UNIVERSIDAD DE LEÓN

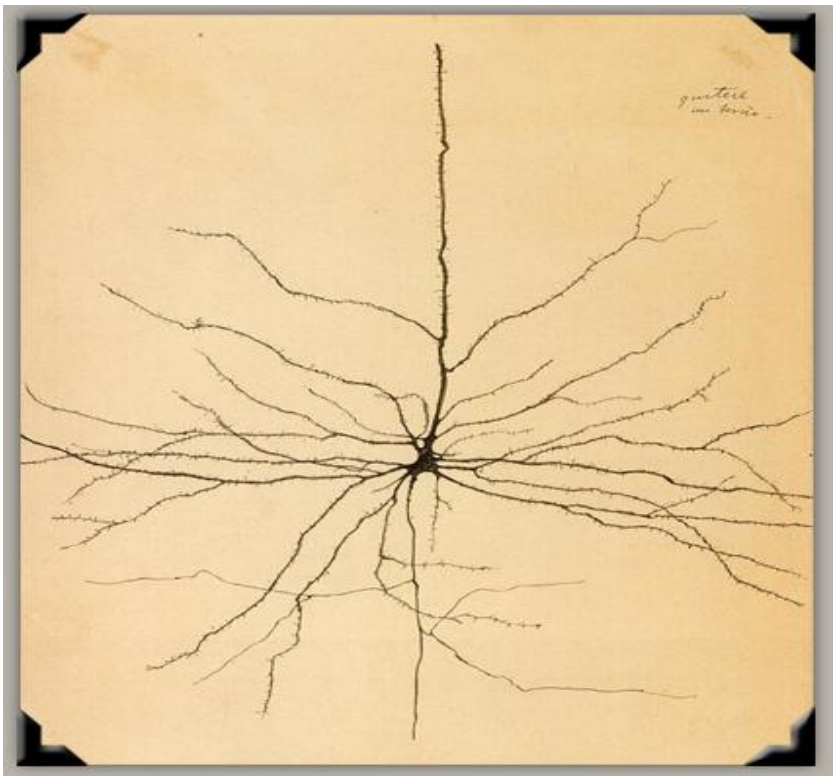
FACULTAD DE C.C. BIOLÓGICAS Y AMBIENTALES

Dpto. de Biología Molecular

Área de Biología Celular

"THE NEUROPROTECTIVE EFFECT OF GABA AND MELOXICAM ON DIFFERENT EXPERIMENTAL MODELS OF CEREBRAL ISCHAEMIA. THE ROLE OF THE GLUTAMATERGIC TRANSPORTERS"

EL EFECTO NEUROPROTECTOR DEL GABA Y MELOXICAM EN DIFERENTES MODELOS EXPERIMENTALES DE ISQUEMIA CEREBRAL. EL PAPEL DE LOS RECAPTADORES GLUTAMATÉRGICOS.



Memoria para optar al Grado de Doctor con Mención internacional presentada por:

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La presente memoria para optar a tesis doctoral ha sido dirigida por los Dres. Arsenio Fernández López (Departamento de Biología Molecular, Universidad de Léon) y Beatriz Martínez Villayandre (Dpto. Fisiología y Farmacología, Universidad de Cantabria).

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“Gracias a todas aquellas personas que de una manera u otra hicieron que mi camino desembocara en la ciencia”

Índice

Abreviaturas / *Abreviations*

1,3BPG: 1,3 bifosfoglicerato / *1,3-bisphosphoglycerate*

3PGK: 3 fosfoglicerato quinasa / *3-phosphoglycerate kinase*

A

ACM: Arteria carótida media / *Middle carotid artery*

ADN: Ácido desoxirribonucleico / *Deoxyribonucleic acid*

ADP: Difosfato de adenosina / *Adenosine diphosphate*

AMPA: Ácido α-amino-3-hidroxilo-5-metil-4-isoxazol-propiónico */ 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid*

AMPc: Adenosina monofosfato-3`,5` ciclico / *Adenosine monophosphate-3 ', 5' cyclic*

ATF6: Factor de transcripción activador 6 / *Activating transcription factor-6*

ATP: Trifosfato de adenosina / *Adenosine triphosphate*

ARN: Ácido ribonucleico / Ribonucleic acid

ARNm: Ácido ribonucleico mensajero / *Messenger ribonucleic acid*

B

BZD: Benzodiacepina / *Benzodiazepine*

C

CA1: Región del hipocampo *Cornu Ammonis* 1 / *Cornu Ammonis 1 hippocampal region*

CA3: Región del hipocampo *Cornu Ammonis* 3 / *Cornu Ammonis 3 hippocampal region*

cDNA: Ácido desoxiribonucleico complementario / *Complementary deoxyribonucleid acid*

CX: Corteza cerebral / *Cerebral cortex*

CHOP: C/EBP proteína homóloga **/** *Growth arrest and DNA damage-inducible gene 153*

CNX: Calnexina / *Calnexin*

COX-2: Ciclooxigenasa II / *Cyclooxygenase*

CRT: Calreticulina / *Calreticulin*

D

DG: Giro dentado / *Dentate gyrus*

E

EAAC-1: Transportador neuronal de aminoácidos excitatorios / *Neuronal excitatory amino acid transporter*

EAATs: Transportadores de aminoácidos excitatorios / *Excitatory amino acid transporters*

ERAD: Degradación de proteínas asociada al reticulo endoplásmico / *ER-associated degradation*

F

FSC: Flujo sanguíneo cerebral / *Cerebral blood flow*

G

GABA: Ácido gamma aminobutírico / *Gamma-aminobutyric acid*

GABA-T: 4-aminobutirato transaminasa / *4-aminobutyrate transaminase*

GAD: Glutamato decarboxilasa / *Glutamate decarboxylase*

Glast-1a: Transportador de glutamato/aspartato / *Glutamate/aspartate transporter*

Glt-1: Transportador de glutamato / Glutamate transporter

GAP: Gliceraldehído-3-fostato / *Glyceraldehyde-3-phosphate*

GAPDH: Gliceraldehído 3-fosfato deshidrogenasa / *Glyceraldehyde 3-phosphate dehydrogenase*

Grp78/bip: Proteína relacionada con glucosa de 78 kilodalton / *Glucose related protein 78*

Grp94: Proteína relacionada con glucosa de 94 kilodalton / *Glucose related protein 94*

GluR: Receptores glutamatérgicos / *Glutamate receptors*

GluRi: Receptores glutamatérgicos ionotrópicos / *Ionotropic glutamate receptors*

GluRm: Receptores glutamatérgicos metabotrópicos / *Metabotropic glutamate receptors*

H

Hsp70: Proteína de choque térmico de 70 kilodalton / *Heat shock protein 70*

Hsp90: Proteína de choque térmico de 90 kilodalton / *Heat shock protein 90*

I

ICG: Isquemia cerebral global / *brain global isquemia*

Incub. Sol. : Solución de incubación / *incubation solution*

IRE1: Proteína1 que requiere inositol */ Inositol-requiring protein-1*

L

LDH: Lactato deshidrogenasa / *Lactate dehydrogenase*

N

NAD^+: Dinucleótido de adenina nicotinamida / *Nicotinamide adenine dinucleotide*

NMDA: N-Metil-D-aspartato / *N-methyl-D-aspartate*

NOS: Sintasas del óxido nítrico */ nitric oxide synthases*

O

OD: Densidad óptica / Optical density

OGD: Privación de oxígeno y glucosa / *Oxygen and glucose deprivation*

OI/R: Ratas envejecidas sometidas a isquemia/reperfusión / *Injured older rats*

OMS: Organización mundial de la salud / *World health organization*

OS: *Sham-operated older rats*

OS+M: *Sham-operated old rats*

P

PAG: Glutaminasa activada por fosfato / *Phosphate activated glutaminase*

PCR: Reacción en cadena de la polimerasa / *Polymerase chain reaction*

PDI: Proteína disulfuro isomerasa / *Protein disulfide isomerase*

PERK: *Protein kinase RNA (PKR)-like ER kinase*

Pi: Fosfato inorgánico / *inorganic phosphate*

R

RE: Retículo endoplásmico / *Endoplasmic reticulum*

RL: Reperfusión / *Reperfusion-like condition*

RT: Transcripción reversa / *Reverse transcription*

S

SEM: Error estándar de la media / *Standard error of the mean*

SNC: Sistema nervioso central / *Central nervous system*

U

UPR: Respuesta a proteínas mal plegadas / *Unfolded protein response*

UPS: Sistema ubiquitina-proteasoma / *Ubiquitin proteasome system*

V

VGLUTs: Transportadores vesiculares de glutamato / *Vesicular glutamate transporters*

Y

YI/R: Ratas jóvenes sometidas a isquemia/reperfusión / *Injured young rats*

YIR+M: Ratas jóvenes sometidas a isquemia/reperfusión con meloxicam / *Injured young rats with meloxicam*

YS: *Sham-operated young rats*

YS+M: *Sham-operated Young rats with meloxicam*

Introducción

Incidencia de ictus en España

La Organización Mundial de la Salud (OMS) en 1987 definió el ictus como: una disfunción neurológica focal o global de inicio súbito, que dura más de 24 horas (o acaba con la vida del paciente), de presumible origen vascular (Ako *et al*., 1980).

La incidencia global del ictus en España no se conoce con precisión, se estima que puede oscilar entre 120-350 casos por cada 100.000 habitantes/año. Esta tasa se ve multiplicada por 10 en la población mayor de 75 años y aproximadamente un 8% de la población mayor de 65 años presenta antecedentes clínicos de ictus (Díaz-Guzmán *et al*., 2008).

La OMS indica un promedio mundial de la enfermedad alrededor de 200 casos nuevos por cada 100.000 habitantes/año (Bonita, 1992). Sin embargo, en España las enfermedades cerebrovasculares están entre las primeras causas de mortalidad específica (primera causa de muerte en mujeres y segunda causa en varones), por detrás del infarto de miocardio (Hervás-Angulo *et al*., 2006). En Europa, las enfermedades cerebrovasculares son la primera causa de invalidez permanente en adultos y la segunda causa de demencia (Olesen *et al*., 2012). En la siguiente tabla se precisan estos datos.

	Mortalidad total		Mortalidad por accidente cerebro vascular	
Edad en años	Mujeres	Varones	Mujeres	Varones
40-44	1541	**3117**	49	**72**
45-49	2330	**4936**	100	**155**
50-54	3161	**7128**	127	**233**
55-59	3950	**9130**	162	**309**
60-64	5017	**12382**	206	**444**
65-69	6692	**15398**	351	**720**
70-74	10802	**20237**	792	**1166**
75-79	21520	**31258**	1999	**2180**
80-84	34286	**36588**	**3596**	2850
85-89	**43303**	31317	**4783**	2684
90-94	**31561**	14937	**3588**	1287
95 en adelante	**16569**	5500	**1715**	436

Tabla1. Mortalidad en España en 2010 según los datos del Instituto Nacional de Estadística.

El ictus es la causa más frecuente de ingreso en los servicios de neurología y requiere una estancia prolongada en el hospital. Su diagnóstico implica el uso de técnicas de elevado coste monetario como resonancia magnética o arteriografía y en muchas ocasiones los tratamientos se mantienen durante toda la vida del paciente, lo que supone en conjunto un coste sanitario muy elevado (Medrano *et al*., 2006).

En la década de 1990 el coste del ictus ya superaba el 3-4% del presupuesto anual para la sanidad en varios países europeos y esta cifra va en aumento. En España, se ha estimado que la mayor parte del gasto (54%) ocurre durante la fase aguda y el otro 46% se produce durante el primer año. Un estudio realizado en 1996 ya cifraba el gasto entre 150 y 12.500 eurospor persona y año, siendo el grado de incapacitación uno de los principales responsables del gasto sanitario. En España, a principios del año 2000, el coste medio por paciente en el primer año se ha estimado en algo más de 5000 euros (Bergman *et al*., 1995; Carod-Artal *et al*., 1999; Martinez-Vila *et al*., 2000; Creutzfeldt *et al*., 2012).

Clasificación etiológica

La mayor parte de los infartos cerebrales son considerados de tipo **isquémico** (en torno a un 85%), mientras que sólo un 15% son considerados de tipo **hemorrágico** (Roda *et al*., 1998).

- ✓ **Ictus isquémicos:** Según la clasificación usada en *la Guía clínica para el diagnóstico y tratamiento del ictus* de la Sociedad Española de Neurología (Arboix *et al*., 2006) se diferencian los siguientes tipos de ictus isquémicos:

  Infarto cerebral aterotrombótico (15-20%): infarto de tamaño medio o grande, cortical o subcortical, de localización carotídea o vertebrobasilar, en el que se cumple alguno de los dos criterios siguientes:

a) Aterosclerosis con estenosis: estenosis > 50% del diámetro luminal u oclusión de las arterias extracraneal o intracraneal de gran calibre (arteria cerebral media, arteria cerebral anterior, arteria cerebral posterior o vertebrobasilar) correspondientes al déficit, en ausencia de otra etiología.

b) Aterosclerosis sin estenosis: presencia de placas o estenosis < 50% de la arteria cerebral media, posterior o tronco basilar, en ausencia de otra etiología y en presencia de, al menos dos de los siguiente factores de riesgo: mayor de 50 años, hipertensión arterial, tabaquismo, diabetes mellitus e hipercolesterolemia.

Infarto cardioembólico (15-20%): Es un infarto de tamaño medio o grande, de localización habitualmente cortical, en el que se pueden apreciar las siguientes cardiopatías de origen: presencia de trombo o tumor intracardíaco, estenosis mitral reumática, prótesis valvulares mecánicas (mitral o aórtica), endocarditis, fibrilación auricular, enfermedad del nodo sinusal, aneurisma ventricular izquierdo o acinesia después de un infarto agudo de miocardio, infarto agudo de miocardio (menos de tres meses), o presencia de hipocinesia cardíaca global o discinesia.

Enfermedad oclusiva de pequeño vaso arterial (infarto lacunar) (25-30%): Es un infarto de pequeño tamaño (< 15 mm de diámetro) en el territorio de una arteria perforante cerebral, que suele ocasionar clínicamente un síndrome lacunar en un paciente con antecedentes de hipertensión arterial u otros factores de riesgo cerebrovascular.

Infarto cerebral de causa inhabitual (3-5%): Es un infarto de tamaño pequeño, medio o grande, cortical o subcortical, de localización carotídea o vertebrobasilar, en un paciente en el que se ha descartado el origen aterotrombótico, cardioembólico o lacunar.

Infarto cerebral de origen indeterminado (30%): Es un infarto de tamaño medio o grande, cortical o subcortical, de localización carotídea o vertebrobasilar, que a su vez puede dividirse en: indeterminado por coexistencia de etiologías, indeterminado por estudio inadecuado y/o insuficiente, y de causa desconocida, cuando tras un exhaustivo estudio se han descartado todas las categorías anteriores.

- ✓ **Ictus hemorrágico**S: La clasificación de este tipo de ictus se estableció en 1998 (Roda *et al.,* 1998) son originados por la rotura de un vaso sanguíneo, bien sea en el parénquima nervioso (hemorragia intracerebral) o de las grandes arterias que irrigan el cerebro (hemorragia subaracnoidea) (Wolf *et al*., 1992) y son consecuencia de determinadas patologías como: Hemorragia por hipertensión arterial, malformación vascular (arteriovenosas, cavernosas….), problemas de anticoagulación, angiopatía amiloide,etc.

Isquemia cerebral

Durante el accidente cerebrovascular se produce una disminución del flujo sanguíneo cerebral (FSC) por debajo de 10 mL/100g/min en un área restringida y como consecuencia se produce una rápida muerte celular (Pulsinelli, 1982). Entre este núcleo isquémico y el parénquima cerebral con irrigación normal (FSC>50 mL/100 g/min) existe una zona con riego reducido cuya extensión depende de la circulación colateral (Heiss y Graf, 1994; Castillo, 2000).

En la zona hipoperfundida se diferencian dos regiones: **zona oligohémica** (FSC > 22 mL/100 g/min) en la cual el riesgo de convertirse en infarto sólo sucede en circunstancias especialmente adversas, y otra, denominada **zona de penumbra** isquémica (FSC < 22 mL/100 g/min), en la que el consumo de oxígeno es todavía suficiente para preservar la supervivencia tisular (Figura 1). Sin embargo, en esta zona de penumbra, el aporte de oxígeno es insuficiente para mantener un adecuado metabolismo oxidativo de la glucosa, lo que produce acidosis láctica. Esto da lugar a una disminución del trifosfato de adenosina (ATP) necesario para mantener en funcionamiento las bombas

iónicas de las membranas celulares (Castillo, 1999; Forbergrovd *et al*., 1992). Si no se controla adecuadamente, la mayor parte de esta zona de penumbra en la fase aguda del ictus isquémico progresa a infarto cerebral (Baron, 2001; Heiss *et al*., 2001).

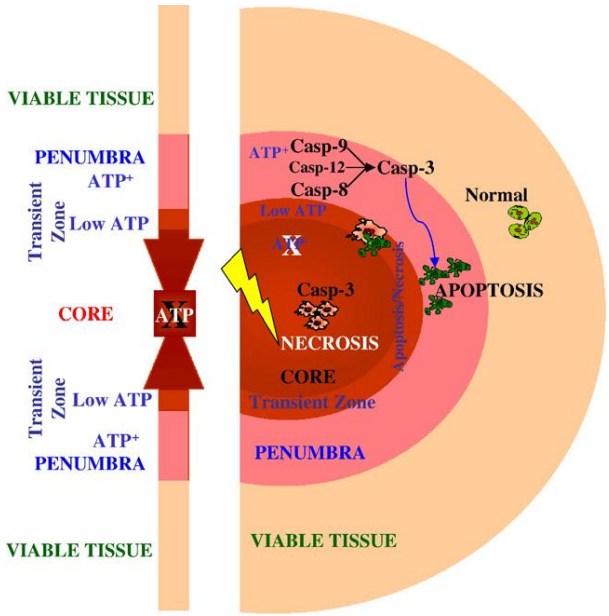

Figura1: Efectos de la reducción del flujo sanguíneo cerebral. (Mehta *et al.*, 2007)

Cascada isquémica neuronal

La alteración del aporte de oxígeno y sustratos, consecuencia del proceso isquémico, genera un déficit energético en la zona infartada que conduce a la pérdida de las funciones de membrana, principalmente la falta de control del gradiente iónico que lleva a la edematización y destrucción celular. El flujo sanguíneo atenuado en la zona de penumbra hace que el déficit de energía sea menor y que la célula responda con una serie de modificaciones sucesivas que se ha denominado **cascada isquémica** (Pulsinelli, 1982) (Figura 2). En las primeras fases de la isquemia, el déficit de oxígeno activa el metabolismo de la glucosa mediante la vía anaerobia, ocasionando acidosis debido al aumento de ácido láctico. Dicha acidosis inhibe la fosforilación oxidativa (Hillered *et al*., 1985), contribuye a la depleción energética, favorece la edematización celular, y aumenta la concentración de calcio libre intracelular al liberarlo de su unión a proteínas (Folbergrova *et al*., 1990). Esto desemboca en lesión endotelial y en

la alteración secundaria de la microcirculación y en la liberación en forma iónica del hierro intracelular unido a proteínas, lo que facilita la formación de radicales OH^- libres (Hurn *et al*., 1995; Lipscomb *et al*., 1998; Davalos *et al*., 2000). También se produce un fallo en las bombas de Na^+ y K^+ originando una depleción del K^+ intracelular que provoca la apertura de los canales de Ca^{2+} dependientes de voltaje (Siesjö, 1992; Ohta *et al*., 2001) y el desbloqueo del tapón de Mg^{2+} en los canales NMDA de Ca^{2+} (dependientes de voltaje y ligando). Estos procesos dan lugar a un incremento de la concentración del calcio iónico intracelular (Pulsinelli, 1982; Heiss y Graf, 1994; Choi, 1988; Ginsberg, 1997) que lleva a la despolarización neuronal masiva.

Como consecuencia de la despolarización neuronal se produce la liberación, desde los terminales presinápticos, de aminoácidos excitadores como glutamato, que abren canales de calcio dependientes de ligando (AMPA y NMDA) en la neurona postsináptica y activan receptores metabotrópicos glutamatérgicos. Este mecanismo contribuye a la liberación de calcio de los depósitos intracelulares, incrementando la concentración de calcio libre en el citosol (Choi, 1988) lo que contribuye a una mayor despolarización neuronal. El aumento de la concentración del calcio iónico intracelular es un factor clave en los procesos que conducen al daño neuronal irreversible. Produce la activación de una serie de quinasas de proteínas, proteasas, endonucleasas, fosfatasas de proteínas y sintasas del óxido nítrico (NOS) (Castillo, 1999), así como la formación de radicales libres derivados como los peroxinitritos (Gursoy-Ozdemir *et al*., 1974). El aumento de calcio intracelular también desacopla la fosforilación oxidativa disminuyendo aun más la disponibilidad energética.

Durante la isquemia cerebral, se liberan otros neurotransmisores al espacio extracelular, principalmente glicina y ácido gamma-aminobutírico (GABA). La glicina es un co-activador del receptor NMDA y su liberación excesiva durante la isquemia origina un aumento de la estimulación del receptor y aumenta el daño neuronal (Castillo *et al*., 1996). La liberación de GABA durante la isquemia es favorecida por el aumento de la concentración del glutamato que es transformado a GABA por activación de la enzima glutamato descarboxilasa (GAD) (que es independiente del ATP y más activa en presencia de acidosis). También se inhibe la enzima transaminasa del GABA (GABA-T) (más activa

con pH elevado) para impedir la formación de más glutamato a partir de GABA lo que contribuye al aumento de los niveles de GABA. Esto hace que la despolarización de la membrana neuronal ocasionada por la isquemia aumente la liberación de GABA al espacio extracelular hasta 250 veces con respecto a las condiciones normales (Nishikawa *et al*., 1994). Un bloqueo de la liberación del GABA, durante o después de la isquemia cerebral puede contribuir a la sobreestimulación de las neuronas vulnerables por el glutamato, facilitando la muerte neuronal (Sternan *et al*., 1989).

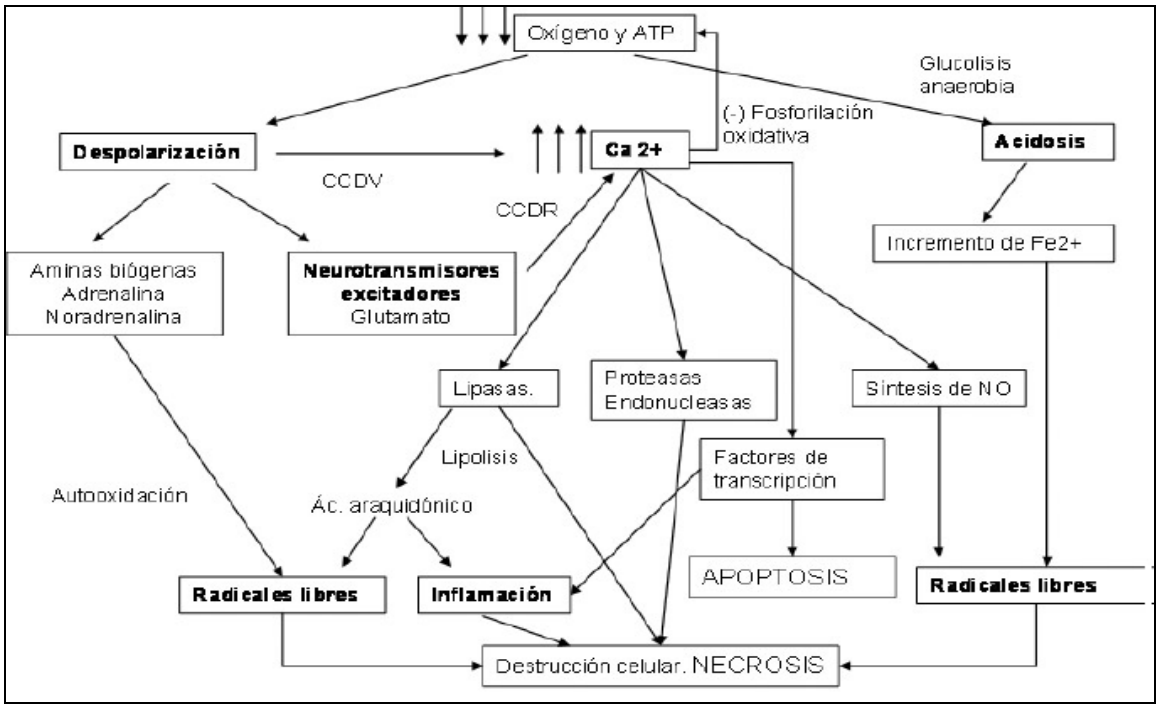

Figura2: Esquema representativo de los mediadores bioquímicos implicados en la cáscada isquémica. (Imagen modificada de Pulsinelli, 1982).

La recaptación de glutamato por astrocitos

No solamente las neuronas juegan papeles clave en la respuesta a la isquemia, las células gliales tienen una importancia crucial en la respuesta isquémica., tanto en la reparación tisular, como en el establecimiento de la lesión definitiva (Ranson y Sontheimer, 1992). La recaptación del glutamato por parte de los astrocitos es realizada, en gran medida, mediante transportadores de

membrana (principalmente **GTL-1** y **GLAST-1a**), lo que permite controlar la concentración adecuada de glutamato presente en la hendidura sináptica (Nichols y Attwell, 1990). El paso de glutamato al interior del astrocito mediado por estos transportadores utiliza como fuente de energía el gradiente de Na^+ de la membrana plasmática. El glutamato recaptado es convertido en glutamina a través de la sintetasa de glutamina; y posteriormente esta glutamina es liberada al medio y recaptada por las neuronas que la reutilizan para la síntesis de glutamato y de GABA (Shigeri *et al*., 2004).

Durante el proceso isquémico se produce el denominado edema astrocitario, siendo éste uno de los factores más importantes en el bloqueo de la recaptación de glutamato, fomentando así el daño excitotóxico mediado por glutamato, producido como consecuencia de la isquemia cerebral (Scheneider *et al*., 1992; Lees, 1993).

Excitotoxicidad

Como consecuencia de la isquemia se desencadenan diferentes procesos de muerte, tanto por necrosis como mediante diversos tipos de muerte neuronal programada. En el "core" isquémico la muerte se produce principalmente por necrosis (Mehta *et al*., 2007) y en la zona de penumbra se produce muerte celular programada, cuyo tipo más estudiado es la muerte neuronal por apoptosis como consecuencia, entre otras causas, de la disminución de ATP (Richer *et al*., 1995; Susin *et al*., 1999).

La reperfusión que sigue al proceso isquémico, provoca un aumento en la liberación del glutamato extracelular que produce la estimulación del receptor NMDA en las neuronas que sobreviven al daño isquémico. Esto lleva a un aumento de la concentración iónica de calcio citosólico, que induce la transcripción de genes de respuesta inmediata (como *cfos* o *c-jun*), que a su vez controlan la expresión de otros genes efectores. Las proteínas producidas (proteínas de estrés, factores de crecimiento neuronal, factor de necrosis tumoral, etc.) promueven la supervivencia y recuperación neuronal, o bien, activan la muerte celular programada (An *et al*., 1993; Lindsay *et al*., 1994).

La muerte por necrosis es una forma de muerte celular asociada con el daño de la membrana plasmática y con el edema citotóxico, y se produce en periodos de tiempo muy cortos, en el rango de minutos (Back, 1998).

Como consecuencia de ambos procesos se produce la mortalidad de un número elevado de células, las cuales, como se ha indicado previamente, vierten al espacio extracelular aminoácidos excitatorios, fundamentalmente glutamato, sobreactivando los receptores glutamatérgicos de NMDA, AMPA y kainato, así como los receptores metabotrópicos. Esto da lugar a una entrada de Na^+, Cl^- y Ca^{2+}, aumentando así el daño y muerte celular, que da lugar a una liberación aun mayor de neurotransmisores excitatorios. Todo este proceso es denominado excitotoxicidad mediada por glutamato (Meldrum, 1985; Dirnagl *et al*., 1999; Meldrum, 2000; Mongin, 2007; Chen *et al*., 2008).

Sistema Glutamatérgico

El glutamato es un importante neurotransmisor excitador en el sistema nervioso central (SNC) de mamíferos, posee receptores en todas las neuronas, lo cual indica que está implicado en gran número de sinapsis y rutas de transmisión del impulso nervioso (Fagni *et al*., 2004).

El glutamato es sintetizado en el terminal presináptico y se almacena en vesículas sinápticas mediante un sistema de recaptación llevado a cabo por **transportadores vesiculares de glutamato** (VGLUTs). Este neurotransmisor es liberado por exocitosis, tanto en las sinapsis como en áreas no sinápticas (Bezzi *et al*., 2001). Además se ha señalado que puede liberarse mediante un transportador “antiport” que intercambia cistina y glutamato (Bakar *et al*., 2002) y por canales aniónicos regulados por volumen (Haskew *et al*., 2002). El glutamato liberado a la hendidura sináptica actúa sobre **receptores glutamatérgicos,** tanto **ionotrópicos** como **metabotrópicos,** situados en ambos terminales pre- y postsinápticos. El glutamato liberado por exocitosis es recaptado por **transportadores de aminoácidos excitatorios** (EAATs) localizados en las membranas de ambos terminales sinápticos y células gliales. Estos transportadores llevan a cabo una rápida eliminación del glutamato

extracelular y manteniendo su concentración por debajo de los niveles neurotóxicos (Shigeri *et al*., 2004; Tzingounis y Wadiche, 2007). El glutamato recaptado por las células gliales se metaboliza en glutamina, a través del ciclo glutamato-glutamina, que es posteriormente transportada de regreso a las neuronas y convertida de nuevo en glutamato, el cual es captado e introducido en las vesículas sinápticas por los VGLUTs cerrando así el ciclo de la neurotransmisión glutamatérgica (Hamberger *et al*., 1979; Thanki *et al*., 1983) (Figura 4).

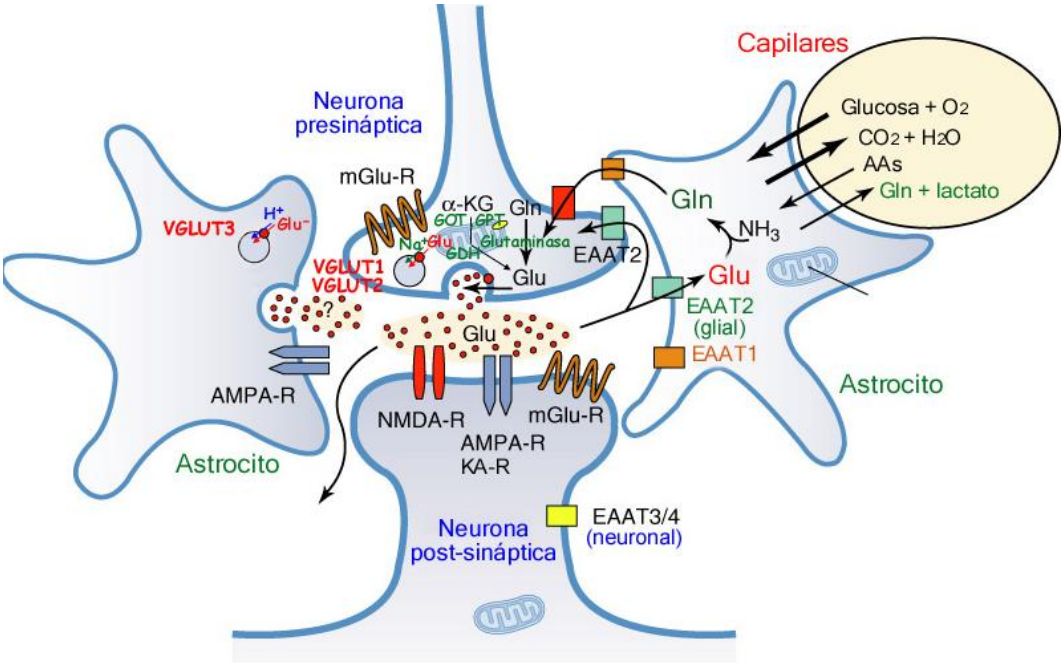

Figure4: Transmision glutamatergica. Imagen modificada de Siegel, G. J (ed.): "Basic Neurochemistry"

Transportadores vesiculares de glutamato

La contribución de los receptores de glutamato a la neurodegeneración después de la isquemia ha sido extensamente descrita (Choi, 1994), pero los mecanismos responsables de la excesiva concentración extracelular de glutamato no se conocen con exactitud. El mecanismo de transporte utilizado por los VGLUTs para introducir el glutamato en las vesículas sinápticas depende del gradiente electroquímico de protones (Lee *et al*., 1999; Bellocchio

et al., 2000; Bai *et al*., 2001; Takamori *et al*., 2001). La regulación de la concentración citosólica del glutamato por estos transportadores, permite a su vez el control de los niveles extracelulares de glutamato, manteniéndolo en niveles suficientemente bajos para evitar la sobreestimulación de los receptores de glutamato. De esta forma se restringe el daño neuronal provocado por la excitotoxicidad (Struzynka *et al*., 2005). La disfunción de los VGLUTs puede ser el inicio o parte de la cascada que lleve a la muerte celular (Torp *et al*., 1995; Rao *et al*., 2000; Fukamachi *et al*., 2001; Rao *et al*., 2001; Kim *et al*., 2006).

Se han descrito tres subtipos de transportadores vesiculares de glutamato (VGLUTs 1-3) (Hisano *et al*., 1997; Lee *et al*., 1999; Aihara *et al*., 2000; Bellocchio *et al*., 2000; Takamori *et al*., 2001; Bai *et al*., 2001; Herzog *et al*., 2001; Kim *et al*., 2006). Están formados por unos 600 aminoácidos y comparten más de un 70% de homología entre sí. Presentan 10 dominios de transmembrana (Figura 5), con un sitio de glicosilación altamente conservado entre los dominios de transmembrana 1 y 2, y numerosas secuencias consenso para la fosforilación por diferentes quinasas de proteínas.

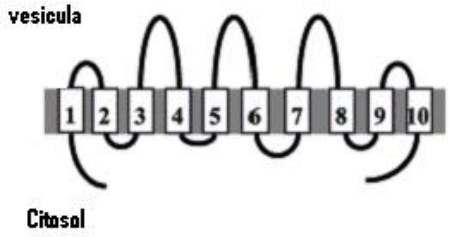

Figura 5: Estructura de los transportadores vesiculares de glutamato (Shigeri *et al*., 2004).

Los distintos subtipos de VGLUTs se expresan diferencialmente en el cerebro adulto de rata. Los VGLUTs 1 y 2 tienen una distribución amplia y complementaria, expresándose en los terminales de todas las sinapsis glutamatérgicas. El VGLUT1 es más abundante en la corteza cerebral, hipocampo y corteza cerebelosa, mientras que el VGLUT2 se localiza preferentemente en el diencéfalo y rombencéfalo (Fremeau *et al*; 2001; Kaneko *et al*., 2002; Li *et al*., 2003). El VGLUT3 se expresa en poblaciones de neuronas que promueven la liberación de otros neurotransmisores clásicos,

como interneuronas colinérgicas del cuerpo estriado, interneuronas gabérgicas de la corteza y el hipocampo y en las neuronas serotonérgicas, y tiene una distribución subcelular tanto axonal como somatodendrítica (Santos *et al*., 2009) (Figura 6). Además, el VGLUT3 se expresa también en tejidos fuera del SNC incluyendo el hígado y el riñón (Fremeau *et al*., 2002; Gras *et al*., 2002; Schafer *et al*., 2002).

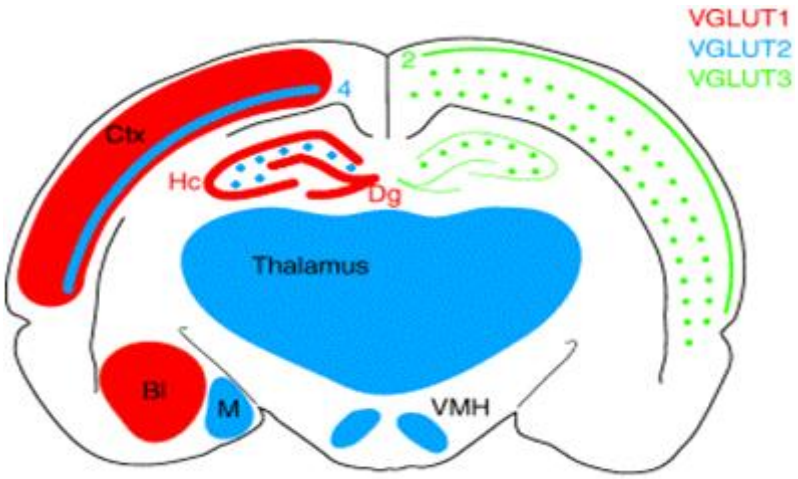

Figura 6: Distribución de los transportadores vesiculares de glutamato. Imagen modificada de: (Fremeau *et al*., 2004).

El gradiente electroquímico de H^+ a través de la membrana de las vesículas que utilizan los VGLUTs, es generado por una ATPasa vesicular que transporta H^+ en contra de gradiente al interior de las mismas. Se ha demostrado que el sistema gliceraldehído 3 fosfato deshidrogenada / 3 fosfoglicerato quinasa (GAPDH-3PGK) localizado en la membrana de la vesícula es utilizado para mantener el suministro de ATP para la bomba H^+/ATPasa. Tras la hidrólisis del ATP, el ADP se recicla rápidamente a ATP por el sistema GAPDH-3PGK durante la glicolisis. El gliceraldehído-3-fosfato (GAP) producido en el metabolismo de la glucosa se convierte en 1,3-bifosfoglicerato (1,3-BPG) a expensas de NAD^+ y Pi presentes en la membrana vesicular sináptica por la gliceraldehído-3-fosfato deshidrogenasa (GAPDH). El 1,3-BPG transfiere su fosfato de alta energía al ADP, reacción catalizada por la GAPDH/3-PGK, formando ATP en la superficie de las vesículas (Figura 7) (Ikemoto *et al*., 2003).

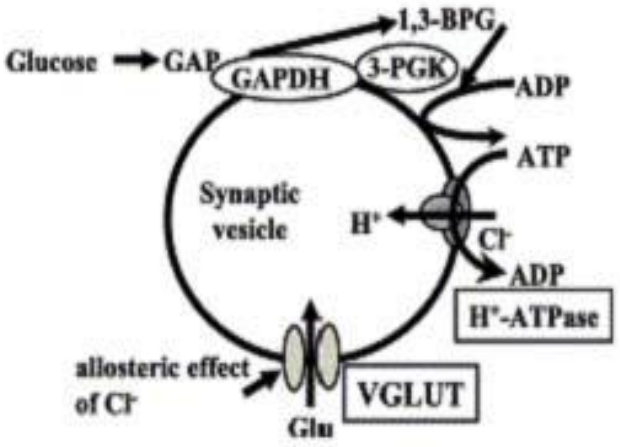

Figura 7: Funcionamiento de los transportadores vesiculares de glutamato. (Shigeri *et al*., 2004).

Receptores glutamatérgicos

Estos receptores juegan un papel muy importante en la plasticidad sináptica, en la capacidad de aprendizaje y en la memoria (Kind y Neumann, 2001; Sheng y Kim, 2002). Las respuestas postsinápticas al glutamato, son mediadas a través de receptores glutamatérgicos metabotrópicos (GluRm) e ionotrópicos (GluRi) (Figura 8).

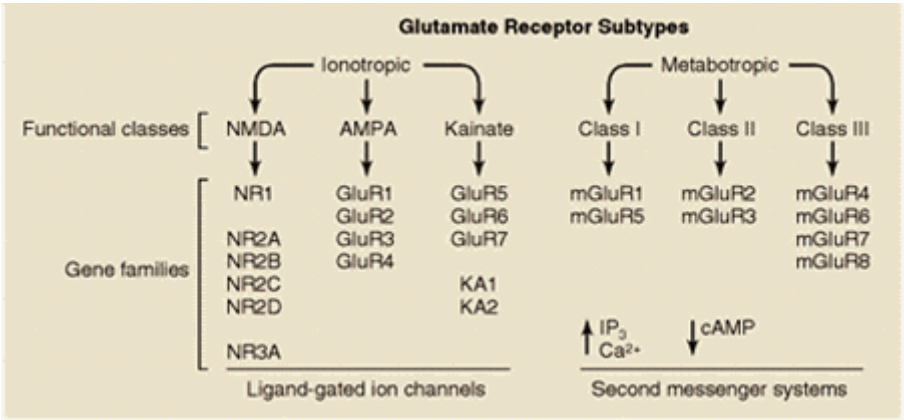

Figura 8: Clasificación de los receptores de aminoácidos excitatorios (imagen modificada de: Watkins y Jane, 2006).

GluRm

Son receptores formados por un polipéptido de siete dominios transmembrana acoplado a proteínas G. La proteína G activada actúa sobre enzimas que catalizan la síntesis o la degradación de segundos mensajeros, que a su vez activan otros procesos bioquímicos. Esto da lugar a una cascada metabólica (Watkins y Jane, 2006). Se han descrito ocho subtipos de estos receptores glutamatérgicos metabotrópicos, los cuales son subdivididos en tres grupos (Figura 8) (Shin *et al*., 2008).

- Grupo I: La activación de estos receptores activa la fosfolipasa C que da lugar a un aumento de Ca^{2+} citosólico. En este grupo se incluyen los GluRm1 y GluRm5.
- Grupo II: Este tipo de receptores son presinápticos y controlan la liberación de un gran número de neurotransmisores entre ellos: glutamato y ácido α-aminobutírico (GABA). En este grupo se encuentran los GluRm2 y GluRm3.
- Grupo III: Incluye los receptores que inhiben la actividad de la adenilato ciclasa, diminuyendo así la concentración de monofosfato cíclico de adenosina (AMPc) intracelular. En este grupo se engloban los GluRm 4, GluRm 6, GluRm 7 y GluRm8.

Los receptores GluRm han sido implicados en numerosos procesos fisiológicos y patológicos como el aprendizaje, la formación de la memoria, procesos neurodegenerativos, isquemia, dolor o epilepsia (Carlton y Neugebauer, 2002; El Far y Betz, 2002).

Los receptores englobados en el grupo I son fundamentalmente postsinápticos, y aumentan la excitabilidad neuronal modulando los receptores NMDA y AMPA, mientras que los del grupo II y III, son mayoritariamente presinápticos y se les relaciona con una disminución de la actividad sináptica, inhibiendo la liberación de glutamato (Bruno *et al*., 2001; Watkins y Jane, 2006).

GluRi

Estos receptores son complejos heteroméricos formados por cinco subunidades peptídicas, cada una de ellas formada por cuatro dominios transmembrana. El canal iónico formado por la agrupación de dichas subunidades, presenta un elevado grado de impermeabilidad a cationes en ausencia del neurotransmisor (Zigmond *et al*., 1999). La unión de ligandos específicos da lugar a un cambio conformacional que produce la apertura del canal iónico, incrementando así el flujo de iones a través de la membrana neuronal estimulada (Hollmann y Heinemann, 1994). Los GluRi se subdividen farmacológicamente en distintas subfamilias según su afinidad por el N-metil-D-aspartato (NMDA), el ácido α–amino-3-hidroxilo-5-metil-4-isoxazol-propiónico (AMPA) o el ácido kainico (Wisden y Seerburg, 1993; Cull-Candy *et al*., 2001; Madden, 2002; Brockie *et al*., 2006) (Figura 8).

Mucha de la toxicidad asociada con el glutamato se ha relacionado con la sobreestimulación de los receptores ionotrópicos (Choi, 1988). En la isquemia transitoria, el metabolismo energético aparece rápidamente modificado en la zona isquémica, provocando la liberación de glutamato (Benveniste *et al*., 1984). Este aumento en la concentración de glutamato extracelular da lugar a la activación de los receptores glutamatérgicos, especialmente los receptores NMDA y AMPA (Choi, 1994). De acuerdo con esto, el bloqueo de estos receptores proporciona neuroprotección en modelos de isquemia tanto *in vivo* como *ex vivo*.

- **Receptores de NMDA**

Este tipo de receptores presentan una alta permeabilidad a Ca^{2+}, Na^{+} y K^{+} (Ichinose *et al*., 2003) y son bloqueados por Mg^{2+} extracelular mediante un proceso dependiente de voltaje (Mayer *et al*., 1984; Nowak *et al*., 1984; Wollmuth y Sobolevsky, 2004). Para la activación de estos receptores se necesita simultáneamente la presencia de glutamato y glicina, co-agonista del glutamato, así como la despolarización neuronal que provoca la liberación del Mg^{2+} que bloquea el receptor (Figura 9).

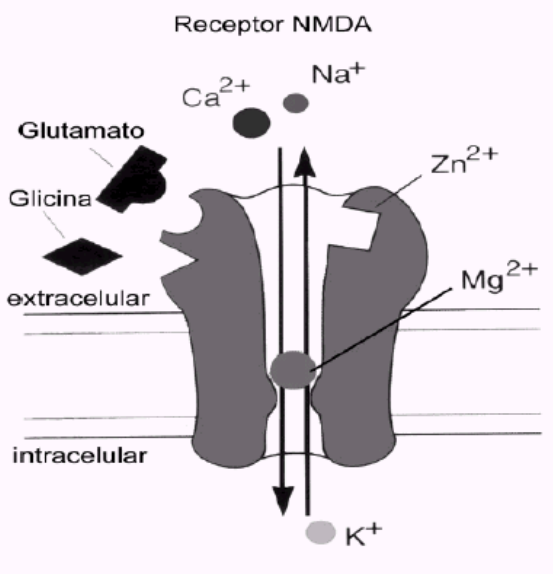

Figura 9: Modelo estructural del receptor NMDA. Tomado de Siegel, G. J (ed.): "Basic Neurochemistry"

Actualmente, se cree que los receptores NMDA son heterotetrámeros (Collingridge *et al*., 2013) formados por distintos tipos de subunidades. La subunidad NR1 contiene el sitio de unión a la glicina y se combina con, al menos, una de las cuatro subunidades NR2 que contienen el sitio de unión al glutamato (A, B, C y D) (Stephenson, 2006). También existen 2 subunidades NR3 (A y B) que no forman receptores funcionales por sí mismas, pero pueden asociarse a complejos NR1/NR2 (Das *et al*., 1998; Moriyoshi *et al*., 1991; Sugihara *et al*., 1992). Los receptores funcionales se asocian normalmente como tetrámeros formados por dos subunidades NR1 y dos subunidades NR2 (Cull-Candy *et al*., 2001; Furukawa *et al*., 2005). Todas estas subunidades presentan un número variable de sitios de maduración alternativa, lo que da lugar a multitud de isoformas distintas, a excepción de la subunidad NR2A (Zukin y Bennett, 1995; Rafiki *et al*., 2000; Cull-Candy *et al*., 2001).

Cada una de las subunidades del receptor NMDA está formada por un dominio C-terminal intracelular, tres dominios de transmembrana (1, 3 y 4), una región situada entre los dominios 1 y 3, que aparece embebida en la membrana formando una estructura en horquilla, y un dominio N-terminal extracelular con dos secuencias (S1 y S2) que forman conjuntamente el sitio de unión al ligando

(Figura 10) (Villmann y Becker, 2007). Cada subunidad cumple funciones específicas (Al-Hallaq *et al*., 2007) y las propiedades farmacológicas y electrofisiológicas del receptor dependen de las distintas isoformas resultantes de la diferente composición de subunidades (Kutsuwada *et al*., 1992; McBain y Mayer, 1994; Monyer *et al*., 1992).

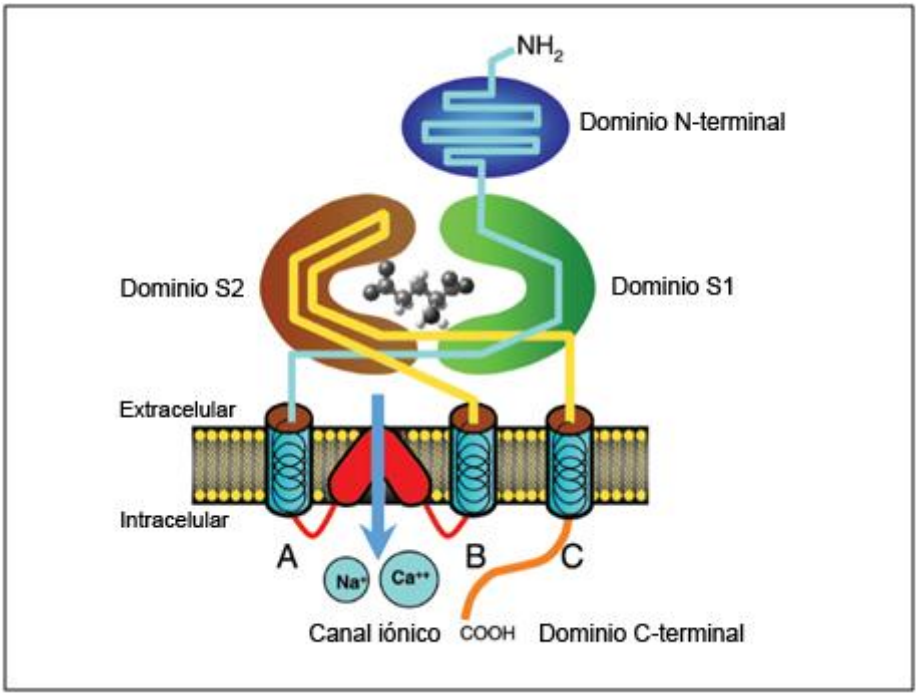

Figura 10: Estructura de las subunidades del receptor de NMDA. (Villmann and Becker 2007).

Las diferentes isoformas de NMDA presentan distinta distribución en el SNC (Monyer *et al*., 1994). Se ha demostrado que la distribución de las diferentes subunidades NR2 también está condicionada por la edad (VanDongen, 2009). Las subunidades NR2B y NR2D predominan en el encéfalo neonatal y son substituidas por NR2A y NR2C en el cerebelo a lo largo del desarrollo (Akazawa *et al*., 1994).

- **Receptores de AMPA**

Estos receptores están formados por tetrámeros de distintas subunidades (GLUR1-4), que pueden combinarse de distintas maneras, generando así canales iónicos con propiedades funcionales diferentes (Kwak y Weiss, 2006).

Se cree que la estructura tetramérica está formada por dos díméros (Greger *et al*., 2007; Mayer, 2006).

Los receptores de AMPA que carecen de subunidad GluR2 son permeables al Ca^{2+}, sin embargo la presencia de al menos una subunidad GluR2 los hace impermeables al mismo (Seeburg *et al*., 2001). En condiciones normales, las neuronas hipocampales expresan receptores AMPA impermeables al Ca^{2+}, mientras que en procesos isquémicos se produce una *down-regulation* en la expresión del ARNm y de la proteína de la subunidad GluR2. Esto produce un cambio en el fenotipo de las neuronas hipocampales (Gorter *et al*., 1997; Pellegrini-Giampietro *et al*., 1997; Opitz *et al*., 2000), que comienzan a expresar mayoritariamente receptores de AMPA permeables a Ca^{2+}, contribuyendo así a la muerte celular retrasada dependiente de Ca^{2+} (Liu *et al*., 2006; Peng *et al*., 2006).

Las propiedades especiales que posee la subunidad GluR2, son debidas a una modificación del ARNm citosólico por ADAR-2, que da lugar a la sustitución de la glutamina (Q) en la posición 607 por un residuo arginina (R). De este modo, la carga positiva adicional en el poro del canal, como consecuencia de la presencia de la arginina, impide el paso de iones divalentes como Ca^{2+} y Zn^{2+} (Jonas y Burnashev, 1995; Swanson *et al*., 1997) (Figura11).

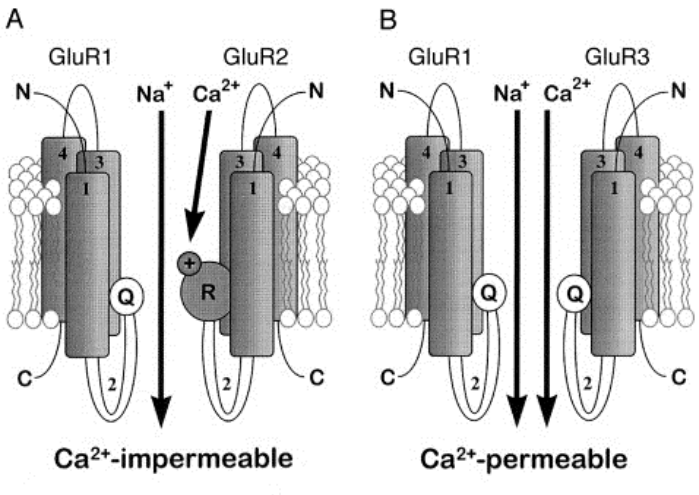

Figure 11: La subunidad GluR2 es la responsable de la permeabilidad al Ca^{2+} de los receptores AMPA (Pellgrini-Giampietro *et al*., 1997).

Todas las subunidades de este tipo de receptor tienen cuatro regiones hidrofóbicas (M1-M4) y un extremo N-terminal extracelular, que ayuda en la formación del dímero y está implicado en la formación del tetrámero (Wu *et al*., 1996; Rosenmund *et al*., 1998). La región M2 se encuentra embebida en la membrana formando una estructura en horquilla, de forma análoga a la descrita en las subunidades de NMDA, que está implicada en la formación del poro del canal. El extremo C-terminal intracelular de estos receptores, interacciona con varias proteínas reguladoras de transmembran,a que son necesarias para la maduración del receptor, el tráfico intracelular o la propia función del canal proteico (Chen *et al*., 2000; Ziff, 2007). El sitio de unión del ligando lo constituyen una secuencia (S1) del extremo N-terminal y otra secuencia (S2) del bucle extracelular (Ayalon *et al*., 2005; Jiang *et al*., 2006) (Figura 12).

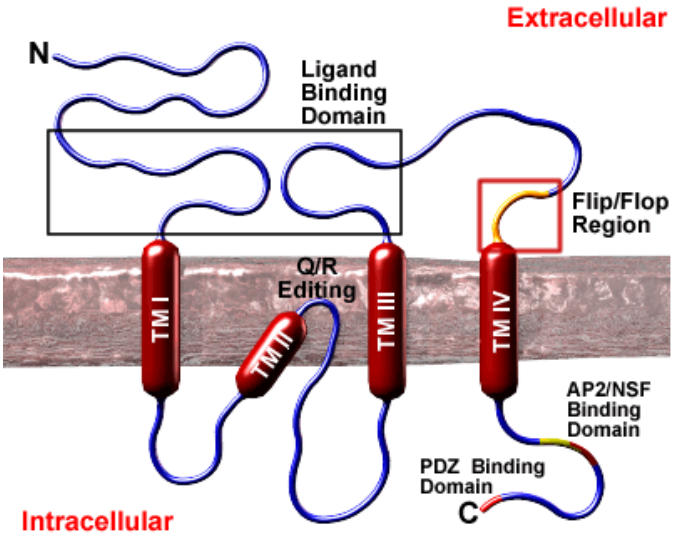

Figura 12: Estructura de las subunidades del receptor de AMPA. (Imagen modificada de Jiang *et al*., 2006).

Los receptores de AMPA se encuentran extensamente distribuidos en el SNC, tanto en neuronas como en células gliales (Belachew y Gallo, 2004). En el cerebro anterior, que incluye hipocampo y neocórtex cerebral, las subunidades predominantes son GluR1 y GluR2 y existen niveles bajos de GluR3 y GluR4 (Monyer *et al*., 1991; Craig *et al*., 1993; Sans *et al*., 2003). La expresión de GluR2 es baja en el desarrollo postnatal temprano comparado con la de GluR1,

pero esta expresión se incrementa rápidamente durante la primera semana postnatal (Monyer *et al*., 1991).

Transportadores de aminoácidos excitatorios

Los transportadores de aminoácidos excitatorios (EAATs) son homómeros compuestos por tres subunidades y todas ellas presentan sitios de unión a glutamato. Presentan dominios N- y C- terminal intracelulares. Su topología de transmembrana no está bien definida y se han propuesto dos modelos. Ambos coinciden en que los EAATs poseen ocho dominios de transmembrana, pero no concuerdan en los tipos de dominios situados entre las regiones transmembrana 6 y 8 (Shigeri *et al*., 2004) (Figura 13). Estos transportadores se expresan en células glutamatérgicas y gliales, recaptando el glutamato liberado en la hendidura sináptica, lo que permite mantener la concentración del glutamato extracelular en niveles que no llegan a ser neurotóxicos (Tzingounis y Wadiche, 2007). Además permiten la reutilización del glutamato intracelular e incluso utilizarlo como precursor del neurotransmisor GABA (Berger y Hediger, 1998).

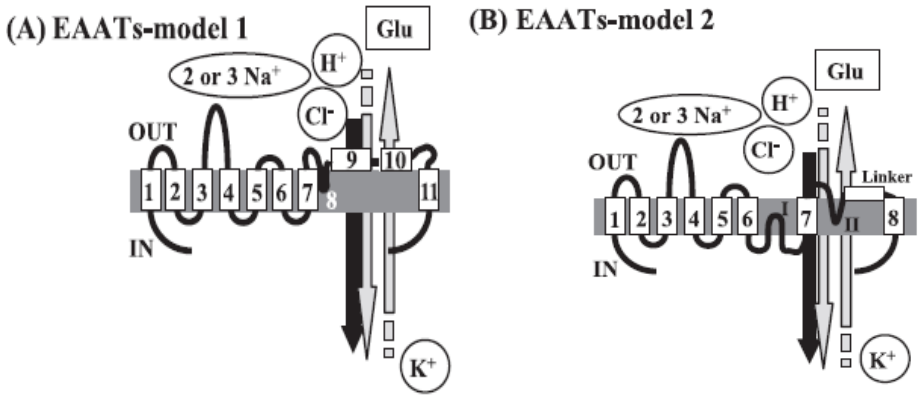

Figura 13: Diferentes modelos de topología de transmembrana de los transportadores de amoniácidos excitatorios (Shigeri *et al*., 2004).

Los EAATs se clasifican en cinco tipos:

- EAAT1 o GLAST (Glutamate/aspartate transporter), cuyo ARNm aparece en las células astrogliales y ependimarias (Storck *et al*., 1992; Berger y Hediger, 1998).
- EAAT2 o GLT-1 (Glutamate transporter-1), en los que el ARNm se expresa en astrocitos y en neuronas (Pines *et al*., 1992; Berger y hediger, 1998).
- EAAT3 o EAAC-1 (Excitatory aminoacid carrier 1), cuyo ARNm se localiza principalmente en neuronas (Rothstein *et al*., 1994; Fairman *et al*., 1995).

Estos 3 tipos de receptores presentan una amplia distribución en el SNC, con niveles elevados en corteza cerebral, hipocampo y estriado (Tzingounis y Wadiche, 2007).

- EAAT4, con una distribución de ARNm restringida al cerebelo (Fairman *et al*., 1995)
- EAAT5, cuyo ARNm se ha detectado en fotorreceptores y en células bipolares de la retina (Arriza *et al*., 1997).

Los EAATs presentan 2 conformaciones. En una de ellas, el sitio de unión de glutamato está expuesto al entorno extracelular, y en la otra, el sitio de unión es accesible desde el citoplasma. La interconversión entre ellas permite la acumulación de glutamato intracelular contra gradiente en un proceso que requiere ATP (Kanner y Schuldiner, 1987; Tzingounis y Wadiche, 2007). Este ATP se utiliza para suministrar energía a las ATPasas Na^+/K^+ y generar un gradiente electroquímico de Na^+ (Masson *et al*., 1999). La generación de este gradiente permite la entrada de una molécula de glutamato que se co-transporta con dos iones Na^+ y un protón, liberando al espacio extracelular un ión K^+ simultáneamente. Posteriormente se produce la salida de tres iones Na^+ conjuntamente con la entrada de dos K^+. Este proceso es necesario para la recuperación del gradiente de Na^+ que requiere el consumo de ATP por la ATPasa Na^+/K^+ (Figura 14).

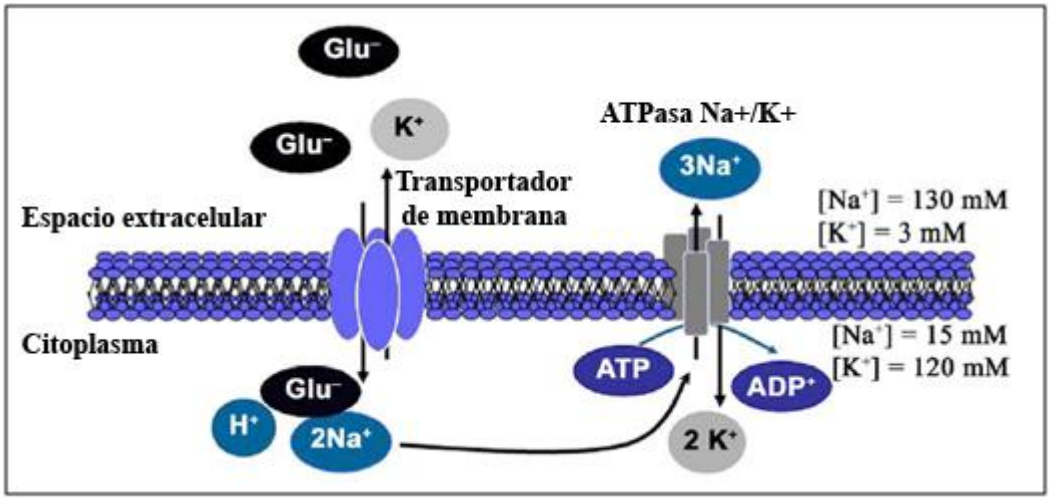

Figura 14: Recaptación de glutamato extracelular mediante los transportadores de aminoácidos excitatorios (Camacho y Massie, 2006).

Sistema GABAérgico

El GABA es el principal aminoácido neurotransmisor inhibidor del SNC de mamíferos, y está presente aproximadamente en el 40% de las neuronas (Krnjevic y Schwartz, 1987). El GABA se encuentra en todo el encéfalo, pero su mayor concentración se localiza en el cerebelo. Posiblemente todas las neuronas inhibidoras cerebelosas utilizan GABA como neurotransmisor, sin embargo, también existen neuronas GABAérgicas localizadas en la corteza cerebral, hipocampo y las estructuras límbicas (Paul, 1995). Los sistemas GABAérgicos más conocidos, son los formados por las fibras que llegan a la substancia nigra procedentes de neuronas localizadas en el estriado y las proyecciones al núcleo dorsal de Deiters, así como a los núcleos intercerebelares, interpósito y fastigial procedentes de las células de Purkinje de la corteza cerebelosa (Curtis *et al*., 1968; Kelly *et al*., 1969; Bowery *et al*., 1981).

La síntesis del GABA se realiza a través del denominado circuito del GABA ("GABA shunt"), que comienza con la transaminación del α-cetoglutarato formado en el ciclo de Krebs por la α-oxoglutarato transaminasa (GABA-T), dando lugar a ácido L-glutámico. Éste es descarboxilado por la enzima glutamato descarboxilasa (GAD) dando lugar a GABA. Una vez sintetizado, el

GABA es almacenado en vesículas en los terminales sinápticos. Cuando se produce el estímulo nervioso, el neurotransmisor es liberado mediante exocitosis, desde la neurona presináptica, a la hendidura sináptica (Zigmond *et al*., 1999).

El GABA liberado en la hendidura sináptica es recaptado, bien por la célula presináptica, o por las células gliales. En las neuronas GABAérgicas, el GABA recaptado es degradado a semialdehído succínico y glutamato mediante el enzima GABA-T que, en este caso, cataliza la reacción inversa a la formación del GABA indicada previamente en el circuito del GABA. El semialdehído succínico es convertido posteriormente a succinato, lo que le permite entrar en el ciclo de Krebs. Mediante la sintetasa de glutamina, el glutamato recaptado en las células gliales es convertido en glutamina, que es transferida a la neurona GABAérgica. Allí es hidrolizada por una glutaminasa activada por fosfato (PAG), para producir el glutamato que se incorpora al circuito del GABA, al ser decarboxilado por GAD para formar GABA, cerrándose así el ciclo (Figura 15).

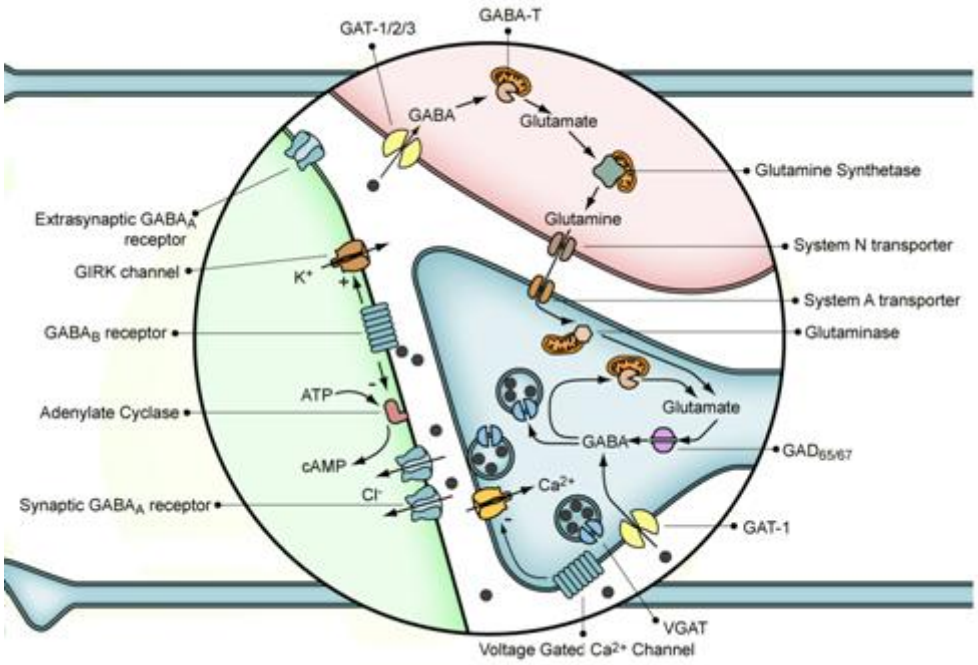

Figura 15: Sistema GABAérgico. Imagen modificada de Bliz0r, 2010.

Receptores GABAérgicos

Existen varios receptores para GABA; **receptores ionotrópicos** o de acción rápida ($GABA_A$) y **receptores metabotrópicos** o de acción lenta ($GABA_B$). Existe un tercer tipo de receptores ionotrópicos ($GABA_C$) que presenta propiedades farmacológicas y electrofisiológicas diferentes, y ha sido aislado de células de la retina (Ortell and Lunt, 1995; Kaupman *et al*., 1997; Qian *et al*., 2009).

Existen agonistas de GABA que reconocen específicamente los diferentes receptores. Esto permitió diferenciar los receptores $GABA_A$ y $GABA_B$. Uno de ellos, el baclofén (beta-p-cloro fenil GABA), es un análogo del GABA que activa los receptores $GABA_{B,}$ pero es inactivo sobre los receptores $GABA_A$ (Akaike *et al*., 1987; Mathers, 1987).

- **RECEPTORES $GABA_A$**

Los receptores $GABA_A$ se localizan en las membranas plasmáticas de los terminales post-sinápticos. Son canales de cloro que, al abrirse, permiten la hiperpolarización de la célula en la mayor parte de los casos, disminuyendo así su excitabilidad (Roberts, 1986; Paul, 1995).

Los receptores $GABA_A$ son complejos oligoméricos que presentan sitios de unión a diferentes ligandos:

- ✓ Sitios de reconocimiento de baja afinidad para el GABA, preferentemente antagonizado por las benzodiazepinas.
- ✓ Existe además un sitio de reconocimiento de alta afinidad para el GABA, que es una forma desensibilizada del receptor.
- ✓ Sitio del agonista exógeno: sería el sitio de las benzodiacepinas (BZD), que aumenta la unión del GABA con el sitio de reconocimiento del receptor $GABA_A$.
- ✓ Sitio de los agonistas inversos: son las beta carbolinas y reducen el flujo de cloro inducido por GABA.

- ✓ Sitio de los agonistas parciales: poseen afinidad y actividad menor que el agonista total, son las ciclopirrolonas.
- ✓ Sitio del coagonista, glicina, que tiene un efecto inhibitorio.
- ✓ Sitio de los antagonistas selectivos: **bicuculina** y SR95531.
- ✓ Sitio de los antagonistas no selectivos: tienen afinidad pero su actividad es nula, no influyendo sobre el canal de cloro, pero si antagonizando las acciones de los agonistas. Es el flumazenil.

(Figura 16)

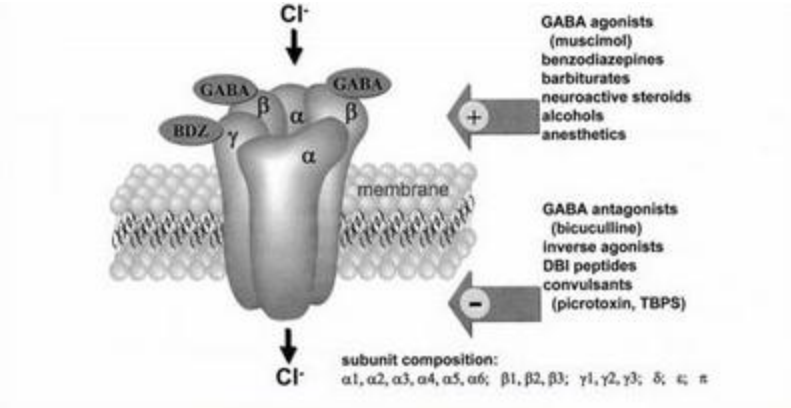

Figura 16: Modelo estructural del receptor de $GABA_A$ unido al canal de Cl-. Tomado de Siegel, G. J (ed.): "Basic Neurochemistry".

Los receptores de $GABA_A$ son pentaméricos y pueden estar formados por cinco subunidades diferentes: α, β, ρ, Δ y δ, todas ellas presentan diferentes isoformas y son necesarias la combinación de cinco de estas subunidades para la formación de los canales de cloro (Zigmond *et al*., 1999). Cada subunidad contiene cuatro dominios de trasmembrana: M1, M2, M3 y M4 (Figura 16). Existen diferentes isoformas compuestas por distintas subunidades, en consecuencia presentan diferencias en la afinidad de los sitios alostéricos adicionales y en la eficacia del sitio de unión al GABA (Seighart, 1992).

- **RECEPTORES $GABA_B$**

Estos receptores se encuentran en la membrana plasmática, tanto del terminal presináptico como del terminal postsináptico. Estos receptores no están relacionados con canales de cloro como el receptor $GABA_A$, sino que modulan canales de Ca^{2+} y de K^+ como consecuencia de la interacción de la proteina G y la adenilato ciclasa. La unión de un agonista al receptor $GABA_B$ presináptico disminuye la entrada de Ca^{2+} y aumenta la entrada de K^+, originando de esta forma menor liberación del neurotransmisor. La unión de un agonista al receptor $GABA_B$ postsináptico aumenta la salida de K^+ al medio extracelular, produciendo un potencial postsináptico inhibitorio lento (Zigmond *et al*., 1999) (Figura 17).

La estructura del receptor $GABA_B$ es similar a la de los receptores GluRm, y presenta una región N-terminal extracelular grande, que contiene el área de unión del ligando, 7 dominios de transmembrana y una región citosólica con el dominio C-terminal. (Kaupman *et al*., 1997; Zigmond *et al*., 1999)

Se han descrito diversos sitios de unión para agonistas y antagonistas en el $GABA_B$:

- ✓ Sitio para el agonista no selectivo GABA.
- ✓ Sitio para el agonista selectivo 3APPA.
- ✓ Sitio para el antagonista no selectivo BACLOFEN.
- ✓ Sitio para el antagonista selectivo CGP35384.

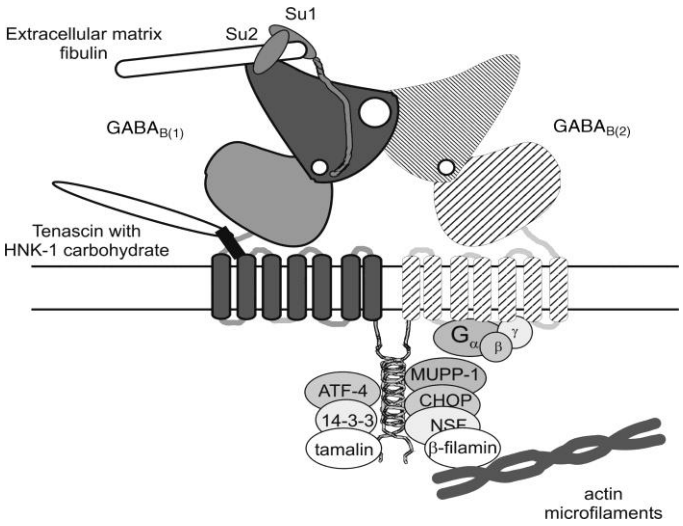

Figura 17: Estructura del receptor GABA$_B$. (Bettler *et al*., 2004).

- **Receptores GABA$_C$**

El receptor GABA$_C$ ha sido el último en ser identificado, se encuentra en las células de la retina. A diferencia de los receptores GABA$_A$ no es bloqueado por la bicuculina y a diferencia de los GABA$_B$ no es modulado por el baclofen. Estos receptores homómeros formados por subunidades ρ (Figura 18), son unas 10 veces más sensibles que los GABA$_A$ al GABA, y muestran una conductancia baja y tiempos de apertura bastante largos, además de una alta selectividad para el Cl^-. (Bormannn y Fiegenspan, 1995; Qian *et al*., 2009)

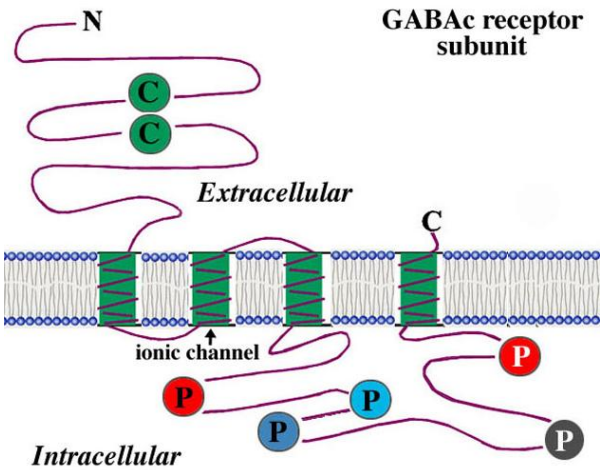

Figura 18: Estructura del receptor de GABA$_C$. (Qian, 2009).

Isquemia y envejecimiento

Se ha descrito que el envejecimiento aumenta la vulnerabilidad del cerebro al daño isquémico o anóxico (Roberts *et al*., 1990; Yao *et al*., 1991), y que los individuos adultos experimentan una proporción más alta de infarto y presentan una tasa menor de recuperación funcional que los jóvenes (Adams *et al*., 1999; Johnston *et al*., 2000; Badan *et al*., 2004). El accidente cerebro-vascular es una de las principales causas de muerte en todo el mundo. Aproximadamente la tercera parte de los supervivientes a un ictus mayores de 65 años desarrollan demencia vascular 3 meses después del episodio isquémico. De hecho es muy probable que muchos de los pacientes diagnosticados con enfermedad de Alzheimer sean en realidad pacientes con demencia vascular (Roman, 2002).

A pesar de esto, existen pocos estudios en los que se compare la respuesta diferencial a la isquemia entre individuos jóvenes y adultos. Además existe cierta controversia ya que algunos autores señalan un mayor daño isquémico asociado a la edad (Davis *et al*., 1995; Nagayama *et al*., 1999; Kharlamov *et al*., 2000; Kövari *et al*., 2004; Ay *et al*., 2005), mientras que otros no detectan cambios asociados al envejecimiento tras el daño isquémico (Badan *et al*., 2003; Wang *et al*., 2003).

También se ha señalado que los animales adultos muestran diferencias en los mecanismos de respuesta a la isquemia con respecto a los jóvenes, lo que puede explicar, al menos en parte, la falta de éxito en la predicción de la eficacia clínica de agentes neuroprotectores (Rosen *et al*., 2005). El efecto principal de la isquemia en animales adultos está asociado a una respuesta reducida al estrés del tejido intacto que rodea la zona infartada (Li *et al*., 2005). También se ha señalado que el incremento de la edad en la conversión de tejido isquémico en tejido infartado, podría considerarse como un marcador biológico para la variabilidad observada en el daño isquémico humano, ya que con la edad se observa un incremento en la transformación del tejido isquémico a tejido infartado (Ay *et al*., 2005). Por tanto, las diferencias encontradas con relación a la edad en la isquemia cerebral deben tomarse en cuenta en el tratamiento de la misma (Baltan *et al*., 2008).

Isquemia e inflamación

Numerosos estudios indican que la inflamación juega un importante papel en la lesión cerebral isquémica (Kriz, 2006; Taoufik y Probert, 2008). El daño cerebral inducido por la isquemia desencadena un aumento de la actividad microglial y astrocitaria, un incremento en la producción de citoquinas, quimioquinas, moléculas de adhesión y metaloproteinasas, además de la infiltración de monocitos y leucocitos en las regiones cerebrales dañadas (Brouns y De Deyn, 2009; Iadecola y Alexander, 2001; Wang *et al*., 2007). El papel de la inflamación en el proceso isquémico es controvertido, ya que una proporción significativa de la respuesta inflamatoria parece agravar la lesión isquémica (Kriz, 2006; Taoufik y Probert 2008), mientras que ciertas respuestas inflamatorias son beneficiosas (Brouns y De Deyn, 2009). Por todo ello, es necesario identificar las respuestas inflamatorias perjudiciales y beneficiosas para diseñar estrategias terapéuticas, que inhiban selectivamente las respuestas perjudiciales al tiempo que mejoran las respuestas beneficiosas (Xia *et al*., 2010).

Tras la isquemia se produce la denominada "cascada inflamatoria" que comienza con el aumento de la expresión de COX-2 (Nogawa *et al* 1997; Nogawa *et al*., 1998). Esto desencadena un aumento en la producción de prostaglandinas, leucotrienos y especies reactivas al oxígeno (ROS), todos ellos implicados en la disfunción de la barrera hematoencefálica, el edema y la muerte neuronal (Nogawa *et al*., 1997; Tomimoto *et al*., 2002; Manabe *et al*., 2004; Kawano *et al*., 2006; Brouns y De Deyn 2009). No obstante, muchas de las células activadas como consecuencia del daño isquémico liberan un número muy elevado de sustancias, que pueden ser citotoxicas y/o citoprotectoras. Debido a ello el uso de antiinflamatorios como tratamientos al daño isquémico debe ser revisado meticulosamente.

El meloxicam [4-hidroxi-2-metil-N-(5-metil-2-tiazolil)-2H-1,2-benzotiazina-1,1-dioxida] es un inhibidor de la enzima COX-2. Es utilizado habitualmente, tanto en clínica veterinaria como humana, como agente antiinflamatorio no esteroideo con una vida media de 20 horas, y se elimina tras ser biotransformado en cuatro metabolitos inactivos (Davies y Skjodt 1999; Gates

et al., 2005). Dado su uso habitual en clínica como antiinflamatorio se ha utilizado en este trabajo para evaluar el efecto de los antiinflamatorios en la respuesta a la isquemia.

Respuesta a proteínas mal plegadas (UPR)

Una gran parte de las proteínas celulares son sintetizadas, ensambladas y plegadas en el retículo endoplásmico (RE) y al menos un tercio del total de proteínas posteriormente serán destinadas al espacio extracelular, a la membrana plasmática y a los compartimentos exo/endocíticos (Kaufman, 2002; Schröder *et al*., 2005; Zhang y Kaufman, 2006).

La viabilidad y el normal funcionamiento de la célula son dependientes de un correcto plegamiento de las proteínas. Para ello existe un sistema enzimático que se une a las proteínas nacientes y promueve su correcto plegamiento y maduración, en un proceso dependiente de ATP. Las foldasas, chaperonas y lectinas son las proteínas encargadas del correcto plegamiento en el RE (Ma *et al*., 2004¸ Schröder *et al*., 2005).

- **Foldasas:** catalizan el plegamiento de las proteínas, aumentando la velocidad del mismo. Un ejemplo es la **proteína disulfuro isomerasa** (PDI) que oxida residuos de cisteína de las proteínas nacientes y cataliza la formación de puentes disulfuro en forma correcta.
- **Chaperonas moleculares:** facilitan el plegamiento de las proteínas impidiendo que las regiones hidrófobas de las proteínas se asocien formando agregados insolubles. Dentro de este grupo de proteínas se consideran dos grandes familias: Hsp70 (cuyo miembro más conocido en el RE es la chaperona **Grp78/Bip**) y Hsp90 (siendo **Grp94** la más conocida). Además existe otra familia formada por proteínas que actúan regulando la actividad ATPasa de Grp78/Bip.

- **Lectinas:** están implicadas en el plegamiento de glicoproteínas y reconocen la presencia de glúcidos específicos unidos a la proteína que se utilizan como indicadores de plegamientos incorrectos de la misma. Entre estas, cabe destacar las chaperonas Calnexina (CNX) y Calreticulina (CRT).
- **Chaperoninas:** esta familia de proteínas es muy ubicua, está formada por subunidades de uno 60 kDa, compuestas por dos anillos heptaméricos, y son capaces de inducir el plegamiento de proteínas, en un proceso que requiere ATP.

Existe gran variedad de estímulos que dan lugar a un aumento en el número de proteínas mal plegadas en el interior del RE: la inhibición de la glicosilación, la reducción de los puentes disulfuro, la bajada de los niveles de Ca^{2+} en el lumen del RE, episodios de hipoxia/isquemia o hipoglucemia, entre otros. Cuando el número de proteínas mal plegadas aumenta se produce el denominado estrés de RE (Kaufman *et al*., 2002; Yoshida, 2007) que puede comprometer el correcto funcionamiento del RE como consecuencia de la acumulación y agregación de proteínas mal plegadas (Liang, 2006; Paschen y Mengesdorf, 2005a y 2005b). Esto es debido a que las proteínas mal plegadas exponen residuos de aminoácidos hidrofóbicos que deberían localizarse en el interior de la proteína correctamente plegada, cuando no ocurre esto, los residuos hidrofóbicos tienden a asociarse y se forman grandes agregados proteicos.

Numerosas situaciones patológicas incluyendo isquemia, enfermedades cardiovasculares, cáncer, diabetes mellitus y diversas enfermedades neurodegenerativas dan lugar a estrés de retículo y su consiguiente aumento de agregados proteicos. En estas condiciones, la célula desencadena mecanismos de respuesta para tratar de eliminar la sobrecarga de estas proteínas, la denominada respuesta a las proteínas mal plegadas ("Unfolded Protein Response" o UPR) (Schröde y Kaufman, 2005) que si no resuelve el estrés de RE induce la muerte celular mediante mecanismos de muerte celular programada. En la mayoría de las enfermedades citadas, la formación de agregados proteicos es responsable de los aspectos patológicos de la enfermedad (Carrard *et al*., 2002; Martinez-Vicente *et al*., 2005).

La UPR detecta y responde ante las distintas situaciones de estrés, siendo esencial para el mantenimiento de la homeostasis celular, y consiste en cuatro respuestas fundamentales que se ponen en marcha simultáneamente:

1. Inhibición generalizada de la síntesis proteica (DeGracia *et al*., 2002), evitando así acumulación de nuevas proteínas mal plegadas.

2. Aumento en la expresión de genes que codifican enzimas residentes en el RE, implicadas en el correcto plegamiento de las proteínas (chaperonas como Grp78, Grp94, PDI y otras), aumentando la capacidad de plegamiento.

3. Inducción transcripcional de genes correspondientes a miembros del complejo de degradación asociado al RE (ER-associated degradation, **ERAD**). Las proteínas que no pasan el control de calidad en este orgánulo son transportadas desde el RE al citosol, ubiquitinadas y degradadas rápidamente por el sistema ubiquitina-proteosoma (UPS) (Hiller *et al*., 1996; Lord *et al*., 2000, Muchowski, 2002; Meusser *et al*., 2005)

La función de estas tres primeras fases, es aliviar a la célula del exceso de proteínas acumuladas en el RE y, por tanto, solucionar la situación de estrés de retículo. En caso de que la situación de estrés no pueda ser resuelta de manera satisfactoria, la UPR es capaz de una cuarta fase:

4. Inducción de muerte celular programada (fundamentalmente por apoptosis) de las células dañadas, con el fin de asegurar la supervivencia del resto. Uno de los marcadores más importantes de este proceso es el factor pro-apoptótico **CHOP** (Schröde y Kaufman, 2005; Ron y Walter, 2007)

EL RE es el mayor almacén de calcio intracelular, la función de las chaperonas del RE está fuertemente influenciada por su concentración y las modificaciones de la misma (Schröder *et al*., 2005). Igualmente, los niveles de glucosa así como los de oxígeno pueden modificar los niveles de expresión de algunas de estas chaperonas, principalmente Grp78/Bip y PDI (Fredman *et al*., 1994; Ferrari, 1999; Ellgaard y Helenius, 2003). La respuesta a la isquemia cerebral genera grandes modificaciones en las concentraciones de Ca^{2+} intracelular, ATP, glucosa y también da lugar a una importante reducción de oxigeno. Por tanto la UPR parece uno de los mecanismos críticos que siguen a proceso

isquémico y obviamente una diana terapéutica clave en terapias de reparación y supervivencia celular.

Modelos experimentales de isquemia cerebral

Los modelos experimentales permiten adquirir conocimientos y comprender mejor la fisiopatología del daño cerebral isquémico. Se utilizan para probar compuestos nuevos capaces de minimizar dicho daño, lo que es de especial interés para su aplicación clínica (Prieto-Arribas *et al*., 2008). Los modelos experimentales también nos permiten estudiar la seguridad y eficacia de los agentes terapéuticos, así como el desarrollo de nuevas técnicas de diagnóstico.

El principal objetivo de los modelos animales es producir una lesión de forma reproducible, minimizando la variabilidad entre individuos. Para ello, la monitorización y el control de todas las variables fisiológicas que puedan modificar el efecto en la lesión tisular es de vital importancia. Las variables que se deben mantener dentro del rango normal a lo largo de todo el experimento son: temperatura, presión arterial, glucemia y gases arteriales. Todos los animales incluidos en un estudio experimental deben ser del mismo sexo y pertenecer al mismo grupo de edad (Prieto-Arribas *et al*., 2008).

Los modelos experimentales de isquemia suelen dividirse en dos grupos principales: ensayos *in vivo,* que al realizarse en animales de experimentación son más proximos a la patología humana, y ensayos *ex vivo,* que son llevados a cabo a nivel tisular o celular y nos permiten controlar las condiciones a ensayar más estrictamente. A continuación se detallan los principales modelos utilizados en el estudio de la isquemia cerebral:

Modelos *in vivo* de isquemia cerebral

- **Modelos de isquemia cerebral global**

  Este modelo reproduce el daño cerebral que se origina tras la obstrucción de las arterias carótidas o un paro cardíaco.

  - **Mediante parada cardíaca**

    La parada cardíaca se puede realizar mediante fibrilación ventricular (Hossman y Hossman, 1973), inyección intracardíaca de cloruro potásico u otros agentes cardiopléjicos (Kofler *et al*., 2004), exanguinación (Behringer *et al*., 2000), ahogamiento (Makarenko, 1972) o asfixia (Liachenko *et al*., 1998).

  - **Mediante interrupción selectiva de la circulación cerebral**

    - **Oclusión de dos vasos en jerbos**

      En el caso de los jerbos se puede conseguir una isquemia cerebral global (ICG) completa ligando las dos arterias carótidas sin necesidad de asociar oclusión de las arterias vertebrales (Levine y Payan, 1966).

    - **Oclusión de dos vasos en ratas**

      Este modelo está basado en la oclusión de las dos arterias carótidas conjuntamente con una hipotensión sistémica (50 mm de Hg aproximadamente), que puede ser alcanzada mediante sangrado controlado o farmacológicamente (Eklöf y Siesjö, 1972). Posteriormente se retiraran las ligaduras carotídeas para recuperar la presión arterial normal. Si se ha llevado a cabo un sangrado controlado, se reemplazará la sangre previamente extraída.

- **Oclusión de cuatro vasos en ratas**

  Este modelo está basado en la oclusión completa de las arterias vertebrales posteriores y las arterias carótidas comunes (Pulsinelli y Brierley, 1979).

- **Estrangulación cervical**

  Consiste en la compresión externa de los vasos mediante estrangulamiento, llevado a cabo por un collarín neumático en torno al cuello. En este modelo hay que tener en cuenta que aparte de interrumpir el flujo arterial también se interrumpe el retorno venoso (Prieto-Arribas *et al*., 2008)

- **Decapitación**

  Se lleva a cabo mediante de la conexión de la cabeza decapitada a un sistema de circulación extracorpórea (Hinzen *et al*., 1972)

- **Modelos de isquemia cerebral focal**

  Este modelo está basado en la oclusión del flujo distal de una sola arteria cerebral.

  - **Oclusión de la arteria carótida común**

    La oclusión de la arteria carótida común asociada a hipoxia produce un daño focal cerebral ipsilateral a la oclusión (Levine, 1960).

  - **Oclusión de la arteria cerebral media**

    Sobre este modelo existen multitud de variantes:

    Oclusión transorbital de la arteria cerebral media (ACM), oclusión transcraneal de la ACM, oclusión intraluminal de la ACM mediante embolia y la más utilizada de todas: **oclusión intraluminal de la ACM con filamento** (Prieto-Arribas *et al*., 2008).

    La oclusión intraluminal con filamento de la ACM se desarrolló a finales de los 80 (Zea *et al*., 1989) y es la más utilizada de las cuatro, ya que es la menos invasiva y la que menor riesgo de mortalidad

presenta, además se puede monitorizar el flujo sanguíneo cerebral de la zona infartada mediante una sonda láser *doppler*.

- **Oclusión microvascular de la arteria cerebral media**
  - **Oclusión fototrombótica**

    Este modelo está basado en la producción de una trombosis intravascular mediante inducción fotoquímica, esto es llevado a cabo mediante la administración de un contraste fotosensible, como el rosa de bengala, por vía intravenosa seguido de una posterior iluminación del área cortical, mediante un láser de argón (Watson *et al*., 1985).

  - **Microembolización de agregados plaquetarios**

    Existen diferentes variantes de este modelo, uno de los más importantes es la inducción de agregados plaquetarios, mediante la inyección intracarotídea de difosfato de adenosina o ácido araquidónico (Fiseschi *et al*., 1975).

  - **Inyección intracraneal de endotelinas**

    Es llevado a cabo mediante la administración directa de un vasoconstrictor (endotelina I o III) (Fiseschi *et al*., 1975; Henshall, 1999).

  - **Infartos espontáneos en ratas hipertensas**

    Las ratas son expuestas a una dieta rica en sal desde las seis semanas de vida y suelen presentar infartos de forma consistente a partir de la duodécima semana (Yamori *et al*., 1976).

Modelos *ex vivo* de isquemia cerebral

Whittingham comenzó a utilizar modelos *ex vivo* en la década de los 80, estos modelos están basados en la incubación de secciones cerebrales en soluciones fisiológicas en ausencia de oxígeno y glucosa, intentando

reproducir, de la manera más exacta posible, la verdadera interrupción que sucede después del proceso isquémico. Estos modelos experimentales presentan tres grandes ventajas con respecto a los modelos animales explicados anteriormente: mayor control de las variables que influyen en la lesión isquémica, mayor facilidad en el estudio de las alteraciones moleculares producidas por la isquemia, y menor coste económico. También presentan desventajas: en primer lugar, debido a las técnicas que se utilizan para la obtención de las secciones, éstas presentan un importante daño traumático, además las secciones son incubadas en un medio diferente al extracelular. Aunque el oxígeno y la glucosa son los principales factores que se pierden en la isquemia, la sangre contiene muchos otros factores que se pierden en los modelos *ex vivo*. La ausencia del cráneo hace que el efecto de edema celular esté muy minimizado. Además, estas secciones se obtienen, mayoritariamente, en individuos jóvenes (Prieto-Arribas *et al*., 2008). Los modelos "ex vivo" se utilizan en procedimientos cortos usando **secciones cerebrales** (Whittingham *et al*., 1984) y relativamente más largos con el uso de **cultivos organotípicos** (Vornow *et al*., 1994) o **cultivos primarios neuronales** (Goldberg y Choi, 1993) extraídos de diferentes estructuras cerebrales

Aims

Stroke is one of the main health problems in the developed countries, both in its clinic and social faces. The search for therapeutic targets and putative therapies either palliative o regenerative is the basic aim of research teams that work on stroke. As previously described through the Introduction Section, the glutamatergic system plays a crucial role in the damage following the ischaemic event and its subsequent reperfusion. This system sems to be the main target to alleviate or prevent the damage derived from stroke. GABA is the main inhibitory neurotransmitter in the brain and, given its ability of modulating the glutamate release, is considered a possible target for modifying the excitotoxic effects of glutamate. Inflammation also plays a critical role in modulating the excitotoxic activity. The role of glutamatergic receptors in different models of brain ischaemia has been widely studied, however, the literature about the role of the glutamatergic transporters in the ischaemic models is scarce and focused on the cerebral focal ischaemia. Therefore this study was addressed mainly in the role of the glutamatergic transporters.

For these reasons, the main **aim of this study is to get insight on the modulatory effect of GABA and inflammation on the glutamatergic response to the ischaemia-reperfusion** and it has been divided in the following objectives:

1) To characterize the response of membrane and vesicular glutamatergic transporters in an in vivo model of global cerebral ischaemia. In contrast with focal cerebral ischaemia, the animal models here studied only present penumbra area (but not ischemic core) and therefore its damage is more homogeneus than the characteristic gradient in the penumbra area in focal ischaemia. The study of the glutamatergic transporters complements previous

studies performed in our laboratory on the AMPA and NMDA glutamatergic receptors. The role of age and the presence of antiinflammatory agents are also included in this objective.

2) To determine the neuroprotective effect of GABA on:

2a) Cell mortality and glutamatergic transporter expression in an oxygen and glucose deprivation (OGD) model of brain slices.

2b) Endoplasmic reticulum stress (ER stress) measuring the unfolded protein response (UPR) in the OGD model of brain slices.

3) To determine the neuroprotective role of antiinflammatory agent meloxicam on cell mortality and glutamatergic transporters expression in an OGD model of hippocampal organotypic culture.

Aim 1:

"Age and inflammation modify the response of the glutamate vesicular transporters (VGLUTs) to the transient global cerebral ischaemia in the rat brain"

INTRODUCTION

Glutamate has long been recognized to play key roles in the pathophysiology of cerebral ischaemia (Castillo et al. 2003), one of the most relevant causes of death in industrialized countries (Rosamond et al. 2008). Glutamate levels are regulated at the synaptic cleft both by plasmatic membrane and vesicular transporters. Different types of excitatory aminoacid transporters (EAATs), which are located in the plasmatic membrane either of neurons, glial cells or both have been described (Danbolt 2001). EAAT1 and EAAT2 have been reported to be present in astrocytes (Chaudhry et al. 1995), whereas EAAT3 and EAAT4 are considered to be neuronal (Furuta et al. 1997). Biophysical properties of EAAT3 and 4 allow them to act as glutamate buffers and seem to maintain normal extracellular glutamate concentration, excessive glutamate being withdrawn from the synapse by EAAT1 and 2, ultimately preventing excitotoxicity (Tzingounis and Wadiche 2007). Vesicular glutamate transporters (VGLUTs) 1, 2 and 3 play a role in the regulation of intracellular glutamate levels by modulating its incorporation into synaptic vesicles (Shigeri et al., 2004). Thus, these transporters may have an important role in the excitotoxicity following an ischaemia-reperfusion (I/R) challenge.

Regional differences in the expression of VGLUTs have been described by in situ hybridization showing that VGLUT1 mRNA can be found in cerebral cortex, cerebellar cortex, hippocampus and thalamus while VGLUT2 appears mainly in mesencephalon and the brainstem (Fremeau et al. 2001; Herzog et al. 2001; Hisano et al. 2000; Ni et al. 1994). Thus, VGLUT1 and 2 have been reported to present a complementary distribution in the cortex (Fujiyama et al. 2001) and

they are also present in the caudate-putamen (Herzog et al. 2004). VGLUT3 is less abundant than VGLUT1 and VGLUT2 and has been found in cortex and caudate-putamen, among other structures (Herzog et al. 2004). The distribution of VGLUT1 and VGLUT2 accounts for the ability of most established excitatory neurons to release glutamate by exocytosis. The complementary pattern of expression in adult brain might reflect differences in membrane trafficking. By contrast, VGLUT3 differs from VGLUT1 and VGLUT2 in its subcellular location (Fremeau et al. 2004).

An increase of VGLUTs in synaptosomes leads to higher levels of glutamate in the synaptic cleft (Wilson et al. 2005). However, data on the role of VGLUTs in the pathophysiology of ischaemia are scarce. Evidences of ischaemia-dependent changes in the expression of VGLUTs as well as their possible interplay with EAATs in the regulation of glutamate levels have been reported recently in a focal ischemia model (Sánchez-Mendoza et al. 2010).

Both age and inflammation play critical roles in the response to ischaemia and, therefore, they should be analyzed for an insight into the role of VGLUTs after an I/R insult. Data on the effect of inflammation on VGLUTs are lacking but they are reported to be modified by age (Canas et al., 2009). We have previously described ischemia-induced damage and modifications in the glutamatergic receptors at 48h after global ischemia and in similar conditions to those described in this report (Dos-Anjos et al. 2009a, b; Montori et al. 2010a, b, c). In an attempt to understand how the whole glutamatergic system responds to ischemia we here report for the first time that the control of the expression of the vesicular glutamate transporters in both mRNA and protein levels changes depends on age and inflammation.

MATERIALS AND METHODS

Animals

Twenty-three 3-month-old and twenty 18-month-old Sprague-Dawley male rats weighing 350-450 g and 650-800 g respectively were housed under standard temperature (22 ± 1 °C) in a 12 h light/dark controlled environment with “ad

libitum" food and water intake. Rats were divided randomly into ischaemic and sham groups. Experiments were carried out in accordance with the Guidelines of the European Union Council (86/609/EU), following Spanish regulations (RD 1201/2005, BOE 252/34367–91, 2005) for the use of laboratory animals, and were approved by the Scientific Committee of the University of León. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Transient global ischaemia

Each animal was placed in the anaesthesia induction box supplied with 4% halothane (Sigma-Aldrich, Madrid, Spain) at a rate of 3 L/minute in 100% oxygen. After induction, anaesthesia was maintained with 1.5% to 2.5% halothane (Fluothane, AstraZeneca1) at 800 mL/minute in 100% oxygen, delivered via a rat face mask. Both common carotid arteries were exposed through a midline incision and transient global ischaemia was induced by bilateral common carotid artery occlusion for 15 min with traumatic aneurysm clips and a moderate hypotension (40-50 mm Hg). The femoral artery was exposed and catheterized to administrate 15 mg/mL, 0.3 mg/min of the hypotensor agent trimetaphan (kindly provided by Roche) and allow continuous recording of arterial blood pressure. Furthermore, the intrinsic hypotensive effect of halothane (Bendel *et al.* 2005) was also used to control the hypotension. A feedback-regulated heating pad was used to maintain the rat temperature at 36 ± 1 °C during surgery. When animal arterial blood pressure recovered, animals were sutured and, after regaining consciousness, they were maintained in an air-conditioned room at 22 ± 1 °C during 48 hours (time of reperfusion). On sham-operated rats, all procedures were performed just like on ischaemic animals, except the carotid arteries were not clamped.

Anti-inflammatory treatment

Two days after global ischaemia, the animals were decapitated and their brains were rapidly removed. Sagital slices of approximately 1 mm were obtained with

a surgical blade and CA1, CA3 and dentate gyrus (DG) were dissected from each slice using a dissecting microscope. The different samples as well as the whole cerebral cortex were then frozen and kept at -80 ºC until used.

Tissue processing

Two days after the global ischaemia, the animals were decapitated and their brains were rapidly removed. Sagital slices of approximately 1 mm were obtained with a surgical blade and CA1, CA3 and dentate gyrus (DG) were dissected from each slice using a dissecting microscope. The different samples as well as the whole cerebral cortex were then frozen and kept at -80 ºC until used.

RNA extraction and reverse transcription (RT)

Total RNA was extracted using the Tripure™ Isolation Reagent (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. The contaminating DNA in the RNA samples was removed by incubation with DNase (Sigma-Aldrich, Madrid, Spain) and confirmed by PCR analysis of total RNA samples prior to RT. After isolation, the integrity of the RNA was assessed using the Experion RNA HighSens Analysis Kit (Biorad Laboratories, Wilmington, USA), following the manufacturer's instructions. The yield of total RNA was determined by measuring the absorbance (260/280 nm) using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). RNA obtained was comparable in young and older animals (0.5-0.7 µg/mg of tissue). RT was performed using random hexamers primers, 600 ng of total RNA as a template and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the recommendations of the manufacturer.

Real time PCR

After RT, cDNA was diluted in sterile water and used as template in real time PCR assays. All mRNA sequences were obtained from GenBank. Primers were

designed using Primer Express software (Applied Biosystems, Foster City, CA, USA) and all of them flanked an intronic sequence to ensure the absence of genomic contamination. The forward and reverse primers used in this study are shown in Table 1. Real time PCR was performed on an ABI PRISM 7000 real time thermal cycler using the SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). In order to determine the optimal cDNA quantity and primer concentration, a standard curve was constructed using increasing amounts of cDNA and different concentrations of primers. The optimal PCR conditions were obtained using 2 µL of 1/10 cDNA dilution as template and 300 nM of each primer. The gene used as reference was the glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Gene	**5´-3´ Forward Primer**	**5´-3´ Reverse Primer**	**Accession Number**
VGLUT1	TGGGTTTCTGCATCAGCTTTG	TGTACTGTTGTTGACCATGGATACG	**NM_053859**
VGLUT2	CGTGAAGAATGGCAGTATGTCTTC	TGAGGCAAATAGTGCATAAAATATGACT	**NM_053427**
VGLUT3	CCTTCCTGGTGCTTGCTGTAG	GGCATATCGTGGAGCAATGTC	**NM_153725**
GAPDH	GGGCAGCCCAGAACATCA	TGACCTTGCCCACAGCCT	**NM_017008**

Table 1. Primers used in real time PCR studies and the GenBank accession numbers.

Increases in the amount of SYBR® Green reporter dye fluorescence during the amplification process were analyzed with a Sequence Detector Software (SDS version 1.6, Applied Biosystems). The mRNA levels of each gene GAPDH-normalized were expressed as $2^{-\Delta Ct}$, while the relative change in the mRNA levels of the genes studied following I/R with respect to the sham-operated group was determined by the equation:

Fold change = $2^{-\Delta\Delta Ct}$

ΔCt = (Ct target – Ct GAPDH);

$\Delta\Delta Ct$ = (Ct target – Ct GAPDH) ischaemic animals – (Ct target – Ct GAPDH) sham-operated animals (Livak and Schmittgen, 2001).

Western blot

Total protein was extracted using Tripure™ Isolation Reagent (Roche Applied Science,Mannheim, Germany) following the manufacturers' instructions. Protein concentrations were determined using the Bradford method (Biorad, Hercules, CA, USA) and stored in urea 8 M, 4% SDS in the presence of a protease inhibitor (Complete proteases inhibitor cocktail, EDTA Free; Roche Applied Science, Mannheim., Germany). Protein samples (20 µg per lane) were loaded an run on a 12% polyacrylamide gel (SDS PAGE, BioRad, Hercules, CA, USA) at 110V for 120 minutes and then transferred to a nitrocellulose membrane using a dry transfer system (Invitrogen) at 20V for 7 minutes. Membranes were blocked in 5% non-fat milk, 0.2% Tween-20 in Tris-buffered saline (TBS-T) for 60 min at room temperature and incubated overnight at 4ºC, with the following primary antibodies: a rabbit polyclonal antibody against the VGLUT1 (1:3000) (Synaptic Systems, Goettingen, Germany); a rabbit polyclonal antibody against the VGLUT2 (1:3000) (Systems, Goettingen, Germany) and a mouse monoclonal antibody anti-β-actin clone AC-74 (1:5000) (Sigma-Aldrich, Madrid, Spain). Then, membranes were incubated with the appropriate secondary antibodies (Dako, Glostrup, Denmark), anti-rabbit or anti-mouse horseradish peroxidase-conjugated, at a dilution of 1:3000 and developed using the Chemiluminescence Luminol Reagent (Santa Cruz Biotech, Madrid, Spain). Following the recommendations of Synaptic Systems proteins, samples were not boiled to label VGLUTs.

Statistical analysis

All results are expressed as mean ± SEM and our data fit significantly to a normal distribution according to a standardized Kurtosis test. Two-way ANOVA tests were conducted to look for interactions between age and ischaemia or between meloxicam treatment and ischaemia. This test was followed by the pos-hoc non parametric Bonferroni test for the data comparison at each age (3 months or 18 months old) or treatment (meloxicam treated or non-treated

animals) between the ischaemic group and its corresponding sham-operated group. Bonferroni test were also used to compare the changes of mRNA levels and protein amounts for each gene by comparing the effects of age, I/R and meloxicam treatment. The significance was set at the 95% confidence level. The statistical analysis was carried out using Graph Pad Prism 4 (Graph Pad software, San Diego, CA, USA).

RESULTS

Age-dependent VGLUT mRNA levels

Comparisons between VGLUT1, VGLUT2 and VGLUT3 mRNA levels showing ratios OS/YS and ratios OI/R/YI/R can be observed in Figure 1. We failed to find age-dependent differences in VGLUT1 and VGLUT3 mRNA levels in any of the structures studied, although we observed an age-dependent decrease in VGLUT2 mRNA levels. The age-dependent response to the ischemic insult measured as the ratio OI/R/YI/R revealed that levels of VGLUT1 and VGLUT3 mRNAs are significantly high in the hippocampus of the old animals while the levels of VGLUT2 mRNAs are significantly low. This contrast in the response of VGLUT genes in the hippocampus was not detected in the cerebral cortex.

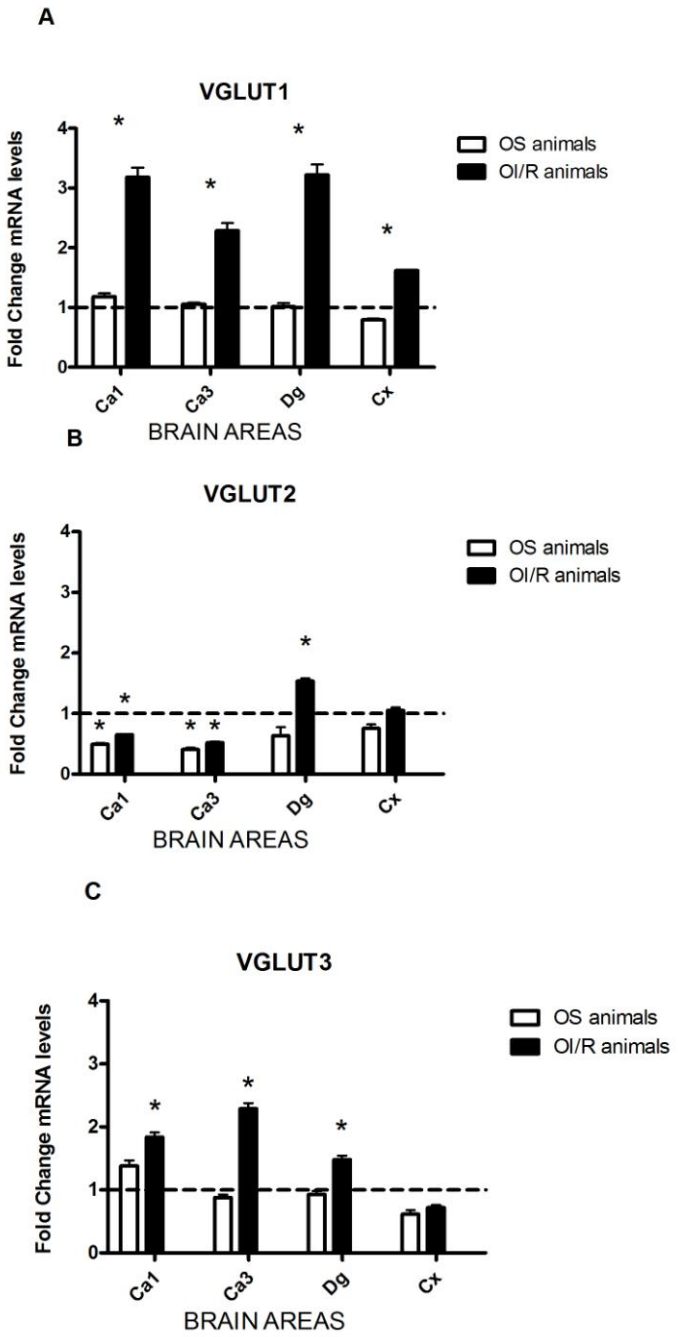

Figure 1. Effect of age. Fold change ($2^{-\Delta\Delta Ct}$) in the VGLUT1 (A), VGLUT2 (B) and VGLUT3 (C) mRNA levels between old and young sham-operated animals (OS/YS) and old and young injured animals (OI/R/YI/R) (YS and YI/R are represented by value 1, indicated by the dotted horizontal line) in the hippocampal CA1, CA3 and dentate gyrus (DG) areas and in cerebral cortex (CX).Notice the outstanding different response of VGLUT1 and VGLUT3 compared to those of VGLUT2. Significant differences are indicated by * ($p<0.05$). (Two-way ANOVA; n=5).

I/R effect on VGLUT mRNA levels

Comparisons between insulted and sham-operated animals can be observed in Figure 2, where sham-operated animals are indicated as value 1 (dotted line). The I/R elicits a decrease of VGLUT1, VGLUT2 and VGLUT3 in all of the hippocampal areas and cerebral cortex in young animals. The I/R-insult in older animals results in different responses depending on the structure and the gene analyzed, but the differences between the mRNA levels of the OI/R animals with respect to the OS ones were less outstanding than those observed in the comparison between YI/R and YS groups. In cerebral cortex, we failed to detect significant changes between OI/R and OS in any of the genes analyzed following the challenge. In the hippocampus, most of the VGLUT1, VGLUT2 and VGLUT3 transcripts were significantly lower in OI/R than in OS. However, those of VGLUT2 in CA1 were similar in OI/R and OS, and VGLUT3 mRNA levels in CA3 were significantly higher in OI/R than in their corresponding sham animals.

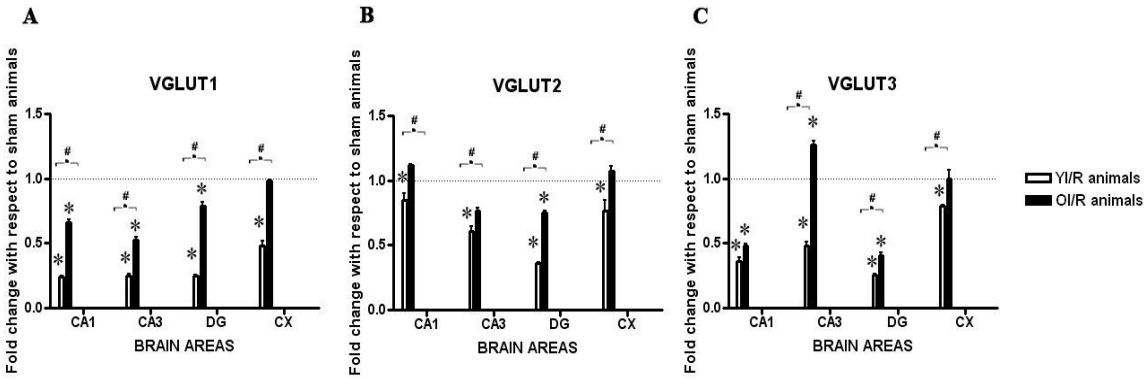

Figure 2. Effect of ischaemia/reperfusion. Fold change ($2^{-\Delta\Delta Ct}$) in the VGLUT1 (A), VGLUT2 (B) and VGLUT3 (C) mRNA levels between injured animals and sham-operated animals (value 1, indicated by the dotted horizontal line) in the hippocampal CA1, CA3 and dentate gyrus (DG) areas and in cerebral cortex (CX) for each age group. Significant differences with respect to sham-operated animals are indicated by * ($p<0.05$). Notice the age-dependent pattern of response. Significant differences between YI/R and OI/R animals are indicated by # ($p<0.05$). Two-way ANOVA (n=5).

VGLUT 1 and VGLUT2 protein levels

Data from Western blot assays show lower VGLUT1 amounts in YI/R with respect to YS in the hippocampus, but similar amounts were observed in both experimental conditions in the cerebral cortex. Increases in VGLUT1 amounts in

the cerebral cortex and CA1, no changes in CA3 and decreases in DG were observed when comparing OI/R and O/S. VGLUT2 amounts in CA1, CA3 and CX of YI/R were higher than those of YS but lower in DG. Interestingly, VGLUT2 amounts in CA3, DG and CX of OI/R were lower than those of OS but higher in CA1 (Figure 3 y 4).

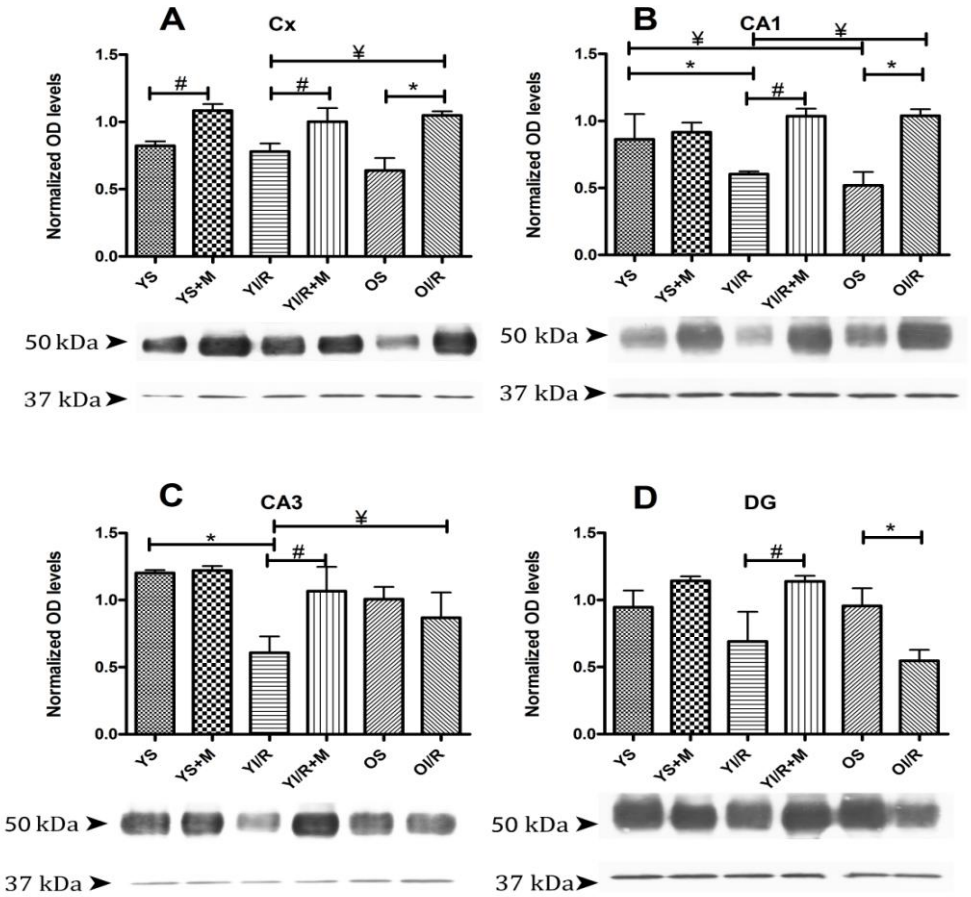

Figure 3. Effect of I/R, age and meloxicam on VGLUT1 (50 kDa) amounts in Cx (A), CA1 (B), CA3 (B) and GD (D). Western blot assays show the protein levels of VGLUT1 and their respective quantifications (Mean ± SEM) normalized with β-actin (37 KDa) in young and older sham (lanes 1 and 5; YS and OS respectively) and injured animals (lanes 3 and 6; YI/R and OI/R respectively). The figure also shows the protein levels of VGLUT1 in young sham and injured meloxicam treated animals (lanes 2 and 4; YS+M and YI/R+M respectively) in the different structures studied. ¥ shows age-dependent significant differences, * indicates I/R significant effect and # shows significant differences as a consequence of meloxicam treatment. ($p<0.05$). Two-way ANOVA (n=5).

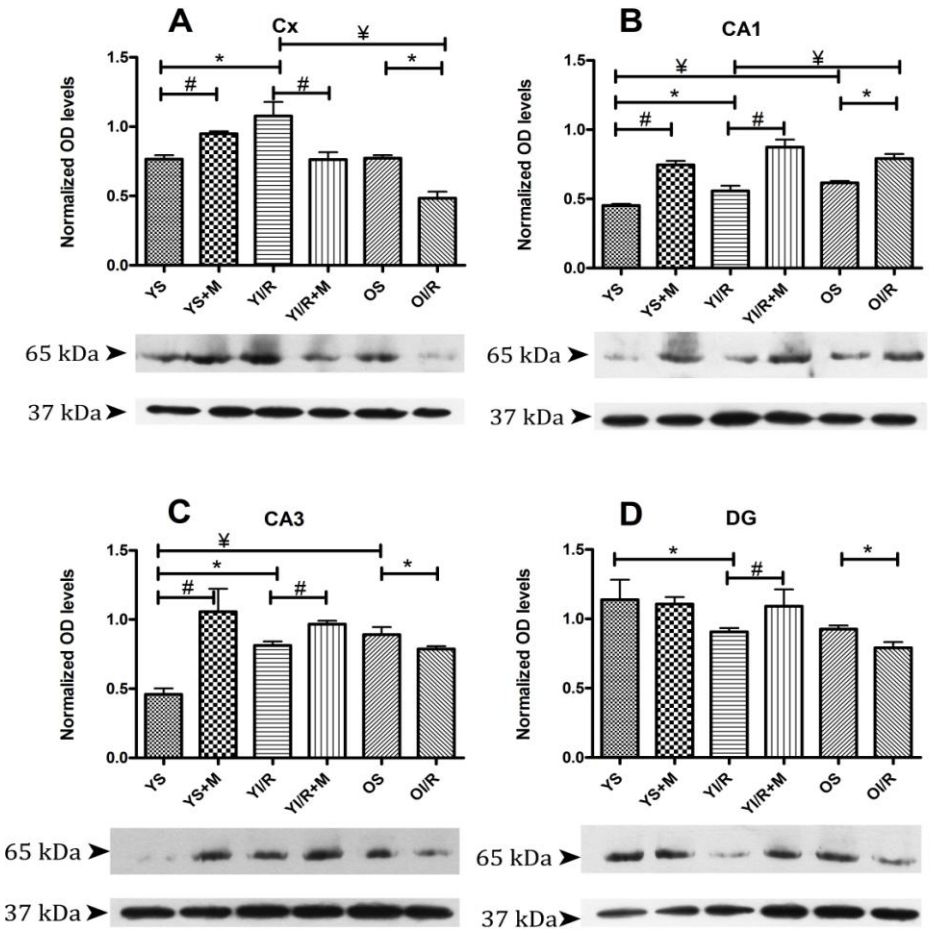

Figure 4. Effect of I/R, age and meloxicam on VGLUT2 (65 kDa) amounts in Cx (A), CA1 (B), CA3 (B) and GD (D). Western blot assays show the protein levels of VGLUT1 and their respective quantifications (Mean ± SEM) normalized with β-actin (37 KDa) in young and older sham (lanes 1 and 5; YS and OS respectively) and injured animals (lanes 3 and 6; YI/R and OI/R respectively). The figure also shows the protein levels of VGLUT2 in young sham and injured meloxicam treated animals (lanes 2 and 4; YS+M and YI/R+M respectively) in the different structures studied. ¥ shows age-dependent significant differences, * indicates I/R significant effect and # shows significant differences as a consequence of meloxicam treatment. ($p<0.05$). Two-way ANOVA (n=5)

Effect of the meloxicam treatment on VGLUT mRNA levels

The treatment with meloxicam after the ischaemic insult results in noticeable differences for the different VGLUT gene mRNAs (Figure 5). Thus, VGLUT1 mRNAs in animals treated with meloxicam displayed similar levels (no significant differences) between insulted and sham-operated animals, with the exception of the hippocampal CA1, where a significant decrease is also observed, yet it is not so pronounced as in non meloxicam-treated animals (see Figure 5A).

VGLUT2 mRNA levels from insulted animals treated with meloxicam displayed significant increases in CA1 and DG with respect to the sham-operated ones, while CA3 and CX did not show significant differences after challenge (Figure 5B).

VGLUT3 mRNA levels in animals treated with meloxicam tend to decrease following I/R, although differences were not always significant with respect to the sham-operated ones (Figure 4C). VGLUT3 mRNA levels in the hippocampus of insulted untreated animals were significantly lower than those of meloxicam-treated ones, but no differences were observed in the VGLUT3 mRNA levels in CX. Two-way ANOVA analyses show significant interactions between meloxicam treatment and ischaemia for most of the structures and genes studied.

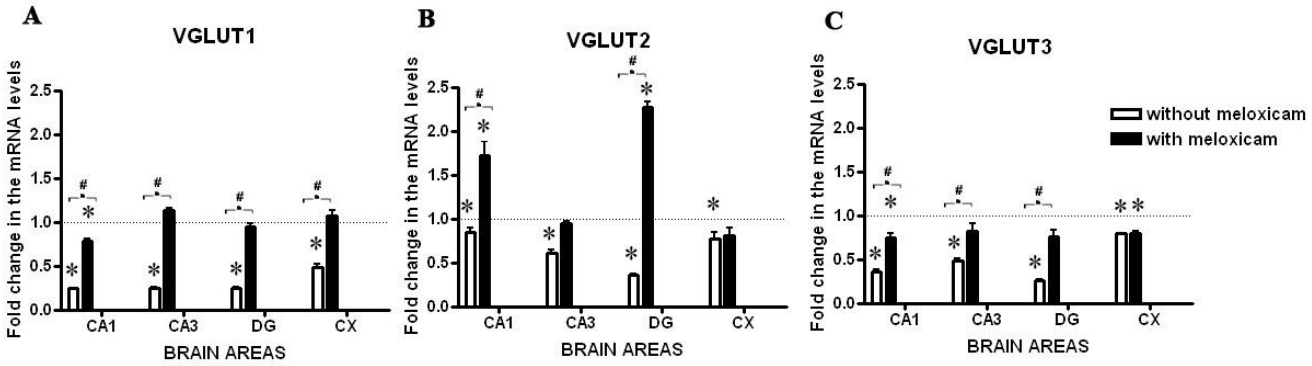

Figure 5. Effect of the meloxicam treatment. Fold change ($2^{-\Delta\Delta Ct}$) in the VGLUT1 (A), VGLUT2 (B) and VGLUT3 (C) mRNA levels between injured animals and sham-operated animals (value 1, indicated by the dotted horizontal line) in the hippocampal CA1, CA3, dentate gyrus (DG) areas and in cerebral cortex (CX) for meloxicam-treated or non-treated rats. Significant differences with respect to sham-operated animals are indicated by * ($p<0.05$). Significant differences between treated and untreated injured animals are indicated by # ($p<0.05$). Two-way ANOVA (n=5).

Effect of the meloxicam treatment on VGLUT protein levels

Data analyses obtained from Western blot assays for animals treated with meloxicam show significant increases in the amounts of VGLUT1 for injured and sham-operated animals when compared with their respective untreated

animals, but we failed to detect this increase in CA1 and CA3 in YS+M. Increases of VGLUT2 amounts in young sham animals treated with meloxicam can be observed in the cerebral cortex, CA1 and CA3 when compared to YS. The treatment with meloxicam in injured animals resulted in a significant increase in all hippocampal structures contrasting with a decrease in the cerebral cortex with respect to YI/R (Figure 3 y 4).

DISCUSSION

Regional distribution differences in the expression of VGLUTs have been previously described by in situ hybridization, although partial overlapping is observed in some brain areas and VGLUT1 and VGLUT2 are considered to have a complementary distribution (Fremeau *et al.* 2001; Herzog *et al.* 2001; Hioki *et al.* 2003; Hisano *et al.* 2000; Ni *et al.* 1994; Sakata-Haga *et al.* 2001). It is worth noting that glutamatergic neurons express VGLUT1 and VGLUT2 isoforms (Bellocchio *et al.* 1998; Fremeau *et al.* 2001; Fujiyama *et al.* 2001; Herzog *et al.* 2001; Kaneko *et al.* 2002; Sakata-Haga *et al.* 2001; Varoqui *et al.* 2002), although the expression of these isoforms by astrocytes has been proved in cultured and freshly isolated astrocytes from rat visual cortices (Montana *et al.* 2004). This could be explained by the PCR amplification. PCR allows the detection of mRNA levels lower than those detectable by *in situ* hybridization, indicating that most of the VGLUT2 and VGLUT3 transcription mainly takes place in areas other than cerebral cortex and hippocampus, in agreement with the literature reports mentioned above, but it also indicates that some of the expressions of these genes can be observed in these areas.

Age-related changes

Levels of VGLUT1 and VGLUT2 have been reported to decrease with aging (Canas *et al.* 2009), however our data for sham operated animals from both PCR and VGLUT1 protein show that this decrease occurs in the cerebral cortex but not in the hippocampus, as shown in figure 1A and 4A. VGLUT3 mRNA levels roughly behave as VGLUT1, also contrasting with the decrease in the

expression of VGLUT2 mRNA levels in all structures. It must be noted that there are some discrepancies between VGLUT2 mRNAs and VGLUT2 protein amounts, however, it should also be born in mind that VGLUT2 protein amounts reflect changes in both hippocampus and subcortical neurons projecting to the hippocampus, while mRNA VGLUT2 protein amounts indicate changes limited to the hippocampus. Anyway, differences in the expression of these genes seem to be age and structure dependent. The comparison between young and aged animal response to the ischaemic insult also shows that VGLUT1 and VGLUT3 behave in a similar way in hippocampus (figure 1A and 1C) as opposed to VGLUT2, consistently with data from OS/YS ratios. However, the different VGLUTs behave differently in Cx, contrasting with OS/YS ratios. Data here presented indicate a lessened age-dependent hippocampal response to ischaemic insult in a similar way to that reported for EAATs or some glutamate receptor subunits (Montori *et al.* 2010a, b, c). It must be noticed that the response in cerebral cortex is lessened compared to that observed in the hippocampus, in agreement with previous data of glutamatergic receptors and EAATs (Dos_Anjos *et al*. 2009a,b; Montori *et al*. 2010a,b,c), evidencing that differential responses to ischemic insult in cerebral cortex and hippocampus observed in young animals remain in the aged animals.

Ischaemia/reperfusion

Some controversy appears in the literature regarding the effect of ischaemia on VGLUTs. Previous data using an ischaemia focal model report variations in the VGLUT1 and VGLUT2 amounts during the time following the insult, indicating changes in VGLUT1 in the cerebral cortex at 72 hours after challenge but not at 24 hours (Sanchez-Mendoza *et al.* 2010). However, these authors describe VGLUT2 and VGLUT3 decreases in the cerebral cortex at 1, 3 and 7 days after insult. They also report changes in the expression pattern of VGLUTs in the cerebral cortex (Sanchez-Mendoza *et al.* 2010). Unaltered VGLUT1 and VGLUT2 immunoreactivities have been described in the hippocampus during the first 3 days after ischaemia-reperfusion in a gerbil model of transient

ischaemia. In our model, modifications in the VGLUT gene transcription can already be observed at 48 hours after insult.

VGLUT1 protein amounts decrease in YI/R animals with respect to YS in the hippocampus, in agreement with PCR data, thus confirming the down-regulation in the expression of this gene in this area 48 hours after the challenge. We failed to detect significant differences in VGLUT1 amounts in the cerebral cortex, despite decreases in VGLUT1 mRNA levels had been observed, which suggests differences in the VGLUT1 protein turnover in hippocampus and cerebral cortex following ischaemic damage. This would agree with the previously reported differential vulnerability to ischaemia of these areas (Gee *et al.* 2006; Jiang *et al*. 2004; Kumari Naga *et al*. 2007; Stanika *et al*. 2010; Vallet and Charpiot, 1994). Interestingly, age noticeably modifies the response of VGLUT1 to ischaemic insult. In contrast with the decreases observed for young animals, we here detected an increase in this protein in OI/R, which does not fit their corresponding OI/R transcripts. Both mRNA and protein response indicate that the control mechanisms of VGLUT1 expression following ischaemia are modified by age and differences between mRNA levels and protein amounts suggest that the mechanism of degradation of this protein has been slowed down.

VGLUT2 western blot assays in YI/R show increases in protein amounts in some structures (CA1 and CA3) with respect to YS that do not fit with mRNA level decreases observed in all of the structures studied. Some discrepancies also appear between the OI/R protein amounts and the mRNA levels when these are compared to those of the OS. As previously mentioned, discrepancies between mRNA and proteins are not strange and reflect differences in the control of the gene expression at different stages (Gorospe *et al*. 2011; Mitchell and Tollervary, 2001; Wilusz *et al.* 2001a,b). Protein post-translational modifications, such as acetylation by acetyltransferases and deacetylases, modify the protein turnover (Sadoul *et al.* 2008). However, the general picture displays an age-dependent lessened effect of VGLUT2 following ischaemic insult. This response is less outstanding than that observed for VGLUT1, although this may stem from the differences in the expression level of VGLUT1 and VGLUT2 (100:1 in mRNA levels).

Since the lack of a suitable antibody prevents us from comparing transcripts and protein levels, we can only assume that VGLUT3 expression pattern is similar to that of VGLUT1. We should note that the low amounts of VGLUT2 probably make them undetectable by in situ hybridization as indicated by Fremeau *et al*. 2004. However, real time PCR magnification allows us to detect the presence of cells expressing both VGLUT2 and VGLUT 3 in the hippocampus.

Meloxicam

Western blots confirm the results of PCR showing that the treatment with meloxicam increases the expression of VGLUT1 in injured animals. Therefore, the COX2 inhibition seems to control the gene expression of VGLUT1. Western blot assays for VGLUT2 also confirm the meloxicam treatment-dependent increases observed in real time PCR data in the hippocampus and therefore the influence of COX2 on VGLUT2 gene expression control in the hippocampus. The results of this study also show that meloxicam treatment following insult results in a lessened response in both VGLUT1 and VGLUT2 mRNA levels as well as in increases in protein amounts. These results show the role of inflammation in the control of the expression of these transporters as previously described for other glutamatergic genes (Montori *et al.* 2010a, b). The results for VGLUT1 do not seem to be related to differential vulnerability, as described for other genes (Berger and Hediger 1998; Chen *et al*. 2004; Montori *et al.* 2010c; Torp *et al*. 1994). However, in spite of the low amount of VGLUT2, the outstanding increases observed in VGLUT2 mRNA levels and protein amounts in CA1 and DG may be an reflection of the higher vulnerability to ischaemia of these hippocampal areas compared to the lesser vulnerability of CA3 and cerebral cortex (Davolio and Greenamyre 1995; Ordy *et al.* 1993; Wilde *et al.* 1997; Yang *et al.* 2000).

When the respective responses of VGLUTs and EAATs to I/R, age and meloxicam are compared, the patterns of VGLUTs transcriptional response are similar to those observed in the EAATs expressed in neurons, and differ from the outstanding I/R-induced increases described for glial transporter GLAST1a

(Montori *et al.* 2010c), which supports the idea that I/R-induced changes in the expression of VGLUTs are mainly related to changes in neurons rather than in glial cells.

CONCLUSIONS

Our data indicate an age-dependent response to the ischaemic damage of VGLUT1 and VGLUT3 while VGLUT2 response presents an age and structure-dependent response to this challenge. The inhibition of COX2 by the treatment with the anti-inflammatory agent meloxicam following ischaemic challenge results in an increase of the protein expression of VGLUT1 and VGLUT2 and a lessened response to ischaemia of all of the VGLUT genes.

Aim 2a:

"$GABA_A$ provides neuroprotection in the cerebral cortex but not in the hippocampus in an OGD brain slices model"

INTRODUCTION

Cerebral ischemia induces an imbalance of excitation and inhibition, which impairs normal brain function. Glutamate is the major excitatory neurotransmitter and is considered one of the key factors in cerebral ischemia-induced cell death. γ- aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system (CNS). Shortly after the onset of ischaemia, GABA and glutamate rapidly accumulate in the intracellular space (Luccini *et al*., 2010). High extracellular glutamate contributes to excitotoxicity primarily through overexcitation of the N-Methyl- D-aspartate (NMDA) glutamatergic receptors, resulting in calcium overloading, ultimately causing necrosis or apoptosis (Lobo *et al*., 2011). It has been postulated that the simultaneous increase in the levels of GABA, may be an intrinsic protective mechanism to elevate synaptic inhibition and counteract this excitotoxicity (Green *et al*., 2000; Clarkson *et al*., 2010). However, there is evidence demonstrating that glutamate-evoked depolarisation of cell membranes during ischaemia induces a substantial Cl^- influx through $GABA_A$ receptor channels, thereby creating a strong osmotic gradient, ultimately resulting in water influx and swelling of the cell (Allen *et al*., 2004; Ricci *et al*., 2011). Shortly after ischaemia, the high levels of GABA in brain can inactivate, or reduce, the number of surface $GABA_A$ receptors (Alicke and Schwartz-Bloom, 1995; Allen *et al*., 2004; Ricci *et al*., 2011). Chronic exposure to GABA decreases

transcription of $GABA_A$ receptor mRNAs (Baumgartner *et al*., 1994; Fénelon and Herbison., 1996) and reduce GABA synthesis (Sheikh and Martin, 1998). This potentially reduces further damage from depolarisation and swelling (although it should be noted that Cl^- entry is not soley attributable to $GABA_A$ receptors, therefore inactivation of $GABA_A$ receptors alone will not completely abolish swelling; Ricci *et al*., 2011).

Glutamate levels are regulated at the synaptic cleft both by plasmatic membrane and vesicular transporters. Five different types of excitatory amino-acid transporters (EAATs) are located at the plasma membrane of either, neurons, glial cells, or both in humans and rodents (Danbolt, 2001). EAAT1 and EAAT2 (the rodent orthologues are GLAST and GLT-1 respectively) are present in astrocytes (Chaudhry *et al*., 1995), whereas EAAT3 (the rodent orthologue is EAAC1) and EAAT4 are predominantly neuronal (Furuta *et al*., 1997). EAAT3 and EAAT4 function as glutamate buffers, maintaining normal extracellular glutamate concentration, while excess of glutamate is removed from the synaptic cleft by EAAT1 and EAAT2, thereby, preventing excitotoxicity (Tzingounis and Wadiche, 2007). Vesicular glutamate transporters (VGLUT1-3) regulate intracellular glutamate levels by modulating its incorporation into synaptic vesicles (Shigeri *et al*., 2004). As build up of glutamate results in excitotoxicity and ultimately cell death (Lobo *et al*., 2011), these transporters may have an important role in the excitotoxicity that follows an ischaemia-reperfusion (I/R) challenge.

Gene expression control is regulated at different levels which in many cases results in a lack of correlation between mRNA and protein levels (Wilusz *et al*., 2001a,b ; Wouters *et al*., 2005; Sadoul *et al*., 2007; Gorospe *et al*., 2011). Thus,

the expression of different proteins may not completely reflect the early cell response following ischaemic damage and the analysis of transcriptome would provide a more accurate insight of the first response of the cell. Thus, the aim of this study is to demonstrate that blocking $GABA_A$ receptors with bicuculline during oxygen glucose deprivation (OGD) and reperfusion (RL) elicits a differential early cell response between hippocampus and cortex in the transporter transcripts that control the glutamate levels at the synaptic gap. As a control of the effect of OGD and RL on the post and presynaptic GABAergic system, we included in this study the transcript levels from some of the most relevant $GABA_A$ receptor subunits in both cerebral cortex and hippocampus (alpha1, beta2 and gamma2) which usually appear in the postsynaptic cell, as well as one of the typical markers of the presinaptic GABA terminals (GAD65). Finally, we correlated this early response with neuronal survival.

MATERIALS AND METHODS

Animals

Experiments were carried out in accordance with the Guidelines of the European Union Council (86/609/EU), following Spanish regulations (BOE 67/8509–12, 1988) for the use of laboratory animals, and were approved by the Ethics Committee of the University of León. All efforts were made to minimize animal suffering and to reduce the number of animals used. In total, 10 male rats were used. They were housed at 22 ± 1°C in a 12-h light/dark controlled environment with free access to food and water.

Preparation and incubation of slices (induction of OGD and reperfusion)

Two-month-old Sprague–Dawley rats were sacrificed by decapitation, the forebrain removed and the rostral, caudal and lateral portions of the hippocampus excised. The hippocampus was cut sagitally along the interhemispheric fissure and sagittal sections comprising hippocampus and cortex (350 μm) were taken from the lateral to the medial part using a vibratome. Prior to sectioning, tissue was submerged in a cold (4°C) cutting solution (CS) bubbled with carbogen (95% O2 and 5% CO2), containing 120 mM NaCl, 2 mM KCl, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11 mM glucose and 20 mM sucrose, pH 7.4 (Merck, Madrid, Spain). After cutting, slices were transferred into pre-incubation solution (PS) at 25°C (identical composition to CS but without sucrose), and bubbled with carbogen for a minimum of 30 min before transfer into an incubation solution (IS, containing; 120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.19 mM $MgSO_4$, 1.18 mM KH_2PO_4 and 11 mM glucose, pH 7.4; Merck, Madrid, Spain) at 25 °C, aerated with carbogen. Sections were transferred from the IS into individual cell strainers (100 micron; BD Falcon,Bedford, MA, USA) and submerged in 2 ml of the solutions under one of four different experimental conditions: 1) IS alone; 2) IS + 100 μM GABA , 3) IS + 1 μM bicuculline (to block voltage-dependent chloride channels). Osmolarity in all the solutions was 300±5 mOsm. Two different experimental procedures were carried out on tissue for each treatment: A) OGD procedure (30 minutes) and B) RL procedure, in order to compare to the reperfusion stage that follows ischemia "in vivo" (for 180 min after the 30 min OGD). The OGD procedure was carried out for each treatment using IS without glucose and aerated with 95% N_2 + 5% CO_2.

Bubbling with this gas mixture must be performed at least for an hour prior to the incubation to ensure that oxygen in the solution is less than 10%, which was measured (HI 3818; Hanna Instruments, Spain). Parallel tissues of each treatment, but not subjected to OGD conditions were maintained as respective controls.

After the appropriate incubation times, under the chosen experimental conditions, samples were collected from the IS to determine lactate dehydrogenase (LDH) activity, after which slices were collected and immediately frozen over dry ice.

LDH activity assay

LDH is a commonly used marker of cell death that is released from damaged cells into the IS. LDH activity was measured spectrophotometrically at 492 nm, using the Citotoxicity detection Kit (Roche Diagnostics, Mannheim, Germany) in a HT Synergy microplate reader (Synergy HT, Bio-Tek, Winooski, VT, USA) Data are expressed as optical density (OD) and show LDH release as a percentage of the IS condition.

Real time PCR

RNA extraction

Total RNA was extracted using the Tripure™ Isolation Reagent (Roche Applied Science, Barcelona, Spain), according to the manufacturer's instructions. Any

contaminating DNA in the RNA samples was removed by incubation with DNase (Sigma Aldrich, Madrid, Spain) and confirmed by PCR analysis of total RNA samples prior to reverse transcription (RT). Total RNA concentrations were determined using a NanoDrop ND-3300 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Isolated RNA was maintained at -80 °C until further processing.

Reverse transcription (RT)

RT was completed with the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), following manufacturers instructions. Reactions were performed for 10 min at 25 °C, 2 h at 37 °C and terminated by heating for 5 sec at 85 °C. The reaction mixture was maintained at -20 °C until further use.

Quantitative real-time PCR

Quantitative real-time PCR of the different mRNAs was performed in triplicate using gene-specific primers and SYBR® Green. Oligonucleotide primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). The primer sequences and corresponding GenBank accession numbers are given in table 1. As an internal control for normalization, PCR reactions were performed concurrently with the amplification of a reference gene, 18S ribosomal RNA (rRNA) that proved to be stable in all the conditions studied.

Target gene	Forward primer (5`-3`)	Reverse primer (5`-3`)	Genbank
18S	GATTAAGTGCCCTTTGTA	GATCCGAGGGCCTCACTAAAC	v01270
VGLUT-1	TGGGTTTCTGCATCAGCTTTG	TGTACTGTTGTTGACCATGGATACG	NM_053859
GLAST 1[a]	CATCCAGGCCAACGAAACA	GAGTCTCCATGGCCTCTGACA	NM_019225
GLT-1	AACCGAGGGTGCCAACAA	TGAGGTGGCTGTCGTGCAT	NM_017215
EAAC-1	GGTTCAAGCCCTAAAGCAGAA	AGGGAGCTTGACCTTAGATGT	NM_013032
GABA α1	TTCACCAAGAGAGGGTATGC	TAGCAATAGTTGCCAAGCCG	NM_183326
GABA β2	TGGCGATGACAATGCAGTCA	AAGCTTAGGGACAATCTGGG	NM_012957
GABA γ2	TTGTGAGCAACCGGAAACCA	CTTCATCCCTCTCTTGAAGG	NM_183327
GAD 65	TCTTTTCTCCTGGTGGTGCC	CCCCAAGCAGCATCCACAT	NM_012563

Table 1. Primers used in real time PCR studies and the GenBank accession numbers.

Real time-PCR was performed on an Step-one plus (Applied Biosystems, Foster City, CA, USA) real-time thermal cycler using the SYBR® Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA) with the following thermal cycler settings: one cycle of 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Cycle thresholds for both genes were selected immediately above the baseline and within the linear range on log scaling. Each reaction (20 µl) consisted of a 2 µl cDNA aliquot, 300 nM of each primer and 10 µl of SYBR® Green PCR Master Mix containing AmpliTaq gold DNA polymerase.

Increases in the amount of SYBR® Green reporter dye fluorescence during the amplification process were analyzed with Sequence Detector software (SDS version 1.6 Applied Biosystems, Foster City, CA, USA). Relative change in expression of the target genes was determined by the following equation:

Fold change=$2^{-\Delta Ct}$,

ΔCt=(Ct target–Ct 18S ribosomal RNA (rRNA)) (Livak and Schmittgen, 2001).

The Ct value is the cycle number at which the fluorescence signal crosses the designated threshold.

Propidium Iodide Assay

Slices (225 μm) were obtained with a vibratome and were incubated in the different conditions (as per the test slices) but 20 min prior to the end of the respective incubations, they were treated with 5 μg/ml propidium iodide (Sigma-Aldrich, Madrid, Spain). Subsequently, sections were fixed in 4% paraformaldehyde in 50 mM phosphate buffered saline (PBS) for 30 min at 4 ºC.

Following this, slices were washed three times, for 10 min each, with 10 μg/ml ribonuclease A (Thermo Scientific, Opelstrasse, Germany) in PBS (50 mM) and stained with 1 μg/ml DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich, Madrid, Spain). Slices were mounted using the anti-fading mounting media DABCO® (1,4- Diazabicyclo[2.2.2]octane solution; Sigma-Aldrich, Madrid) for confocal observation using a Nikon EclipseTE-2000 (Nikon Instruments, Melville, New York, USA).

Statistical analysis

Two animals were sacrificed for each assay and at least two hippocampal and cortical slices from each animal were used in each experimental condition. Five

different assays of each experimental procedure were carried out. Since the data from each assay represented the average of two rats, we considered n=5 (although 10 animals were used in total). Statistical significances of the relative mRNA levels as well as the LDH released between the different experimental groups were analyzed using one-way ANOVA followed by the Student–Newman–Keuls (SNK) post-hoc test. Significance was set at the 95% confidence level. Relative variability between reference genes was analyzed using the coefficient of variation and 18S RNA (rRNA) was considered the most appropriate reference gene (Banda *et al*., 2008).

RESULTS

Effects of OGD and reperfusion on expression of $GABA_A$ receptor and glutamate transporter genes

A uniform, 20% decrease in $GABA_A$ receptor subunit mRNA levels (α1, β2 and γ2) was observed in the hippocampus following OGD, a slightly larger decrease in mRNAs encoding α1 and β2 subunits was visible following reperfusion (~30%); this was not seen with γ2-subunit mRNA, which decreased the same magnitude after both OGD and reperfusion (Figure 1, E-G). In cortex, OGD and reperfusion resulted in a similar decline in α1- and β2-subunit mRNAs, but interestingly the γ2-subunit mRNA levels were unchanged by OGD and were further reduced by ~20% following reperfusion (Figure 1, E-G). In contrast, GAD65 transcript levels in the hippocampus were completely unaffected by OGD and reperfusion (Figure 1, H). However, in the cortex, GAD65 mRNA was significantly decreased following OGD (~20%) and reperfusion (~70%).

Glutamate transporter gene expression was also reduced following OGD and reperfusion in both hippocampus and cortex (Figure 1 A-D). The greatest decreases were found in cortex following reperfusion, whereby GLAST1a, EAAC-1, GLT-1 and VGLUT1 mRNA levels were all reduced by ~60% (they were only reduced by 30- 40% in this region following OGD). In the hippocampus all glutamate gene transcripts were decreased following OGD and in a lesser degree during the RL.

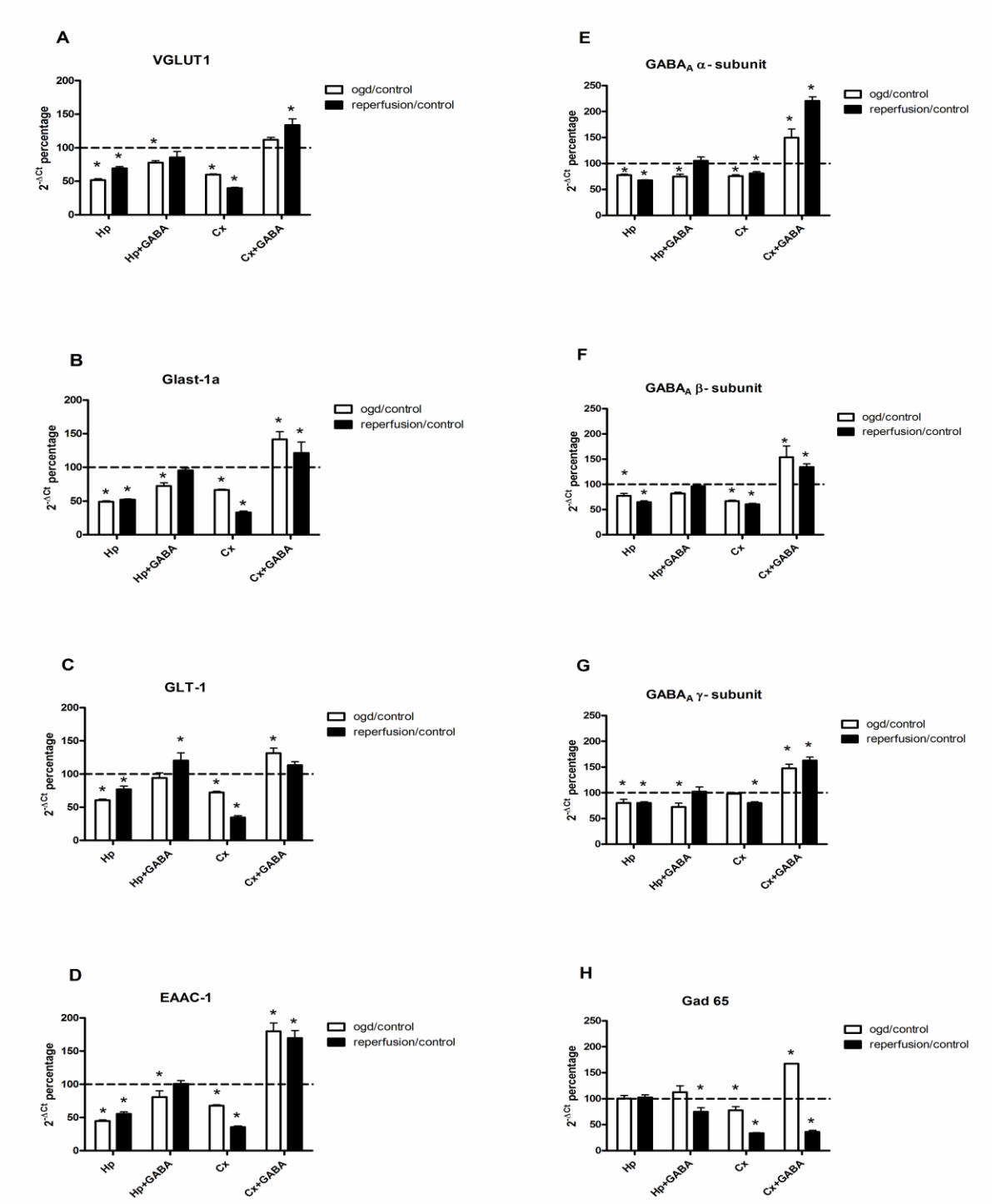

Figure 4. Effect of OGD and reperfusion in the presence (and absence) of GABA (100 μM) on expression of genes encoding: glutamate transporters (A-D), $GABA_A$ receptor subunits (E-G) and GAD65 (H), in hippocampus and cortex. Data are shown as mean ± SEM of the %-fold change ($2^{-\Delta Ct}$) in mRNA levels following OGD and reperfusion relative to untreated controls. (*) $p < 0.05$.

Effects of OGD and reperfusion on expression of $GABA_A$ receptor and glutamate transporter genes in the presence of GABA

The decreases in $GABA_A$ receptor subunit gene expression elicited by OGD in hippocampus were generally lessened in the presence of GABA. In fact, GABA prevented any decrease at all in α1-, β2- and γ2-subunit mRNA levels in hippocampus following reperfusion (Figure 1, E-G). Interestingly, after OGD and reperfusion in hippocampus, GAD65 mRNA levels were unaffected, but in the presence of GABA, the transcript levels were unaffected following OGD and decreased after reperfusion (Figure 1, H). In cortex, the effect of GABA was even more profound, whereby there was an increase in all subunit mRNAs following both OGD and RL (40-50% increase). Interestingly, GAD65 transcripts appeared strongly decreased following reperfusion (Figure 1H)

Similar effects were observed with the glutamate transporter genes (Figure 1, A-H). The hippocampal decreases in glutamatergic gene expression following OGD and reperfusion were lessened in the presence of GABA. In fact, GLT-1 mRNA was increased following reperfusion in the presence of GABA. Again mRNAs in the cortex showed a stronger response to the presence of GABA, all being increased by at least 10% (GLT-1), up to 70% (EAAC-1).

In general, the presence of GABA lessened the decrease in gene expression of all the genes in the hippocampus and completely reversed the response in the cortex in that all genes showed increased expression following OGD and reperfusion.

Effects of OGD and reperfusion on expression of $GABA_A$ receptor and glutamate transporter genes in the presence of bicuculline

The effect on gene expression following OGD and reperfusion in the presence of bicuculline was analyzed (Figure 2, A-H). Similar to the action of GABA, in the hippocampus the decrease in mRNA levels after OGD was lessened by bicuculline; interestingly the presence of bicuculline during RL in hippocampus, resulted in an increase in α1-, β2- and γ2-subunit mRNA. However, in the cortex the effect was quite different. A substantial reduction (of up to 90%) in mRNA levels was observed, demonstrating a differential response to bicuculline between the two regions. GAD65 mRNA was significantly increased in the presence of bicuculline (60-70%) in the hippocampus following OGD and reperfusion, and also to the cortex (by 30%), in contrast to the $GABA_A$ receptor subunit mRNAs. However, the presence of bicuculline in the medium resulted in a substantial decrease (40%) in the cortex (Figure 2, H).

Bicuculline also lessened the OGD-induced decrease in GLAST1a, EAAC-1, GLT-1 and VGLUT1 mRNAs in hippocampus (Figure 2, A-D). Following reperfusion, in the hippocampus, EAAC-1 and VGLUT1 mRNAs were increased (by 70% and 10% respectively). In the cortex, bicuculline administration elicited different effects on the glutamatergic gene transcripts. Its presence in the OGD condition resulted in a less number of Glast-1a and EAAC-1 transcripts (by 10%) (Figure 2, B, D, columns 7) compared to those observed in its absence (by 30%) (Figure 2, B, D, columns 5). In contrast, bicuculline attenuated the reduction of GLT-1 and VGLUT1 transcripts in the OGD condition (by 14-20%) (Fig. 2, A, C, columns 7) compared to the results observed in its absence (by 30-40%) (Figure 2, A, C, columns 5). In the RL condition the presence of

bicuculline resulted in lower transcript levels in all the glutamatergic genes studies (compare columns 8 versus columns 6 in Figure 2).

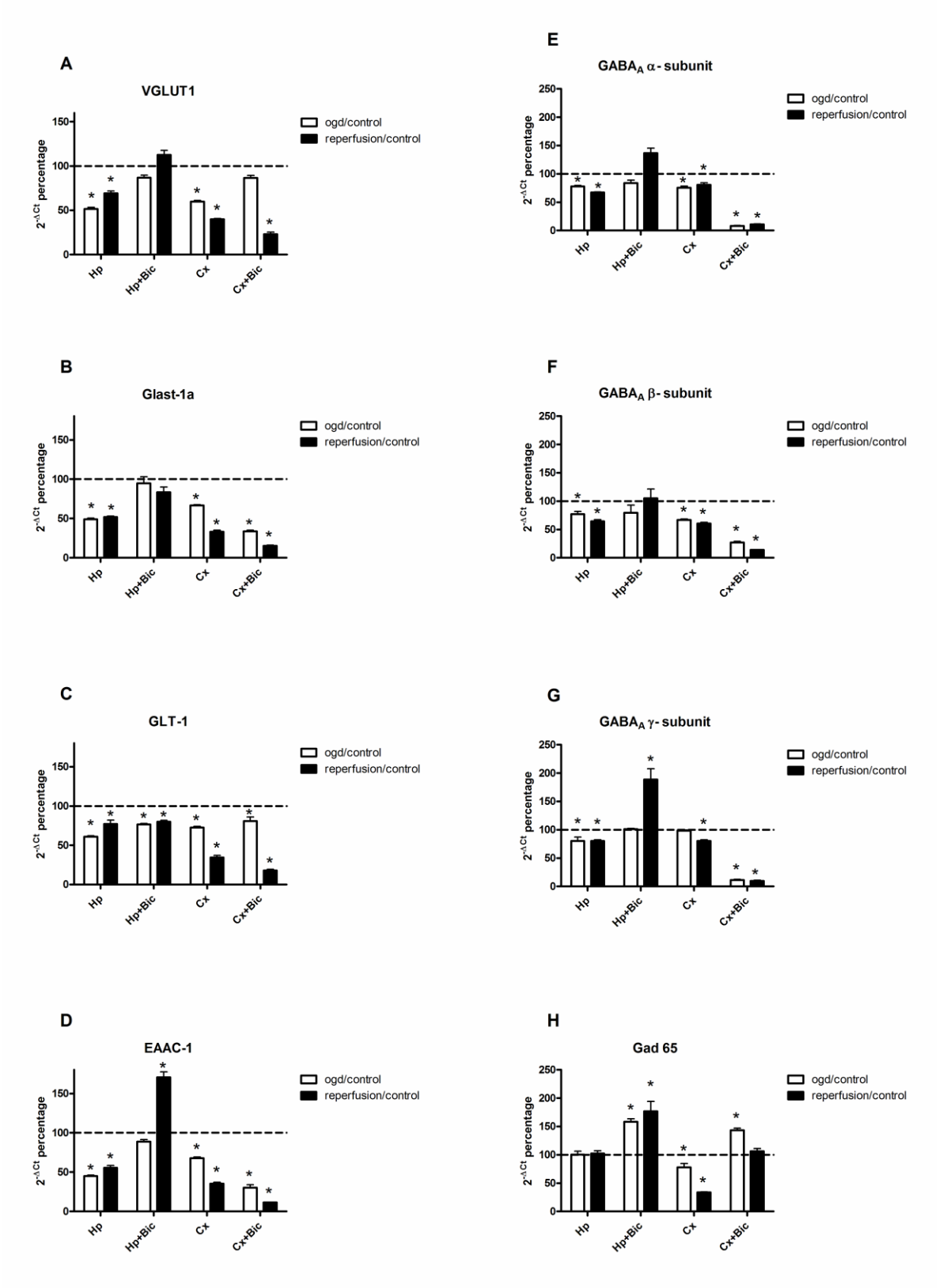

Figure 2. Effect of OGD and reperfusion in the presence (and absence) of bicuculline (1 μM) on expression of genes encoding: glutamate transporters (A-D), $GABA_A$ receptor subunits (E-G) and GAD65 (H), in hippocampus and cortex. Data are shown as mean ± SEM of the %-fold change ($2^{-\Delta Ct}$) in mRNA levels following OGD and reperfusion relative to untreated controls. (*) $p < 0.05$.

Cell mortality assays

LDH levels in individual experimental conditions are shown in Figure 3, where different responses can be observed between hippocampus and cerebral cortex. In both regions there is an OGD-induced increase in the mortality level, and this level is further increased during reperfusion, but by much more in the hippocampus compared to the cerebral cortex. The presence of 100 μM GABA in the incubation media significantly reduced the mortality, both during OGD and reperfusion, most notably in the hippocampus. Interestingly, the presence of bicuculline, increased mortality during OGD and in the reperfusion-like in cerebral cortex, but decreased mortality in the hippocampus in both conditions. However, it should be noted that bicuculline enhances mortality when compared with the effects of GABA in both structures and mortality in the cerebral cortex was significantly higher than in the hippocampus. The data obtained for the LDH assay were further confirmed by propidium iodide assays and confocal microscopy (Figures 4 and 5).

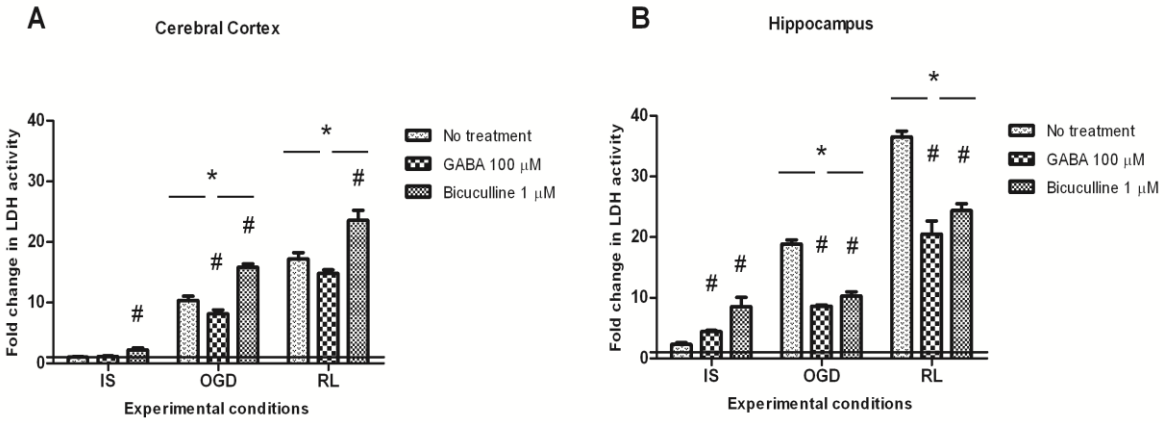

Figure 3. Lactate dehydrogenase (LDH) values showing the effects of OGD and reperfusion-like (in the presence of GABA and/or bicuculline) on cell survival in the hippocampus (A) and cortex (B). Data are shown as the percentage of the activity of LDH released to the medium. Data were compared to LDH activity of tissues maintained in IS in the absence of drugs (GABA and/or bicuculline) in the cerebral cortex (dotted line) which was considered as a control condition and shows the mortality resulting from the standard procedure previous to OGD assays in the cerebral cortex. Notice that LDH levels in the reperfusion should be added to the LDH levels of the OGD for a complete measurement of the mortality. * indicates significant changes between the presence of a drug treatment with respect to the same treatment in normoxic condtions. # indicates significant differences elicited by the presence of the distinct drugs in control, OGD and reperfusion-like conditions with respect to control, OGD and reperfusion-like conditions in absence of drugs. ($p< 0.05$; $n= 10$).

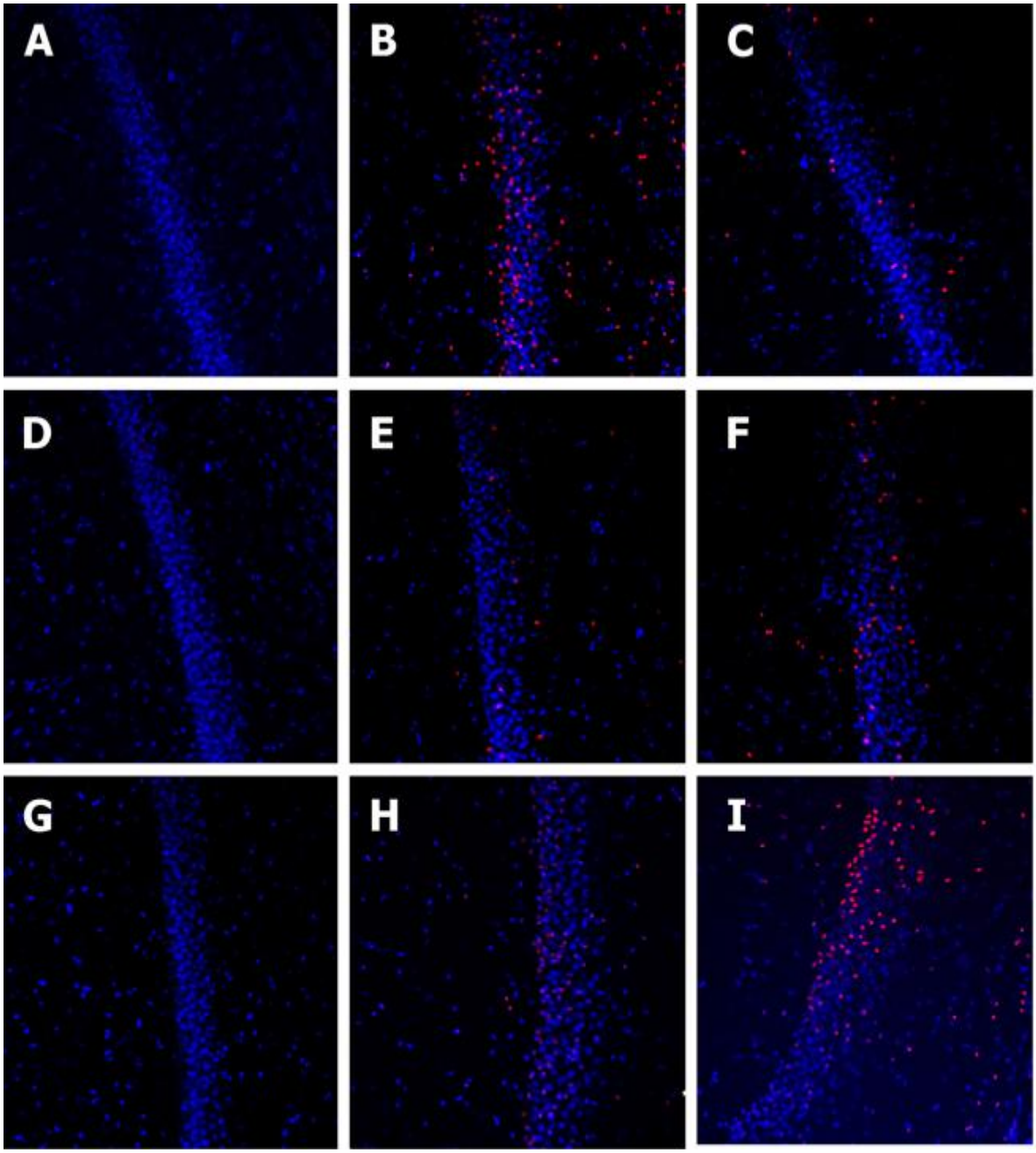

Figure 4. Cortical slices (225 µm thick) labeled with propidium iodide (damaged cells) and DAPI (total cells). Image shows control (A, D, G, J), OGD (B, E, H and K) and reperfusion-like (C, F, I, L) tissues in the absence of drugs (A, B, C), in the presence of 100 µM GABA (D, E, F), 1 µM bicuculline (G, H, I) and 100 µM GABA + 1 µM bicuculline (J, K, L). Bar = 50 µm.

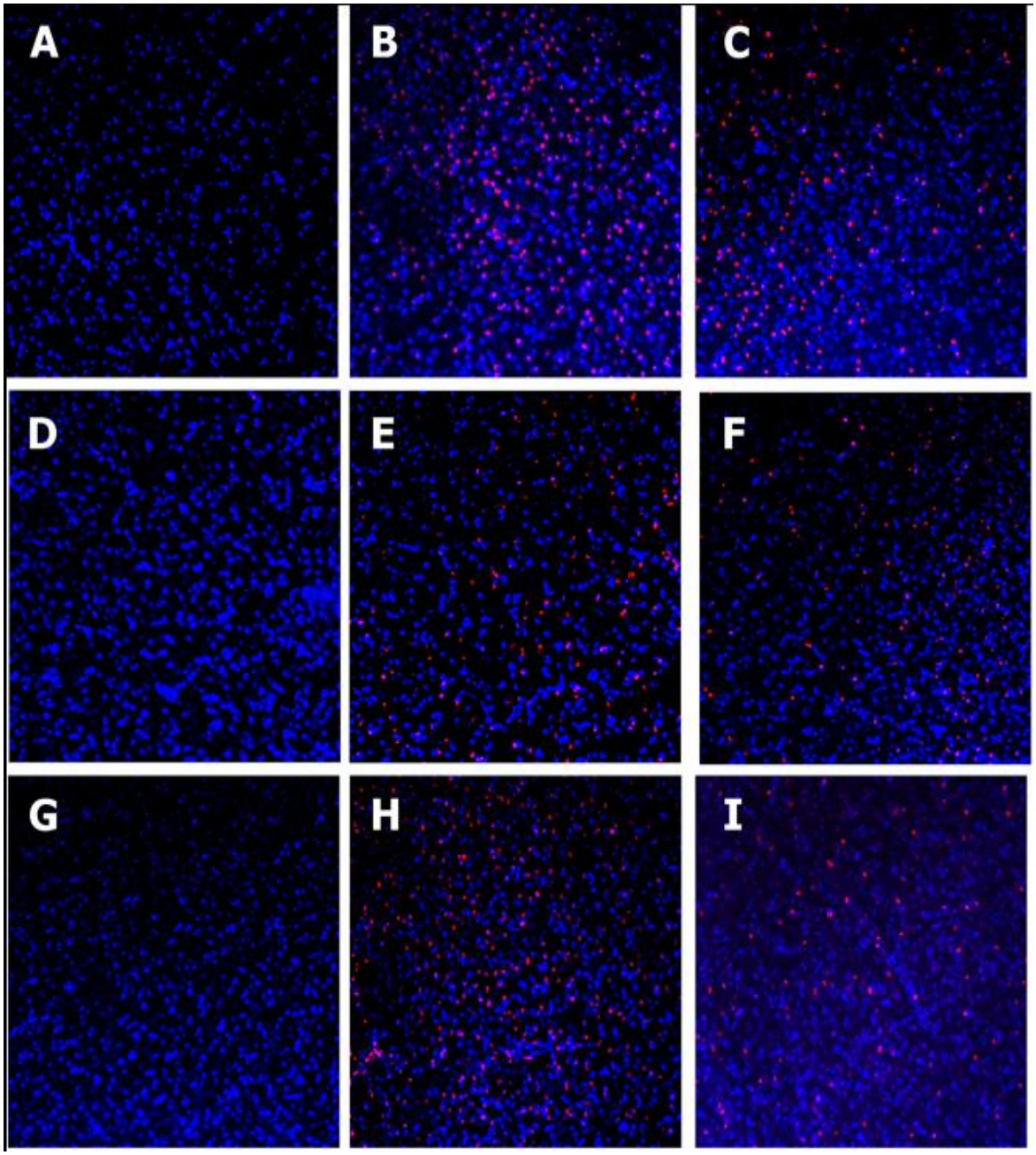

Figure 5. CA1 in hippocampal slices (225 μm thick) labeled with propidium iodide (damaged cells) and DAPI (total cells). Image shows control (A, D, G, J), OGD (B, E, H and K) and reperfusion-like (C, F, I, L) tissues in the absence of drugs (A, B, C), in the presence of 100 μM GABA (D, E, F), 1 μM bicuculline (G, H, I) and 100 μM GABA + 1 μM bicuculline (J, K, L). Bar = 50 μm.

DISCUSSION

Cell Mortality

Glutamate-evoked Ca^{2+} entry through NMDA receptors causes membrane depolarization and subsequent influx of Cl^- ions, which elicits cytotoxicity, as shown in neuronal cell culture assays (Rothman, 1985; Olney et al., 1986; Choi, 1987). A number of studies that support this hypothesis have shown protective effects of chloride-free media against NMDA-induced toxicity in organotypic cultures and brain slices (Takahashi et al., 1995; Gröndahl et al., 1998).The beneficial or detrimental effects of the ischemia-induced chloride influxes through ligand-operated chloride channels such as $GABA_A$ receptors have been discussed at length (Erdo *et al*., 1991; Hasbani *et al*., 1998; Inglefield and Schwartz-Bloom, 1998; Chen *et al*., 1999; Galeffi *et al*., 2004; Babot *et al*., 2005; Kumar *et al*., 2006). GABA-mediated neuroprotection following OGD, in rabbit cortical slices, has been previously described in a range of concentration between 30-1000 μM (Ricci *et al*., 2011). Our results, both from LDH and propidium iodide confirm that GABA neuroprotective effect also occurs during the reperfusion time we analyzed. The GABA-mediated neuroprotection observed in the hippocampus is also observed in the presence of bicuculline which suggest that, in this structure, $GABA_A$ chloride channels do not play an important role in this response. In the cerebral cortex, in contrast, these chloride channels seem to be important in the GABA-mediated neuroprotection since bicuculline was able to increase the cell mortality. The strong decrease mediated by bicuculline in the $GABA_A$ subunit transcripts in the cerebral cortex, but not in the hippocampus, provides additional support for a neuroprotective

role of $GABA_A$ chloride channels in the cerebral cortex, but not in the hippocampus. Thus, neuroprotection induced by GABA is different for hippocampus and cerebral cortex and may underlie the previously described structure-dependent vulnerability to ischemia in the hippocampus and cerebral cortex (Ordy *et al*., 1993; Roberts *et al*., 1997; Yang *et al*., 2000; Jiang *et al*., 2004 Gee *et al*., 2006). Further support to this idea is provided by the higher amounts of transcripts in the presence of GABA observed in the cerebral cortex compared to the hippocampus, in contrast to the increased mortality and strong transcript decreases observed in the presence of bicuculline in the cerebral cortex.

The glutamatergic response

GLT-1, the major glial glutamate transporter in the cerebral cortex and hippocampus (Kanai *et al*., 1997) has been reported to decrease after focal brain ischaemia, both in mRNA and protein levels (Torp *et al*., 1995; Ketheeswaranathan *et al*., 2011) as well as for EAAC1 in the cerebral cortex (Rao *et al*., 2000). However, antisense knockdown of glial GLT-1, but not the neuronal glutamate transporter EAAC1, promotes the neuronal damage following transient ischaemia (Rao *et al*., 2001) which has been confirmed with assays over-expressing GLT-1 in a rat focal ischemia model (Harvey et al., 2011). Our results are consistent with the above mentioned neuroprotective role for GLT-1, however the similar response found in both vesicular and membrane glutamate transporters suggests that other glutamate transporters else than GLT-1 are involved in the mortality.

The decreases in the glutamate transporter transcripts here observed, together with those described for ionotropic glutamatergic receptors (Dos Anjos *et al.,* 2009) provide additional evidences that OGD downregulates the whole glutamatergic gene expression (Ketheeswaranathan *et al*., 2011) including both neuronal and glial glutamatergic components.

Our results also support the hypothesis that the downregulation of the glutamate transporters is responsible for the massive presence of extracellular glutamate during OGD and the subsequent excitotoxicity responsible for the cell mortality (Ketheeswaranathan *et al*., 2011). Additional support to this idea comes from our experiments using GABA, that lessens the downregulation which in turn could account for less cell mortality, and bicuculline that results in the diametric effect.

The presynaptic GABAergic component

Previous studies using *in vitro* hippocampal slices deprived of oxygen and glucose indicate that GABA levels increase rapidly in the superfusate and then normalize within 15 min of reoxygenation (Burke and Taylor 1992; Inglefield and Schwartz-Bloom 1998; Saransaari and Oja 1998). These data suggest that the GABAergic presynaptic component is rapidly stabilized during OGD, which would be consistent with the lack of responsiveness in the presynaptic gabaergic marker GAD65 transcripts. This lack of responsiveness to the ischemia in the GABaergic presynaptic system concurs with reports indicating that GABAergic interneurons are relatively resistant to ischemia (Gonzales *et al*., 1992, Saji *et al*., 1994). This GABAergic presynaptic behaviour contrasts

with the glutamatergic presynaptic component, where a decrease in gene expression is observed.

CONCLUSSION

In summary, the OGD-dependent decrease in membrane and vesicular glutamatergic transporters, together with the decreases observed for the ionotropic glutamatergic receptors, supports that OGD elicits a general decrease in the glutamatergic system. In contrast, the OGD results in a different response for the GABAergic presynaptic and postsynaptic system. GABA elicits a neuroprotective effect in both cerebral cortex and hippocampus by lessening the downregulation in the glutamatergic transporter transcripts however, the neuroprotection is $GABA_A$-dependent in cerebral cortex but not in the hippocampus.

Aim 2b:

"The presence of GABA elicits opposite response in the unfolded protein response gene transcripts in the hippocampus and cerebral cortex in an oxygen and glucose deprivation model of rat brain slices."

INTRODUCTION

Endoplasmic reticulum (ER) stress, following brain ischaemia/reperfusion, has been evidenced by several different markers such as ER Ca^{2+} depletion, decreased protein degradation and increased aggregation of proteins, as well as the accumulation of lipid peroxidation products in ER and Golgi structures (Kohno *et al.*, 1997; Parsons *et al.*, 1999; Shibata *et al.*, 2003; DeGracia *et al.*, 2004). Aggregation of unfolded proteins triggers the ER-stress response and the subsequent unfolded protein response (UPR). This involves increases in different molecular chaperones including the GRP78, an ER-resident BiP (binding immunoglobulin protein) family member (Schroder and Kaufman, 2005). GRP78/BiP binds peptides and maintains them in a folding-competent state, thus preventing aggregation during the folding process (Gething, 1999; Schroder and Kaufman, 2005). Protein disulfide isomerase (PDI) is other protein involved in UPR that catalyses disulfide bond formation and plays an important role in the folding of glycosylated proteins (Wilusz *et al*., 2001a,b ; Wouters *et al*., 2005; Braakman and Bulleid, 2011; Gorospe *et al*., 2011,). The accumulation of misfolded proteins in the ER lumen results in the dissociation of GRP78/BiP, which allows the binding of this chaperone to the unfolded proteins,

for facilitating protein refolding, as well as the activation of the three effectors of UPR, the inositol-requiring kinase (IRE1), the RNA-activated protein kinase-like ER resident kinase (PERK) and the activating transcription factor 6 (ATF6) (Bertolotti *et al*., 2000; Shen *et al*., 2002; Ron and Harding, 2000). Despite an overall decrease in peptide synthesis and a concurrent increase in degradation, the expression of several proteins is increased. These molecules attempt to alleviate the cell of the excess of unfolded protein in the ER, for example, chaperones such as Grp78/Bip, Grp94 and PDI (Sidrauski *et al*., 1998). When the ER stress cannot be overcome (i.e. if it is too extensive or prolonged), the UPR is able to induce the apoptotic cell death through pro-apoptotic mediators such as Chop (C/EBP-homologous protein) (Ron and Walter 2007; Hetz, 2012). Ischaemia has been reported to induce the UPR (Tajiri *et al*., 2004; Oida *et al*., 2008; Bando *et al*., 2012; Ouyang *et al*., 2012) where the consequent increase in GRP78/BiP expression is thought to exert a protective effect, presumably through its chaperone activities (Nakka *et al*., 2010). In fact, increased transcription of GRP78/BiP limits the severity of ischaemia-induced infarcts in brain (Kudo *et al.,* 2008; Oida *et al.*, 2008; Ouyang *et al.*, 2011). Thus, quantification of the expression of UPR-gene transcripts (such as GRP78/BiP, GRP94, PDI and CHOP) is a useful tool for anaylsis of the UPR following ischaemic insult.

Previous observations indicate a neuroprotective effect elicited by GABA on hippocampal slice cultures (deFazio *et al.,* 2008) and in hippocampal and cerebral sections (Llorente *et al.,* submitted for publication) as a result of OGD. In this report we demonstrate that GABA-dependent mechanisms play a crucial role in the OGD-induced UPR although different in the

rat hippocampus and cerebral cortex. This study demonstrate for the first time in a brain slice model the role of chloride channels in the UPR comparing the effect of GABAergic agents on normoxic and OGD conditions.

MATERIALS AND METHODS

Animals

Experiments were carried out in accordance with the Guidelines of the European Union Council (86/609/EU), following Spanish regulations (BOE 67/8509–12, 1988) for the use of laboratory animals, and were approved by the Ethics Committee of the University of León. All efforts were made to minimize animal suffering and to reduce the number of animals used. In total, 10 male rats were used. They were housed at 22 ± 1°C in a 12-h light/dark controlled environment with free access to food and water.

Preparation and incubation of slices (induction of OGD and reperfusion)

Two-month-old Sprague–Dawley rats were sacrificed by decapitation, the forebrain removed and the rostral, caudal and lateral portions of the hippocampus excised. The hippocampus was cut sagitally along the interhemispheric fissure and sagittal sections comprising hippocampus and cortex (350 μm) were taken from the lateral to the medial part using a vibratome. Prior to sectioning, tissue was submerged in a cold (4°C) cutting solution (CS) bubbled with carbogen (95% O2 and 5% CO2), containing 120 mM NaCl, 2 mM KCl, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11 mM glucose and 20 mM sucrose, pH 7.4 (Merck, Madrid, Spain).

After cutting, slices were transferred into pre-incubation solution (PS) at 25°C (identical composition to CS but without sucrose), and bubbled with carbogen for a minimum of 30 min before transfer into an incubation solution (IS, containing; 120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.19 mM $MgSO_4$, 1.18 mM KH_2PO_4 and 11 mM glucose, pH 7.4; Merck, Madrid, Spain) at 25 °C, aerated with carbogen. Sections were transferred from the IS into individual cell strainers (100 micron; BD Falcon,Bedford, MA, USA) and submerged in 2 ml of the solutions under one of four different experimental conditions: 1) IS alone; 2) IS + 100 µM GABA , 3) IS + 1 µM bicuculline (to block voltage-dependent chloride channels). Osmolarity in all the solutions was 300±5 mOsm. Two different experimental procedures were carried out on tissue for each treatment: A) OGD procedure (30 minutes) and B) RL procedure, in order to compare to the reperfusion stage that follows ischemia "in vivo" (for 180 min after the 30 min OGD). The OGD procedure was carried out for each treatment using IS without glucose and aerated with 95% N_2 + 5% CO_2. Bubbling with this gas mixture must be performed at least for an hour prior to the incubation to ensure that oxygen in the solution is less than 10%, which was measured (HI 3818; Hanna Instruments, Spain). Parallel tissues of each treatment, but not subjected to OGD conditions were maintained as respective controls.

After the appropriate incubation times, under the chosen experimental conditions, samples were collected from the IS to determine

Real time PCR

RNA extraction

Total RNA was extracted using the Tripure™ Isolation Reagent (Roche Applied Science, Barcelona, Spain), according to the manufacturer's instructions. Any contaminating DNA in the RNA samples was removed by incubation with DNase (Sigma Aldrich, Madrid, Spain) and confirmed by PCR analysis of total RNA samples prior to reverse transcription (RT). Total RNA concentrations were determined using a NanoDrop ND-3300 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Isolated RNA was maintained at -80 °C until further processing.

Reverse transcription (RT)

RT was completed with the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), following manufacturers instructions. Reactions were performed for 10 min at 25 °C, 2 h at 37 °C and terminated by heating for 5 sec at 85 °C. The reaction mixture was maintained at -20 °C until further use.

Quantitative real-time PCR

Quantitative real-time PCR of the different mRNAs was performed in triplicate using gene-specific primers and SYBR® Green. Oligonucleotide primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). The primer sequences and corresponding GenBank accession numbers are given in table 1. As an internal control for normalization, PCR

reactions were performed concurrently with the amplification of a reference gene, 18S ribosomal RNA (rRNA) that proved to be stable in all the conditions studied.

Target gene	Forward primer (5`-3`)	Reverse primer (5`-3`)	Genbank
18S	GATTAAGTGCCCTTTGTA	GATCCGAGGGCCTCACTAAAC	v01270
PDI	CTGCTGTTCCTGCCCAAGAGTGT	TGGCTCATCAGGTGGGGCTTG	NM_012998
GRP78	CGTCCAACCCGGAGAACA	ATTCCAAGTGCGTCCGATG	NM_013083
GRP94	GTGGGTGCTGGGCCTCT	GACTTCATCGTCAGCTCTCACA	NM_001012197
CHOP	GCATCCCTAGCTTGGCTGACT	ATCTGGAGAGCGAGGGCTTT	NM_001109986

Table 1. Primers used in real time PCR studies and the GenBank accession numbers.

Real time-PCR was performed on an Step-one plus (Applied Biosystems, Foster City, CA, USA) real-time thermal cycler using the SYBR® Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA) with the following thermal cycler settings: one cycle of 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Cycle thresholds for both genes were selected immediately above the baseline and within the linear range on log scaling. Each reaction (20 µl) consisted of a 2 µl cDNA aliquot, 300 nM of each primer and 10 µl of SYBR® Green PCR Master Mix containing AmpliTaq gold DNA polymerase.

Increases in the amount of SYBR® Green reporter dye fluorescence during the amplification process were analyzed with Sequence Detector software (SDS version 1.6 Applied Biosystems, Foster City, CA, USA). Relative change in expression of the target genes was determined by the following equation:

Fold change=$2^{-\Delta Ct}$,

ΔCt=(Ct target–Ct 18S ribosomal RNA (rRNA)) (Livak and Schmittgen, 2001).

The Ct value is the cycle number at which the fluorescence signal crosses the designated threshold.

Statistical analysis

Two animals were sacrificed for each assay and at least two hippocampal and cortical slices from each animal were used in each experimental condition. Five different assays of each experimental procedure were carried out. Since the data from each assay represented the average of two rats, we considered n=5 (although 10 animals were used in total). Statistical significance of the relative mRNA levels between the different experimental groups was analysed using one-way ANOVA followed by the Student–Newman–Keuls (SNK) post-hoc test. Significance was set at the 95% confidence level. Relative variability between reference genes was analyzed using the coefficient of variation and 18S RNA (rRNA) was considered the most appropriate reference gene (Banda *et al*., 2008).

RESULTS

Effects of drugs on the normoxic condition

The presence of the gabaergic agents used in normoxic conditions resulted in significant increases in all the gene transcripts studied compared to the control condition (Figure 1). Thus, in the hippocampus GABA elicit significant increases in the transcripts (about four fold for PDI and GRP 78, two fold for CHOP and ninety fold for GRP94). A striking significant increase with respect to the control

conditions was observed in the transcripts of all the genes studied was observed in the presence of bicuculline (about five fold for CHOP, eight fold for PDI, eleven fold for GRP78 and four hundred fold for GRP94). The increases induced by bicuculline were also significant compared to those observed in the presence of GABA in all the genes studied.

In the cerebral cortex a similar pattern was observed in most of cases. Thus, GABA elicited increases in the transcripts but in a lesser degree than those observed in the hippocampus for all the genes studied except CHOP were higher increases of transcripts were observed (Figure 1). In this structure, the presence of bicuculline elicited noticeable higher levels of the transcripts than those observed in the hippocampus, except for GRP94.

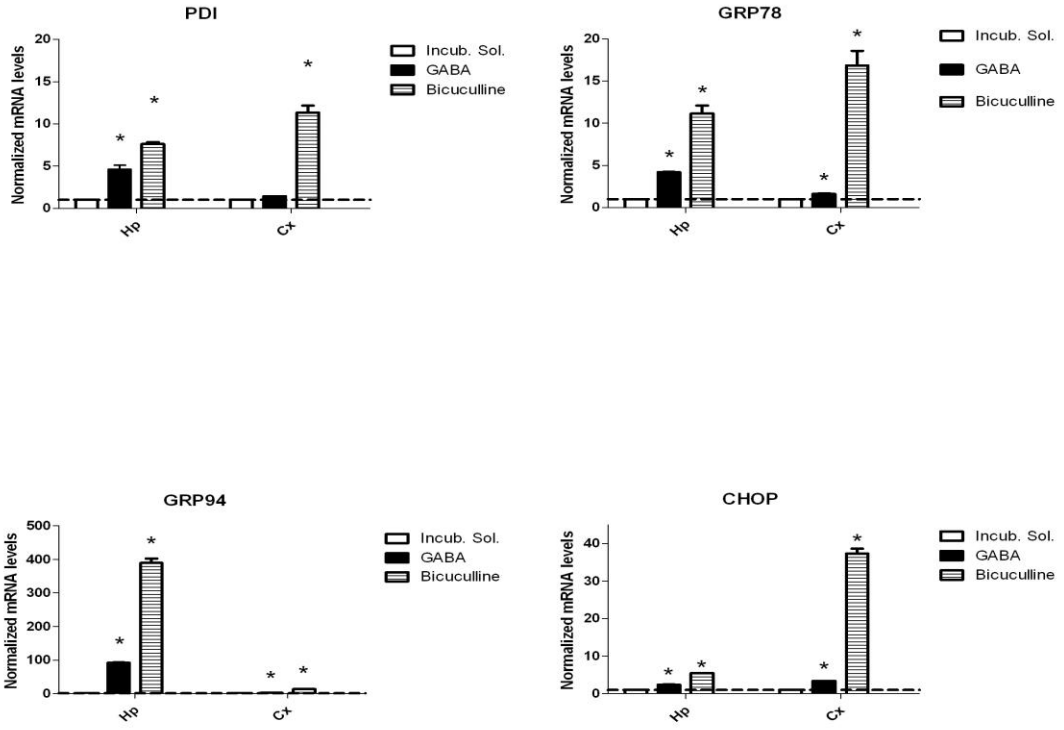

Figure1: Effect of 100 µM GABA and 1 µM bicuculline in normoxic conditions on mRNA levels of genes encoding UPR markers of molecular chaperons (PDI, GRP78 and GRP94) and a widely used marker of apoptosis (CHOP) normalized against 18S and compared with normoxic condition (expressed by the open columns and the dotted line) which is considered as value 1. Data are shown as mean ± SEM of fold change (2-ΔΔCt) in mRNA levels. The presence of both GABA and bicuculline increases the UPR-associated gene transcripts. However, levels

obtained in the presence of bicuculline are higher than those obtained in the presence of GABA in both hippocampus (Hp) and cerebral cortex (Cx). Statistics were made using one-way ANOVA followed by the non-parametric SNK test (*) $p < 0.05$.

Assays of OGD and RL in the absence of drugs

In the hippocampus the effect of OGD in the transcripts studied was none (PDI), small significant increases (GRP78) or significant decreases (GRP94 and CHOP). The RL condition resulted in similar results except for GRP78 where significant decreases were observed (Figure 2 A, C, E and G, IS columns). In contrast, in the cerebral cortex a similar pattern was observed for all the transcripts studied with significant increases elicited by OGD that still significantly increased following RL (Figure 2 B, D, F and H, IS columns).

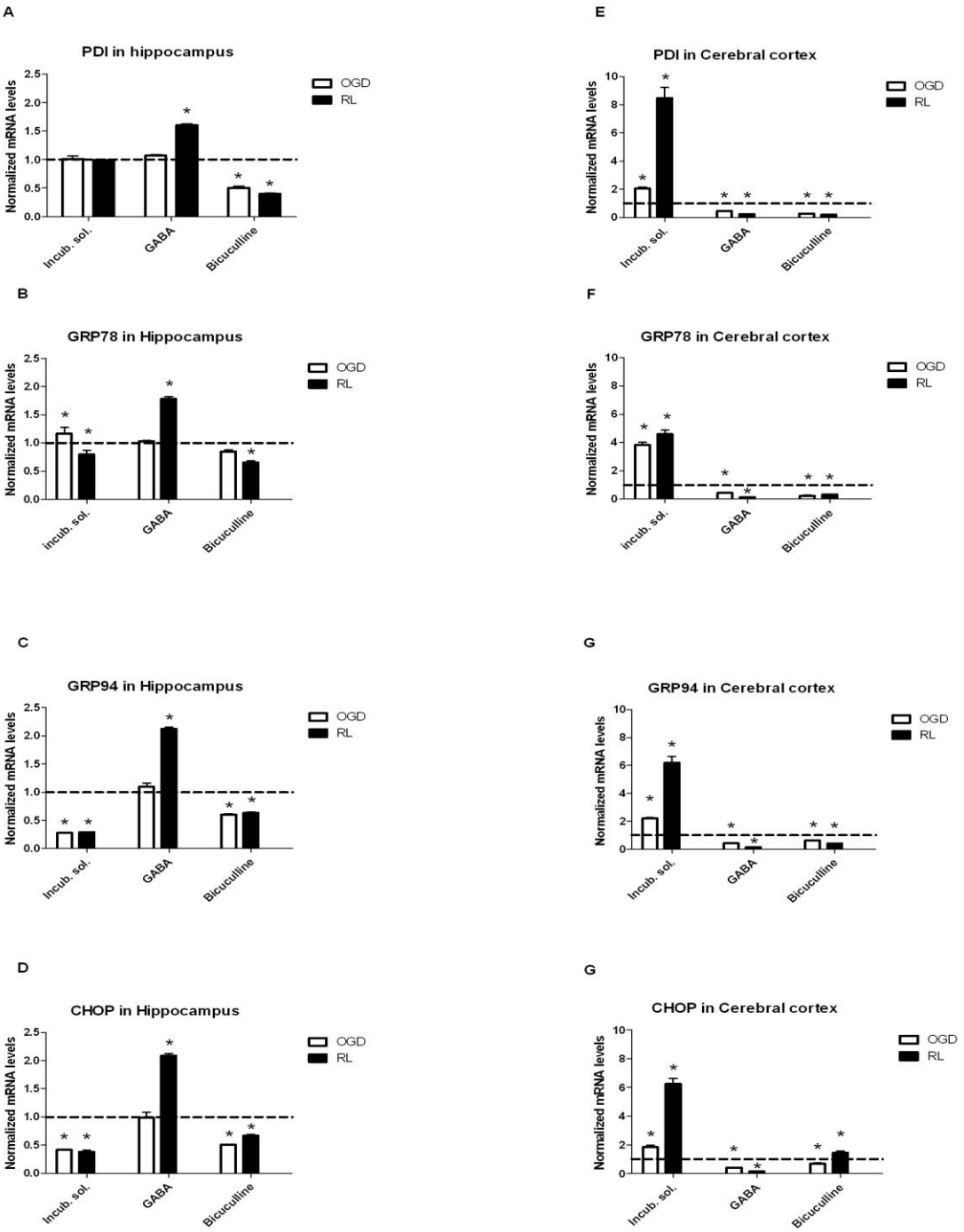

Figure 2. Effect of 100 µM GABA and 1 µM bicuculline in OGD and RL conditions on mRNA levels of genes encoding UPR markers of molecular chaperons (PDI, GRP78 and GRP94) and a the apoptosis marker CHOP normalized against 18S and compared with normoxic condition (indicated as value 1 by the dotted line). Data are shown as mean ± SEM of fold change ($2^{-\Delta\Delta Ct}$) in mRNA levels. Notice the different response in the cerebral cortex (Cx) and the hippocampus (Hp) during OGD and RL conditions (first two columns in the different graphs), the different response of these structures in the presence of GABA (columns 3 and 4 in the different graphs) contrasting with the similar response in the presence of bicuculline. One-way ANOVA followed by the non-parametric SNK test (*) $p < 0.05$.

Effects of the presence of GABAergic agents during OGD and RL assays

The presence of GABA in OGD and RL conditions elicits striking differences between hippocampus and cerebral cortex. Thus, in the hippocampus, GABA prevents OGD-induced changes in the transcript levels in any of the genes studied, however noticeable increases in all the gene transcripts studied were observed following RL (Figure 2 A, C; E and G, GABA columns) compared to normoxic condition in the presence of GABA. In contrast, in the cerebral cortex a significant decrease in the transcripts of all genes studied were observed following OGD that were still stronger following RL (Figure 2 B, D, F and H, GABA columns).

The presence of bicuculline in OGD and RL conditions resulted in decreases of the transcripts in all the genes studied in both hippocampus and cerebral cortex compared to their corresponding normoxic control in the presence of bicuculline, although some differences can be observed. Thus, OGD elicits significant decreases in PDI, GRP94 and CHOP transcripts. Decreases in the GRP78 do not reach significant levels. Following RL, PDI and GRP78 transcripts remained lower than those observed in OGD while CHOP and GRP94 presented similar or slight higher values than those observed after OGD (Figure 2 A, C; E and G, B columns). In the cerebral cortex PDI and GRP94 presented significant lower transcript levels following OGD and still lower levels during RL, while the strong transcript decreases observed in GRP78 elicited by OGD were lessened after RL. CHOP transcripts were significantly decreased by OGD but their levels increased during RL, becoming even significantly higher than those observed in their normoxic control in the presence of this agent (Figure 2 B, D, F and H, B columns).

DISCUSSION

Gene transcript levels reflect the primary response of the cell to OGD and RL conditions and do not always correlate with protein levels. mRNA levels are determined by various mechanisms (such as rate of initiation of transcription and mRNA splicing, stability, location or masking). Independent of these, further mechanisms affect the protein expression (including activity of translation initiation factors and ribosomal proteins) posttranslational modifications of the protein (e.g. phosphorylation, glycosylation or acetylation), trafficking and protein stability (Wilusz *et al*., 2001; Wouters *et al*., 2005; Sadoul *et al*., 2007; Gorospe *et al*., 2011). Thus, the expression of different proteins may not completely reflect the early cell response following ischaemic damage and the analysis of transcriptome would provide a more accurate insight of the first response of the cell which is the aim of this study.

This model does not present an ischaemic core as shown in focal ischaemia (Astrup *et al*., 1981; Baron, 1999; Umegaki *et al*., 2005; Fabricius *et al*., 2006; Mehta *et al*., 2007), therefore it must be considered as a model where only the penumbra area appears. Moreover, the tissue is incubated homogeneously, in contrast with the gradients in the penumbra area that can be observed during focal ischaemia. Thus, brain slices represent a model of homogeneous penumbra area which allows an accurate measurement of penumbra damage dependent on time and structure.

OGD-induced UPR and mortality

Genes here studied (PDI, GRP78 and GRP94) provide a measure of the brain modifications during the anoxic-induced ER stress and subsequent reoxygenation (White *et al*., 2000; Tajiri *et al*., 2004; Oida *et al*., 2008; Ouyang *et al*., 2012). The identical incubation conditions in hippocampus and cerebral cortex revealed distinct OGD-induced UPRs thus indicating intrinsic structural differences, rather than the effect of other global factors indicated for in vivo models such as differences in blood flow (Hu *et al*., 2000; Hu *et al*., 2001; Truettner *et al*., 2009) or differential inflammation (Sundararajan *et al*., 2005; Lakhan *et al*., 2009; Zhang, 2006). Additional support to the intrinsic differences between cerebral cortex and hippocampus come from cell mortality studies using the same OGD model (Llorente *et al*., submitted for publication). Different vulnerability to the ischemia in hippocampus and cerebral cortex has been previously reported (Ordy *et al*., 1993; Roberts *et al*., 1997; Yang *et al*., 2000; Jiang *et al*., 2004 Gee *et al*., 2006) and the different UPRs observed in these structures could underlie this differential sensitivity to the ischemia. The OGD-induced UPR in the cerebral cortex supports its ability of counteracting the ischaemic damage, thus making it more resistant to this challenge than the hippocampus, where no response is observed.

Increases in CHOP transcripts in the cerebral cortex but not in the hippocampus suggest differences in the course time of induction of apoptotic response between these structures or even different cell death types. This early response of CHOP could provide an early measure of the extension of this death; however it is not possible to prove it in the brain slice model given the short

work window. Early measures of CHOP in other models and the corresponding delayed death by apoptosis would be required to prove this hypothesis.

OGD decreases the transcripts of glutamate and GABA receptors, glutamate transporters, both EAATs and VGLUTs in both cerebral cortex and hippocampus (Hinoi *et al*., 2005; Dos Anjos *et al*., 2009; Montori *et al*., 2012; Sanchez-Mendoza *et a*l., 2010; Llorente *et al*., submitted for publication). Results of UPR gene transcripts indicates that in absence of glucose and oxygen cells are still able to increase (cerebral cortex) or maintain (in the hippocampus) the transcription, and support the idea that mechanisms controlling the gene expression are still functional in the ischemic tissue discarding mortality as the main responsible for decreases in the gene transcripts.

GABA effect on OGD-dependent UPR

Increases in the UPR gene transcripts induced by the presence of GABA in normoxic conditions reveal a GABA-dependent mechanism involved in the ER stress. This fact is corroborated by the effect of bicuculline which supports that a decrease in the cytosolic chloride ion concentration increases the ER stress as indicated by all the markers used in this study.

The presence of GABA and the $GABA_A$ antagonist bicuculline during OGD is able to block the increased OGD-induced UPR in the cerebral cortex. This supports the notion that UPR in the cerebral cortex is mainly mediated by a GABAergic mechanism. However, it seems not to be a $GABA_A$-dependent mechanism since $GABA_A$ channel opening and closing result in the same effect.

In contrast, in the hippocampus the presence of GABA during OGD and RL does not change or even increases the UPR while bicuculline slightly decreases the response. This suggests that the GABA-dependent mechanism above suggested for the cerebral cortex has low relevance in the hippocampal UPR.

Finally, our data also show that differences in the ischemic vulnerability described for hippocampus and cerebral cortex (Martone *et al*., 2000; Zou *et al*., 2005; Gee *et al*., 2006; Jamison *et al*., 2008) can be detected early in the transcription control levels and affect a number of different systems such as GABAergic (Llorente *et al.*, submitted for publication) and glutamatergic (Dos Anjos *et al*., 2009, Llorente *et al*, submitted for publication) as well as the UPR-related gene system here described.

CONCLUSSION

In summary, we conclude that in normoxic conditions cytosolic chloride concentration plays an important role in the control of the ER stress, however, during OGD and RL the role of the cytosolic chloride has low relevance in the control of the ER-stress and that different mechanisms control the UPR in cerebral cortex and hippocampus. In the cerebral cortex, a GABA-dependent mechanism (not $GABA_A$) seems to play a critical role while in the hippocampus this mechanism seems to present low relevance.

Aim 3:

"OGD-dependent glutamatergic transcript modifications in hippocampal organotypic culture"

INTRODUCCION

COX2 present a close interdependence with the glutamatergic system. The increased expression of COX2 enhances the production of PGE2 which promotes the probability of glutamate release in the synapsis in cultured hippocampal neurons and in slices (Sang et al., 2005, Yang et al 2008). The inhibition of COX2 results in a suppression of the glutamatergic neurotransmission of both long term depression and long term potentiation in the hippocampus (Chen et al., 2007; Murray and O'Connor, 2003, Slaninan et al., 2005, Yang and Chen, 2008). The prostaglandin G2, that results from the action of the COX2 on the arachidonic acid is converted in prostaglandin H2 in a second peroxidase action of the COX2 which results in the release of free radicals (Strauss and Glass, 2001, Consilvio et al., 2004). On the other hand, the activation of the N-methyl-d-aspartic acid (NMDA) receptor upregulates the COX2 in hippocampal and cortical neurons since the administration of MK801 an antagonist of the NMDA receptor abolish the induction of COX-2 induction (Yamagata et al., 1993) which suggests that the upregulation of COX2 in neurons depends on glutamatergic activity in the synapse (Consilvio et al., 2004). The protective effect of blocking COX2 has been reported in global ischemia and it has been indicated that COX2 activity contributes to CA1 neuronal death after global ischemia (Nakayama et al., 1998). Studies in focal

ischaemia indicate that COX2 is induced in peri-ischemic areas and play a significant role in their pathology (Hara, 1998). COX2 inmunoreactive neurons have been reported to accumulate in peri-infarct regions in human cerebral ischaemia (Tomimoto, 2002). Assays in an in vivo neonatal model of cerebral hypoxia-ischemia concluded that COX-2 inhibition protected neonatal rats against death, progression of brain injury and neurobehavioral deficits after a hypoxic-ischemic insult (Fathali et al., 2010). Assays in organotypic hippocampal culture show that activation of COX-2 by activating NMDA receptors with 10 µM NMDA induced increased cell death that was reduced by the presence 1 nM SC58236, a COX-2 inhibitor. The presence of 100 mM SC58236 also proved be neuroprotectant effect in OGD assays (McCullough et al., 2004).

In this objective we show the neuroprotectant effect of the antiinflammatory agent meloxicam which decreases the mortality in the hippocampal organotypic culture. We analyzed the expression of the protein and mRNA levels of different glutamatergic genes to find a possible explanation of this neuroprotectant effect. We describe for the first time how VGLUT2, NR2B and GluR2 transcripts respond to OGD in a different way than VGLUT1, Glast 1a, GLT-1, EAAC1, NR1, NR2A and GluR1 and these differences are increased by the presence of meloxicam in the incubation medium.

MATERIALS AND METHODS

Experiments and animal use procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH

Publications No. 80-23, revised 1996). The experimental protocols were approved by the Animal Care Committee of the Department of Pharmacology, University of Florence, in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123) and the European Communities Council Directive of 24 November 1986 (86/609/EEC). The authors further attest that all efforts were made to minimize the number of animals used and their suffering.

Materials

Propidium iodide (PI) were purchased from Sigma (St Louis, MO, USA). Tissue culture reagents were obtained from Gibco-BRL (San Giuliano Milanese, MI, Italy) and Sigma (St Louis, MO, USA).

Preparation of rat organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared as previously reported (Pellegrini-Giampietro et al., 1999a; Pellegrini-Giampietro et al., 1999b). Briefly, hippocampi were removed from the brains of 8-10 days old Wistar rats (Harlan, MI, Italy), and transverse slices (420 μm) were prepared using a McIlwain tissue chopper in a sterile environment. Slices were first placed in Hanks' balanced salt solution (supplemented with 5 mg/ml glucose and 3.75 μg/ml amphotericin B) and then transferred onto 30 mm diameter semiporous membranes inserts (Millicell-CM PIC M 03050; Millipore, Italy), which were placed in six-well tissue culture plates containing 1.2 ml medium per well. The slices culture medium

consisted of 50% Eagle's minimal essential medium, 25% heat-inactivated horse serum, 25% Hanks' balanced salt solution, 5 mg/ml glucose, 2 mM L-glutamine, and 3.75 µg/ml amphotericin B. Slices were maintained at 37°C in an incubator in atmosphere of humidified air and 5% CO2 and culture medium was changed twice a week. Slices were kept in culture for 12–14 days and before experiments all slices were screened for viability in a blind fashion by an inverted-phase contrast miroscope; slices displaying signs of neurodegeneration were discarded from the study.

OGD in rat organotypic hippocampal slices

Cultures were exposed to OGD as previously reported in detail (Pellegrini-Giampietro et al., 1999a; Pellegrini-Giampietro et al., 1999b). Briefly, the slices were subjected to OGD by exposing them to a serum- and glucose-free medium saturated with 95% N2 and 5% CO2. Following 30 min of incubation at 37°C in an airtight anoxic chamber equipped with an oxygen gas controller (BioSpherix, New York, USA), the cultures were transferred to oxygenated serum-free medium (75% Eagle's minimal essential medium; 25% Hank's balanced salt solution; 2 mM L-glutamine; and 3.75 µg/ml amphotericin B) containing 5 mg/ml glucose and returned to the incubator under normoxic conditions until neuronal injury was evaluated 24 h later. This experiment was made in absence and presence of 50µM of meloxicam in all conditions here studied.

Assessment of CA1 pyramidal cell injury

Cell injury was assessed using the fluorescent dye PI (5 μg/ml), a highly polar compound which enters the cell only if the membrane is damaged and becomes fluorescent upon binding to DNA. PI was added to the medium at the end of the 24 h recovery period following OGD. Thirty minutes later, fluorescence was viewed using an inverted fluorescence microscope (Olympus IX-50; Solent Scientific, Segensworth, UK) equipped with a xenon-arc lamp, a low-power objective (4X) and a rhodamine filter. Images were digitized using a video image obtained by a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) controlled by software (InCyt Im1TM; Intracellular Imaging Inc., Cincinnati, OH, USA) and subsequently analyzed using the Image-Pro Plus morphometric analysis software (Media Cybernetics, Silver Spring, MD, USA). In order to quantify cell death, the CA1 hippocampal subfield was identified and encompassed in a frame using the drawing function in the image software (ImageJ; NIH, Bethesda, USA) and the optical density of PI fluorescence was detected. There was a linear correlation between CA1 PI fluorescence and the number of injured CA1 pyramidal cells as detected by morphological criteria (Pellegrini-Giampietro et al., 1999a).

Real time PCR

RNA extraction

Total RNA was extracted using the Tripure™ Isolation Reagent (Roche Applied Science, Barcelona, Spain), according to the manufacturer's instructions. Any contaminating DNA in the RNA samples was removed by incubation with

DNase (Sigma Aldrich, Madrid, Spain) and confirmed by PCR analysis of total RNA samples prior to reverse transcription (RT). Total RNA concentrations were determined using a NanoDrop ND-3300 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Isolated RNA was maintained at -80 °C until further processing.

Reverse transcription (RT)

RT was completed with the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), following manufacturer's instructions. Reactions were performed for 10 min at 25 °C, 2 h at 37 °C and terminated by heating for 5 sec at 85 °C. The reaction mixture was maintained at -20 °C until further use.

Quantitative real-time PCR

Quantitative real-time PCR of the different mRNAs was performed in triplicate using gene-specific primers and SYBR® Green. Oligonucleotide primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). The primer sequences and corresponding GenBank accession numbers are given in table 1. As an internal control for normalization, PCR reactions were performed concurrently with the amplification of a reference gene, 18S ribosomal RNA (rRNA).

Gen	Forward primer (5`-3`)	Reverse primer (5`-3`)	Genbank
18 s	GATTAAGTGCCCTTTGTA	GATCCGAGGGCCTCACTAAAC	v01270
VGLUT1	TGGGTTTCTGCATCAGCTTTG	TGTACTGTTGTTGACCATGGATACG	NM_053859
VGLUT2	CGTGAAGAATGGCAGTATGTCTTC	TGAGGCAAATAGTGCATAAAATATGACT	NM_053427
Glast 1a	CATCCAGGCCAACGAAACA	GAGTCTCCATGGCCTCTGACA	NM_019225
Glt-1	AACCGAGGGTGCCAACAA	TGAGGTGGCTGTCGTGCAT	NM_017215
Eaac-1	GGTTCAAGCCCTAAAGCAGAA	AGGGAGCTTGACCTTAGATGT	NM_013032
NR1	CAGCCGTGAACGTGTGGAG	TGCTCTACCACTCTTTCTATCC	NM_017010
NR2A	CAGTGATGTGTATATTTCAGAGCATGTTA	ACACTCGTCTATTGCTGCAGGAA	NM_012573
NR2B	TCCGTCTTTCTTATGTGGATATGC	CCTCTAGGCGGACAGATTAAGG	NM_012574
GLUR1	CGAGTTCTGCTACAAATCCCG	TGTCCGTATGGCTTCATTGATG	NM_031608
GLUR2	CCAAGGACTCGGGAAGTAAGG	CCCCCGACAAGGATGTAGAA	NM_017261

Table 1. Primers used in real time PCR studies and the GenBank accession numbers.

Real time-PCR was performed on an Step one plus real-time thermal cycler using the SYBR® Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA) with the following thermal cycler settings: one cycle of 2 min at 50 °C, one cycle of 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Cycle thresholds for both genes were selected immediately above the baseline and within the linear range on log scaling. Each reaction (20 µl) consisted of a 2 µl cDNA aliquot, 300 nM of each primer and 10 µl of SYBR® Green PCR Master Mix containing AmpliTaq gold DNA polymerase.

Increases in the amount of SYBR® Green reporter dye fluorescence during the amplification process were analyzed with Sequence Detector software (SDS version 1.6 Applied Biosystems, Foster City, CA, USA). Relative change in expression of the target genes was determined by the following equation:

Fold change=2−ΔCt, ΔCt=(Ct target–Ct 18S ribosomal RNA (rRNA)) (Livak and Schmittgen, 2001).

The Ct value is the cycle number at which the fluorescence signal crosses the designated threshold.

Statistical analysis

Data are presented as means ± SE of n experiments. Statistical significance of the relative mRNA levels between the different experimental groups was analysed using one-way ANOVA followed by the Student–Newman–Keuls (SNK) post-hoc test. Significance was set at the 95% confidence level. Relative variability between reference genes was analyzed using the coefficient of variation and 18S RNA (rRNA) was considered the most appropriate reference gene (Banda *et al*., 2008). All statistical calculations were performed using GRAPH-PAD PRISM v. 5 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Mortality assays

OGD induced a noticeable increase in the mortality when measured with propidium iodide. The presence of meloxicam did not significantly changed the mortality in normoxic conditions but resulted in an outstanding neuroprotectant effect during OGD (Figure1).

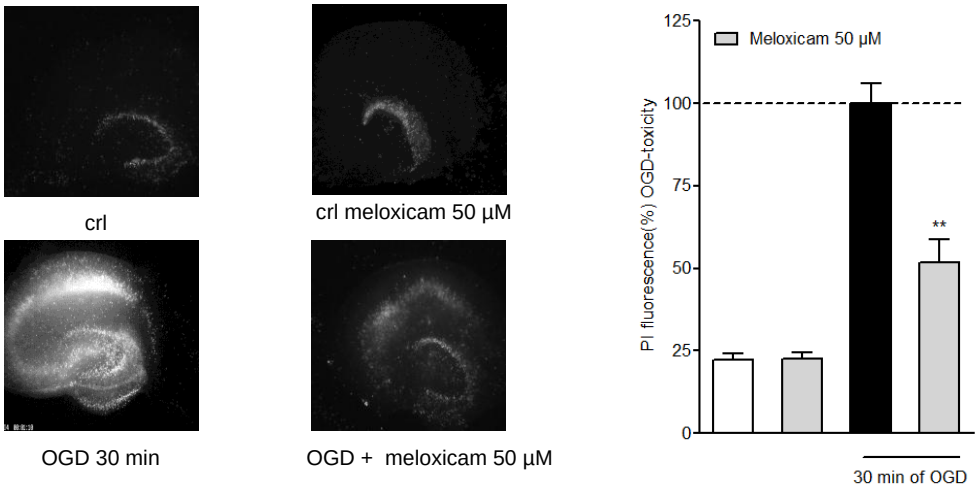

Figure 1. Effect of meloxicam 50 µM on OGD toxicity in rat organotypic hippocampal slices. OGD was applied for 30 min and 24 h later damage in the CA1 region was assessed by measuring the intensity of PI fluorescence. Meloxicam was present in the incubation medium during OGD and the subsequent 24 h recovery period. Meloxicam attenuated CA1 injury induced by 30 min of OGD. Bars represent the mean ± SEM of at least 3 experiments. *P< 0.05 and **P<0.01 vs. OGD (ANOVA + Tukey's test)

Vesicular glutamate transporters

VGLUT1

Both OGD and RL 1 h conditions resulted in a decrease of VGLUT1 mRNA levels compared to those of control condition (60-70%). Interestingly, outstanding significant increases (more than 150%) in the VGLUT1 transcripts were observed in RL 3 condition while in RL 24 h condition the mRNA levels appeared similar to those of the control condition (Figure 2A).

The presence of 50 µM meloxicam did not induced changes in VGLUT1 mRNA levels in normoxic conditions. The presence of meloxicam resulted in a decrease of the mRNA levels in OGD and all RL conditions compared to the normoxic condition. In the presence of meloxicam VGLUT1 mRNA levels where

significantly lower in RL 3 h and RL 24 h compared to those observed in its absence but we failed in (Figure 2B).

VGLUT2

VGLUT2 transcript analysis showed a very different response to that observed in VGLUT1. Thus, significant VGLUT2 mRNA level increases (up to 60% and 40% following OGD and RL 3 h conditions respectively) with respect to the normoxic condition, contrasting with the significant decreases in VGLUT2 mRNA levels (~25%) were observed in RL 1 h and RL 24 h conditions (Figure 2C).

Similar VGLUT2 mRNA levels were observed in normoxic conditions in the presence of the absence of 50 µM meloxicam. However, the presence of meloxicam in the OGD condition resulted in an outstanding significant increase in the VGLUT2 transcript levels compared to OGD in the absence of meloxicam. This result contrasts with the significant decreases induced by the presence of meloxicam in all the reperfusion-like conditions studied compared to their respective conditions in the absence of meloxicam (Figure 2D).

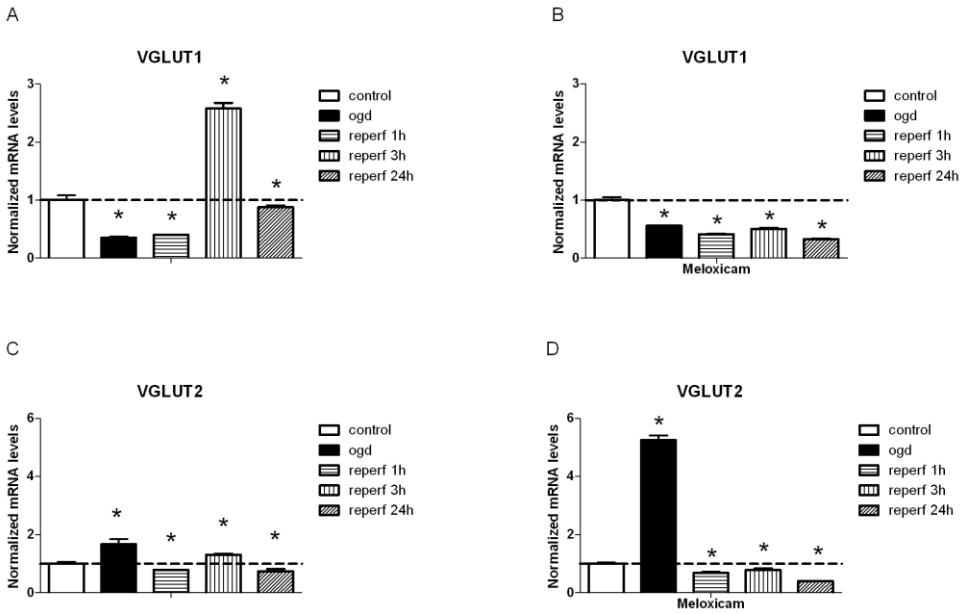

Figure 2. Fold change ($2^{-\Delta\Delta Ct}$) in the VGLUT1 and VGLUT2 mRNA levels between OGD, 1,3 and 24 reperfusion hours and control condition (value 1, indicated by the dotted horizontal line) in the hippocampal slices treated with 50μM meloxicam and non treated conditions. Significant differences with respect to the control condition are indicated by * ($p<0.05$). Two-way ANOVA (n=5).

Membrane glutamate transporters

EAATs presented a similar pattern of response: OGD, RL 1 h and RL 24 h presented significant low mRNA levels compared to those observed in normoxic condition except in EAAC1 where we failed in detecting significant differences in OGD and RL 1h conditions and in GLAST-1a whose mRNA levels were significantly higher in RL 24 h. However, all mRNAs levels of tissues in the RL 3 h condition presented higher or similar levels to those of the control condition.

The presence of meloxicam in the incubation medium lessened the differences between mRNA levels of normoxic, OGD and RL conditions but we could find significant decreases in the mRNA levels in the RL 24 h condition in all these group of genes and also in GLT-1 in RL 1 h conditions. In contrast, increases of

mRNA transcripts were observed in GLAST-1A in RL 1h condition and in EAAC-1 in RL 3h condition (Figure 3A-F).

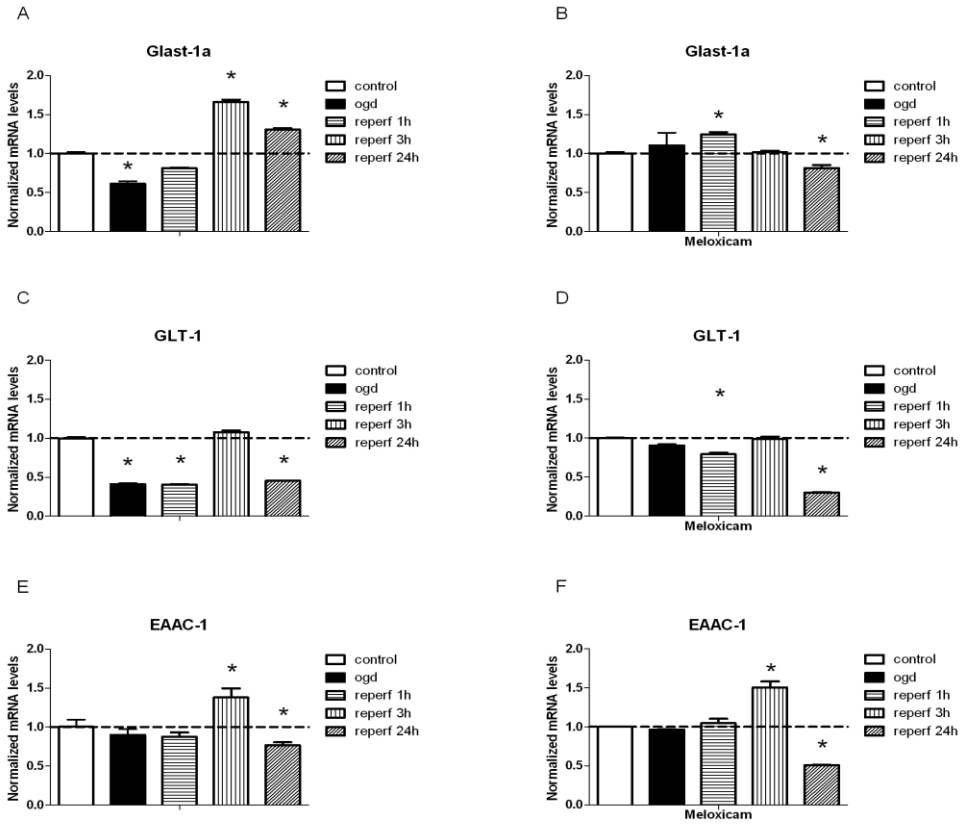

Figure 3. Fold change ($2^{-\Delta\Delta Ct}$) in the Glast-1a. GLT-1 and EAAC-1 mRNA levels between OGD, 1,3 and 24 reperfusion hours and control condition (value 1, indicated by the dotted horizontal line) in the hippocampal slices treated with 50μM meloxicam and non treated conditions. Significant differences with respect to the control condition are indicated by * ($p<0.05$). Two-way ANOVA (n=5).

AMPA AND NMDA GLUTAMATE RECEPTORS

A similar pattern of response was observed for the mRNA levels of the glutamate NMDAR NR1, NR2a and AMPAR GluR1 subunits with significant decreases in OGD, RL 1 h, and RL 24 h conditions. In contrast, an outstanding increase of their transcripts was observed in the RL 3 h condition (Figure 4A, 4C and Figure 5A).

The presence of meloxicam in the incubation medium lessened the response but still we were able to detect the significant differences observed in the absence of meloxicam except in the RL 3 h condition were no differences were observed(Figure 4B, 4D and Figure 5B).

NR2b and GluR2 transcripts presented a different pattern of response than those mentioned above. Thus, significant increased levels in the OGD condition in NR2b (140 %) or no changes for GluR2 were observed compared with the normoxic condition. Both NR2b and GluR2 transcripts were significantly lower in all RL conditions compared with normoxic conditions (Figure 4E and Figure 5C).

The presence of meloxicam resulted in an outstanding increase (10 fold) in the NR2b mRNA levels compared with its corresponding normoxic condition that contrast with the significant decreases in all RL conditions (Figure 4F). GluR2 transcript levels were also modified by the presence of meloxicam resulting in significant increases in OGD, RL 1 h RL 3 h and a significant decrease in RL 24h compared with its corresponding normoxic condition (Figure 5D).

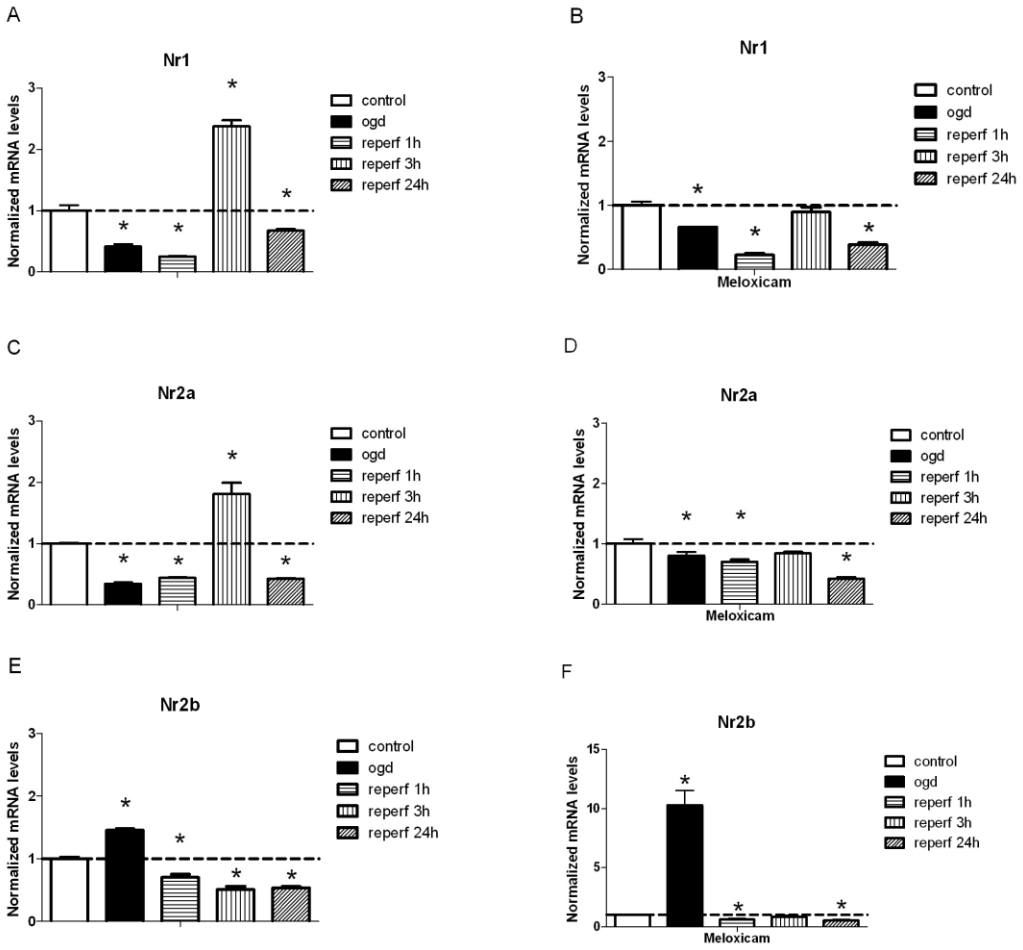

Figure 4. Fold change ($2^{-\Delta\Delta Ct}$) in the NR1, NR2A and NR2B mRNA levels between OGD, 1,3 and 24 reperfusion hours and control condition (value 1, indicated by the dotted horizontal line) in the hippocampal slices treated with 50μM meloxicam and non treated conditions. Significant differences with respect to the control condition are indicated by * ($p<0.05$). Two-way ANOVA (n=5).

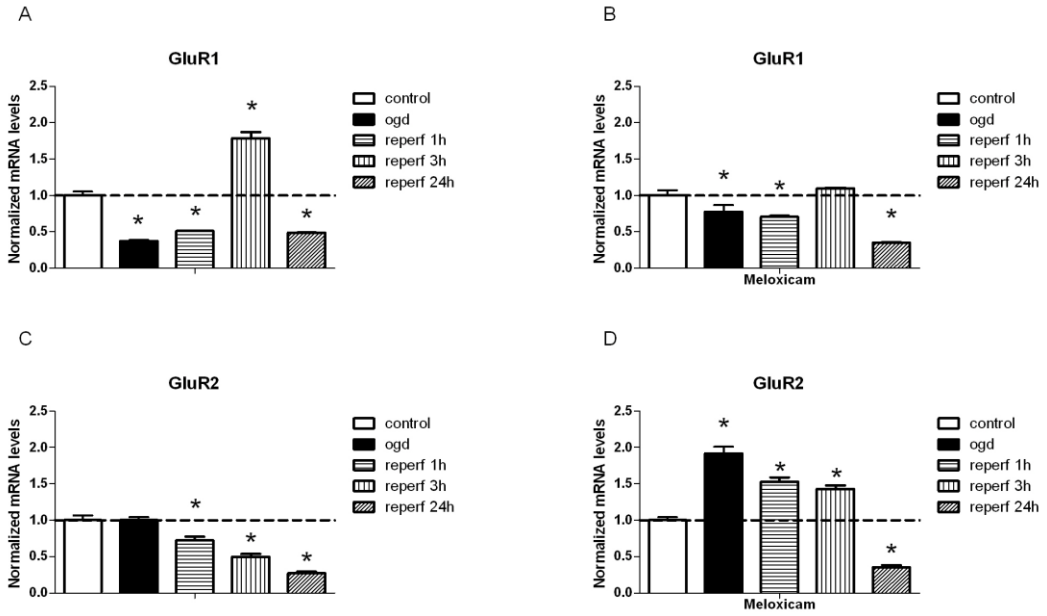

Figure 5. Fold change ($2^{-\Delta\Delta Ct}$) in the NR1, NR2A and NR2B mRNA levels between OGD, 1,3 and 24 reperfusion hours and control condition (value 1, indicated by the dotted horizontal line) in the hippocampal slices treated with 50μM meloxicam and non treated conditions. Significant differences with respect to the control condition are indicated by * ($p<0.05$). Two-way ANOVA (n=5).

DISCUSSION

Our data provide additional evidences to previous reports indicating that COX-2 inhibition results in a neuroprotectant effect on different models of cerebral ischaemia (Nakayama et al., 1998; Fathali et al., 2010, McCullough et al., 2004). Since COX-2 is the first step in the synthesis of different prostanglandins their different roles in neuroprotectant or toxic effects have been hypothesized (Andreasson, 2010). The role of glutamate excitotoxicity in the ischaemic damage is widely accepted (Campos et al., 2011, Lipton 1999) and a role of COX-2 in attenuating glutamate excitotoxicity and, consequently, Ca2+ influx, by indirectly modulating the transcription of AMPA/KA and NMDA receptors has been hypothesized (Caracciolo et al., 2011). Our results provide further

evidences that COX-2 also modulates other genes of the glutamatergic system, particularly the vesicular (VGLUTs) and membrane (EAATs) transporters. In this regard, we detected a similar pattern of response in the VGLUT1, NR1, NR2A and GluR1, Glast-1a, GLT-1 and EAAC1 mRNA levels where the main characteristic is the increased level of mRNA levels in the RL 3h condition which suggests that a time between 1 and 3 hours is critical for the recovery of the expression of this genes after OGD. The inhibition of COX-2 results in a loss of this pattern of response and transcript values at different times are similar.

A different pattern of response was observed for VGLUT2, GluR2b and NR2B transcripts whose main characteristic is that their mRNA levels are not modified and even can be increased, during OGD. This second pattern was more noticeable when COX-2 is inhibited. Thus, COX-2 seems to play different roles in the control of the early expression of some glutamatergic genes.

Differences in the regulation of these two groups of genes could be related with their different roles. Thus NR2B is distributed mainly extrasynaptically and NR2A is confined to synapses of mature neurons (Rumbaugh and Vicini, 1999). A crucial role in synaptic NMDA receptor in the neuronal survival has been indicated whereas extrasynaptic NMDAR is coupled to cell-death pathways (Hardingham et al., 2002). GluR2B has been indicated to play a role in a protective mechanism for the excitotoxicity elicited by Ca2+, and the expression of GluR2 can be used as switch to control the permeability of the AMPARs to Ca2+ (Pellegrini-Giampietro, 1997). Thus, the presence of GluR2 subunit in the AMPAR isoforms results in decreases in the channel permeability to Ca2+/Zn2+ (Hollmann and Heinemann, 1994; Pellegrini-Giampietro, 1997). Differences in the behaviour of VGLUT1 and VGLUT2 have been described in rat

hippocampal culture indicating that VGLUT2 is cleaved by calpains after excitotoxic stimulation while VGLUT1 is less affected (Lobo et al., 2011).

Summary

Stroke is the main reason for most of deaths and disabilities in the developed countries with a high socioeconomic impact. In Spain, stroke is the first cause of death in women and the second one in men, after heart attack.

During stroke, a restrict brain area suffers a decrease in the cerebral blood flow (CBF), under 10 mL/100 g/min, which results in a rapid cell death. Overactivation of the glutamatergic receptors elicited by the increase of glutamate in the extracellular medium is considered one of the most important factors involved in the ischaemic damage. This overactivation results in a strong increase of the intracellular calcium concentration leading to a massive neuronal depolarization. As a consequence, an extraordinary release of excitatory aminoacids, as glutamate, occurs from the presynaptic terminals, a process known as glutamate-mediated excitotoxicity. Inhibitory neurotransmitters such as γ-aminobutiric acid (GABA) and glycine are also released.

Therefore, excitotoxicity results from excessive release and/or inadequate withdrawal of the synaptic glutamate. In normal conditions, the neuronal and glial glutamate membrane transporters (EAATs) eliminate the glutamate from the synaptic cleft avoiding the overstimulation of the glutamatergic receptors. The vesicular transporters (VGLUTs) contribute to decrease the cytosolic glutamate by driving it into synaptic vesicles. Understanding the excitotoxic process is crucial to develop possible therapies for preventing the ischaemic damage. VGLUTs, EAATs, AMPA and NMDA glutamatergic receptors, as well as the main $GABA_A$ receptor subunits, are the main molecules involved in the ischaemic process and the subsequent reperfusion.

Viability and normal cell function depend on the proper protein folding which is monitorized by the cell trough a complex quality control. Accumulation of misfolded proteins in the cell is called endoplasmic reticulum stress (ER stress) that cells try to overcome eliciting a number of molecular processes included in the so named unfolded protein response (UPR).

The basic aim of stroke researchers is to find therapeutic targets and possible palliative or regenerative therapies. In this regard, the present study analyzes some aspects of the modulatory effects of GABA and inflammation on the glutamatergic response to the ischaemia in different experimental models (both in vivo and ex vivo). The expression of vesicular (VGLUTs) and membrane glutamatergic transporters (EAATs), as well as NMDA and AMPA glutamatergic receptors were measured. Also, the expression of a number of molecules involved in the UPR following the ischaemic damage was studied.

All the studies were carried out on male rats (Sprague-Dawley). In vivo ischaemia-reperfusion (I/R) studies were carried out on a global cerebral ischaemia model in animals with different ages. Ex vivo OGD assays were performed in young animals using two different models, brain slices from cerebral cortex and hippocampus and hippocampal organotypic cultures. Expression of the different genes was quantified measuring mRNA and protein levels using real time quantitative PCR and Western blot assays respectively in the different structures. Cell mortality was determined by propidium iodide staining and lactate dehydrogenase released.

In vivo assays showed significant decreases in the ARNm levels of VGLUT1, VGLUT2 and VGLUT3 transporters both in hippocampus (CA1, CA3 areas and

dentate gyrus) and cerebral cortex in 3-month-old animals. However, the response observed in 18-month-old animals was significantly lessened. The treatment with meloxicam also resulted in a lessened response in these transcripts and in an increase in the protein amounts.

OGD assays carried out in the ex vivo model of brain slices analyzed the response in cerebral cortex and hippocampus. Assays evidenced an increased mortality as consequence of both OGD and reperfusion in cerebral cortex and hippocampus. The presence of 100 µM GABA in the medium resulted in a significant decrease in this mortality in OGD and reperfusion in both structures. The use of a selective $GABA_A$ antagonist, bicuculline, in the medium allowed discarding a significant role of chloride calcium $GABA_A$ channels in the hippocampal mortality but they play an important role in the GABA neuroprotective effect in the cerebral cortex. The presence of GABA also resulted in increased transcript levels in all the genes studied (VGLUTs, EAATs, $GABA_A$ subunits and GAD65) in normoxic, OGD and RL conditions in both hippocampus and cerebral cortex. Either the presence of GABA in normoxic conditions or the OGD and RL assays without GABA resulted in increased transcript levels of the chaperons GRP78, GRP94 and PDI as well as the proapoptotic marker CHOP in all the structures studied. The presence of GABA during the OGD and RL assays resulted in a significant decrease in these transcripts in the cerebral cortex, however, a different response was observed in the hippocampus, where transcrips were not modified during OGD but increased during RL condition.

Ex vivo OGD assays on hippocampal organotypic culture evidenced that the antiinflammatory agent meloxicam, a cyclooxygenase 2 inhibitor, resulted in a

decrease of the mortality during OGD. Two different responses in the transcripts of the genes analyzed were observed in these assays. VGLUT1, Glast-1a, GLT-1, EAAC-1, NR1, NR2A and GluR1 transcripts appeared decreased in OGD and increased after 3 hours reperfusion. In contrast, GluR2, NR2B and VGLUT2 transcripts appeared significantly increased during OGD but presented significant decreases along RL with respect to the normoxic condition. The presence of meloxicam enhanced the response of the second group of genes and lessened the response of the first group.

Conclusions

1) In vivo assays demonstrate that glutamate vesicular transporters (VGLUTs) are modified by global cerebral ischaemia. Interestingly, young animals presented decreased transcript levels while aged animals presented increased transcript levels. Protein expression was also modulated in this ischaemia model resulting mainly in increases levels although this modulation was age-structure and VGLUT subtype-dependent.

2) COX-2 inhibition lessened the global cerebral ischemia response induced in VGLUT transcripts in young animals. This inhibition itself induced increases in VGLUTs but we could not detect any ischemia-dependent effect in protein expression.

3) Ex vivo assays in brain slices showed that glutamatergic membrane and vesicular transporter transcripts appear decreased by both OGD and RL in hippocampus and cerebral cortex in the glutamatergic presynaptic neurons, the postsynaptic neurons expressing EAATs and glial cells. In contrast, OGD assays induced decreased transcripts in the GABAergic subunits expressed in postsynaptic neurons but no changes were observed in the transcript expression of the GABAergic presynaptic terminals.

4) The presence GABA during OGD and RL results in a neuroprotective effect in both cerebral cortex and hippocampus, lessening the downregulation in the hippocampus and upregulating in the cerebral cortex the glutamatergic transporter transcripts.

5) The neuroprotective effec of GABA is $GABA_A$ chloride channel receptor-dependent in the cerebral cortex but not in the hippocampus.

6) The presence of GABA in normoxic conditions results in an increased endoplasmic reticulum stress in a $GABA_A$-dependent way. During OGD and RL the role of the cytosolic chloride has low relevance in the control of the ER-stress.

7) In the cerebral cortex, a GABA-dependent mechanism (not $GABA_A$) seems to play a critical role in the OGD-induced UPR while in the hippocampus this mechanism seems to present low relevance.

8) OGD assays in hippocampal organotypic culture allows to discriminate two groups of genes with different patterns of transcript expression. Group 1 includes VGLUT1, Glast-1a, GLT-1, EAAC-1, NR1, NR2A and GluR1 and group 2 includes GluR2, NR2B y VGLUT2.

9) COX-2 inhibition exacerbates the mRNA expression pattern of group 2 and lessened the expression pattern of group 1.

Resumen y objetivos

La isquemia cerebral es una de las principales causas de muerte y discapacidad en los países industrializados, y ocasiona un gran coste socioeconómico. En España, el ictus es la primera causa de muerte en mujeres y segunda en varones, por detrás del infarto de miocardio.

Durante el accidente cerebrovascular se produce una disminución del flujo sanguíneo cerebral (FSC) por debajo de 10 mL/100 g/min en un área restringida y, como consecuencia, se produce una rápida muerte celular. Uno de los factores importantes que afectan la muerte neuronal, promovida tras el daño isquémic,o es la sobreactivación de receptores glutamatérgicos, debida al aumento de glutamato en el espacio extracelular durante el proceso isquémico. Esta sobreexcitación da lugar a un gran incremento del calcio intracelular, que lleva a una despolarización neuronal masiva. Como consecuencia se produce una liberación masiva, desde los terminales presinápticos, de aminoácidos excitadores como glutamato, un proceso conocido **como excitotoxicidad mediada por glutamato**. Además se liberan neurotransmisores inhibidores como ácido gamma-aminobutírico (GABA) y glicina.

La excitotoxicidad resulta, por tanto, de una liberación excesiva y/o de una retirada inadecuada del glutamato sináptico. En condiciones normales, los transportadores de membrana para la recaptación de glutamato (EAATs), presentes en las neuronas y las células de la glía, permiten eliminar el glutamato del espacio sináptico, evitando que se llegue a producir una sobreestimulación de los receptores glutamatérgicos. Los transportadores vesiculares (VGLUTs), reducen el contenido de glutamato citosólico al incorporarlo a las vesículas sinápticas. Comprender el proceso excitotóxico es clave para poder desarrollar posibles terapias para paliar el daño isquémico.

Entre las moléculas que juegan un papel clave en este proceso hay que destacar los **VGLUTs, EAATs**, los receptores de glutamato **NMDA y AMPA**, así como las principales subunidades que conforman el receptor **$GABA_A$**.

La viabilidad y el normal funcionamiento de la célula dependen del correcto plegamiento de las proteínas, para lo cual existe un complejo sistema de control celular, que promueve el correcto plegamiento proteico. En el caso de acumulación de proteínas que no llegan a adquirir la correcta conformación nativa, se desencadena el denominado estrés de retículo, que la célula trata de resolver mediante diferentes mecanismos moleculares que originan la llamada respuesta a proteínas mal plegadas (UPR).

La búsqueda de dianas terapéuticas y posibles terapias paliativas o regenerativas es el objetivo básico de los grupos de investigación que trabajan en el accidente cerebrovascular. En este sentido, el presente estudio analiza algunos aspectos de los efectos moduladores del GABA y la inflamación sobre la respuesta glutamatérgica a la isquemia, en diferentes modelos experimentales in vivo e in vitro, midiendo la expresión de los transportadores glutamatérgicos vesiculares (VGLUTs) y de membrana (EAATs), así como de los receptores glutamatérgicos NMDA y AMPA. También se describe la expresión de algunas de las moléculas implicadas en la respuesta de proteínas mal plegadas (UPR) como consecuencia del daño isquémico.

A partir de estos precedentes se plantearon los siguientes **objetivos:**

1) Caracterizar la respuesta de los recaptadores glutamatérgicos, de membrana y vesiculares, en un modelo de isquemia cerebral global. A diferencia de la isquemia cerebral focal, los modelos utilizados presentan únicamente una zona de penumbra, lo que en principio presenta un daño más homogéneo al no existir un core isquémico de muerte neuronal y el consecuente gradiente de daño en la zona de penumbra típico de la isquemia focal. El estudio de los recaptadores complementa los estudios realizados por nuestro grupo de investigación sobre los receptores glutamatérgicos AMPA y NMDA. En este objetivo, se incluye el papel de la edad y la presencia de agentes antiinflamatorios.

2) Determinar el efecto neuroprotector del GABA midiendo

2a) el efecto neuroprotector del GABA, midiendo la mortalidad celular y su papel sobre los recaptadores glutamatérgicos en la respuesta a la isquemia.

2b) el efecto del GABA sobre el estrés de retículo, midiendo la respuesta a las proteínas mal plegadas (UPR)

3) Determinar el papel neuroprotector de agentes antiinflamatorios (meloxicam), midiendo la mortalidad celular y su papel sobre los recaptadores glutamatérgicos en la respuesta a la isquemia.

Todo el estudio fue realizado en ratas macho de la cepa Sprague-Dawley. Los ensayos de isquemia-reperfusion (I/R) in vivo, se llevaron a cabo usando un modelo de isquemia global transitoria, en ratas de diferentes edades. Para el

estudio de OGD, se realizaron dos tipos de ensayos ex vivo usando secciones hipocampales y de corteza cerebral, y cultivos organotipicos hipocampales, ambos de ratas jóvenes. La expresión de los diferentes genes estudiados en las distintas estructuras cerebrales, se realizó cuantificando sus niveles de ARNm y proteína, mediante las técnicas de PCR en tiempo real y Western blot respectivamente. La medida de la mortalidad celular, se realizó mediante tinción con ioduro de propidio y cuantificando los niveles de lactato deshidrogenasa.

Los ensayos in vivo mostraron un descenso significativo en los niveles de ARNm de los transportadores VGLUT1, VGLUT2 y VGLUT3, tanto en hipocampo (CA1, CA3 y GD) como en corteza cerebral en individuos de 3 meses de edad, mientras que se puede observar una atenuación muy clara de esta respuesta en individuos envejecidos (18 meses). El tratamiento con un agente inhibitorio de la ciclooxigenasa 2 (meloxicam), también dio lugar a una atenuación de la respuesta en la expresión de los ARNm de estos genes, y un incremento en la expresión de sus niveles de proteína.

Los ensayos de OGD ex vivo llevados a cabo en el modelo de secciones hipocampales y de corteza cerebral, mostró un incremento en la mortalidad en ambas estructuras, tanto como consecuencia de la OGD como de la reperfusión. El tratamiento de las secciones con 100 μM de GABA, mostró un descenso significativo en la mortalidad celular, tanto en la OGD como en la reperfusión en ambas estructuras. El uso de un antagonista selectivo de los receptores $GABA_A$, bicuculina, permitió demostrar que los canales de cloro del complejo $GABA_A$, presentan poca relevancia en la mortalidad hipocampal, pero juegan un importante papel en el efecto neuroprotector del GABA en el corteza

cerebral. En presencia de GABA también se observó un aumento en los transcritos de todos los genes estudiados (VGLUTs, EAATs, subunidades $GABA_A$ y el marcador presináptico GAD65) y en todas las condiciones del estudio (normóxicas, OGD y RL), tanto en corteza cerebral como en hipocampo.

Tanto la presencia de GABA en condiciones normóxicas, como los ensayos de OGD y RL en ausencia de GABA, produjeron un incremento en la expresión de los transcritos de las chaperonas GRP78, GRP94 y PDI, así como en el marcador de apoptosis CHOP en ambas estructuras. La presencia de GABA en los ensayos durante la OGD y RL dio lugar a un descenso significativo de estos transcritos en la corteza cerebral, sin embargo, en el hipocampo, la respuesta fue diferente, manteniéndose los niveles de los transcritos durante la OGD y aumentando durante la RL.

Los ensayos de OGD ex vivo, llevados a cabo en el modelo de cultivos hipocampales organotípicos, mostraron que el agente antiinflamatorio meloxicam, un inhibidor de la ciclooxigenasa 2, presenta una disminución de la mortalidad durante la OGD. Se observaron dos respuestas diferentes en los transcritos de los genes analizados. Un grupo de ellos, VGLUT1, Glast-1a, GLT-1, EAAC-1, NR1, NR2A y GluR1, presentaron niveles significativamente más bajos durante la OGD y aumentos a las 3 horas de reperfusión, mientras que otro grupo, GluR2, NR2B y VGLUT2, presentaron aumentos significativos de sus niveles de ARN durante la OGD y niveles significativamente más bajos durante la RL comparados con las condiciones normóxicas. La presencia de meloxicam intensificó la respuesta del segundo grupo de genes y atenuó la respuesta del primer grupo.

Conclusiones

1) Los ensayos in vivo, demuestran que los transportadores vesiculares de glutamato (VGLUTs), son modificados por la isquemia cerebral global. En los animales jóvenes, disminuyen los niveles de transcritos, mientras que los animales viejos aumenta. La expresión de las proteínas también fue modulada en este modelo de isquemia, principalmente con aumentos en la expresión, aunque esta modulación depende de la edad, la estructura y el subtipo de VGLUT.

2) La inhibición de la COX-2, atenúa la respuesta inducida por la isquemia cerebral global, en los transcritos de VGLUT en animales jóvenes. La inhibición dio lugar a aumentos en estos transcritos, pero no se pudo detectar ningún efecto mediado por isquemia en la expresión de los niveles de proteína.

3) En los ensayos ex vivo en secciones de hipocampo y corteza cerebral, los transcritos de los transportadores glutamatérgicos de membrana y vesiculares, aparecieron reducidos, durante la OGD y la RL, en el hipocampo y la corteza cerebral en las neuronas glutamatérgicas presinápticas, las postsinapticas que expresan EAATs y en las células gliales. Por el contrario, los ensayos de OGD dieron lugar a disminuciones en los transcritos de las subunidades de receptores GABAérgicos expresados en neuronas postsinápticas, pero no se observaron cambios en la expresión de la transcripción de los terminales presinápticos GABAérgicos.

4) La presencia de GABA durante OGD y RL, presentó un papel neuroprotector tanto en la corteza cerebral como en el hipocampo. También modificó la respuesta en los transcritos de los transportadores glutamatérgico inducida por

la OGD, atenuándola en el hipocampo e incrementándola en la corteza cerebral.

5) El efecto neuroprotector de GABA, es dependiente de los canales de cloro del receptor $GABA_A$ en la corteza cerebral, pero no en el hipocampo.

6) La presencia de GABA en condiciones normóxicas, da lugar a un aumento del estrés de retículo endoplásmico dependiente de $GABA_A$. Durante la OGD y RL, el papel del cloruro citosólico tiene escasa relevancia en el control del estrés de retículo.

7) En el hipocampo, un mecanismo dependiente de GABA pero no de $GABA_A$, parece jugar un papel crítico en la UPR inducida por OGD, pero en la corteza cerebral este mecanismo parece presentar escasa relevancia.

8) Los ensayos de OGD en cultivos organotípicos hipocampales, permiten discriminar dos grupos de genes con diferentes patrones en la expresión de sus transcritos. El grupo 1 incluye VGLUT1, Glast 1a-, GLT-1, EAAC-1, NR1, NR2A y GluR1, y el grupo 2 incluye GluR2, NR2B y VGLUT2.

9) La inhibición de la COX-2 amplifica el patrón de expresión de ARNm del grupo 2, y atenúa el patrón de expresión del grupo 1.

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