti-GFP (1:500, Invitrogen) overnight at 4°C, washed 4 times with PBS and incubated with the secondary antibody (Alexa Flour goat 488 anti-rabbit; 1:500 Invitrogen). Finally, the cells were washed 4 times with PBS and the

cover slips were mounted on slides with fluorescence mounting medium (DAKO). The coverslips were mounted in a florescence mounting medium and imaging was performed with Axio Observer 2.1 fluorescence microscope**.**

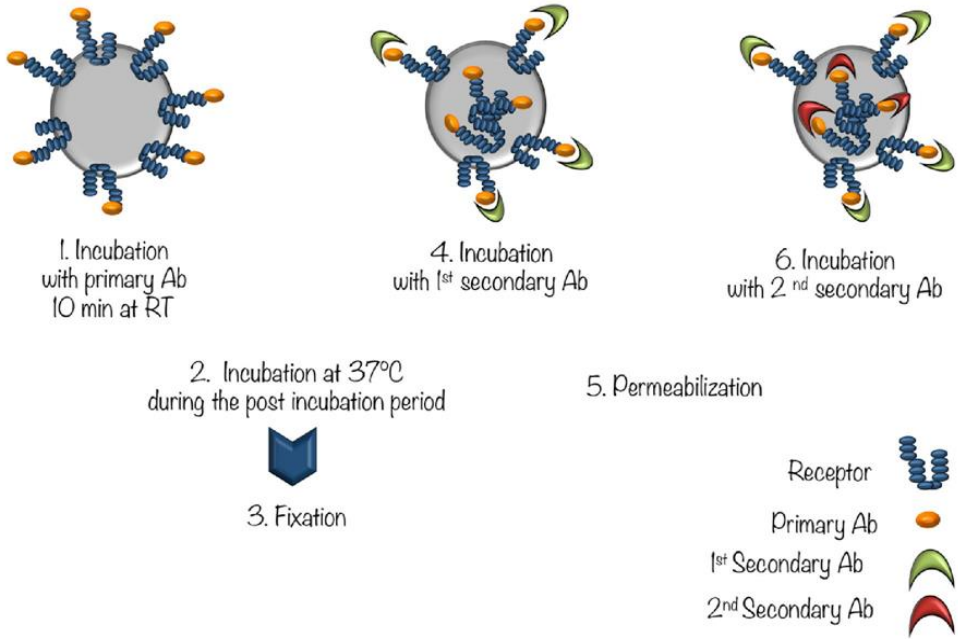

FIGURE 2.3. Schematic representation of the fluorescence assay used to assess receptor internalization.

2.14. RECEPTOR RECYCLING ASSAY

Cultured living hippocampal neurons (15 DIV), transfected with myc-huGABA$_A$R β3, EGFP-HAP1 A or EGFP-HAP1 B, were incubated at RT for 10 min in the presence of a high concentration of first primary antibody, mouse anti-myc (1:300, Cell Signaling) diluted in conditioned medium. The cells were then washed with PBS pre-warmed at 37°C to remove the unbound antibodies and further incubated with conditioned medium at 37°C for 20 min to allow the internalization of antibody-bound receptors. The antibodies remaining on the cell surface were then removed by incubation with a stripping solution (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 20 min to allow the receptor recycling. After recycling, neurons were fixed and myc-

antibody complexes recycling back to the surface were detected by incubation of the cells with the first secondary antibody (Alexa Flour goat 568 anti-mouse; 1:200 Invitrogen). To allow the detection of the receptors that were not recycled, the cells were then permeabilized and incubated with 10% BSA in PBS for 1 h at RT. After this blocking step, the cells were incubated with the secondary antibody for 1 h at RT (goat Alexa Flour 670 anti-mouse; 1:200 Invitrogen). To detect the cells that were transfected with EGFP-HAP1 A or EGFP-HAP1 plasmids, cells were incubated with the primary antibody (rabbit) anti-GFP (1:500) overnight at 4°C. Neurons were the incubated with the secondary antibody (Alexa Flour 488 anti-rabbit 1:400 Invitrogen) for 1h at RT. Finally, the coverslips were mounted on slides with fluorescence mounting medium (DAKO) and imaging was performed with Axio Observer 2.1 fluorescence microscope **.**

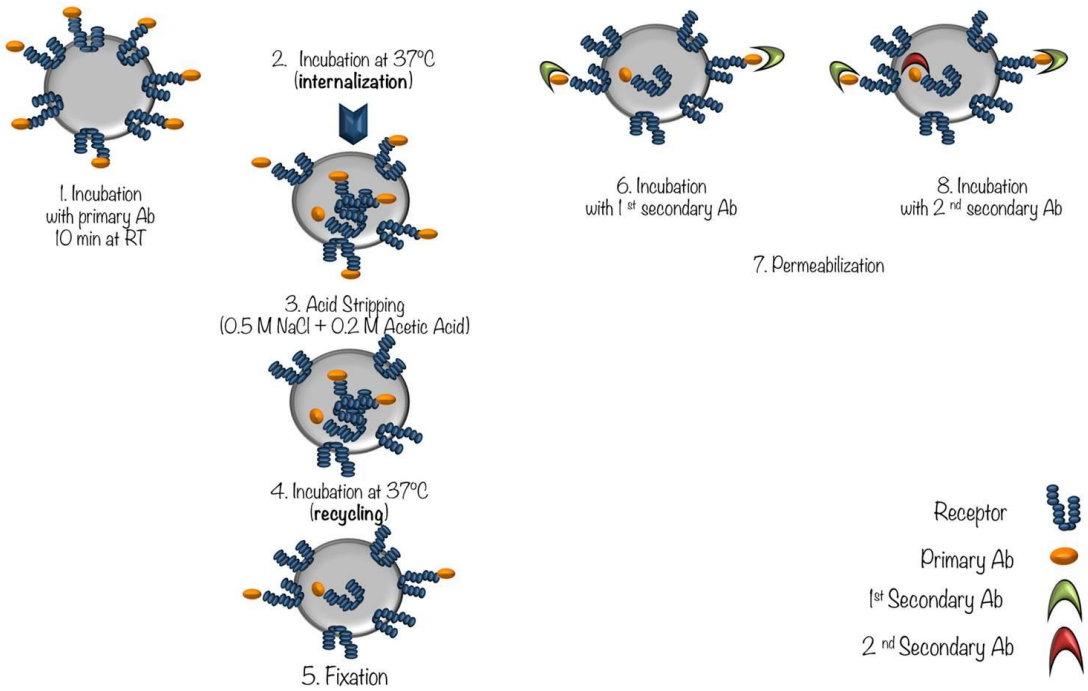

FIGURE 2.4. Schematic representation of the receptor recycling assay.

RESULTS

3.1. CHARACTERIZATION OF CULTURED HIPPOCAMPAL NEURONS

The protocol to culture hippocampal neurons was well established and already used in our laboratory. To reduce the contamination with astrocytes we supplemented the culture medium with 5-fluoro-2'-deoxyuridine (FDU, 10 µM), which inhibits thymidy-late synthase (TS) causing an imbalance of the intracellular deoxyribonucleoside triphosphate (dNTP) pool with consequent inhibition of DNA synthesis (Yoshioka et al. 1987). This inhibitor was added to the cultured hippocampal neurons after two days in culture. After fifteen days in culture the cells were fixed and immunocytochemistry was performed to label the glial fibrillary acidic protein (GFAP) and the microtubule-associated protein 2 (MAP2) that is a somato-dendritic marker. The cells were then counted and the percentage of glial cells was calculated. The average percentage of glial cells in the cultures was 4.2% (n=3) (Figure 3.1).

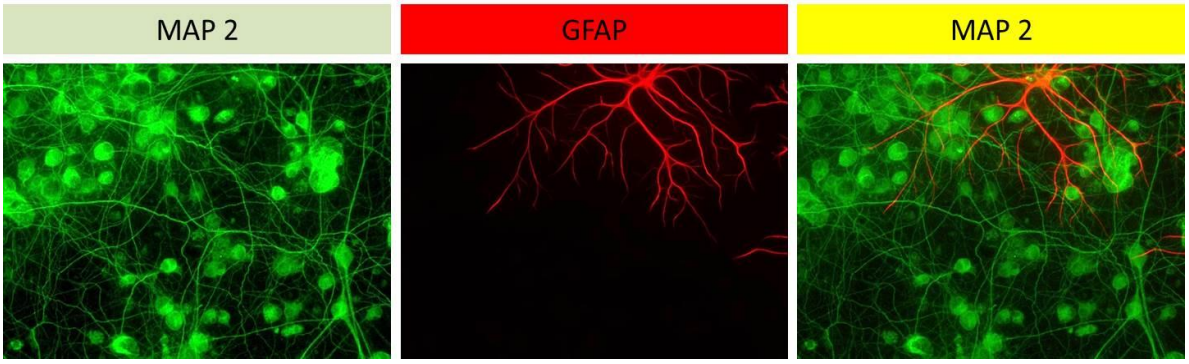

Figure 3.1. Characterization of cultured hippocampal neurons at DIV15.
The culture medium was supplemented with 5-fluoro-2'-deoxyuridine (FDU, 10 µM) at DIV2 to inhibit glial cell growth. At DIV15 the cells were fixed and the neurons were labelled with anti-MAP2 while glial cells were labelled with an anti-GFAP antibody. The glial cells were counted and their abundance was calculated as a percentage of the total number of cells (neurons + glial cells). The experiment was performed three times in independent preparations.

3.2. OGD DECREASES HAP1 TOTAL PROTEIN LEVELS BY A CALPAIN DEPENDENT MECHANISM

The sorting of $GABA_AR$ after internalization is determined by its interaction with the HAP1 protein (Kittler et al. 2004). To assess the effect of OGD, a well-established *in vitro* model of global cerebral ischemia (Dawson et al. 1996; Goldberg and Choi 1993), on HAP1 total protein levels, we subjected hippocampal neurons to 90 min of OGD and the

cells were further incubated in culture-conditioned medium for different time periods. HAP1 total protein levels were analyzed with western blot using a specific antibody against amino acids common to the two isoforms of HAP1, HAP1-A and HAP1-B. The results show no significant differences in HAP1 total protein levels immediately after OGD, but at a decrease of ~20% was observed 2 h after the stimulus, and of 40% at 4 h and 6 h after injury. HAP1 protein levels were further decreased to ~50% 8 h after OGD (Figure 3.2 A).

The absence of oxygen and glucose during OGD induces ATP depletion and the dissipation of cellular ionic gradients. These mechanisms lead to an increase in the intracellular calcium concentration with a consequent activation of calpains (Saido et al. 1994; Vanderklish and Bahr 2000), a family of calcium-dependent enzymes which cleave cytoskeleton proteins and other substrates (Bevers and Neumar 2008). To understand whether calpains are involved in the OGD-induced downregulation of HAP1 protein, hippocampal neurons were subjected to OGD (90 min) in the presence or absence of the chemical calpain inhibitors MDL28170 (50 µM). The results show that MDL28170 abrogates the effect of OGD on HAP1 total protein when evaluated 8 h after the insult, preventing its reduction in hippocampal neurons subjected to OGD (Figure 3.2 B).

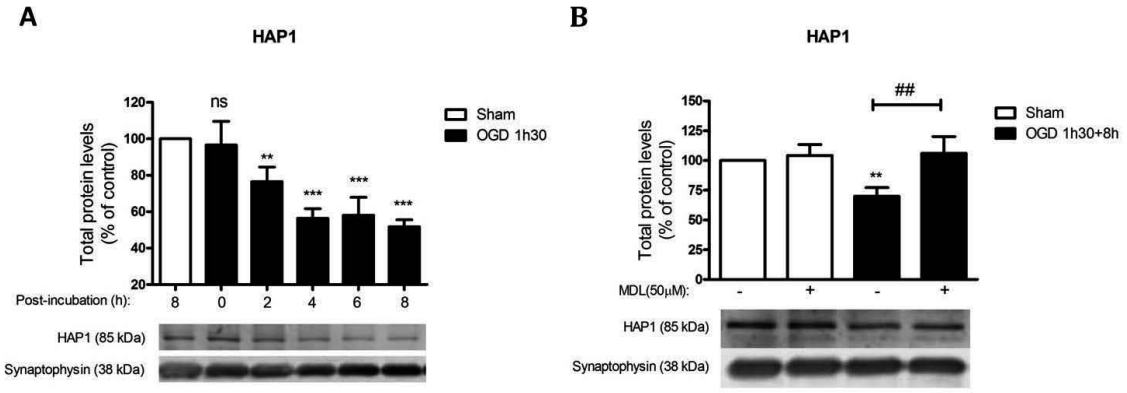

Figure 3.2. HAP1 total protein levels are downregulated *in vitro* ischemia (OGD) by a calpain-dependent mechanism.

(A) Cultured hippocampal neurons (15 DIV) were exposed to OGD (90 min) and were further incubated in culture-conditioned medium for the indicated periods of time: 0 h, 2 h, 4 h, 6 h and 8 h (post-incubation). (B) Cultured hippocampal neurons (15 DIV) were exposed to OGD for 90 min in the presence or absence of MDL28170 (50 µM) and were further incubated in culture-conditioned medium for 8 h (post-incubation) with the same inhibitors. (A-B) HAP1 total protein levels were

analyzed with western blot and the results were normalized with the loading control synaptophysin. Results are the men ± SEM of at least 3 independent experiments performed in distinct preparations. One-way ANOVA was performed as statistical analysis, followed by Dunnett's and/or Bonferroni test. **p<0.01, ***p<0.001, $^{\#\#}$p<0.01- significantly difference when compared to control conditions or for the indicated comparisons. Non-significant differences were indicate as ns.

To further investigate whether calpains act directly on the cleavage of HAP1, we used different algorithms that predict potential proteolytic cleavage sites. Polypeptide sequences enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) called PEST sequences are targeted by proteases for rapid destruction (Rogers et al. 1986). This process may be mediated by calpain proteins (Shumway et al. 1999). The Software EMBOSS (http://sourceforge.net) was used to identify the sites with the highest PEST sequence score in the two rat HAP1 isoforms (HAP1-A and HAP1-B). Figures 3.3 A and B show the presence of putative PEST sequences in both HAP1 isoforms: from amino acid 476 to 532 (score +19.36) and from amino acid 4 to 42 (score +14.8). Using the GPS-CCD program (Calpain Cleavage Detector based on the algorithm for Group-based prediction System, available at http://ccd.biocuckoo.org/), the sites that were retrieved with the highest score were the following: HAP1-A - Gln342 (1.332), Ala367 (1.34), Arg403 (1.079), Ser404 (1.008); HAP1-B - Gln342 (1.332), Ala367 (1.34), Arg403 (1.079), Ser404 (1.008), Asp579 (1.125).

It was proposed that calpains cleave their substrates in disordered segments of the proteins (Tompa et al. 2004). We used the metaPrDOS bioinformatic tool (http://prdos.hgc.jp/cgi-bin/meta/top.cgi) to predict the disorder tendency in the two isoforms of HAP1 protein. The results showed that both HAP1 isoforms presents a considerable degree of disorder in different segments (amino acids in HAP1-A: 1 to 71, 88 to 101, 216 to 257, 361 to 378, 390 to 424, 456 to 520, 564 to 599; amino acids in HAP1-B: 1 to 71, 88 to 101, 106 to 119, 216 to 258, 365 to 378, 380 to 423, 456 to 520, 564 to 593, 601 to 629) (Figure 3.3 C and D). These bioinformatic analyses reinforce the hypothesis that HAP1 protein levels may be regulated by calpain activity.

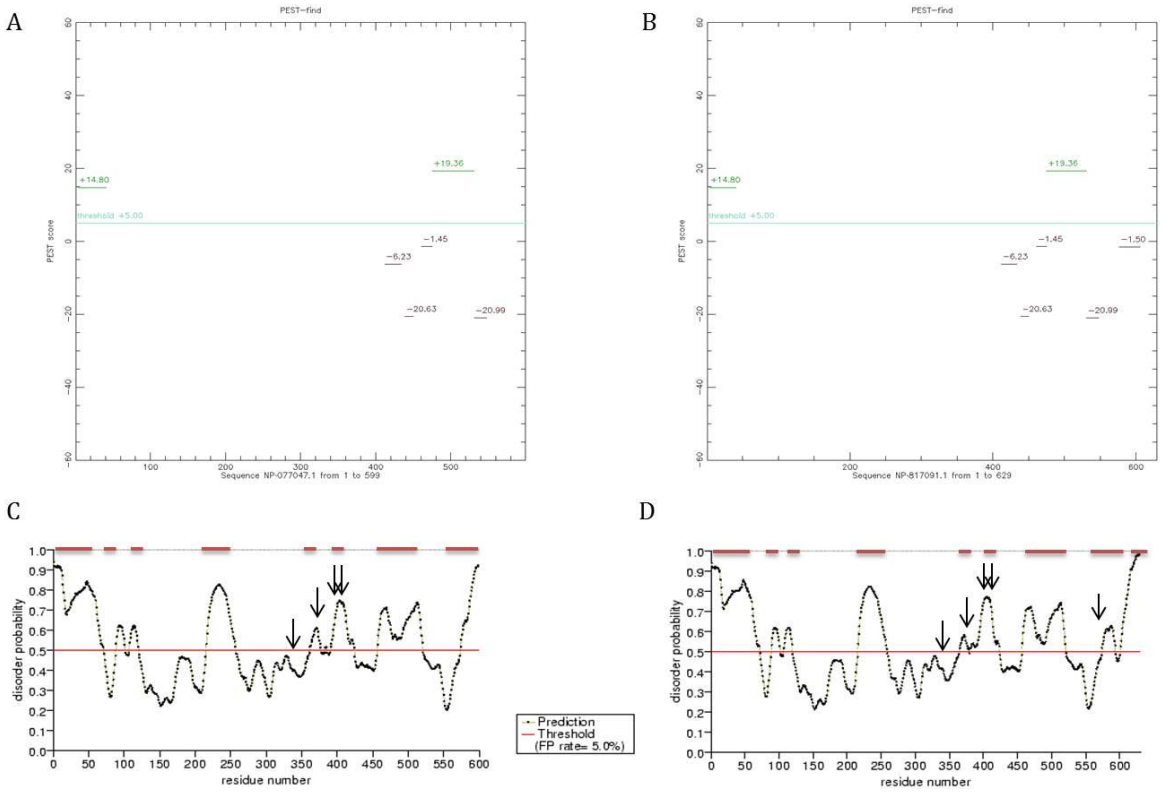

Figure 3.3. Identification of putative sites of cleavage by calpains in HAP1 protein.
Analysis of the putative sites of cleavage by calpains in the HAP1-A (A) and HAP1-B (B), using the software EMBOSS. Positive values above the threshold +5.00 correspond to predicted cleavage sites. Prediction of the disorder tendency in HAP1-A (C) and HAP1-B (D) based on the analysis of amino acids sequences with meta PrDOS bioinformatic tool. Each peak labeled with a red line corresponds to disordered segment. The arrows indicate the cleavage sites.

To confirm the results pointing to a role of calpains in the cleavage of HAP1 in hippocampal neurons exposed to OGD, we also evaluated the effect of calpastatin (endogenous inhibitor of calpains) on the total protein levels of HAP1 after OGD. Calpastatin is a natural specific inhibitor of calpains (Ishida et al. 1991; Lee et al. 1992) which binds to calpain forming a calpain-calpastatin complex (Tompa et al. 2002). Cultured hippocampal neurons (10 DIV) were infected with AAV type 1 virus that express calpastatin or GFP, and five days after infection hippocampal neurons were subjected to OGD (90 min) or incubated in sham conditions. Total HAP1 protein levels were analyzed 8 h after the insult by western blot analysis. The results show a significant decrease of HAP1 total protein levels in non-infected neurons (~40%) after OGD, and a slight but not significant reduction in cell death was observed when the cells overexpressing calpastatin (Figure 3.4). As expected, this protective effect was not observed in the cells transduced

with GFP. These results suggest that an upregulation of calpastatin protein levels reduce the OGD-induced downregulation of HAP1, but additional experiments are required to confirm the role of calpain in the cleavage of HAP1 using this experimental approach.

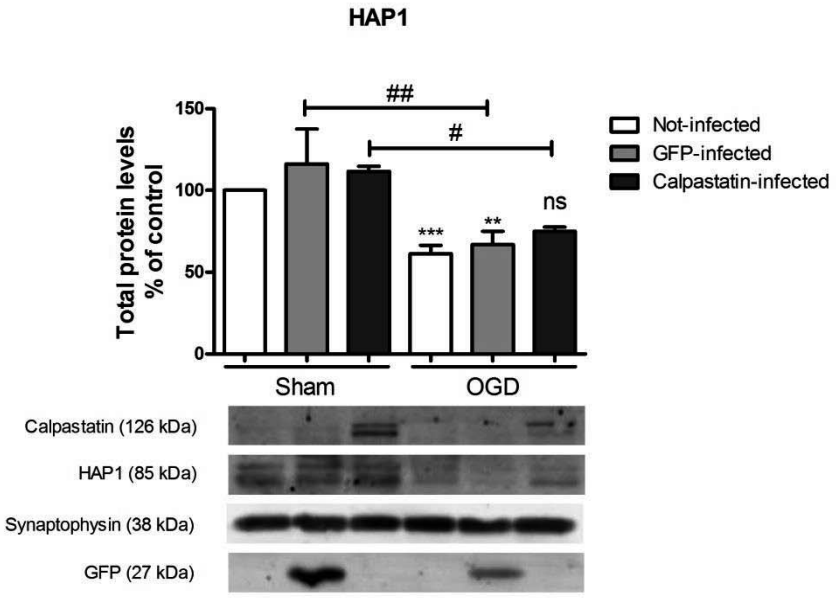

Figure 3.4. Calpastatin prevents the OGD-induced reduction of HAP1 total protein.
Culture of hippocampal neurons infected or not with AAV type 1 virus expressing calpastatin or GFP were exposed to OGD (90 min) and western blot analyses was performed 8 h (post-incubation) after the insult. HAP1 total protein levels were determined and successful infection was confirmed using specific antibodies against calpastatin and GFP. The results of HAP1 protein were normalized with the loading control synaptophysin. Results are the mean ± SEM of 3 independent experiments performed in distinct preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's and Bonferroni test. $^{**}p<0.01$, $^{***}p<0.001$, $^{\#}p<0.05$, $^{\#\#}p<0.01$ - significantly difference when compared to control conditions or for the indicated comparisons. Non-significant differences are indicated as ns.

3.3. INHIBION OF PP1/PP2A PHOSPHATASES PREVENTS OGD-INDUCED REDUCTION OF HAP1 PROTEIN LEVELS

It has been found that HAP1 interaction with several different motor proteins is required for intracellular transport (Rong et al. 2007; Engelender et al. 1997) and these interactions were shown to be affected by protein phosphorylation (Rong et al. 2006). Here we evaluated whether HAP1 protein expression is modulated by phosphorylation/dephosphorylation mechanisms in hippocampal neurons exposed to ischemic conditions. Cultured hippocampal neurons (15 DIV) were subjected to OGD for 90 min, and the cells were further incubated for 8 h in the presence of okadaic acid (0.5 µM) [protein serine/threonine phosphatase 1, 2A inhibitor (Garcia et al. 2003)], or with cyclosporin A (1µM) (calcineurin inhibitor). Incubation of hippocampal neurons with okadaic acid under control conditions upregulated HAP1 protein levels. This robust effect may contribute, at least in part, to the preservation of HAP1 protein levels, as compared with the control conditions, when hippocampal neurons were subjected to transient OGD (Figure 3.5 A). Although cyclosportin A had a small but significant effect on HAP1 protein levels under control conditions, the phosphatase inhibitor was without effect on the downregulation of HAP1 observed after OGD (Figure 3.5 A). The effect of protein phosphorylation on OGD-induced cleavage of HAP1 was further investigated in experiments where hippocampal neurons were incubated with forskolin (FSK), an activator of adenylyl cyclase, or with Phorbol 12-myristate 13-acetate (PMA), a specific activator of group A (α, βI, βII, γ) and group B (δ, ε, η, θ) Protein Kinase Cs (PKCs) (Figure 3.5 B). The results showed no effect of these two compounds on HAP1 protein expression upon OGD, suggesting that HAP1 downregulation induced by the ischemic insult is not affected by PKA and PKC.

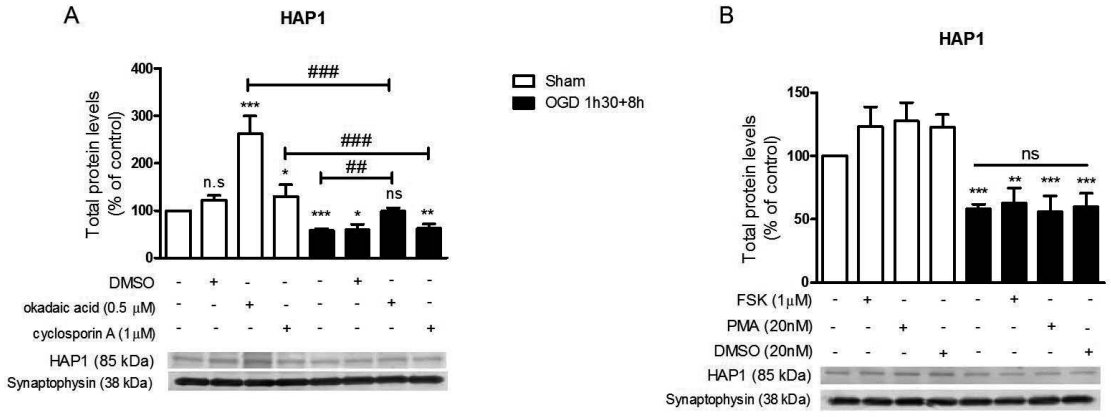

Figure 3.5. HAP1 total protein levels are dowregulated *in vitro* ischemia (OGD) by a mechanism dependent on the activity PP1/PP2A phosphatases.
(A) Cultured hippocampal neurons (15 DIV) were exposed to OGD for 90 min in the presence or in the absence of 0.5 μM okadaic acid or 1μM cyclosporin A. (B) Cultured hippocampal neurons (15 DIV) were exposed to OGD for 90 min in the presence or in the absence of 1 μM FSK or 20nM PMA. (A-B) HAP1 total protein levels were 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 Dunette's or Bonferroni test. *p<0.05, **p<0.01, ***p<0.001, $^{\#\#}$p<0.01 $^{\#\#\#}$p<0.001 - significantly different when compared with control conditions or for the indicated comparisons. Non-significant differences were indicated as ns.

3.4. HAP1 TOTAL PROTEIN LEVELS ARE INCREASED AFTER TRANSIENT MCAO, AN *IN VIVO* MODEL OF CEREBRAL ISCHEMIA

To determine whether HAP1 is also downregulated in brain ischemia *in vivo*, we measured the changes in HAP1 total protein levels in the brain of adult mice subjected to MCAO, a model of focal cerebral ischemia. Different regions of the brain, the infarct core, the penumbra as well as the ipsilateral brain hemisphere, were analyzed 48 h after transient (45 min) MCAO. Surprisingly, western blot analysis for HAP1 showed an increase of total protein levels of about 50%. No significant differences were observed in the penumbra and contralateral cerebrocortical regions (Figure 3.6). These results indicate that MCAO upregulates HAP1 protein in the ischemic core brain region.

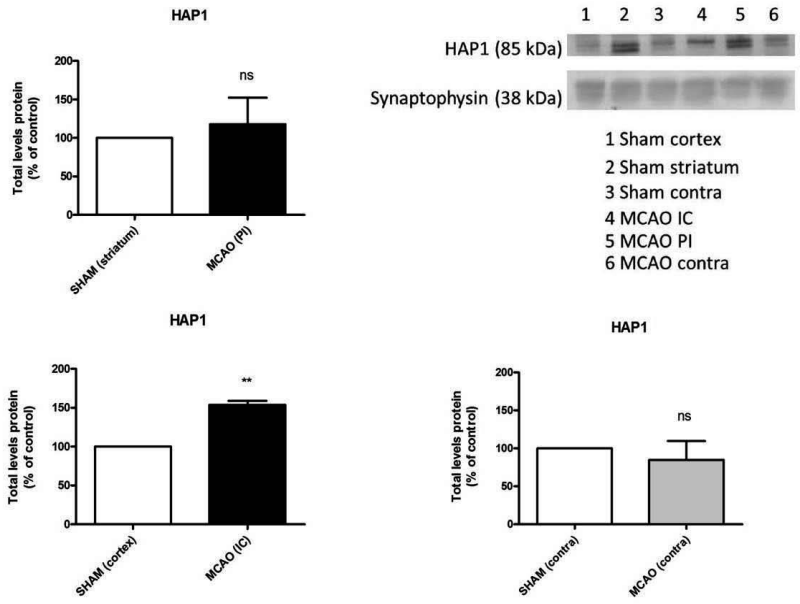

Figure 3.6. HAP1 protein is upregulated in the ischemic core of transient brain ischemia *in vivo.*
Adult male mice (C57BL/6) were subjected to transient occlusion of the right middle cerebral artery (MCA) for 45 min, followed by reperfusion for 48 h. The control (sham) was subjected to surgery for the same times. HAP1 total protein levels were analyzed in different brain regions by western blot: ischemic core (IC) and penumbra (affected by MCAO) and ipsilateral brain hemisfere. HAP1 total protein levels were analyzed also for the control in equivalent brain regions. The results were normalized with the loading control synaptophysin. Results are the men ± SEM of 3 independent experiments, using samples from different animals. Student's *t*-test was performed as statistical analysis. Significant differences (**$p<0.01$) were considered from the ischemic core (IC) and Sham cortex in operated animals. Non-significant (ns) differences were considered comparing the penumbra and controlateral regions to the correspondent region in sham operated animals.

3.5. OGD DECREASES HAP1 TOTAL PROTEIN LEVELS IN CORTICAL NEURONS AND INCREASES ITS LEVELS IN GLIAL CELLS

To understand the differential effect of ischemic injury on HAP1 total protein levels, in the *in vivo* and *in vitro* models of brain ischemia, we investigated the OGD-induced alterations in the abundance of the protein in cultured cortical neurons and in cultured glial cells. In fact, a major difference between the *in vivo* and *in vitro* models of ischemia used in the experiments described above is the cellular heterogeneity of the system: the brain tissue analyzed in the MCAO experiments contains neurons and glial cells while the hippocampal cultures used in OGD studies are highly enriched in neurons.

Cerebrocortical neurons (15 DIV) were subjected to OGD for 90 min and the cells were further incubated in culture-conditioned medium for 8 h. HAP1 total protein levels were analyzed with western blot and the results showed a decrease of HAP1 total protein by about ~25 % in agreement with the results obtained in hippocampal neurons exposed to OGD (Figure 3.7). These results indicate that OGD affects the HAP1 protein levels in cultured cortical neurons as well as in hippocampal neurons. Similar experiments were performed using cultured cerebrocortical glial cells, which were subjected to OGD (90 min) and then incubated in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 15% of fetal bovine serum (FBS) for different periods of post-incubation (Figure 3.8. A). HAP1 total protein levels were evaluated with western blot analysis right after OGD or at 4 h, 8 h, 24 h and 48 h after the stimulus. The results show a time dependent and transient effect of OGD on HAP1 protein levels. An increased in HAP1 was observed starting 8 h after OGD, and the effect was statistically significant at 12 h of post-incubation (~40%). At a later time point, 48 h after the insult, we observed a significant decrease in HAP1 (~60%). These results suggest that the differential effects of *in vivo* and *in vitro* ischemia on HAP1 protein levels may be due to the distinct response by neurons and glial cells.

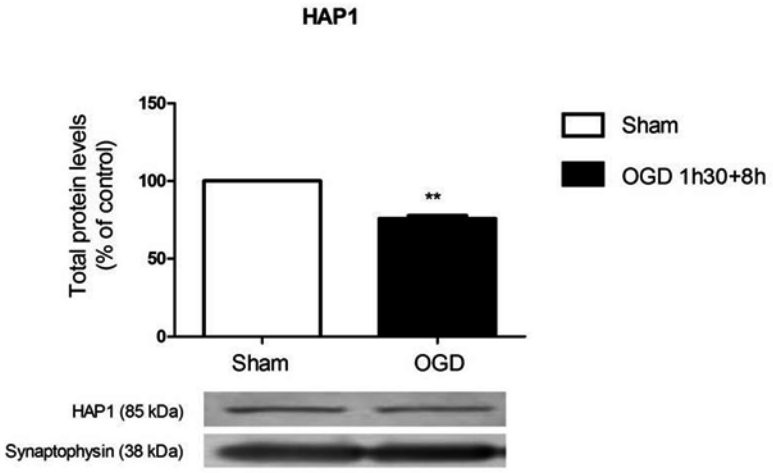

Figure 3.7. HAP1 protein is downregulated in cortical neurons exposed to OGD.
Cultured cortical neurons (15 DIV) were exposed to OGD for 90 min and were further incubated in culture-conditioned medium for 8 h (post-incubation). Total HAP1 protein levels was analyzed by western blot and the results were normalized with the loading control synaptophysin. Results are the mean ± SEM of at least 3 independent experiments performed in distinct preparations, and are expressed as percentage of the control. Statistical analysis was performed by Student's *t*-test. **p<0.01 - significantly different when compared to control condition.

This is the first work in which HAP1 is shown to be present in glial cells. Although previous studies suggested the absence of HAP1 in mature astrocytes (Xiang et al. 2014), the results of the western blot experiments shown in Figure 3.8 B indicate that the protein is also expressed in mature astrocytes. This result was also confirmed with RT-PCR analysis in mature astrocytes using specific primers of HAP1 (data not shown).

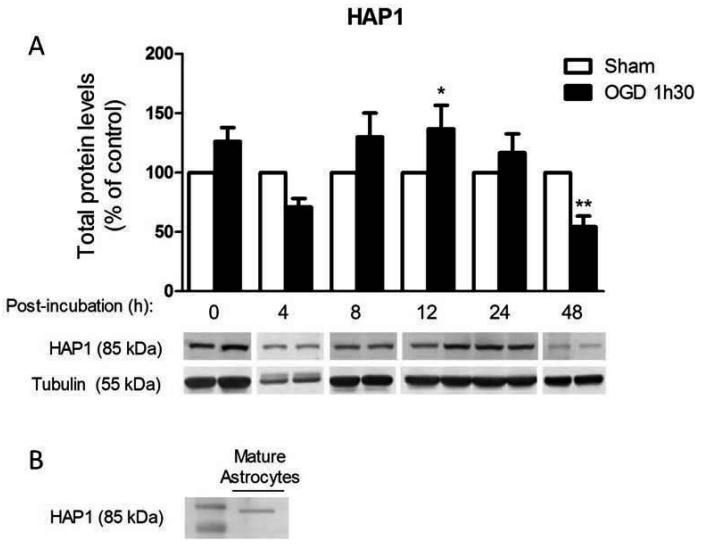

Figure 3.8. HAP1 protein levels are altered in glial cells exposed to OGD.
(A) Cultured of glial cells were exposed to OGD for 90 min and further incubated in DMEM supplemented with 15% of FBS for 0 h, 4 h, 8 h, 12 h, 24 h or 48 h (post-incubation). HAP1 total protein levels were analyzed with western blot and the results were normalized with the loading control tubulin. The results are the mean ± SEM of 3 independent experiments performed in distinct preparations, and are expressed as percentage of the control. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni's test. *$p<0.05$, **$p<0.01$ - significantly different when compared to the respective control condition. (B) Western blot analysis of cultured mature astrocytes showing the presence of HAP1 protein.

3.6. OGD-INDUCED HAP1 mRNA ALTERATION IN GLIAL CELLS

The expression of proteins in the cells is regulated by transcription, translation, biosynthesis and degradation mechanisms. To determine whether the observed OGD-induced upregulation of HAP1 protein levels in glial cells (Figure 3.8) was due to changes in transcription activity we measured the changes in HAP1-mRNA under the same conditions. The levels of HAP1 mRNA were analyzed through PCR analysis using the total RNA extracted from glial cells after 90 min of OGD followed by 12 h of post-incubation. The GenEx software was used to choose the reference genes. Two methods of statistical analysis were used to identify the most suitable reference genes: 1) geNorm that compares the variations in all expression ratios for every candidate gene and recommends to use the last pair as optimum reference genes (with lower variation); 2) Normifinder that calculates both the variation of every gene within the group (intragroup variation) and the variation of every gene between the groups (intergroup variation), recommending the optimum pair of reference genes (Santos and Duarte 2008). Using this statistical methods the actin and tubulin mRNAs were chosen as reference genes to normalize the expression of HAP1 mRNA. The results (Figure 3.9) show a small but not significant decrease of mRNA expression following the *in vitro* ischemic insult, suggesting that the alteration of HAP1 protein levels observed in the same condition is not related to the changes in mRNA. However, the alterations in mRNA are normally faster and precede the changes in protein expression. Therefore the downregulation in HAP1 mRNA observed 12 h after OGD may account for the reduction in HAP1 protein levels observed 48 h after OGD (Figure 3.8). Thus, additional studies should be performed to determine whether the expression of HAP1 gene is increased at early time points after the OGD insult.

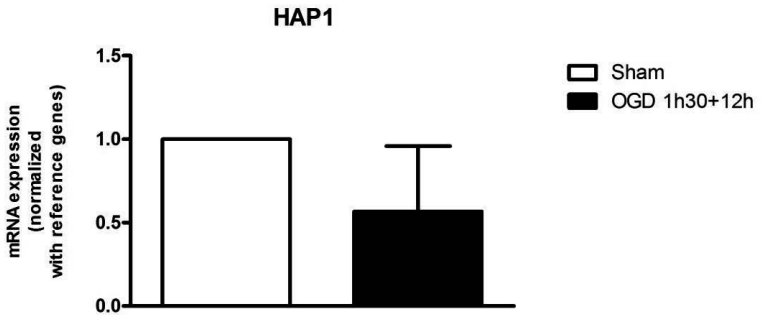

Figure 3.9. OGD-induced alteration of HAP1 mRNA levels in glial cells.
Cultured glial cells were exposed to OGD for 90 min and the mRNA was extracted for qPCR analysis 12 h after the stimulus (post-incubation). Results were normalized with the reference genes (tubulin and actin) and the mean ± SEM of at least 3 different experiments performed in independent preparations was calculated. The results are expressed as percentage of control (Sham). The differences obtained were not statistically significant, as determined by Student's *t*-test.

3.7. OVEREXPRESSION OF HAP1 PROTEIN ABROGATES THE OGD-INDUCED REDUCTION OF $GABA_AR$ β3 SUBUNIT RECYLING RATIO

Privious results from our laboratory showed a decreased $GABA_AR$ β3 subunit recycling rate and a reduction of $GABA_AR$/HAP1 interaction in hippocampal neurons exsposed to OGD. Considering the role of HAP1 in the trafficking of $GABA_AR$ (Kittler et al. 2004), we investigated the effect of HAP1 overexpession on $GABA_AR$ surface expression and recycling. Cultured hippocampal neurons (13 DIV) were transfected with myc-tagged hu$GABA_AR$ β3, GFP-tagged HAP1-A or GFP-tagged HAP1-B, and $GABA_AR$ β3 surface expression and recycling ratio were analyzed 2 days after transfection, following 90 min of OGD. The surface receptors were detected by immunocytochemistry under non-permeabilizing conditions, with an antibody against the extracellular myc-tag. The rate of receptor recycling was determined after labeling of the surface receptors immediately after OGD, and an additional incubation of 30 min after the stimulus was performed to allow the receptor recycling (this incubation period allows the detection of the OGD-induced decrease in the recycling). Immunocytochemistry analysis showed that HAP1-A and HAP1-B overexpression prevents the OGD-induced reduction of $GABA_AR$ β3 subunit surface levels (Figure 3.10 A), as well as the reduction in $GABA_AR$ β3 recycling under the same conditions (Figure 3.10 B). These results indicate that HAP1 overexpression is capable of rescuing the levels of surface $GABA_AR$ β3 subunit during OGD, possibly by improving its recycling

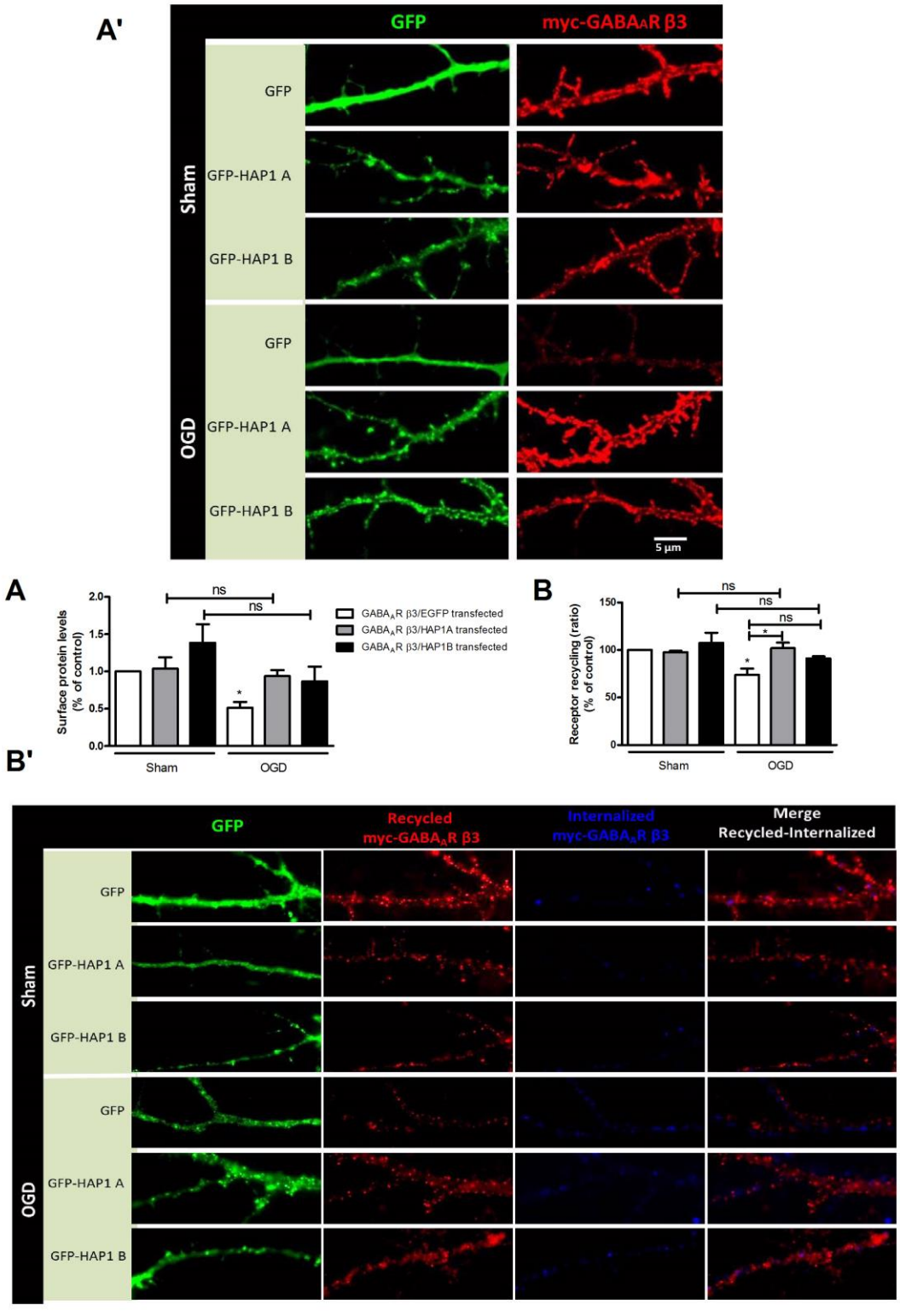

Figure 3.10. Overexpression of HAP1 protein abrogates the effect of OGD on $GABA_AR$ β3 subunit recycling.

(A-A') Cultured hippocampal neurons were transfected with myc-tagged $GABA_AR$ β3 subunit, together with GFP-tagged HAP1A, GFP-tagged HAP1B or EGFP, and subjected to OGD (90 min).

The effect of OGD on the surface expression of the myc-tagged $GABA_AR$ β3 subunits was evaluated in the neuritic compartments by immunocytochemistry, with an anti-myc antibody, under non-permeabilizing conditions. (B-B') Receptor recycling was assessed using an antibody-feeding assay and analyzed by fluorescence microscopy in cells labelled with anti-myc (N-terminus). After quantification of the immunoreactivity in the neuritic compartments, the results were expressed as a ratio of reclycled receptors/total receptor immunoreactivity and as percentage of control. The results were normalized and presented as percentage of the control. The results are the mean ± SEM of 3 independent experiments, performed in different preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunett and Bonferroni's test. *$p<0.05$ - significantly different when compared to the respective control condition. Non-significant (ns) differences were observed for the indicated comparisons.

DISCUSSION AND CONLUSIONS

Huntingtin-associated protein 1 (HAP1), originally identified as a neuronal protein that interacts with the Huntington disease (HD) protein, huntingtin (htt), plays a critical role in the trafficking of intracellular organelles and membrane proteins by interacting with a number of proteins (Li et al. 1998b; Li et al. 1995; Martin et al. 1999; Engelender et al. 1997). Among these, HAP1 binds the $GABA_AR$ β subunit regulating its trafficking (Kittler et al. 2004). $GABA_AR$ regulate neuronal excitability depending of their cell surface availability and stability. During $GABA_AR$ cell trafficking, HAP1 inhibits the receptor lysosomal degradation and facilitates its recycling back to the plasma membrane (Kittler et al. 2004). Accordingly, overexpression of HAP1 increases $GABA_AR$ cell surface number and decrease neuronal excitability (Kittler et al. 2004). Disruption of $GABA_AR$ trafficking alters the balance between excitatory and inhibitory neurotransmission in the brain, contributing to pathological processing in conditions such as epilepsy and ischemia, which are characterized by an acute receptor surface downmodulation and loss of synaptic $GABA_AR$. HAP1 is emerging as an important regulator of $GABA_AR$ membrane expression and as a possible target for future therapeutic strategies in cerebral ischemia treatment. Considering the results previously obtained in our laboratory showing a reduction in $GABA_AR$ recycling rate, as well as a decrease in $GABA_AR$/HAP1 interaction, in hippocampal neurons subjected to OGD, in the present work we aimed at investigating the effect of cerebral ischemia on HAP1 receptor expression and its contribution to the alteration of $GABA_AR$ trafficking observed in OGD. Our results show a downregulation of HAP1 in hippocampal and cortical neurons subjected to OGD, and the provide evidence for the role of the protein in the regulation of $GABA_AR$ surface expression and intracellular traffic under ischemic conditions.

Exposure of hippocampal neurons to 90 min of OGD, an experimental condition that induces the death of about ~35% of the neurons (Mele et al. 2014), reduced the expression of HAP1 protein when tested 2 h after the stimulus, and this effect was maintained during the re-oxygenation period for up to 8 h. Since the downregulation of the protein was only observed at 2 h after OGD it is unlikely to account for the decrease in the interaction between $GABA_AR$ β3 and HAP1 observed in hippocampal neurons immediately after the

ischemic insult (Mele et al. 2014). Therefore, these results suggest that the downmodulation of HAP1 is not the initial cause of $GABA_AR$ β3 recycling impairment but is rather a factor that can exacerbate the reduction of surface $GABA_AR$ expression. Accordingly, we observed a protective effect of HAP1 overexpression on the OGD-induced reduction of membrane $GABA_AR$ β3 subunits, as shown by live staining of $GABA_AR$ β3 subunits in hippocampal neurons subjected to OGD (90 min). Moreover using the antibody feeding assay we demonstrate that HAP1 overexpression also rescued hippocampal neurons from the impairment of $GABA_AR$ recycling.

The regulation of the $GABA_AR$/HAP1 interaction is crucial for the alteration of $GABA_AR$ trafficking observed in cerebral ischemia (Mele et al. 2014). HAP1 interacts with a region in the intracellular domain of $GABA_AR$ β subunit through an amino acid sequence of its central domain (residues 220-520), but the molecular mechanisms involved in this interaction were not characterized. HAP1 phosphorylation may play a role in the modulation of $GABA_AR$/HAP1 interaction. In fact, this post-translational modification has an important role in regulating HAP1 binding with some microtubule-associated proteins involved in retrograde and anterograde transport, such as p150Glued and kinesin light chain (Aniento et al. 1993; Lin et al. 2002). Our results indicate that protein phosphorylation (possibly HAP1 phosphorylation) is also involved in the control of total HAP1 protein levels in hippocampal neurons, both under control conditions and following transient OGD. Incubation of hippocampal neurons with the PP1 and PP2A inhibitor okadaic acid significantly upregulated HAP1 protein levels under control conditions, and this effect may account, at least in part, for the preservation of HAP1 protein levels, as compared with the control conditions, when hippocampal neurons were subjected to transient OGD. In contrast, although calcineurin inhibition slightly increased HAP1 protein levels under control conditions, it was without effect on the downregulation of the protein after transient OGD. Taken together, these results suggest that: i) PP1/PP2A and calcineurin phosphatases contribute to the maintenance of physiological levels of HAP1, and ii) PP1/PP2A inhibition prevents the OGD-induced downregulation of HAP1, possibly by stabilizing the protein in the cells. These results are in agreement with the observed increase of HAP1 levels following stimulation of hypothalamic cultured neurons with PP1/PP2A phosphatase inhibitor (okadaic acid) (Rong et al. 2006). On the other hand, PKA and PKC activation did

not alter HAP1 levels both in control and OGD condition, suggesting a specific role of phosphatases in this process. Alternatively, other protein kinases may be involved in the regulation of HAP1 protein levels. Furthermore, the decrease in ATP levels following OGD (Choi 1996) may also impair protein kinase activity.

Inhibition of calpains with MDL28170 was found to prevent OGD-induced downregulation of HAP1, showing a role for these Ca^{2+}-dependent proteases in HAP1 degradation after OGD. 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; Mele et al. 2014; Lobo et al. 2011). Surprisingly these results were not confirmed in the MCAO *in vitro* model of ischemia, where an increased HAP1 protein expression was observed in the cerebral area corresponding to ischemic core. We hypothesized two possible reasons for this discrepancy. First, the OGD *in vitro* model of ischemia was performed using cultured hippocampal neurons while the ischemic core of MCAO is located in the cerebral cortex; therefore, there is a difference between the cell types subjected to the insult in the two models. The second hypothesis is related to the presence of glial cells in the cerebral tissue (that is the case of MCAO samples), which are not represented in the hippocampal cultures used for OGD. The results showing i) an upregulation of HAP1 in cultured glial cells following OGD and ii) the decrease in HAP1 protein levels in cultured cortical neurons subjected to OGD, similarly to the results obtained in hippocampal neurons, favour the second hypothesis.

To further investigate the effect of OGD on HAP1 protein in glial cells we analysed the alterations in the mRNA levels to determine the role of transcription regulation in the process. Quantitative PCR data from glial cells subjected to OGD showed a small but not significant decrease of HAP1-mRNA levels, showing that the alteration of HAP1 protein levels in glial cells during OGD are not correlated with modifications at transcriptional level. However, since the alterations in mRNA are normally faster and precede the changes in protein expression, the results obtained from mRNA analyses in glial cells 12 h after OGD may be related to the decrease of HAP1 protein observed 24 h after the ischemic insult and that is exacerbated at 48 h.

The present study also showed for the first time the presence of HAP1 in glial cells. Previous studies suggested the absence of the protein in glial cells, as shown both with

western blot analysis in mature astrocytes obtained from cultured mouse neurospheres (Xiang et al. 2014) and with immunolabeling cerebral slices (Gutekunst et al. 1998). However, and in contrast with these observations we demonstrated by western blot analysis and with qPCR, the expression of this protein and the correspondent mRNA in glial cells as well as in mature astrocytes.

Taken together our results indicate that ischemic condition down-modulates HAP1 protein by a calpain-dependent mechanism, and a role for protein phosphatases-1/-2A in the regulation of HAP1 protein stability was also identified. Moreover, in this work was uncovered a novel role for HAP1 protein in the regulation of $GABA_AR$ trafficking during cerebral ischemia. Considering the protective effect of $GABA_AR$ surface stabilization against ischemic-induced neuronal death (Smith et al. 2012; Mele et al. 2014), HAP1 may constitute an important therapeutic target for cerebral ischemia.

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