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EXPRESIÓN DE LOS RECEPTORES GLUTAMATÉRGICOS DE NMDA Y AMPA TRAS LA ISQUEMIA CEREBRAL. EFECTO DE LA PRIVACIÓN DE OXÍGENO Y GLUCOSA SOBRE LA EXPRESIÓN DEL RECEPTOR DE NMDA.

Expression of glutamatergic NMDA and AMPA receptors after cerebral ischemia. Effect of oxygen and glucose deprivation on NMDA receptor expression.

Memoria para optar al Grado de Doctor con Mención europea
presentada por

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(Art. 11.3 del R.D. 56/2005)

El Dr. D. Arsenio Fernández López y la Dra Dña Beatriz Martínez Villayandre como Directores[1] de la Tesis Doctoral titulada **"Expresión de los receptores glutamatérgicos de NMDA y AMPA tras la isquemia cerebral. Efecto de la privación de oxígeno y glucosa sobre la expresión del receptor de NMDA"** realizada por **D. Severiano Dos Anjos Vilaboa** en el Departamento de Biología Molecular, informan favorablemente el depósito de la misma, dado que reúne las condiciones necesarias para su defensa.

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ABREVIATURAS

- **ADAR2:** Adenosina deaminasa dependiente de RNA 2, RNA dependent adenosine deaminase 2
- **AMPA:** Ácido α-amino-3-hidroxi-5-metil-4-isoxazol-propiónico, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
- **AMPAR:** Receptores de AMPA, AMPA receptors
- **ARN:** Ácido ribonucleico, ribonucleic acid
- **ARNm:** ARN mensajero, messenger RNA
- **ATP:** Adenosina trifosfato, adenosine triphosphate
- **CA1:** Región del hipocampo *Cornu Ammonis* 1, *Cornu Ammonis* 1 hippocampal region
- **CA3:** Región del hipocampo *Cornu Ammonis* 3, *Cornu Ammonis* 3 hippocampal region
- **CC:** Corteza cerebral, Cerebral cortex
- **C-Pu:** Caudado-Putamen, Caudate-Putamen
- **CNQX:** 6-ciano-7-nitro-quinoxalina-2,3-diona, 6-cyano-7-nitroquinoxaline-2,3-dione
- **DMEM:** Medio basal de Eagle modificado por Dulbecco, Dulbecco´s Modified Eagle´s Medium
- **ERK:** Kinasa regulada por señales extracelulares, Extracellular Signal-Regulated Kinase
- **FSC:** Flujo sanguíneo cerebral, cerebral blood flow
- **GAPDH:** Gliceraldehído 3-fosfato deshidrogenasa, Glyceraldehyde 3-phosphate dehydrogenase
- **GD:** Giro dentado, Dentate gyrus
- **GluR:** Receptor de glutamato, Glutamate receptor
- **IL:** interleucina, interleukin
- **iNOS:** Óxido nítrico sintasa inducible, Inducible Nitric Oxide Synthase
- **I/R:** Isquemia/Reperfusión, Ischemia/Reperfusion
- **LDH:** Lactato deshidrogenasa, Lactate Dehydrogenase
- **Mac-1 (*CD11b*/*CD18*):** Molécula de adhesión de macrófagos 1, Macrophage adhesion molecule-*1*
- **MAGUK:** Guanilato kinasas asociadas a membrana, Membrane-Associated Guanylate Kinase
- **MAPK:** Proteína kinasa activada por mitógenos, Mitogen-Activated Protein Kinase
- **MAST2:** Serina/treonina kinasa2 asociada a microtúbulos, Microtubule associated serine/threonine kinase2
- **NMDA:** N-metil-D-Aspartato, N-methyl-D-Aspartate
- **NMDAR:** Receptores de NMDA, NMDA receptors
- **nNOS:** Óxido nítrico sintasa neuronal, Neuronal Nitric Oxide Synthase
- **OGD:** Privación de oxígeno y glucosa, Oxygen Glucose Deprivation
- **PBS:** Tampón fosfato salino**,** Phosphate Buffered Saline
- **PCR:** Reacción en cadena de la polimerasa, Polymerase chain reaction
- **PSD:** Densidad postsináptica, Post synaptic density
- **REST:** Factor de transcripción de silenciamiento de RE1, RE1-silencing transcription factor
- **TARPs:** Proteínas transmembrana asociadas al receptor de AMPA, Transmembrane AMPA receptor-associated protein

- **TBS:** Tampón tris salino, Tris Buffered Saline
- **TGF-β:** Factor de crecimiento transformante beta, Transforming growth factor beta
- **TNF-α:** Factor de necrosis tumoral alfa, Tumor necrosis factor alpha
- **VO:** Oclusión arterial, Vessel Occlusion

I. INTRODUCCIÓN

1. ENFERMEDADES CEREBROVASCULARES

Los accidentes cerebrovasculares agudos (ictus) son trastornos neurológicos originados por la disminución brusca del aporte sanguíneo cerebral. La incidencia del ictus es de unos 200 casos por cada 100.000 habitantes (Bonita, 1992), lo que origina en España cada año entre 120000-130000 casos de accidente cerebrovascular. Más de la mitad de los pacientes quedan discapacitados o fallecen (Prieto-Arribas *et al.*, 2008).

Según el Instituto Nacional de Estadística, el ictus representa en España la segunda causa de muerte en los varones y la primera en las mujeres (INE, 2008). Además constituye la causa más importante de invalidez permanente por las secuelas que quedan en los supervivientes (Gil de Castro y Gil-Núñez, 2000). El accidente cerebrovascular es la primera causa de ingreso hospitalario por enfermedad neurológica y la causa más frecuente de demanda asistencial en los servicios de urgencias por enfermedad neurológica grave, lo que conlleva elevados gastos económicos (Hervás-Angulo *et al.*, 2006).

Existen algunos factores de riesgo que aumentan la probabilidad de sufrir un accidente cerebrovascular, entre los que destacan: la edad, el género (mayor prevalencia en hombres, pero mortalidad más alta en mujeres), factores genéticos (relacionados con antecedentes familiares), y otros como la alta presión sanguínea, tabaquismo, alcoholismo o sedentarismo (Lainez, 1999).

La mayoría de los accidentes cerebrovasculares (85%) son de tipo isquémico. Un accidente cerebrovascular isquémico ocurre cuando una arteria que proporciona sangre al cerebro queda obstruida, reduciendo o interrumpiendo el flujo de sangre en el mismo. Si la gravedad de la isquemia cerebral produce una pérdida celular irreversible con lesión destructiva localizada, se denomina infarto cerebral (García, 1984). La causa más común de este bloqueo y del consecuente infarto son los coágulos de sangre. El resto de accidentes cerebrovasculares (aproximadamente un 15%) son de tipo hemorrágico, 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).

El fenómeno que caracteriza a todas las enfermedades cerebrovasculares es la isquemia cerebral, la cual se define como la reducción del aporte sanguíneo hasta unos niveles insuficientes para mantener el metabolismo y funcionamiento normales de las células cerebrales. Las causas más frecuentes de isquemia son el estrechamiento gradual (ateroesclerosis), la oclusión súbita (trombosis), la microembolia en vasos de pequeño calibre o la hipertensión intracraneal (Hossmann, 1982). Otras causas de isquemia cerebral son la parada cardiaca y también el shock e hipotensión sistémica, que originan el descenso del aporte sanguíneo al cerebro en su totalidad (isquemia global). La isquemia cerebral focal corresponde a situaciones en las que la reducción del flujo sanguíneo se circunscribe al territorio irrigado por una determinada arteria del cerebro (Brouns y De Deyn, 2009).

La obstrucción de un vaso sanguíneo cerebral produce una disminución del gradiente de flujo de sangre en el territorio vascular afectado que origina una zona hipoperfundida (núcleo isquémico) y una zona circundante con un flujo reducido pero que permite el mantenimiento funcional de las neuronas, denominada penumbra isquémica (Obrenovitch, 1995).

2. MODELOS EXPERIMENTALES DE ISQUEMIA CEREBRAL

Los modelos experimentales han sido una herramienta fundamental para el conocimiento de la fisiopatología de los accidentes cerebrovasculares, ya que han permitido estudiar de una forma relativamente simple un proceso tan complejo como la isquemia cerebral, que se caracteriza por una gran heterogeneidad. Además el uso de estos modelos permite el desarrollo de nuevos compuestos con posible efecto terapéutico para su posterior aplicación clínica (Prieto-Arribas *et al.,* 2008).

Los modelos experimentales se pueden dividir en dos categorías principales: modelos *in vivo* que permiten reproducir la patología humana en animales de experimentación (principalmente roedores) y modelos *in vitro* llevados a cabo a nivel celular o tisular en los cuales se controlan estrictamente las condiciones a ensayar. A continuación se detallarán los principales modelos utilizados en el estudio de la isquemia cerebral.

2.1. Modelos *in vivo* de isquemia

2.1.1. Modelos de isquemia global

Estos modelos se basan en la oclusión de las grandes arterias que irrigan el cerebro y reproducen el daño cerebral que se origina tras un paro cardiaco o la obstrucción de las arterias carótidas.

- **Modelo de oclusión de 4 vasos:**

En este modelo se produce una interrupción completa de las arterias vertebrales posteriores (normalmente por cauterización), seguida de la oclusión de las arterias carótidas (Pulsinelli y Brierley, 1979).

- **Modelo de oclusión de 2 vasos:**

Este modelo se basa en el bloqueo de las dos arterias carótidas acompañado de una hipotensión sistémica (alrededor de 50 mm de Hg), que puede ser alcanzada farmacológicamente o mediante un sangrado controlado del animal. Este modelo se desarrolló por primera vez en la década de los 70 (Eklöf y Siesjo, 1972). Utilizando este modelo se reduce parcialmente el flujo sanguíneo pero de forma suficiente para causar daño cerebral, principalmente en las neuronas piramidales del área hipocampal CA1, y también aunque en menor medida en corteza cerebral y caudado-putamen (Smith *et al.*, 1984).

2.1.2. Modelos de isquemia focal

- **Oclusión de la arteria cerebral media:**

La mayor parte de los modelos de isquemia focal se basan en la oclusión de la arteria cerebral media, lo cual da lugar a una reducción del flujo sanguíneo en el estriado y la corteza cerebral (García, 1984). Esta oclusión puede ser permanente o temporal (con reperfusión). Sobre este modelo existen multitud de variantes. Una de las técnicas más utilizadas desarrollada a finales de la década de los 80 (Longa *et al.*, 1989) y relativamente poco invasiva, es aquella que utiliza un filamento intraluminal para realizar la oclusión de la arteria cerebral media. Mediante una sonda láser *doppler* se puede monitorizar el flujo sanguíneo cerebral en la zona infartada (Riyamongkol *et al.*, 2002).

● **Oclusión unilateral de la arteria carótida común:**

La oclusión de la arteria carótida común asociada a hipoxia en ratas produce un daño focal cerebral ipsilateral a la oclusión. Las lesiones se producen en casi todas las regiones cerebrales, principalmente hipocampo (el área más afectada), corteza cerebral, estriado, y tálamo. La sustancia gris se ve más afectada que la sustancia blanca (Levine, 1960).

● **Oclusión microvascular de la arteria cerebral media:**

-Oclusión fototrombótica: Consiste en la administración de un contraste fotosensible como el rosa de bengala que provoca una trombosis intravascular mediante inducción fotoquímica. Este modelo se ha desarrollado para producir lesiones isquémicas focales corticales (Watson *et al.*, 1985).

-Microembolización de agregados plaquetarios: Este modelo se ha desarrollado para reproducir los ataques isquémicos transitorios y consiste básicamente en la formación de agregados plaquetarios que se inducen con la inyección intracarotídea de adenosina difosfato o ácido araquidónico (Fieschi *et al.*, 1975).

-Infartos espontáneos en ratas hipertensas: Existe una cepa de ratas hipertensas que son propensas a presentar infartos cerebrales (Yamori *et al.*, 1976). Estas ratas se someten a una dieta rica en sales desde las seis semanas de vida y empiezan a presentar infartos de forma consistente a partir de la duodécima semana. Este modelo se asemeja mucho al que se produce en clínica humana pero presenta una gran variabilidad tanto en la localización como en las características patológicas.

2.2. Modelos *in vitro* de isquemia

2.2.1. Modelo de privación de oxígeno y glucosa (*Oxygen glucose deprivation*, OGD)

Este modelo empezó a utilizarse en la década de los 80 (Whittingham *et al.*, 1984) y consiste en la incubación de secciones de tejido cerebral en soluciones fisiológicas que carecen de oxígeno (mediante burbujeo con nitrógeno) y glucosa, para reproducir la interrupción del aporte de oxígeno y nutrientes al parénquima cerebral que ocurre tras un proceso isquémico. Aunque no son verdaderos experimentos de isquemia cerebral, sus respuestas ayudan a comprender los mecanismos celulares y moleculares que se producen en condiciones similares a isquemia. La principal ventaja de este modelo es que permite un control muy estricto del microambiente extracelular, la duración del proceso de anoxia, etc, y mantiene las características histológicas y electrofisiológicas del tejido cerebral. Este modelo puede utilizarse en procedimientos cortos utilizando secciones cerebrales, relativamente largos mediante el uso de cultivos organotípicos (Vornov *et al.*, 1994) o usando cultivos primarios neuronales (Goldberg y Choi, 1993) procedentes de distintas estructuras encefálicas.

2.2.2. Otros modelos:

Existen algunos modelos que tratan de reproducir el fenómeno de excitotoxicidad que se produce por la isquemia mediante adición de glutamato o NMDA (Kim *et al.*, 2009).

También se usan modelos de isquemia química que producen hipoxia utilizando sustancias como la oligomicina que inhibe la ATP sintasa o a través de azida sódica (Djali y Dawson, 2001), y se pueden acompañar también de hipoglucemia.

En el desarrollo de este trabajo se han utilizado 2 modelos experimentales distintos, ambos desarrollados en ratas de la cepa Sprague-Dawley: el modelo de isquemia global transitoria *in vivo* mediante oclusión de las arterias carótidas e hipotensión seguido de reperfusión, y el modelo *ex vivo* de OGD utilizando secciones hipocampales de cerebro de rata.

2.3. Importancia de la caracterización de la expresión génica en modelo *ex vivo*

El uso de secciones cerebrales en ensayos de órgano aislado en el campo de la neurociencia es ampliamente utilizado desde que a principios de la década de los 60 se usó por primera vez para realizar estudios electrofisiológicos y bioquímicos (McIlwain, 1961). Este modelo se utiliza habitualmente para el estudio de receptores de neurotransmisores y también en ensayos de OGD (Jourdi *et al.*, 2009; Zhou y Baudry, 2006). Una de sus ventajas principales es que permite un control estricto del medio en el que se encuentra la sección de cerebro, regulando las concentraciones de las diferentes moléculas que actúan sobre las células, al mismo tiempo que mantiene en gran medida la morfología y complejidad celular del tejido, y al menos en parte, las interacciones neuronales que se presentan *in vivo*.

A pesar de su uso para estudios de carácter bioquímico, farmacológico y electrofisiológico, existen muy pocos estudios en los cuales se determine la expresión génica utilizando este modelo. En particular son muy escasos los estudios que analizan la expresión génica *ex vivo* de distintos receptores de neurotransmisores, en concreto de los receptores de glutamato (Pérez-Velázquez y Zhang, 1994; Small *et al.*, 1997). En los últimos años la técnica de PCR en tiempo real se ha convertido en una herramiento muy utilizada, ya que permite medir con gran exactitud y sensibilidad los niveles de ARN mensajero de distintos genes. Los niveles de ARNm de los genes de interés se normalizan con respecto a genes de referencia, habitualmente genes *housekeeping* (Gilsbach *et al.*, 2006). Aunque los genes *housekeeping* se suelen mantener constantes tienen cierto grado de variabilidad y pueden verse influidos por el tratamiento experimental. Por ello, antes de proceder a realizar ensayos de PCR en tiempo real es necesario comprobar que grado de variabilidad presentan los genes que van a ser utilizados como referencia en las distintas muestras y condiciones para seleccionar los que presentan menos variabilidad (Banda *et al.*, 2008). Además es preciso asegurarse de un buen nivel de preservación de la integridad del ARN de las muestras a estudiar para descartar su posible influencia en los resultados obtenidos (Fleige y Pfaffl, 2006; Perez-Novo *et al.*, 2005).

Los datos que existen hasta el momento de los modelos *ex vivo* de sistema nervioso no presentan un estudio sistemático de la integridad del ARN y niveles de ARNm a lo largo del tiempo, así como de la influencia de la temperatura y los medios de incubación utilizados. Por tanto se hace preciso poner a punto el modelo y definir una ventana de trabajo adecuada, lo que constituye uno de los objetivos de este estudio.

3. FISIOPATOLOGÍA DE LA ISQUEMIA CEREBRAL

Durante la isquemia cerebral se producen una serie de alteraciones hemodinámicas, bioquímicas y neurofisiológicas que ocurren de forma simultánea, y que dependen básicamente de la intensidad y duración del daño isquémico. A continuación, se describen los fenómenos básicos que se producen durante un proceso isquémico y que se relacionan más directamente con este trabajo.

Para que se produzca la actividad eléctrica de las neuronas y la preservación de sus funciones biosintéticas se requiere un flujo sanguíneo normal de 50 a 55 ml/min/100 g de tejido cerebral en humanos. Tras la aparición de una isquemia cerebral (especialmente de tipo focal), debido a que la reducción del flujo sanguíneo no es homogénea en el territorio isquémico, es posible observar dos regiones morfológicas que tienen gran importancia fisiológica en el desarrollo del daño tisular: núcleo isquémico y zona de penumbra (Lee *et al.,* 1999*;* Lipton, 1999; Sweeney, 1997).

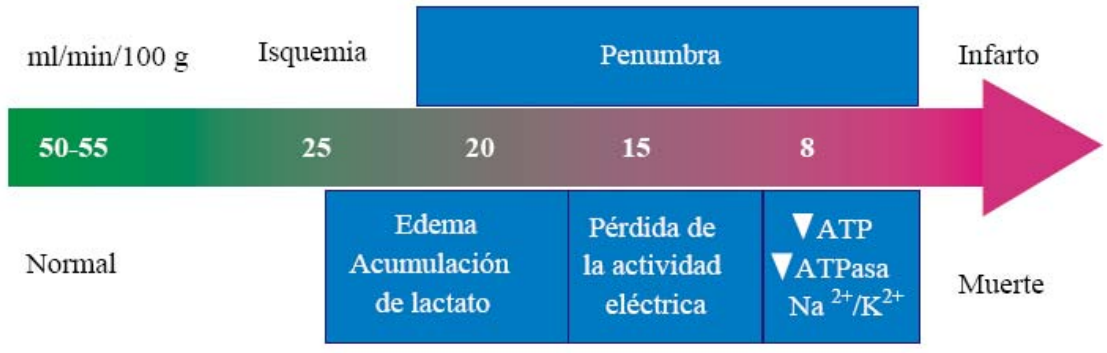

Figura 1. Efectos de la reducción del flujo sanguíneo cerebral.

La región más afectada es denominada núcleo isquémico, donde el flujo sanguíneo cerebral (FSC) disminuye a 10 ml/min/100 g de tejido que corresponde a menos del 20% del FSC normal. El daño neuronal puede ser irreversible después de 30 minutos, las neuronas comienzan a perder gradientes iónicos, se despolarizan y mueren por procesos necróticos inmediatos. La segunda región localizada en la periferia del núcleo es denominada zona de penumbra isquémica donde el flujo sanguíneo cerebral es de 10 a 25 ml/min/100 g de tejido (≤ 50% del FSC normal) y la evolución del daño cerebral es menos rápida y en muchos casos reversible (Dirnagl *et al.,* 1999; Lipton, 1999). En las neuronas localizadas en esta segunda región se deteriora la capacidad de generar potenciales de acción, pero se mantienen gradientes iónicos intactos hasta que la producción de adenosina trifosfato (ATP) cae por debajo del 50% de sus niveles normales (Iadecola, 1999).

La zona de penumbra es potencialmente recuperable, la integridad de la membrana celular se preserva y puede ser rescatada antes de su conversión a núcleo isquémico (Back, 1998).

3.1. Procesos celulares y bioquímicos que ocurren durante la isquemia cerebral y la reperfusión

Durante la isquemia cerebral, las fuentes de almacenamiento de ATP se reducen rápidamente, mientras los sistemas de transporte activo (como las bombas de calcio, sodio y potasio) siguen funcionando por un cierto tiempo, contribuyendo a la disminución de las reservas de energía intracelular (Lee *et al.*, 1999; Lipton, 1999; Sweeney, 1997, White *et al.*, 2000). Las bombas de calcio de la membrana plasmática y del retículo endoplásmico se mantienen activas para conservar la concentración de calcio libre intracelular en niveles fisiológicos, con un valor cercano a 100 nM (Banasiak *et al.*, 2000; Brini y Carafoli, 2000). Las bombas de sodio y potasio localizadas en la membrana plasmática también contribuyen al consumo de ATP para mantener el potencial eléctrico de la membrana y los gradientes iónicos (Banasiak *et al.*, 2000; Dirnagl *et al.*, 1999). Con la entrada masiva de Ca^{2+} al espacio intracelular y la pérdida del gradiente iónico se produce una despolarización permanente de la membrana celular. Inmediatamente después se presenta acidosis y liberación presináptica del neurotransmisor glutamato, el cual se une a receptores ionotrópicos, fundamentalmente del tipo NMDA y AMPA generando su activación y una mayor entrada de calcio. Esta activación se ve

favorecida debido a la liberación del Mg^{2+} que mantenía bloqueados los receptores como consecuencia de la despolarización de la membrana. Estos hechos contribuyen a generar un mayor daño neuronal debido a la excitotoxicidad por glutamato (como se describe más adelante en las secciones 4.2 y 4.4) además de los daños producidos por la alteración del metabolismo celular (Dirnagl *et al.*, 1999; Lee *et al.*, 1999; Michaelis, 1998).

La activación de receptores de NMDA es responsable en gran medida del importante incremento en los niveles de calcio intracelular y del inicio de la cascada isquémica que originará finalmente muerte celular (Ginsberg, 2003). Se ha demostrado que el incremento en la concentración intracelular de calcio libre promueve la muerte neuronal ya sea por procesos necróticos o apoptóticos (Banasiak *et al.*, 2000; Brini y Carafoli, 2000; Lipton, 1999; Tymianski *et al.* 1993; White *et al.*, 2000). El incremento del calcio en el interior celular activa una gran variedad de enzimas (proteasas, fosfolipasas, endonucleasas, etc.) y promueve a su vez la expresión de varios genes de respuesta inmediata temprana (Trump y Berezesky, 1995).

Después de un proceso isquémico se produce también una reducción importante en el pH intracelular, hasta valores de 6,4 a 6,7, y se ha demostrado que la hiperglucemia agrava los daños inducidos por la isquemia cerebral (Kagansky *et al.*, 2001). Esto se debe principalmente a la producción de ácido láctico durante la glucolisis anaeróbica, y parece que esta acidosis incrementa el daño isquémico y que los astrocitos podrían tener un papel neuroprotector (Lascola y Kraig, 1997).

La isquemia cerebral y especialmente la reperfusión (restablecimiento de la circulación sanguínea) son responsables del daño oxidativo por generación de radicales libres (Nita *et al.*, 2001), que provocan graves efectos sobre la supervivencia neuronal (Chan, 2001; Lewen *et al.*, 2000). El cerebro es especialmente susceptible al daño mediado por radicales libres debido a su alto contenido en ácidos grasos poliinsaturados y a su elevado metabolismo oxidativo. Además las neuronas son especialmente vulnerables a especies reactivas de oxígeno debido a que presentan niveles de antioxidantes bajos (Coyle y Puttfarcken, 1993). Durante la reperfusión el aporte de oxígeno es el principal responsable de la

generación de radicales libres que contribuyen a aumentar el daño celular (Chan, 2001).

Otros cambios bioquímicos que se detectan durante la isquemia cerebral incluyen la acumulación de ácidos grasos libres y la metabolización de fosfolípidos. Muchos de los ácidos grasos libres derivan de lípidos de membrana que son degradados como resultado de la activación de lipasas y fosfolipasas. Ciertas fosfolipasas, como la fosfolipasa A_2, son dependientes de calcio y se activan en situaciones de isquemia (Muralikrishna y Hatcher, 2006). Además uno de los aspectos más sensibles a la reducción del flujo sanguíneo cerebral es la síntesis proteica que se inhibe profundamente durante la isquemia (Neumar *et al.*, 1998), ya sea focal o global, a pesar de que no se observan cambios en la morfología de los polirribosomas que permanecen agregados durante el periodo de la isquemia. Esto impide la nueva síntesis de proteínas que, de prolongarse durante el tiempo suficiente, podría provocar un déficit en proteínas esenciales para la supervivencia celular (Planas, 1997).

Existe un importante componente inflamatorio en la patogénesis de la isquemia cerebral. Los principales mediadores del daño inflamatorio son citoquinas (IL-1, IL-6, TNF-α y TGF-β), moléculas de adhesión (selectinas, integrinas e inmunoglobulinas), eicosanoides y la óxido nítrico sintasa inducible (iNOS) que se producen inmediatamente después de la aparición de la isquemia y contribuyen al daño neuronal (Wang *et al.*, 2007). Estas moléculas son producidas por células endoteliales, astrocitos, células microgliales y leucocitos (granulocitos, macrófagos y linfocitos).

El papel de la microglía parece fundamental tras un proceso isquémico. Las células microgliales son los macrófagos residentes del sistema nervioso central (Kreutzberg, 1996) y una vez activadas se transforman en fagocitos. Esta activación está asociada a la expresión de una β-integrina de membrana (CD11b o Mac-1). Se ha descrito que tras la isquemia existe un importante aumento en la expresión de CD11b (Pforte *et al.*, 2005), y parece que anticuerpos frente a esta proteína actúan como neuroprotectores tras la isquemia (Chopp *et al.*, 1994).

La suma de sucesos intracelulares y extracelulares mencionados hace que las neuronas localizadas en el núcleo isquémico mueran por un proceso necrótico en un intervalo corto de tiempo (minutos). Sin embargo, las neuronas localizadas en

la zona de penumbra podrán recuperarse o morir por procesos apoptóticos (incluso varios días después) (Banasiak *et al.*, 2000; Dirnagl *et al.*, 1999; Lipton, 1999). El daño neuronal observado después de isquemia global o focal se produce varios días después provocando la denominada muerte neuronal retrasada. Dado que la zona de penumbra isquémica tiene un menor daño que el núcleo isquémico y mantiene unos niveles de ATP razonables, en esta región predomina el proceso de apoptosis (Hata *et al.*, 2000; Kerr *et al.*, 1972).

4. RECEPTORES DE GLUTAMATO

Las evidencias presentadas en las secciones anteriores indican que la activación de los receptores de glutamato es un factor muy importante que desencadena el inicio de la muerte neuronal debida a isquemia, y en este apartado realizaremos una revisión de los mismos, con especial atención en los receptores de NMDA y AMPA, así como su relación con la isquemia cerebral.

Los receptores de glutamato (GluRs) son responsables en gran medida de la neurotransmisión excitatoria en el sistema nervioso central de los mamíferos. Los receptores glutamatérgicos son de dos tipos: receptores ionotrópicos (iGluRs) y metabotrópicos (mGluRs). Los receptores ionotrópicos están formados por varias subunidades proteicas que forman canales iónicos específicos de catión normalmente cuya apertura se regula por la unión de ligandos específicos (Hollmann y Heinemann, 1994; Nakanishi, 1992). Los mGluRs son proteínas con siete dominios de transmembrana que se acoplan a proteínas G y regulan la producción de mensajeros intracelulares, también son regulados por la unión de ligandos (Gudermann *et al.*, 1995).

Los receptores ionotrópicos se subdividen en tres grupos según su capacidad para reaccionar frente a distintos agonistas: los receptores de AMPA (α-amino-3-hidroxi-5-metil-4isoxazolpropionato ácido), receptores de kainato y receptores de NMDA (N-metil-D-aspartato). Hasta ahora se han descrito 18 genes en mamíferos que codifican las subunidades que forman los receptores ionotrópicos (Dingledine *et al.*, 1999) (Figura 2).

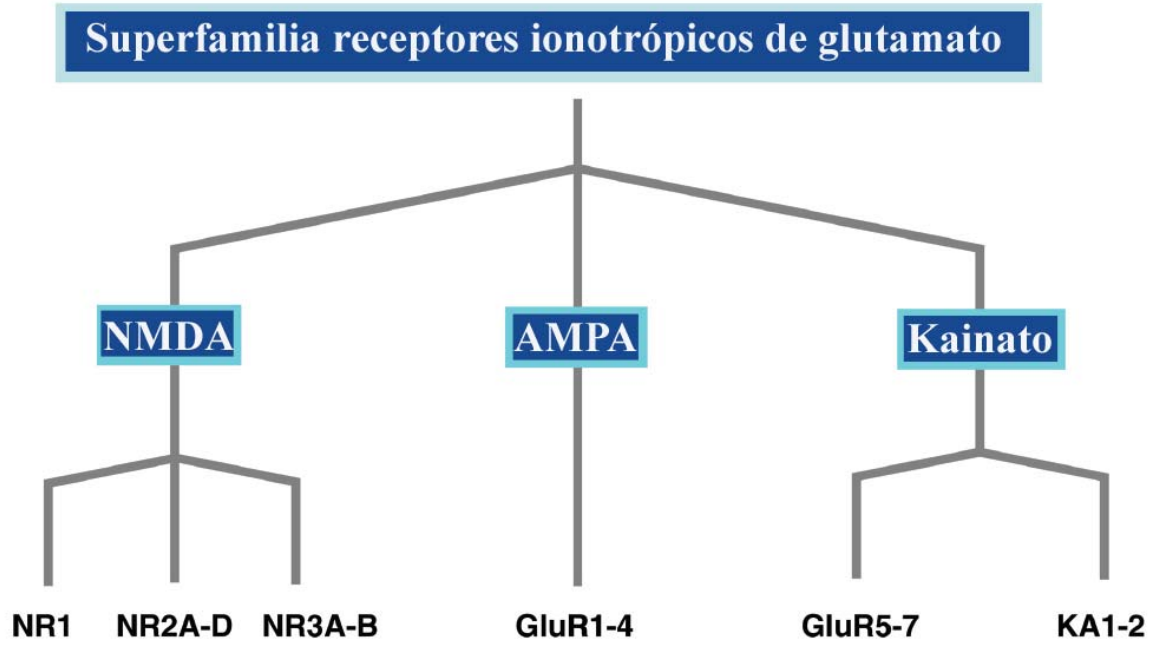

Figura 2. Subfamilias de receptores ionotrópicos de glutamato.

4.1. Receptores de NMDA

Los receptores de NMDA participan en funciones muy importantes como la transmisión sináptica excitatoria, el aprendizaje o la memoria (Ozawa *et al.*, 1998). Además juegan un papel fundamental en los fenómenos de excitotoxicidad en la isquemia cerebral y en enfermedades neurodegenerativas como el Alzheimer (Mishizen-Eberz *et al.*, 2004). Los receptores de NMDA presentan una serie de características únicas, entre las que se incluyen: alta permeabilidad al Ca^{2+}, una cinética de activación/desactivación lenta, permeabilidad para iones monovalentes como el Na^{+} y K^{+} también muy elevada, incluso superior a la del Ca^{2+} (Ichinose *et al.*, 2003), y un bloqueo de su actividad por Mg^{2+} extracelular sensible a cambios de voltaje (Wollmuth y Sobolevsky, 2004). Para que estos receptores puedan ser activados por glutamato se requiere que la glicina esté unida al receptor actuando como co-agonista (Moriyoshi *et al.*, 1991), además de la liberación del Mg^{2+} que se produce tras la despolarización de la célula. La presencia de otras moléculas en el espacio extracelular como poliaminas y Zn^{2+} pueden modificar el comportamiento del receptor (Cull-Candy *et al.*, 2001).

A lo largo de los años 90 se descubrieron la mayoría de las subunidades que forman la familia de receptores de NMDA, la subunidad NR1, las 4 subunidades

NR2 (A, B, C y D) y 2 subunidades NR3 (Das *et al.*, 1998; Moriyoshi *et al.*, 1991; Sugihara *et al.*, 1992). La subunidad NR1, presenta 3 sitios de maduración alternativa lo que da lugar a 8 isoformas distintas (A-H) (Zukin y Bennett, 1995). También las subunidades NR2 (excepto NR2A) y NR3 pueden presentar maduración alternativa y distintas isoformas. Así la subunidad NR2C presenta 2 sitios de maduración alternativa (Rafiki *et al.*, 2000), y las subunidades NR2D y NR3A un único sitio (Cull-Candy *et al.*, 2001).

Varios estudios de hibridación *in situ* han determinado que las subunidades de los receptores de NMDA se distribuyen de manera diferencial a lo largo del encéfalo, con patrones de expresión que cambian a lo largo del desarrollo (Monyer *et al.*, 1994). Se ha demostrado que las subunidades NR2B y NR2D predominan en el encéfalo neonatal pero durante el desarrollo van siendo reemplazadas por la subunidad NR2A, y en algunas regiones como el cerebelo por la subunidad NR2C (Akazawa *et al.*, 1994).

Como se muestra en la figura 3, cada una de las subunidades que conforman el receptor de NMDA contiene 3 segmentos de transmembrana (M1, M3 y M4), un segmento embebido en la membrana (M2) a modo de horquilla, un dominio amino terminal extracelular y un dominio carboxilo citoplásmico (Ozawa *et al.*, 1998). Cada una de las subunidades presenta un dominio de unión a ligando, en el cual intervienen 2 secuencias, llamadas S1 y S2 que forman una estructura a modo de concha (Villmann y Becker, 2007). Esta estructura general también aparece en el resto de miembros de la superfamilia de receptores ionotrópicos glutamatérgicos.

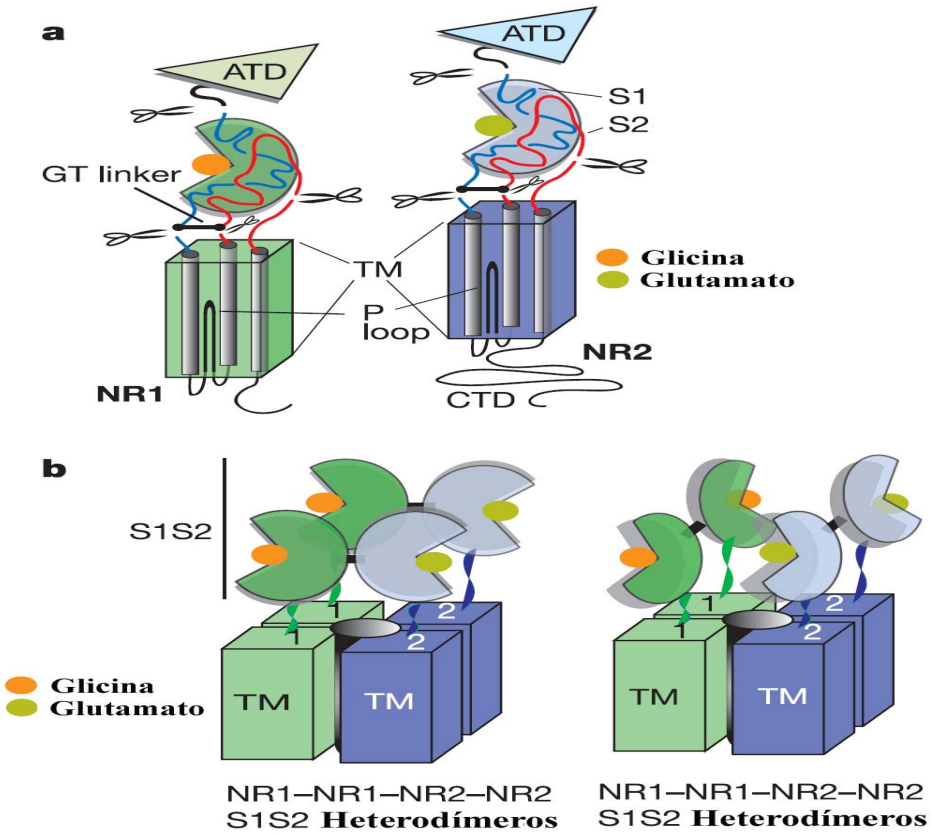

Figura 3. La figura muestra la estructura de las subunidades NR1 y NR2, cuyo dominio de unión de ligando puede ser aislado y cristalizado (a) y su disposición en la membrana celular para formar canales tetraméricos formados por dos subunidades NR1 y dos subunidades NR2 (b). Se muestran dos posibilidades de dimerización (modificado de Furukawa *et al.*, 2005).

Todos los receptores de NMDA se agrupan para formar isoformas heteroméricas compuestas por subunidades NR1 que se combinan con al menos un tipo de subunidad NR2. Las subunidades NR3 no forman receptores funcionales por sí mismas, pero se pueden unir a complejos NR1/NR2. Los receptores de NMDA funcionales se asocian normalmente como tetrámeros formados por dos subunidades NR1 y dos subunidades NR2 (Cull-Candy *et al.*, 2001; Furukawa *et al.*, 2005). Algunos autores han descrito que en hipocampo de rata, las subunidades NR2A y NR2B existen principalmente como complejos diheteroméricos que interaccionan de manera similar con proteínas PSD-95, pero forman distintos complejos proteicos uniéndose a distintas proteínas (Al Hallaq *et al.*, 2007). Incluso podrían existir como complejos triheteroméricos formados por subunidades NR1, NR2A y NR2B en neuronas del cerebro anterior (Luo *et al.*, 1997) y complejos NR1/NR2A/NR2C en cerebelo (Brickley *et al.*, 2003).

Numerosos estudios han demostrado que la composición de subunidades del receptor de NMDA determina las propiedades electrofisiológicas y farmacológicas de la isoforma (Kutsuwada *et al.*, 1992; McBain y Mayer, 1994; Meguro *et al.*, 1992). La subunidad determina el grado de fosforilación (Chen y Roche, 2007; Salter y Kalia, 2004) y la asociación con proteínas intracelulares (Wenthold *et al.*, 2003). En este sentido, las subunidades NR2 determinan las propiedades fisiológicas del canal iónico, como la conductancia, el tiempo de apertura o la sensibilidad al bloqueo por Mg^{2+} (Monyer *et al.*, 1992). Además, los canales formados por subunidades NR1 y NR2 son muy permeables al Ca^{2+} (Burnashev, 1998), lo cual determina su importante papel en la neurotoxicidad mediada por estos receptores (Sattler *et al.*, 1999).

4.2. Receptores de NMDA e isquemia cerebral

Está demostrado que las neuronas inmaduras en cultivo son más resistentes al daño neurotóxico mediado por receptores de NMDA (Marks *et al.*, 2000). Esta vulnerabilidad reducida se relaciona con el patrón de expresión diferencial de las distintas subunidades NR2, observándose que la expresión de NR2B y NR2D empieza en el día 14 del período embrionario, mientras que NR2A y NR2C se detectan por primera vez en el período perinatal (Monyer *et al.*, 1994; Wenzel *et al.*, 1996).

La subunidad NR2A se ha relacionado con el daño promovido por glutamato tras la isquemia cerebral. En este sentido, el desarrollo de ratones *knock-out* para la subunidad NR2A (Morikawa *et al.*, 1998) ha permitido concluir que estos ratones presentan una importante reducción de la zona cerebral infartada.

Existen muchas proteínas citoplásmicas que interaccionan con las subunidades que componen los receptores de NMDA (Scannevin y Huganir, 2000), entre ellas, las proteínas pertenecientes a la superfamilia de proteínas MAGUK (guanilato kinasas asociadas a membrana) que aparecen concentradas en las sinapsis. Otro grupo de proteínas que presentan interacciones directas con subunidades de los receptores de NMDA son miembros de la familia PSD-95/SAP90 (Gomperts, 1996); estas interacciones se producen con el extremo C-terminal citoplásmico de las subunidades NR2 y algunas variantes de procesamiento alternativo de la subunidad NR1 (Kornau *et al.*, 1995) y juegan un papel muy

importante en la localización sináptica y el agrupamiento de los receptores de NMDA (Niethammer *et al.*, 1996).

El importante papel que poseen los receptores de NMDA en la excitotoxicidad se ha demostrado mediante transfección de receptores de NMDA clonados en líneas celulares, ya que la expresión de los mismos promueve la muerte celular (Anegawa *et al.*, 1995; Rameau *et al.*, 2000). Parece que los receptores de NMDA no funcionales (por ejemplo los receptores NR1 homoméricos) no producen muerte celular, a diferencia de las combinaciones funcionales NR1/NR2A y NR1/NR2B. Además, los niveles de muerte celular son mucho más bajos en presencia de las combinaciones NR1/NR2C y NR1/NR2D, a pesar de que las corrientes a través de estos receptores funcionan normalmente (Lynch y Guttmann, 2002). Por tanto, las subunidades NR2A/NR2B actúan como mediadores de la muerte celular y confieren a las células que las expresan más susceptibilidad al daño excitotóxico. En este sentido, la eliminación de los 400 aminoácidos finales de la subunidad NR2A disminuye el nivel de muerte celular en células transfectadas, a pesar de que la respuesta a agonistas y el calcio intracelular se mantienen constantes (Anegawa *et al.*, 2000). Esto indica que los procesos excitotóxicos están mediados por eventos intracelulares que ocurren selectivamente a través del extremo C-terminal (Tymianski *et al.*, 1993).

En las neuronas, la entrada de Ca^{2+} a través de los receptores de NMDA provoca la muerte celular de una manera más eficiente que a través de otros canales de Ca^{2+} (Arundine y Tymianski, 2004), lo que sugiere que las proteínas responsables de la excitotoxicidad dependiente de Ca^{2+} están asociadas a la vía intracelular desencadenada por los receptores de NMDA. Entre las proteínas implicadas en esta señalización está la proteína PSD-95 que se une al dominio PDZ terminal de las subunidades NR2, y también a la óxido nítrico sintasa neuronal (nNOS). Cuando se interrumpe la interacción entre la subunidad NR2B y la proteína PSD-95, el receptor de NMDA se desacopla de la activación de la nNOS, reduciendo en gran medida la excitotoxicidad mediada por estos receptores (Aarts *et al.*, 2002; Cui *et al.*, 2007).

Todos estos precedentes demuestran la importancia de los receptores de NMDA en la muerte celular promovida por la excitotoxicidad mediada por glutamato

tras un proceso isquémico, así como su importancia como diana terapéutica en un futuro tratamiento frente a la isquemia cerebral.

4.3. Receptores de AMPA

Los receptores de AMPA (AMPAR) participan en la transmisión sináptica excitatoria rápida. Estos receptores están formados por tetrámeros a partir de distintas subunidades (GluR1-4), que se combinan de distintas maneras (ensamblajes homo o hetero-oligoméricos) dando lugar a diferentes propiedades funcionales del receptor (Kwak y Weiss, 2006).

Todas las subunidades del receptor tienen un extremo N-terminal extracelular, un extremo C-terminal intracelular y cuatro dominios hidrofóbicos asociados a membrana (M1-M4), uno de los cuales forma un *loop* (M2). Además existen proteínas reguladoras de transmembrana de receptores de AMPA (TARPs) que son necesarias para la maduración del receptor, el tráfico intracelular o la propia función del canal proteico (Chen *et al.*, 2000; Ziff, 2007).

Los AMPAR se encuentran ampliamente distribuidos en el sistema nervioso central, tanto en neuronas como en células gliales (Belachew y Gallo, 2004). La gran mayoría de estos receptores se presentan como estructuras heteroméricas que contienen la subunidad GluR2 (Greger *et al.*, 2002; Wenthold *et al.*, 1996). Los AMPAR son tetrámeros (Rosenmund *et al.*, 1998; Wu *et al.*, 1996) que se forman en el retículo endoplásmico como un dímero de dímeros (Mayer, 2006). En el cerebro anterior, que incluye hipocampo y neocórtex cerebral, las subunidades predominantes son GluR1 y GluR2 mientras que GluR3 y GluR4 presentan niveles bajos (Craig *et al.*, 1993; Monyer *et al.*, 1991; Sans *et al.*, 2003). Durante el desarrollo postnatal temprano, la expresión de GluR2 es baja comparada con la de GluR1, pero se incrementa rápidamente durante la primera semana postnatal (Monyer *et al.*, 1991).

Los AMPAR que contienen al menos una subunidad GluR2 son impermeables al Ca^{2+}, mientras que aquellos que carecen de ella son permeables al mismo (Seeburg *et al.*, 2001). De este modo parece que la subunidad GluR2 es crucial en la determinación de la función de los AMPAR, y es la subunidad más regulada a todos los niveles. Algunos estudios de manipulación genética de la

subunidad GluR2 en ratones muestran un fenotipo muy afectado con alteraciones neurológicas, demostrando la gran importancia que parece tener para el funcionamiento normal del cerebro (Feldmeyer *et al.*, 1999; Hartmann *et al.*, 2004).

Estas propiedades y características especiales de la subunidad GluR2 vienen determinadas post-transcripcionalmente mediante edición del ARN en el sitio Q/R del segmento M2 (Burnashev *et al.*, 1992.). La mayor parte de proteínas GluR2 maduras están editadas y contienen un residuo arginina (R) en la posición 607 en lugar de una glutamina (Q) que está codificada genéticamente. Este cambio es el resultado de una edición hidrolítica en una base adenosina en el pre-ARNm, que se transforma en inosina por la enzima ADAR2 (adenosina desaminasa R2) (Higuchi *et al.*, 1993). De este modo la carga positiva adicional introducida en el poro del canal por la presencia de esta arginina impide el paso de iones divalentes (Ca^{2+} y Zn^{2+}) y el bloqueo endógeno intracelular por poliaminas (Jonas y Burnashev, 1995; Swanson *et al.*, 1997) (Figura 4).

4.4. Receptores de AMPA e isquemia cerebral

Los AMPAR permeables al Ca^{2+} tienen un papel muy importante en los fenómenos de excitotoxicidad que se asocian a algunas enfermedades y desórdenes neurológicos. En la isquemia global transitoria que se genera como consecuencia de una parada cardíaca o que se induce experimentalmente en modelos animales, se produce una muerte neuronal retrasada y selectiva, principalmente en las neuronas piramidales del área hipocampal CA1. Un rasgo clave de este proceso, es el aumento intracelular de calcio durante el episodio isquémico, además de un incremento de Zn^{2+} en esta área después de 24-48 horas tras la isquemia cerebral, y antes de la muerte celular (Liu y Zukin, 2007).

En condiciones fisiológicas, las neuronas hipocampales expresan receptores de AMPA impermeables al calcio, dada la abundancia de subunidades GluR2. Los procesos isquémicos promueven una importante *down-regulation* en la expresión de la subunidad GluR2 tanto a nivel de ARNm como de proteína, en las neuronas vulnerables del área CA1, lo cual cambia completamente el fenotipo de las mismas (Gorter *et al.*, 1997; Opitz *et al.*, 2000; Pellegrini-Giampietro *et al.*, 1997), y estas células pasan a expresar principalmente receptores de AMPA que carecen de la subunidad GluR2, lo cual les confiere características muy distintas, como pueden

ser su alta permeabilidad a iones divalentes y sensibilidad a poliaminas (Liu *et al.*, 2004) (Figura 4).

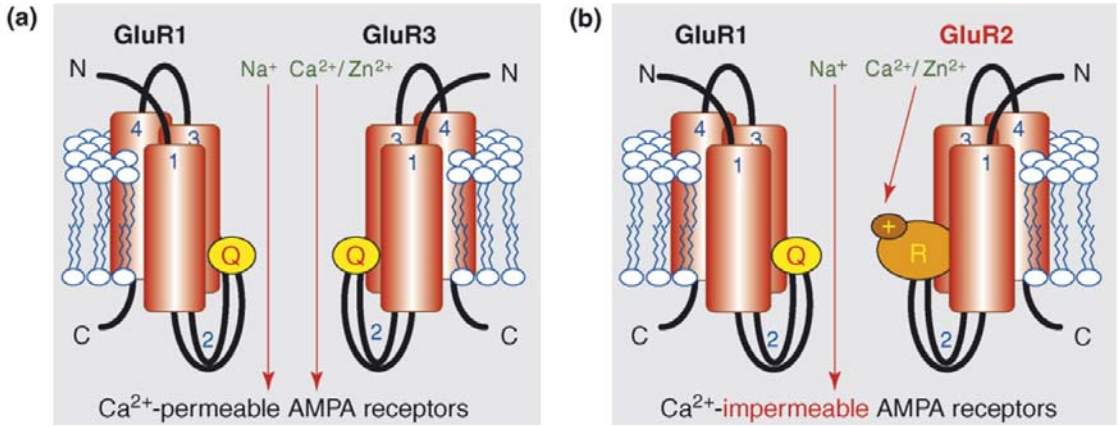

Figura 4. Imagen que muestra la dependencia de la permeabilidad al calcio según la composición de subunidades del receptor de AMPA. Los AMPAR que contienen la subunidad GluR2 editada son impermeables al calcio, mientras que aquellos que carecen de ella son permeables. Modificada de Liu y Zukin, 2007.

Todas las evidencias previas indican que los receptores de AMPA están directamente relacionados con la muerte neuronal que acontece tras la isquemia. Los ratones *knock-down* usando oligonucleótidos antisentido para la subunidad GluR2, presentan muerte celular de las neuronas piramidales incluso en ausencia de daño isquémico (Oguro *et al.*, 1999). Del mismo modo, la sobreexpresión de receptores de AMPA permeables al calcio promueve muerte neuronal en las neuronas piramidales del área CA3, que son normalmente resistentes a la isquemia, mientras que la sobreexpresión de receptores impermeables al Ca^{2+} en CA1, protege las neuronas del daño isquémico (Liu *et al.*, 2004).

Algunos estudios recientes señalan un papel del factor de transcripción silenciador de RE1 (REST) en el cambio del fenotipo del receptor de AMPA. El daño isquémico promueve la activación del represor transcripcional REST en las neuronas vulnerables del área CA1 (Calderone *et al.*, 2003). REST se une al promotor de GluR2 y suprime la expresión génica en neuronas destinadas a morir. Resultados recientes indican que no sólo la expresión de GluR2, sino también el tráfico del receptor y la edición de su ARN pueden estar alterados en respuesta al daño neuronal. En este sentido, la isquemia provoca internalización de receptores de AMPA que contienen subunidad GluR2 mediante endocitosis mediada por clatrina (Liu *et al.*, 2006). Asimismo, se ha demostrado que la isquemia global

inhibe la actividad de la enzima adenosina desaminasa ADAR2, provocando disminución en la edición de GluR2 en el sitio Q/R (Peng *et al.*, 2006), lo cual contribuye a la vulnerabilidad neuronal en la isquemia cerebral, haciendo a los receptores de AMPA más permeables a iones divalentes.

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II.HIPÓTESIS DE TRABAJO Y OBJETIVOS

Dado que la heterogeneidad en la combinación de distintas subunidades de los receptores de NMDA y AMPA da lugar a receptores con distintas propiedades funcionales nuestra hipótesis es que esta heterogeneidad podría ser la causa de la distinta vulnerabilidad observada en diferentes áreas del encéfalo tras la isquemia cerebral. Es decir, la susceptibilidad de las células frente a procesos excitotóxicos podría depender de la diferente composición en las isoformas de los receptores de AMPA o NMDA. Para verificar si esta hipótesis es cierta se propusieron los siguientes objetivos:

1.- Determinación del efecto del proceso de isquemia/reperfusión tras un tiempo dado (48 horas) en estructuras cerebrales con distinta vulnerabilidad al daño isquémico estudiando:

1a.- El nivel de daño neuronal.

1b.- Los cambios en la expresión génica de las subunidades del receptor de NMDA y de AMPA.

1c.- Establecer la posible relación entre los niveles de inflamación (a través de activación microglial) y la expresión de los receptores de NMDA y de AMPA en las distintas estructuras cerebrales estudiadas.

2.- Caracterización y puesta a punto de un modelo *ex vivo* usando secciones de cerebro de rata para el desarrollo de ensayos de PCR a tiempo real, y su posterior utilización en experimentos de privación de oxígeno y glucosa (OGD). Evaluación de distintos genes de referencia.

3.- Aplicación del modelo de OGD para la cuantificación de la expresión génica (niveles de ARNm) de las subunidades del receptor de NMDA en secciones hipocampales de rata, así como el estudio de los posibles mecanismos implicados en los cambios observados (utilización de antagonistas de receptores glutamatérgicos y de glutamato *per se*).

III. TRABAJOS

TRANSIENT GLOBAL ISCHEMIA IN RAT BRAIN PROMOTES DIFFERENT NMDA RECEPTOR REGULATION DEPENDING ON THE BRAIN STRUCTURE STUDIED.

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ABSTRACT

The mRNA expression of the major subunits of N-methyl-D-aspartate receptors (NR1, NR2A and NR2B) following ischemia-reperfusion were studied in structures with different vulnerabilities to ischemic insult in the rat brain. The study was performed using quantitative real-time PCR on samples from 3 month-old male Sprague-Dawley rats after global transient forebrain ischemia followed by 48 hours of reperfusion. Expression of NMDA receptor subunits mRNAs decreased significantly in all structures studied in the injured animals as compared to the sham-operated ones. The hippocampal subfields (CA1, CA3 and dentate gyrus) as well as the caudate-putamen, both reported to be highly ischemic-vulnerable structures, showed outstandingly lower mRNA levels of NMDA receptor subunits than the cerebral cortex, which is considered a more ischemic-resistant structure. The ratios of the mRNA levels of the different subunits were analyzed as a measure of the NMDA receptor expression pattern for each structure studied. Hippocampal areas showed changes in NMDA receptor expression after the insult, with significant decreases in the NR2A with respect to the NR1 and NR2B subunits. Thus, the NR1:NR2A:NR2B (1:1:2) ratios observed in the sham-operated animals became (2:1:4) in insulted animals. This modified expression pattern was similar in CA1, CA3 and the dentate gyrus, in spite of the different vulnerabilities reported for these hippocampal areas. In contrast, no significant differences in the expression pattern were observed in the caudate putamen or cerebral cortex on comparing the sham-operated animals with the ischemia-reperfused rats. Our results support the notion that the regulation of NMDA receptor gene expression is dependent on the brain structure rather than on the higher or lower vulnerability of the area studied.

1. INTRODUCTION

NMDA receptors (NMDAR) are ionotropic voltage-dependent glutamate channels that are considered to play an important role in excitotoxicity and neuronal damage. This is of huge interest since the neuronal damage that occurs after a stroke is considered to be a major cause of morbidity and mortality and hence has considerable socio-economic costs in today's society. NMDARs appear as macromolecular assemblies in which several subunits form the channel. Seven NMDAR subunits have been identified so far: one NR1, four NR2 (A–D), and two NR3 (A, B). Most native NMDARs appear to function as heterotetrameric assemblies composed of two glycine-binding NR1 and two glutamate-binding NR2 subunits (Köhr, 2006; Parsons *et al.*, 2007). Different NMDAR subunits confer distinct electrophysiological and pharmacological properties to the receptors and couple them with different signaling machineries (Liu *et al.*, 2007). Thus, the response to glutamate depends on NMDAR stoichiometry.

The neuronal damage that follows stroke has been associated with the excitotoxicity induced by overactivation of NMDARs as a consequence of the release of high glutamate levels into the extracellular space during ischemia (Iadecola, 1999; Cai and Rhodes, 2001, Nishizawa, 2001). However, NMDAR activation has been reported to be able to produce either neuronal survival or death-promoting actions and it has been claimed that this dual action is mediated by receptor subunit composition. Such opposing actions may explain, at least in part, the failure of NMDAR antagonism-based clinical trials on stroke and provide a scientific basis for developing novel and effective NMDA receptor-based stroke therapies (Liu *et al.*, 2007). Understanding the molecular mechanisms that mediate stroke-related brain damage and use of this knowledge in the development of effective therapeutics are urgently required to address this problem (Liu *et al.*, 2007), but large-scale clinical trials have failed to find the expected efficacy of NMDAR antagonists in reducing brain injuries (Lee *et al.*, 1999; Ikonomidou and Turski, 2002; Kemp and McKernan, 2002). The reasons underlying the apparent contradiction between basic research results and those of clinical trials remain unknown but are likely multifactorial (Albensi *et al.*, 2004).

The presence of neuronal damage can be detected from 24 hours after ischemia in the hippocampus (Zhang *et al.*, 1997), but ischemia-reperfusion (I/R)-

dependent vulnerability varies in the different brain areas (Kirino, 1982; Pulsinelli, 1985; Schmidt-Kastner and Freund, 1991**;** Sugawara *et al.*, 1999; Yang *et al.*, 2000; Ikegaya and Matsuki, 2002). Thus, knowledge of NMDAR gene regulation in the different areas should contribute to understanding the mechanisms underlying the different vulnerabilities of cells to I/R. However, differences in damage following ischemia varies depending on the area studied, thus NR1 subunit mRNA of CA1 hippocampal region has been reported to decrease after the first 24 hours after insult while NR1 subunit mRNA of CA3 region has been reported to change 72 hours after challenge (Sugimoto *et al.*,, 1994). To obtain a picture of the ischemia-dependent damage in different areas of the brain we chose 48 hours after challenge to ensure that at least one structure exhibited ischemia-dependent expression changes in some NMDAR subunit genes. The role of different subunits in the cell damage following I/R has been widely studied (Morikawa *et al.*, 1998; Liu *et al.*, 2007), but as far as we know no comprehensive studies quantifying the balance of the different NMDARs subunits and comparing areas with different susceptibility to ischemic damage have been reported. The NR1, NR2A and NR2B subunits are the major components of the heteromeric NMDAR channel complex distributed in the mature forebrain (Watanabe *et al.*, 1992) and we therefore assume that modifications in NMDAR gene expression could be detected by analyzing the changes in gene expression in these subunits. The present report compares the mRNA expression of the major NMDAR subunits after I/R in areas reported to present different vulnerabilities, such as the hippocampus, cerebral cortex (CC) and caudate-putamen (C-Pu), in an attempt to determine whether modifications in NMDAR regulation play a role in the different degrees of damage observed in different structures.

2. MATERIALS AND METHODS

2.1. Animals

Ten Sprague-Dawley male rats weighing 350-400 g were housed under standard temperature (22 ± 1°C) and in a 12-h light/dark controlled environment with free access to food and water. The animals were divided randomly into ischemic and sham groups. 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 Scientific Committee of the University of León. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Transient global ischemia

Rats were placed in the anesthesia induction box supplied with 4% halothane and 100% oxygen. After induction, anesthesia was maintained with 1.5% halothane, delivered via a face mask that was specially devised to fit the animal's frontal part of the head.

Both common carotid arteries were exposed through a midline incision and transient global ischemia was induced by bilateral common carotid artery occlusion for 15 min with atraumatic aneurysm clips and moderate hypotension using trimetaphan (kindly provided by Roche) as a hypotensor agent (15mg/ml, 0.3 mg/min). Rats were maintained with mean arterial blood pressure of about 40-50 mm Hg. Rectal temperature was controlled at 37.0 ± 0.5°C during surgery with a feedback-regulated heating pad. The femoral artery was exposed and catheterized to allow continuous recording of arterial blood pressure and the administration of trimetaphan. After recovery of arterial blood pressure, the catheter was removed and the animals were sutured. After regaining consciousness, they were maintained in an air-conditioned room at 22°C. For sham-operated rats, all procedures were performed exactly as for ischemic animals with the exception that the carotid arteries were not clamped.

2.3. Tissue processing

Two days after 15 minute-global ischemia, the animals were sacrificed by decapitation and their brains were rapidly removed. CA1, CA3, the dentate gyrus (DG), CC and C-Pu were obtained using a dissecting microscope and immediately frozen in liquid nitrogen until used. Those used in the assessment of ischemic neuronal damage were removed and postfixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h before cryoprotecting in 30% sucrose/PBS for 48 h. Brains were frozen in powdered dry ice and serial 40-µm cryosections throughout the entire extent of the hippocampal formation were cut on a sliding microtome on the sagittal plane, placed in PBS, and mounted onto gelatin-coated slides. The slides were dried on a slide warmer for 1 h and then processed for cresyl violet and Fluorojade-B (FJB) staining.

2.4. Cresyl violet and Fluorojade-B

Neuronal damage was visualized using cresyl violet staining. Slides were hydrated from absolute alcohol to distilled water and incubated with a 0.1% cresyl violet solution for 2 min. Excessive staining was removed by briefly immersing the slides in a 95% alcohol solution and dehydrating them. After clearing the sections with 2 series of xylene solution, they were mounted with entellan and observed under light microscopy (Olympus, Tokyo, Japan).

Neuronal degeneration was studied using Fluorojade-B staining. The protocol was performed using slight modifications of the technique described by Schmued *et al.*, (1997). Sections were first incubated immersed in 100% ethyl alcohol for 3 min, followed by a 1-min change in 70% alcohol and a 1-min change in distilled water. Then, they were incubated in a solution of 0.06% potassium permanganate for 10 min on a rotating stage, rinsed in distilled water for 2 min, and incubated in a 0.00035% solution of FJB (Histo-Chem Inc., Jefferson, AR). The sections were then rinsed in distilled water (3x2 min), air-dried, and placed on a slide warmer until fully dry (5–10 min). The dry slides were cleared in xylene (2x5 min) and coverslipped with entellan. Sections were examined with an epifluorescence microscope using a filter system suitable for visualizing fluorescein.

2.5. RNA extraction

Total RNA was extracted using the Tripure™ Isolation Reagent (Roche), according to the instructions of the manufacturer. This procedure allows the isolation of total RNA, DNA and protein fractions from a single sample. The contaminating DNA in the RNA samples was removed by incubation with DNase (Sigma) and confirmed by PCR analysis of total RNA samples prior to reverse transcription (RT). The yield of total RNA was determined by measuring the absorbance (260/280 nm) of ethanol-precipitated aliquots of the samples. Total RNA concentrations were determined using a NanoDrop ND-3300 spectrophotometer (NanoDrop Technologies, USA). Isolated RNA was finally frozen at −80°C until further processing.

2.6. Microcapillary gel electrophoresis

This technique was used to check RNA integrity using the Experion™ Automated Electrophoresis System (BioRad Laboratories, USA). We analyzed 100 ng of total RNA from all samples studied using the Experion RNA HighSens Analysis Kit (Biorad Laboratories, Wilmington, USA), following the manufacturer's instructions. The 28S/18S rRNA ratio was used to assess RNA quality, using the respective areas under the 28S and the 18S peaks (Kerman *et al.*, 2006).

2.7. Reverse transcription

The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used. The 20 µl transcription mix for each sample contained 0.6 µg total RNA (10 µl), 2 µl 10x RT buffer, 0.8 µl dNTP 25x (100 mM), 2 µl 10x RT Random Primers, and 1 µl MultiScribe™ Reverse Transcriptase (50 units/µl).

Reactions were performed for 10 min at 25°C, 2 h at 37°C and terminated with 5 s at 85°C. Subsequently, the reaction mixture was maintained at -20°C until used for PCR amplification.

2.8. Quantitative real-time PCR

Real-time PCR quantification of NMDAR subunit mRNA was performed in triplicate using gene-specific primers and SYBR® Green. Oligonucleotide primers were designed using Primer Express software (Applied Biosystems). The primer sequences and the GenBank Accession Numbers are given in Table 1.

Gene	Forward primer (5´-3´)	Reverse primer (5´-3´)	GenBank
NR1	CAGCCGTGAACGTGTGGAG	TGCTCTACCACTCTTTCTATCC	NM 017010
NR2A	CAGTGATGTGTATATTTCAGAGCATGTTA	ACACTCGTCTATTGCTGCAGGAA	NM 012573
NR2B	TCCGTCTTTCTTATGTGGATATGC	CCTCTAGGCGGACAGATTAAGG	NM 012574
GAPDH	GGGCAGCCCAGAACATCA	TGTCCGTATGGCTTCATTGATG	NM 017008

Table 1. Primer sequences and Genbank accession numbers for the different genes studied.

As an internal control for normalization, PCR reactions were performed concurrently with the amplification of a reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Real time-PCR was performed on an ABI PRISM 7000 real-time thermal cycler using the SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA) and the following thermal cycler settings: 1 cycle of 2 min at 50ºC, 1 cycle of 10 min at 95ºC, 40 cycles of 15 s at 95ºC and 1 min at 60ºC. Cycle thresholds for both genes were selected immediately above the baseline and within the linear range on log scaling. The reaction mixture (20 µl) consisted of 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). The relative change in the mRNA expression of the subunits studied following global ischemia was determined by the equation:
Fold change = $2^{-\Delta Ct}$, ΔCt = (Ct target–Ct GAPDH) (Livak and Schmittgen, 2001). The Ct value is the cycle number at which the fluorescence signal crosses the threshold.

2.9. Statistical Analysis

All results are expressed as means ± SEM of the fold-change values of at least two different experiments for each PCR assay. Statistical significance among mRNA levels of the different experimental groups (ischemia and sham-operated rats) was analyzed with the unpaired Student´s t test (SPSS). The unpaired Student's t test between the different mRNA ratios (NR2A:NR1, NR2A:NR2B and NR2B:NR1) were also used to compare the expression NMDAR patterns between the sham-operated and chemically insulted animals. The significance was set at the 95% confidence level.

3. RESULTS

3.1. Morphological modification induced by I/R.

Figure 1 shows images indicating the damage in different brain areas of the sham-operated and 48 h post-insult animals. Staining with cresyl violet reflects the degree of disorganization and Fluorojade-B mirrors degenerating neurons in the structures studied. In both staining modes, CA1 displays the most marked modifications while CC shows fewer differences between the insulted and sham-operated animals.

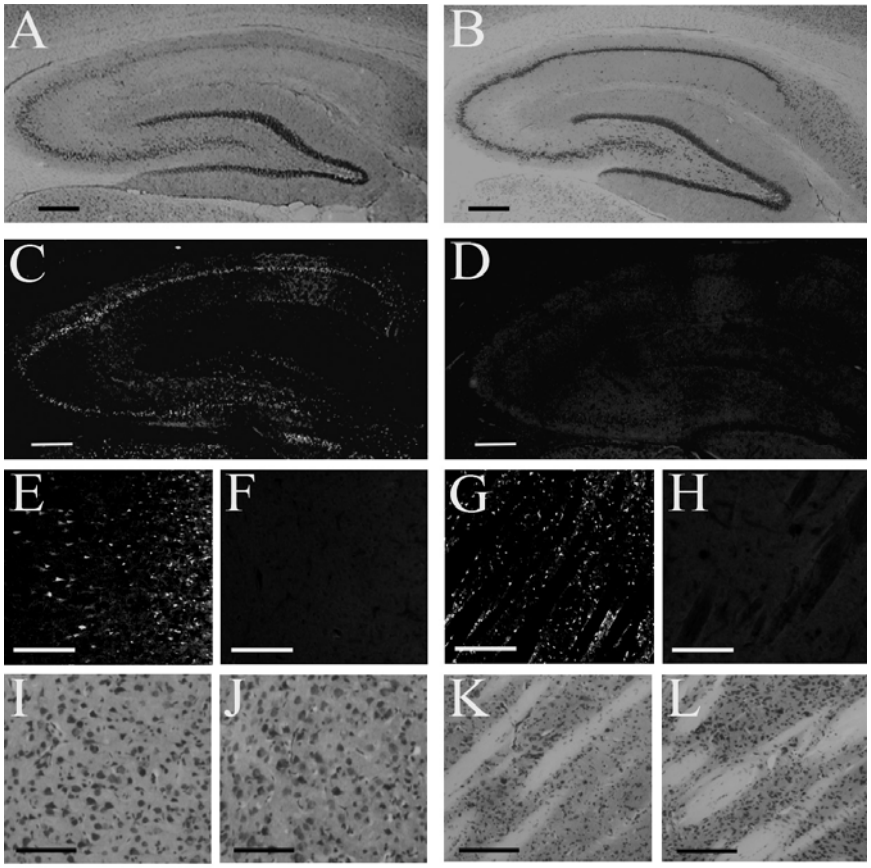

Figure 1. Hippocampus, CC and C-Pu stained with cresyl violet in insulted (A, I, K) and sham-operated (B, J, L) animals respectively. CA1 appears less stained and with smaller neuronal somata in the insulted animals. These differences are less outstanding in CA3, DG and C-Pu and barely noticeable in CC. Degenerating neurons, as indicated by Fluorojade B, are more outstanding in CA1 and CA3 than in DG, CC or C-Pu on comparing insulted (C,E,G) with sham-operated animals (D,F,H). Scale bars: 0.6 mm (A-D) and 200 μm (E-L).

3.2. Amounts of RNA

In CA1, the animals with ischemia showed significantly ($p<0.05$, $n=5$) lower total RNA values (250 ± 19 ng/µl) with respect to those of the CA1 in the sham-operated animals (300 ± 13 ng/µl). In contrast, no significant differences were observed in total RNA values in CA3 and DG of the insulted animals (146 ± 13 ng/µl, and 345 ± 20 ng/µl respectively) in comparison with those of sham-operated animals (141 ± 10 ng/µl, and 351 ± 54 ng/µl respectively). Likewise, no significant differences were observed in total RNA values in CC and C-Pu of the injured animals (1623 ± 95 ng/µl, and 523 ± 41 ng/µl respectively) as compared with those of the sham-operated animals (1725 ± 132 ng/µl, and 614 ± 34 ng/µl respectively).

The same amounts of RNA of the different samples (transformed into cDNA) were amplified by real time PCR, showing the following Ct values for GAPDH: CA1:19.48 ± 0.09; CA3: 19.81 ± 0.12; DG: 19.64 ± 0.12; CC: 19.60 ± 0.13; C-Pu: 19.90 ± 0.11. No significant differences were observed among any of the structures studied.

3.3. RNA integrity

The 28S/18S ratios displayed similar values in both the control and treated animals in all structures studied, with no significant differences in the integrity of RNA among samples (unpaired Student´s t test).

3.4. NMDAR subunit mRNA expression after transient global ischemia in the rat brain

The levels of mRNA of the different NMDAR subunits normalized with respect to GAPDH mRNA in each structure are indicated in table 2. Significant decreases in mRNA expression of the injured animals with respect to that of the sham-operated animals were observed in the hippocampus, CC and C-Pu. Thus, in CA1, the NR1 expression of animals after ischemia-reperfusion decreased to levels close to 32%; NR2A to about 15%, and NR2B to about 26% with respect to those of the sham-operated animals. In CA3, expression decreased to levels close to 18% for NR1; 13% for NR2A, and 22% for NR2B. In DG, the values were about 43% for NR1; 23% for NR2A, and 33% for NR2B. In CC, the decreases were less outstanding but still significant, with levels close to 53% for NR1; 61% for NR2A, and 71% for NR2B. C-Pu also exhibited outstanding significant decreases, with levels close to 15% for

NR1; 18% for NR2A, and 19% for NR2B. A comparison of the expression patterns is showed in figure 2.

	CA1	CA3	DG	CC	C-Pu
NR1 sham	0.107 ± 0.007	0.141 ± 0.141	0.139 ± 0.011	0.058 ± 0.002	0.133 ± 0.008
NR2A sham	0.110 ± 0.012	0.113 ± 0.113	0.112 ± 0.015	0.051 ± 0.005	0.039 ± 0.008
NR2B sham	0.226 ± 0.030	0.189 ± 0.189	0.235 ± 0.038	0.199 ± 0.003	0.781 ± 0.210
NR1 exp.	0.033 ± 0.017	0.027 ± 0.027	0.059 ± 0.007	0.030 ± 0.008	0.020 ± 0.011
NR2A exp.	0.015 ± 0.008	0.012 ± 0.012	0.025 ± 0.012	0.031 ± 0.005	0.007 ± 0.002
NR2B exp.	0.060 ± 0.035	0.039 ± 0.039	0.088 ± 0.015	0.141 ± 0.026	0.141 ± 0.054

Table 2. Relative gene expression levels for the different NMDAR subunits normalized with respect to the GAPDH mRNAs of each animal. Data represent means ± S.E.M (n=5). Statistically significant differences ($p<0.05$) were found for all the subunits and structures when sham-operated and insulted animals (exp.) were compared (Student's unpaired t-test).

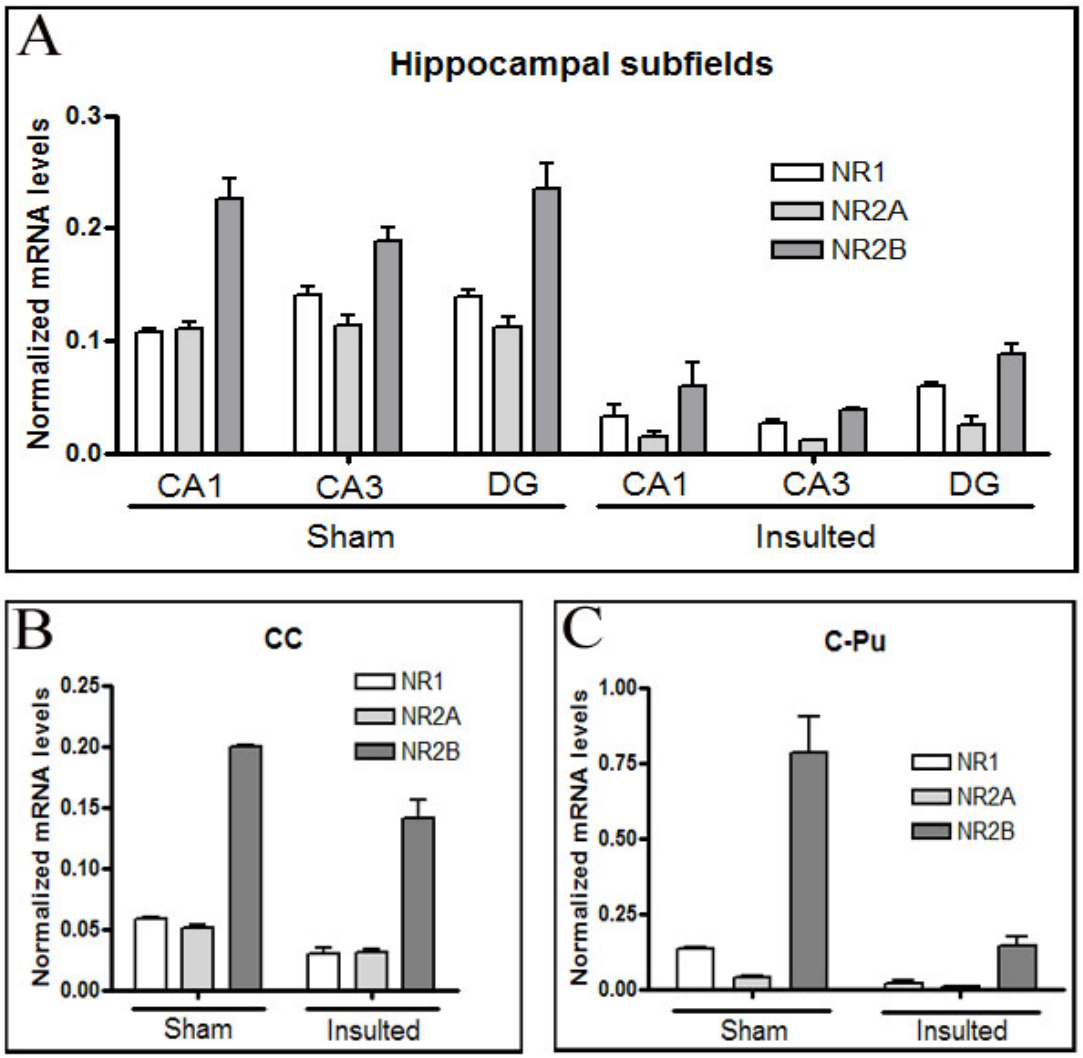

Figure 2. Normalized mRNA levels of NR1, NR2A and NR2B subunits from sham-operated and insulted animals in hippocampus (A), cerebral cortex (B) and caudate-putamen (C). Expression pattern (ratio NR1:NR2A:NR2B) in the different areas of hippocampus, roughly 1:1:2 modifies into 2:1:4 following 48 h I/R, while expression patterns in CC and C-Pu maintain their respective ratios in the sham-operated and insulted animals.

4. DISCUSSION

The significant decrease observed in CA1 total RNA levels matches the changes observed in cell morphology, revealing an important degree of cell damage in this area (Figure 1). The better preserved cell morphology in CA3, DG, CC and C-Pu after the insult also matches the similar total RNA amounts in these areas in both the sham-operated and insulted animals. These data are in agreement with previous reports indicating that CA1 is a more vulnerable area than CA3, DG, CC or C-Pu (Kirino, 1982; Pulsinelli, 1985; Schmidt-Kastner and Freund, 1991**;** Sugawara *et al.*, 1999; Yang *et al.*, 2000; Ikegaya and Matsuki, 2002). Changes in morphology are difficult to quantify since cells show differences in size and shape, as well as in number (Espanol *et al.* 1998; Gadamski *et al.*, 2001; Olson and McKeon, 2004; Bendel *et al.*, 2005). Modifications in total amounts of RNA could represent a straight way to quantify the tissue damage. Thus, the approximately 15-20% decrease in the total amount of RNA observed in CA1 could reflect the damage to ischemia/reperfusion-vulnerable cells, while in CA3, DG, CC and C-Pu most cells would be ischemia/reperfusion-resistant cells (at least for this time of study).

The values of GAPDH mRNA levels did not show differences between the samples from the sham-operated and insulted animals. Accordingly, the outstanding post-insult decreases observed in NR1, NR2A, and NR2B mRNA normalized levels (using GAPDH as reference) indicate that ischemia elicits a strong decrease in the expression of NMDA receptor genes in all brain structures studied. Although these decreases have been widely described in the hippocampus (Zhang *et al.*, 1997; Hsu *et al.*, 1998; Sugimoto *et al.*, 1994), comparison with other structures, such as the cerebral cortex and C-Pu, shows that transient forebrain ischemia followed by 48 hours of reperfusion elicits a general down-regulation of NMDAR gene expression in the different brain structures. The decreased gene expression could be explained in terms of a compensatory mechanism aimed at protecting neuronal cells against the excitotoxicity induced by glutamate through the down-regulation of NMDARs, which would make cells less responsive to the activation by glutamate (Wong *et al.*, 2001).

Our data show that outstanding differences in the down-regulation of NMDAR gene expression (considered as a whole) seem to be related to the vulnerability of the different structures studied. Thus, the hippocampus and

striatum -which are reported to be highly ischemic-vulnerable structures- show lower levels of NMDAR expression than the cerebral cortex, which has been reported to be a less vulnerable structure (Kirino, 1982; Kawai *et al.*, 1992). Since these data were obtained 48 hours after insult, we measured the expression levels of ischemic-resistant cells at this time. Thus, the global NMDAR expression could be used as a measure of ischemia-reperfusion vulnerability in the different regions. In this sense, CC, with a less marked decreased NMDAR expression, would be less vulnerable than the hippocampus or striatum.

Our data also indicate that cells surviving ischemia-reperfusion in the hippocampus show a change in the expression pattern of NMDARs. In approximate terms, we can affirm that the NR1:NR2A:NR2B ratio in the sham-operated animals (1:1:2) changes to (2:1:4) after insult. However, no significant changes were observed in the expression pattern of CC (1:1:4) after ischemia or in C-Pu, where the NR1:NR2A:NR2B ratio in the sham-operated animals was 4:1:26 and was modified to 3:1:20 in insulted rats.

The present findings also reveal that in contrast with CC and C-Pu, all hippocampal areas 48 hours post-insult exhibit half the NR2A expression of that observed in the sham-operated animals with respect to NR1 and NR2B, indicating that the regulation of NMDAR subunit expression depends upon the structure studied, affording different expression patterns. NR2A is always the subunit with the lowest mRNA levels and also seems to be responsible for the changes in the expression pattern of NMDAR, which suggests that NR2A could be critical in the regulation of NMDAR expression. Different hypotheses have been advanced regarding the role of the different NMDAR subunits in excitotoxicity (Morikawa *et al.*, 1998; Hardingham *et al.*, 2002; Liu *et al.*, 2007). Controversial statements have been made about the role of the NR2A subunit in excitotoxicity after I/R. Some data suggest that increases in NR2A subunit expression would promote excitotoxicity damage. Thus, mice deficient in NR2A have been reported to show an attenuation of focal ischemic brain injury, and it has been speculated that a decrease in the number of functional NMDAR receptor channels on the cell membrane of forebrain neurons in knockout mice renders cells more resistant to glutamate-induced neurotoxicity (Morikawa *et al.*, 1998). In contrast, other authors have described that the activation of NR2A-containing receptors appears to be neuroprotective after stroke (Liu *et al.*, 2007). However, generalizations concerning the roles of the

different subunits in mediating NMDAR-mediated neuronal survival and death should be taken with caution. Roles of NMDAR may vary, depending on the developmental stage, brain area, and disease model (Liu *et al* 2007). Our data show that the NR1:NR2A:NR2B ratios are not modified by I/R in CC or C-Pu. On the other hand, modification of the NR1:NR2A:NR2B pattern in the hippocampus following I/R shows a striking decrease in NR2A expression. However, NR2A is regulated in the same way in highly vulnerable hippocampal areas (CA1) as in less vulnerable ones (CA3 and DG), suggesting that the regulation of NR2A expression is similar throughout the hippocampus, regardless of the different vulnerabilities of CA1, CA3 or DG. Thus, it seems that the regulation of NMDAR gene expression depends on the brain structure rather than on the greater or lesser vulnerability of the area studied.

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GLOBAL ISCHEMIA-INDUCED MODIFICATIONS IN THE EXPRESSION OF AMPA RECEPTORS AND INFLAMMATION IN RAT BRAIN.

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ABSTRACT

Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPAR) and inflammatory processes have been related to ischemia-induced damage, but there are few studies addressing their response in different brain areas. Here we compare AMPAR expression after ischemia in several brain areas (hippocampus, cerebral cortex and caudate-putamen) in an attempt to correlate it with their different vulnerabilities. We found outstanding decreases in GluR1 and GluR2 mRNA levels after global ischemia and 48 hours reperfusion (I/R) in all the areas studied, however, protein levels maintained in some areas such as CA3, suggesting different post-transcriptional control in different areas of the brain. To characterize the inflammatory response in these areas, we measured the mRNA levels of CD11b/CD18 membrane integrin (a reactive microglia marker), which showed an important but similar up-regulation in all brain areas studied, which was confirmed by immunohistochemistry. We conclude that the down-regulation of AMPAR gene expression following I/R does not explain differences in the vulnerability of different areas. Additionally, our data indicate that the level of inflammation is independent of the vulnerability of the different brain areas and does not explain differences in the AMPAR expression observed in the brain areas studied.

1. INTRODUCTION

Cerebral ischemia is one of the main causes of death in industrialized countries. Each year around 700,000 individuals suffer stroke in US and the health care of the survivors cost 62 billion dollars annually (Rosamond *et al.*, 2008). Among the different mechanisms involved in ischemia-induced neuronal damage, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARs) seem to play a role in the response to ischemia and they have been described to be down-regulated after the insult (Gorter *et al.*, 1997), especially in the CA1 hippocampal area (Opitz *et al.*, 2000), which is highly vulnerable to ischemic insult (Kirino, 1985). The role of AMPARs in other brain areas with different vulnerabilities is poorly understood and knowledge of how AMPARs respond to ischemic insults should provide insight into the molecular mechanisms underlying the neuronal damage.

AMPARs mediate the greater part of fast excitatory synaptic transmission (Collingridge and Lester, 1989). These receptors appear on the membrane as tetramers made up of combinations of four possible different subunits: GluR1-4 (Rosenmund *et al.*, 1998; Dingledine *et al.*, 1999; Greger *et al.*, 2007). Differences in the isoform composition of AMPARs have been described throughout the brain. Thus, the cerebellum has been reported to present very low levels of the GluR1 subunit, which is only expressed in Bergmann glia, while GluR2 subunit is located in Purkinje and granule cells (Martin *et al.*, 1993).

In contrast, GluR1 and GluR2 are the most abundant subunits in the forebrain (Monyer *et al.*, 1991), and it has been described that pyramidal cells mainly express heterotetramers of GluR1 and GluR2 subunits (Wenthold *et al.*, 1996; Sans *et al.*, 2003).

How might AMPAR channels be involved in the ischemia-induced damage? AMPA channels are permeant to Na^+ and to a lesser extent to divalent cations such as Ca^{2+} or Zn^{2+}, which have been reported to play an important role in neuronal injury after ischemia (Kwak and Weiss, 2006). In this regard, the GluR2 subunit is of special interest since it has been reported to be critical in determining Ca^{2+} permeability through the channel (Hollmann *et al.*, 1991). Mature GluR2 subunits are almost all edited at position 607, where the genomically encoded glutamine (Q)

is replaced by arginine (R) (Burnashev *et al.*, 1992) in a reaction catalyzed by the adenosine deaminase ADAR2 (Higuchi *et al.*, 1993). The presence of this arginine residue introduces a positive charge into the pore, which prevents the passage of divalent cations, including Ca^{2+} (Jonas and Burnashev, 1995). Based on the properties of the GluR2 subunit in permeability to these cations, the "GluR2 hypothesis" of ischemia has been proposed, suggesting that delayed neuronal death of CA1 pyramidal cells could be due to the reduced GluR2 expression found after ischemia and the increased entry of Ca^{2+} through AMPA receptor channels (Pellegrini-Giampietro *et al.*, 1997). The rise in cytosolic Ca^{2+} lead to generation of reactive oxygen species, activation of phospholipases, proteases and endonucleases and thereby producing neuronal death (Kwak and Weiss, 2006; Bano and Nicotera, 2007).

Thus, one of our goals was to determine if the AMPAR expression was modified in structures with different vulnerabilities after ischemic injury.

The inflammation process has been also reported to play an important role after ischemia (Chamorro and Hallenbeck, 2006), where microglial cell activation is increased (Abraham and Lazar, 2000). A microglial marker, CD11b/CD18 (also called Mac-1), a β2 membrane integrin (McGeer and McGeer, 1995; Kim and De Vellis, 2005), is widely used to measure this activation. We wondered whether areas with different vulnerabilities had different degree of inflammation, and also whether CD11b might be used as a marker of vulnerability.

We report for the first time a comparative study of AMPAR GluR1 and GluR2 subunits in response to ischemia-reperfusion at both mRNA and protein levels, as well as the inflammatory response in some forebrain areas (cerebral cortex, caudate-putamen and hippocampus) whose different vulnerability following 48h hours of reperfusion after ischemia we have previously described (Dos-Anjos *et al.*, 2008).

2. EXPERIMENTAL PROCEDURES

2.1. *Animals*

Ten Sprague-Dawley male rats weighing 350-400 g were housed under standard conditions of temperature (22 ± 1°C) in a 12-h light/dark controlled environment with free access to food and water. The animals were randomly divided into ischemic and sham groups. 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 Scientific Committee of the University of León. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Transient global ischemia

The animals were placed in an anesthesia induction box supplied with 4% halothane and 100% oxygen. After induction, anesthesia was maintained with 1.5% halothane, delivered via a face mask that was specially devised to fit the frontal part of the animals' heads.

Both common carotid arteries were exposed through a midline incision and transient global ischemia was induced by bilateral common carotid artery occlusion for 15 min with atraumatic aneurysm clips and moderate hypotension using Trimetaphan (kindly provided by Roche) as a hypotensive agent (15 mg/ml, 0.3 mg/min). Rats were maintained with a mean arterial blood pressure of about 40-50 mm Hg. Rectal temperature was controlled at 37.0 ± 0.5°C during surgery with a feedback-regulated heating pad. The femoral artery was exposed and catheterized to allow continuous recording of arterial blood pressure and the administration of Trimetaphan. After recovery of arterial blood pressure, the catheter was removed and the animals were sutured. After regaining consciousness, they were maintained in an air-conditioned room at 22°C. For sham-operated rats, all procedures were performed exactly as for ischemic animals with the exception of the carotid artery clamping.

2.3. Tissue processing

Two days after global ischemia, the animals were sacrificed by decapitation and their brains were rapidly removed. CA1, CA3, the dentate gyrus (DG), cerebral

cortex (CC) and Caudate-Putamen (C-Pu) were obtained using a dissecting microscope and immediately frozen in liquid nitrogen until used. Those brains used for immunohistochemistry were removed and immersed in 4% paraformaldehyde in phosphate buffer saline (PBS), pH 7.4, for 48H. They were then immersed in 30% sucrose in PBS, pH 7.4, until they sank (2–5 days). Tissues were finally frozen in dry ice and maintained at -80ºC.

2.4. RNA extraction

Total RNA was extracted using the Tripure™ Isolation Reagent (Roche), according to the manufacturer´s instructions. This procedure allows the isolation of total RNA, DNA, and protein fractions from a single sample. The contaminating DNA in the RNA samples was removed by incubation with DNase (Sigma) and confirmed by PCR analysis of total RNA samples prior to reverse transcription (RT). The yield of total RNA was determined by measuring the absorbance (260/280 nm) of ethanol-precipitated aliquots of the samples. Total RNA concentrations were determined using a NanoDrop ND-3300 spectrophotometer (NanoDrop Technologies, USA). Isolated RNA was finally frozen at −80°C until further processing.

2.5. Microcapillary gel electrophoresis

This technique was used to check RNA integrity using the ExperionTM Automated Electrophoresis System (BioRad Laboratories, USA). We analyzed 100 ng of total RNA from all samples studied, using the Experion RNA HighSens Analysis Kit (Biorad Laboratories, Wilmington, USA) and following the manufacturer's instructions. The 28S/18S rRNA ratio was used to assess RNA quality (data not shown), using the respective areas under the 28S and the 18S peaks (Kerman et al., 2006).

2.6. Reverse transcription

The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used. The 20-µl transcription mix for each sample contained 0.6 µg total RNA (10 µl), 2 µl 10x RT buffer, 0.8 µl dNTP 25x (100 mM), 2 µl 10x RT Random Primers, 1 µl MultiScribeTM Reverse Transcriptase (50 units/µl) and 4.2 µl of DEPC water.

Reactions were performed for 10 min at 25°C, 2 h at 37ºC and terminated with 5 s at 85ºC. Subsequently, the reaction mixture was maintained at -20ºC until used for PCR amplification.

2.7. Quantitative real-time PCR

Real-time PCR quantification of AMPAR subunits and CD11b/CD18 mRNA levels was performed in triplicate using gene-specific primers and SYBR® Green. Oligonucleotide primers were designed using Primer Express software (Applied Biosystems). The primer sequences and the GenBank Accession Numbers are given in Table 1.

Gene	Forward primer	Reverse primer	Reference Sequence
GluR1	5′- CGAGTTCTGCTACAAATCCCG	5′-TGTCCGTATGGCTTCATTGATG	NM_031608
GluR2	5′-CCAAGGACTCGGGAAGTAAGG	5′-CCCCCGACAAGGATGTAGAA	NM_017261
CD11b/CD18	5′-GATGCTTACTTGGGTTATGCTT	5′-CGAGGTGCCCCTAAAACCA	NM_012711
GAPDH	5′-GGGCAGCCCAGAACATCA	5′-TGACCTTGCCCACAGCCT	NM_017008

Table 1: Primers used for Real-time PCR studies.

As an internal control for normalization, PCR reactions were performed concurrently with the amplification of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Real time-PCR was performed on an ABI PRISM 7000 real-time thermal cycler using the SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA) and the following thermal cycler settings: 1 cycle of 2 min at 50°C, 1 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. The reaction mixture (20 μl) consisted of a 2-μl cDNA (3 ng), 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). The relative change in the mRNA expression of the subunits studied following global ischemia was determined by the equation:
Fold change = $2^{-\Delta Ct}$, ΔCt = (Ct target–Ct GAPDH) (Livak and Schmittgen, 2001). The Ct value is the cycle number at which the fluorescence signal crosses the threshold.

2.8. Immunohistochemistry of CD11b/CD18

Sagittal sections 30 micrometers thick were cut on a freezing microtome for free-floating processing. Sections were first washed in PBS and endogenous peroxidases were inhibited with 3% H2O2/3% methanol in PBS, pH 7.4, for 20 min, after which endogenous biotin was inhibited with Blocking Kit (Vector Labs, Burlingame,CA, USA). Non-specific binding was blocked by incubating the sections in 0.2 % Triton X-100, 10 % goat serum in PBS. Section immunolabeling was performed using a monoclonal mouse anti-rat CD11b primary antibody (1:1000) (AbD Serotec) overnight at 4°C, which was detected by incubating for 1 h with a biotinylated secondary antibody (1:500 dilution, Goat Anti-mouse, Vector Laboratories) followed by incubation for 90 min with streptavidin-conjugated horseradish peroxidase (Sigma–Aldrich) diluted 1:2000. The peroxidase reaction was visualized with 0.05% diaminobenzidine tetrahydrochloride (DAB, Sigma–Aldrich), 0.03% nickel ammonium sulfate and 0.01% hydrogen peroxide in PBS for 3 minutes. Sections were mounted on gelatin-coated slides, air dried, dehydrated in a graded ethanol series, cleared in xylene, and coverslipped with Entellan mounting medium (Merck). The specificity of the immune reactions was controlled by omitting the primary antibody.

2.9. Western blotting

Western blotting was used for quantifying GluR1 and GluR2 protein expression in the cerebral cortex, caudate putamen, and CA1, CA3 and DG hippocampal regions from ischemia-injured and sham-operated animals. Total protein was extracted using Tripure Isolation Reagent (Roche Diagnostics) following the manufacturers' instructions. Protein concentrations were determined using the Lowry method (Biorad). Electrophoresis of equal amounts of protein (30 µg per lane) was run on 10 % SDS-PAGE gel and then transferred to a nitrocellulose membrane. Membranes were incubated in 0.2% Tween 20 in Tris-buffered saline (TBS-T) containing 5% non-fat milk for at least 60 min at room temperature to block non-specific binding. Then, the membrane was incubated overnight at 4°C in polyclonal goat anti-GluR2 IgG (Santa Cruz Biotechnology) for GluR2 AMPA subunit staining and polyclonal rabbit anti-GluR1 IgG (Chemicon) for GluR1 staining. The following day, the membrane was washed in TBS-T, incubated for 1 h in the HRP-conjugated secondary antibodies (donkey anti-goat and goat anti-rabbit respectively) diluted 1:3000 in TBS-T, and detected by Chemiluminescent HRP substrate (Millipore). Photographic films (GE Healthcare) were analyzed with a GS-800 computing

densitometer using Quantity One software (Biorad), choosing the mean band densities as parameter. The optical density values of GluR1 or GluR2 were normalized with respect to their corresponding β-actin (Sigma-Aldrich) values and expressed as means ± SEM.

2.10. Statistical Analyses

All Real-time PCR results are expressed as means ± SEM of the fold-change values of at least two different experiments for each assay. Statistical significance among mRNA levels of GluR1 and GluR2 AMPA subunits of the different experimental groups (ischemia and sham-operated rats) was analyzed with the unpaired Student´s t test.

Western blot results were also analyzed with the unpaired Student's t test to determine significance between the sham and I/R (15 minutes of global ischemia followed by 48 hours of reperfusion) animals.

Data analysis was carried out using GraphPad Prism version 4.0 (GraphPad Software, San Diego, USA). Significance was set at the 95% confidence level.

3. RESULTS

3.1. Transient global brain ischemia decreases GluR1/2 mRNA levels.

In all structures studied, the data from real-time PCR studies revealed a significant down-regulation of GluR1 and GluR2 AMPAR subunits mRNA levels at 48 hours of reperfusion after global brain ischemia (I/R) (Fig.1). In the injured animals, the lowest mRNA levels were found in hippocampal areas and caudate-putamen (these levels representing 20-30 % of those of the sham animals), while the cerebral cortex was less affected (with levels up to 70 % for GluR1 and 60 % for GluR2 with respect to those of sham animals).

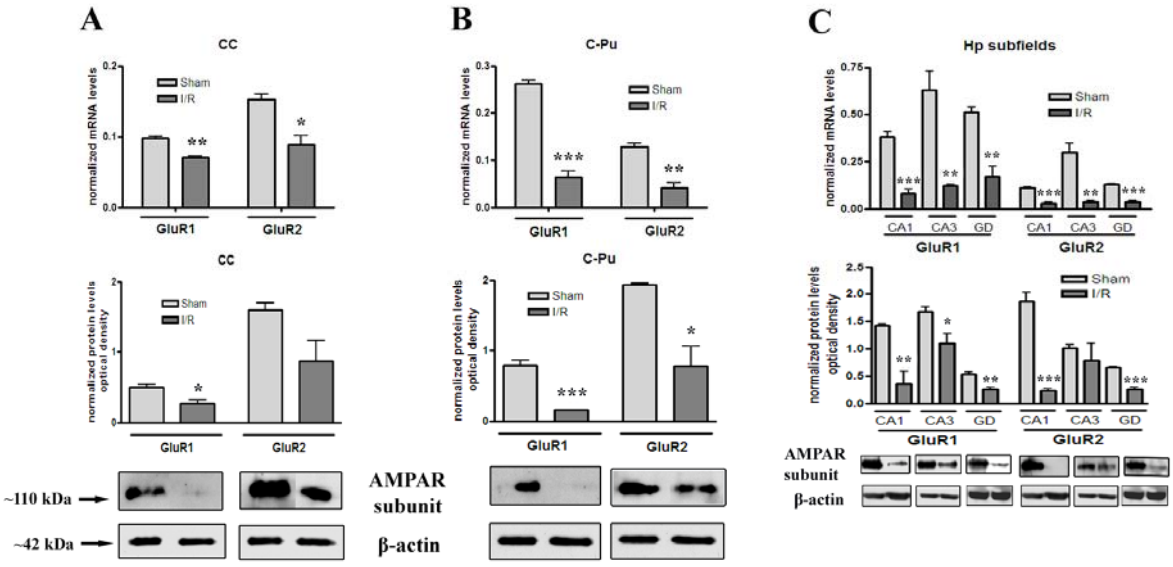

Figure 1: Real-time PCR and Western blot results of different forebrain structures of sham and I/R (ischemia/reperfusion) animals. mRNA and protein levels of GluR1 and GluR2 AMPAR subunits in cerebral cortex (CC) **(A)**, caudate-putamen (C-Pu) **(B)** and hippocampal areas **(C)**. Representative images of Western blot assays are also shown. GD: Dentate Gyrus. *Significant differences ($p<0.05$) with respect to sham animals, ** ($p<0.01$), *** ($p<0.001$) (Student´s t-test, n=5).

3.2. GluR1 protein decreases following transient global brain ischemia-reperfusion while GluR2 protein does not decreases in all structures.

In most cases, significantly decreased protein levels were observed at 48 hours of reperfusion after ischemic insult, although no significant differences were found for the GluR2 AMPAR subunit in cerebral cortex and CA3 hippocampal area after I/R. Significant decreases were also observed for GluR1 levels in all the structures studied (Fig. 1).

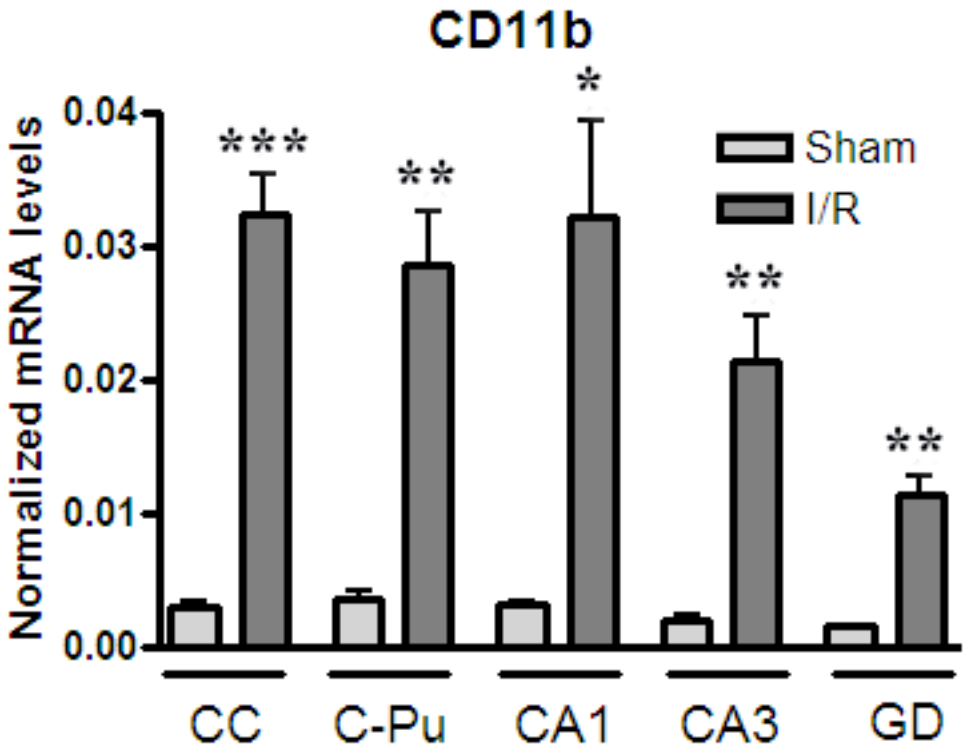

Figure 2: Effect of transient global ischemia followed by 48 hours of reperfusion on CD11b/CD18 mRNA levels in the different structures studied. CC: Cerebral cortex, C-Pu: Caudate-Putamen, GD: Dentate Gyrus. *Significant differences ($p<0.05$) with respect to sham animals, ** ($p<0.01$), *** ($p<0.001$) (Student´s t-test, n=5).

3.3. The inflammatory response is similar in all structures studied

The mRNA levels of the CD11b microglia-activated marker showed outstanding increases (around ten fold) in insulted rats as compared to those of the sham animals (Fig. 2). Likewise, CD11b/CD18 integrin-immunoreactive cells studied by immunohistochemistry displayed noticeable morphological changes after ischemia-reperfusion. The resting microglial cells of the sham animals appeared widely ramified with a moderate degree of labeling. In contrast, the microglial cells of the insulted animals displayed an ameboid appearance, with an important increase in size and intense labeling (Fig. 3-4). Comparable results were found among the different forebrain areas studied, without apparent differences.

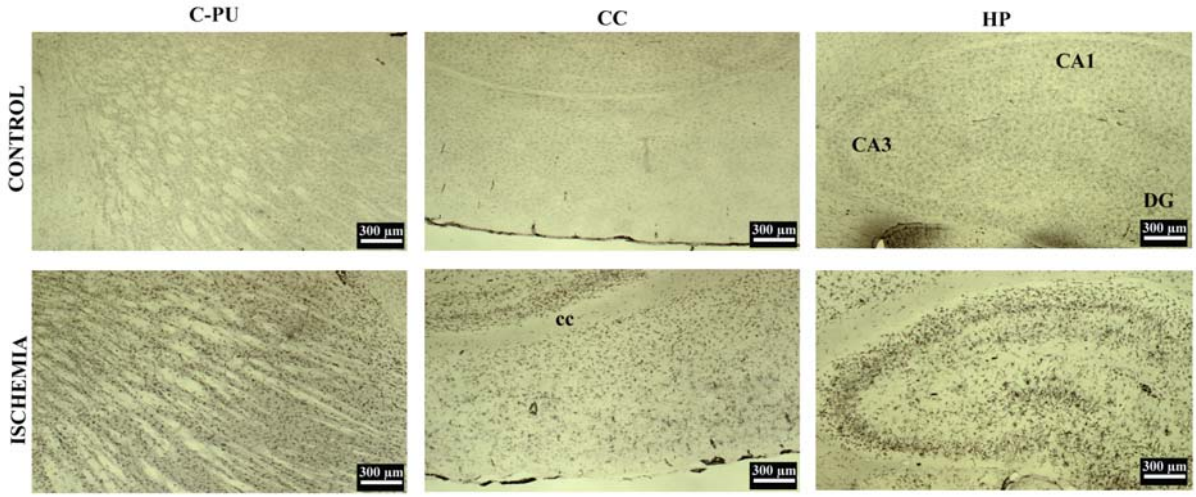

Figure 3: Representative images of cerebral cortex, caudate-putamen and hippocampus from injured and sham animals immunolabelled with CD11b. Notice the strong labeling of the activated microglia following ischemia. CC: Cerebral cortex, C-Pu: Caudate-Putamen, HP: Hippocampus.

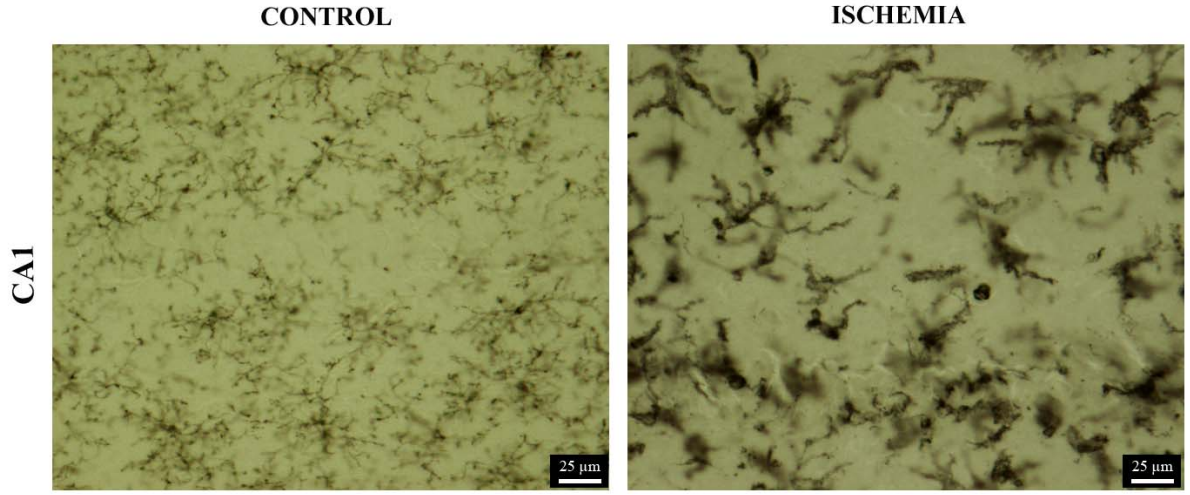

Figure 4: Detailed images of microglial cells of injured and sham animals in CA1 hippocampal area. Notice the strong labelling and ameboid morphology of the microglia-reactive cells of the insulted animals as compared with the widely ramified and low-moderate intensity of the microglial cells in the sham animals.

4. DISCUSSION

4.1. Differential vulnerability and AMPAR subunit expression

The data reported here show that under the conditions studied, ischemia-reperfusion elicits significant decreases in both the GluR1 and the GluR2 AMPAR subunits, although outstanding differences are observed in the different structures. Thus, both GluR1 and GluR2 mRNA decreases in cerebral cortex were less noticeable than those of the caudate-putamen and the hippocampal areas. Regional differences in susceptibility to ischemic damage have been widely reported (Globus *et al.*, 1990; Cervos-Navarro and Diemer, 1991) and it has been indicated that the decrease in the expression of NMDAR subunits is related to the different structures studied (Dos Anjos *et al.*, 2008). Given the tight relationships between NMDA and AMPA receptors (Burnashev *et al.*, 1998), it is not surprising that the regulation of both NMDA and AMPA receptor genes is modified by the ischemic insult however it is not clear if the expression of these genes accounts for the differences in I/R vulnerability of different brain areas.

One of the hypothesis correlating vulnerability with AMPA gene expression is the "GluR2 hypothesis" in which high levels of GluR2 have been related to the ischemia resistance (Pellegrini-Giampietro *et al.*, 1997). The presence of the AMPAR GluR2 subunit in the whole receptor has been reported to decrease calcium permeability through the channel (Hollmann *et al.*, 1991, Washburn *et al.*, 1997). Thus, the “GluR2 hypothesis”, proposes that the down-regulation of GluR2 gene expression may serve as a "molecular switch" leading to the formation of Ca^{2+}-permeable AMPA receptors and enhanced toxicity of endogenous glutamate following a neurological insult (Pellegrini-Giampietro *et al*, 1997). Our data support in some way this hypothesis in the hippocampal CA1 where the GluR2 decreases in CA1 are very noticeable and even more important than those of GluR1 in both mRNA levels and protein levels (figure 1C). In contrast, in the more I/R resistant hippocampal CA3 (Schmidt-Kastner and Freund, 1991), we observed similar GluR2 protein levels in sham and injured animals (despite the strong decrease in the GluR2 mRNA levels observed) (figure 1C). Furthermore, our data in cerebral cortex and striatum do not fit the GluR2 hypothesis, since the low I/R vulnerable cerebral cortex present similar decreases in GluR1 and GluR2 (in both protein and mRNA levels) in a similar way than the more I/R vulnerable striatum (figure 1A and B). We cannot discard that GluR2 play a role in the vulnerability but our results suggest

that other mechanisms different than the presence of GluR2 are involved in the vulnerability of different areas.

A second hypothesis indicates a correlation between persistent translation arrest (TA) and delayed neuronal death of vulnerable neurons following brain ischemia and reperfusion, although the mechanisms and significance are poorly understood (DeGracia *et al.*, 2008). Our data support that different areas present different mechanisms in the control of the expression of the AMPAR subunits. For instance, in cerebral cortex and CA1 decreases in the mRNA levels observed in GluR1 and GluR2 correlates with the protein levels of these subunits, however, this correlation in CA3 is clearly different. Thus, GluR2 subunit levels in CA3 measured by Western blot were similar in sham and insulted animals (in contrast the results in CA1 and dentate girus), however, the study of gene expression revealed a similar down-regulation in CA3, CA1 and the dentate gyrus by measuring the GluR2 and GluR1 mRNA levels. These results suggest that ischemia induces a similar down-regulation in the gene expression of the AMPAR subunits in the whole hippocampus but differences in the control at translational or post-translational levels, including for example modifications in the protein turnover, exist and could be involved in the different ischemia vulnerability. Our data indicating similar or low decreases in protein levels of GluR1 and GluR2 in CA3 versus the decrease of these subunit in other areas such as CA1 could be explained as persistent differential TA in these areas of the hippocampus, however the hypothesis cannot be applied when other areas with less vulnerability as cerebral cortex are included in the analysis.

4.2. Inflammation, vulnerability and AMPAR subunit expression

It has been reported that inflammation plays an important role in the pathophysiology of ischemia-reperfusion injury (Chamorro and Hallenbeck, 2006; Rodríguez-Yáñez and Castillo, 2008) and Cd11b/CD18 (Mac-1) is considered one of the most reliable marker of activated microglia and therefore an inflammation parameter (González-Scarano and Baltuch, 1999, Kim and De Vellis, 2005). Global ischemia is followed by an acute and a prolonged inflammatory response characterized by activation of resident microglial cells (Mac-1 expression), and production of inflammatory cytokines such as TNF-alpha (Uno *et al.*, 1997). Since TNF-alpha is involved in AMPAR trafficking and subunit expression (Leonoudakis *et al.*, 2004) we hypothesized that if differences in inflammation in areas with different vulnerability exist they could lead to differences in the AMPAR expression. We

found very high levels of Cd11b in cerebral cortex, hippocampus, and caudate-putamen, indicating a strong inflammatory response to the ischemia in all these structures. However, the lack of differences among the different areas studied in inflammation levels at 48 hours of I/R using CD11b as a marker, suggests that the degree of vulnerability to ischemia depends on the actual properties of the neuronal type rather than on environmental factors. Moreover, we failed in finding a correlation between inflammation and AMPAR gene expression suggesting that other factors than inflammation are involved in the regulation of the AMPAR subunits.

We must point out that data from the global ischemia model should be treated with caution in terms of comparison with data from stroke. Rather, this model should be compared with other pathologies producing global brain ischemia which are more common in humans, such as cardiac arrest. In this regard, the information here presented contributes to the understanding of the mechanisms involved in cerebrovascular diseases showing how the I/R modifies the expression control of the AMPA glutamatergic receptors and, in a more limited way, the inflammatory response.

On the other hand, the general decrease here reported in the AMPAR expression in the different structures studied, and the results previously reported for the NMDA receptor (Dos-Anjos *et al.*, 2009) indicate that a reduction in the blood flow followed by reperfusion induces a strong decrease in the mRNA levels of the glutamatergic receptors and an increase in at least one inflammatory marker. We expect similar results for experiments carried out using other brain ischemia models such as 4-VO or focal ischemia since these models bring about a global or focal blood flow reduction followed by reperfusion.

In conclusion, this study shows that after 48 hours of ischemia, a down-regulation of the AMPAR subunits GluR1 and GluR2 can be observed both at mRNA and protein levels, but this down-regulation seems not to be correlated to areas with more vulnerability. This study suggest that the expression of these genes does not explain the differences in I/R vulnerability of different brain areas and the observed differential vulnerability seems to be independent of inflammation, at least at this time point.

Acknowledgements

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QUANTITATIVE GENE EXPRESSION ANALYSIS IN A BRAIN SLICE MODEL: INFLUENCE OF TEMPERATURE AND INCUBATION MEDIA.

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ABSTRACT

We describe the RNA integrity (28S/18S ratio) and the mRNA expression of genes encoding GAPDH, Mast2 and β-actin in cortical brain slices incubated for up to 24 hours in Ringer solution and DMEM media at 25ºC and 37ºC. Our data reveal an optimal temporal working window between 1 and 6 hours when slices are incubated in Ringer solution at 25ºC that allows experiments related to gene expression dynamics to be performed more suitably than those carried out at 37ºC. Additionally, we show that reference gene expression may be modified in dynamic experiments and may compromise studies of gene expression.

Keywords:

Real-time PCR, RNA integrity, reference genes, in vitro model

Abbreviations:

Mast2 (microtubule associated serine/threonine kinase2), GAPDH (glyceraldehyde 3-phosphate dehydrogenase), DMEM (Dulbecco´s Modified Eagle´s Medium)

1. INTRODUCTION

Brain slices are widely used as a model on neurotransmitters and receptors studies and have the advantage of being used in highly controlled *in vitro* environments, thus preserving much of the cellular complexity and interactions inherent to the brain *in vivo*. The temporal availability of brain slices for experimental procedures is short (less than 24 hours), but it does allow study the gene expression in acute experimental assays [1]. The first step for checking the suitability of this model is the RNA integrity maintenance of the slices over time. The use of capillary gel electrophoresis technology allows easy and fast determination of RNA quality, a previous step to measuring mRNA levels, whose suitability is compromised in low-quality RNA samples [2,3]. Moreover, the expression of the reference genes used must be validated under the experimental conditions employed [4]. Electrophysiological recordings are usually performed in a balanced salt medium, such as Ringer solution. Some studies addressing the responses of brain slices to oxidative stress use complex culture media (DMEM-F-12) [5], but it remains unknown how the differences in different media affect mRNA levels in acute experimental procedures. Previous studies carried out on liver have demonstrated the importance of temperature in RNA integrity and mRNA levels, and their relationships [2]. Here we report the behavior of RNA integrity and mRNA levels of housekeeping genes encoding β-actin, Mast2 and GAPDH from brain slices along 24 hours of incubation in Ringer solution and DMEM complex media, as well as the effect of different temperatures.

2. MATERIAL AND METHODS

This study was carried out on vibratome sagittal sections (350 μm) from the parietal cortex of nineteen male Sprague-Dawley rats, 2-3 months old. Sections were incubated in oxygenated Ringer´s solution (119 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 26.2 mM $NaHCO_3$, 1.3 mM $MgSO_4$, 11 mM glucose) or DMEM/F12 culture medium in a chamber saturated with 95% O_2/5% CO_2. Assays were carried out at 25ºC and 37 ºC and different times of incubation (up to 24 hours). After the incubation time, slices were frozen in liquid nitrogen, homogenized in Tripure, and processed for RNA isolation following Roche's recommendations. Total RNA concentrations were determined using a NanoDrop ND-3300 spectrophotometer and RNA samples maintained at −80 ◦C until further processing. RNA integrity was determined by microcapillary gel electrophoresis using the Experion™ Automated Electrophoresis System (Biorad), following the manufacturer´s instructions. The 28S/18S rRNA ratio was used to assess RNA quality using the respective areas under the 28S and the 18S peaks [6].

Studies on mRNA were performed from 1 μg of RNA template that was converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems). The primer sequences used for real-time RT-PCR analysis were: rat GAPDH, forward primer 5´-GGGCAGCCCAGAACATCA-3´ and reverse primer 5´-TGACCTTGCCCACAGCCT-3´ (GenBank accession no. **NM_017008**); for β-actin, we used a forward primer 5´-CCCTGGCTCCTAGCACCAT-3´and reverse primer 5´-TAGAGCCACCAATCCACAGA-3´ (**NM_031144**); for rat Mast2, forward primer 5´-AGATATTGCGGAAGCGGTTAT and reverse primer 5´-AGCACAAACGAG GCACATCTG (**NM_001108005**). PCRs were performed in an ABI PRISM 7700 thermal cycler using the SYBR Green PCR Master Mix kit (Applied Biosystems) and the following settings: 2 min at 50ºC, 10 min at 95ºC and 40 cycles of 15 s at 95ºC and 1 min at 60ºC. Results were analyzed with Sequence Detector software. To determine the effect of temperature and incubation time on the gene expression levels, the $2^{-\Delta Ct}$ method was used, where ΔCt= Ct time x – Ct time 0 [7]. Data represent mean fold-change ± SEM in mRNA levels relative to control samples (obtained immediately after sectioning on the vibratome).

Slice Cell viability was determined by measuring the LDH release of the slices using the Cytotoxicity detection kit following the manufacturer's instructions. The results are shown as percentages of total LDH release.

3. RESULTS

Significant decreases (~30 %) were observed in the RNA integrity of immediately obtained slices with respect to cortical tissue just removed from the brain (fig 1A and B). The modification of RNA integrity along the incubation time with respect to control slices under the different incubation conditions are shown in figure 1C. The effect of temperature was observed in the different incubation media. Thus, up to 6 hours of incubation at 25 ºC, the 28S/18S ratio had similar values when tissues were incubated in Ringer solution, while significant increases were observed in DMEM culture media. However, 24 hours of incubation at this temperature resulted in a decrease of up to 65% in the 28/18S ratio in both incubation media. In contrast, the study carried out at 37ºC revealed outstanding differences in the 28S/18S ratio values throughout the incubation period in both media (Ringer solution and DMEM). Moreover, incubation at 37ºC induced decreases in the 28S/18S ratios of up to 25% after 24 hours of incubation in DMEM, and of up to 20% in Ringer solution (fig.1C).

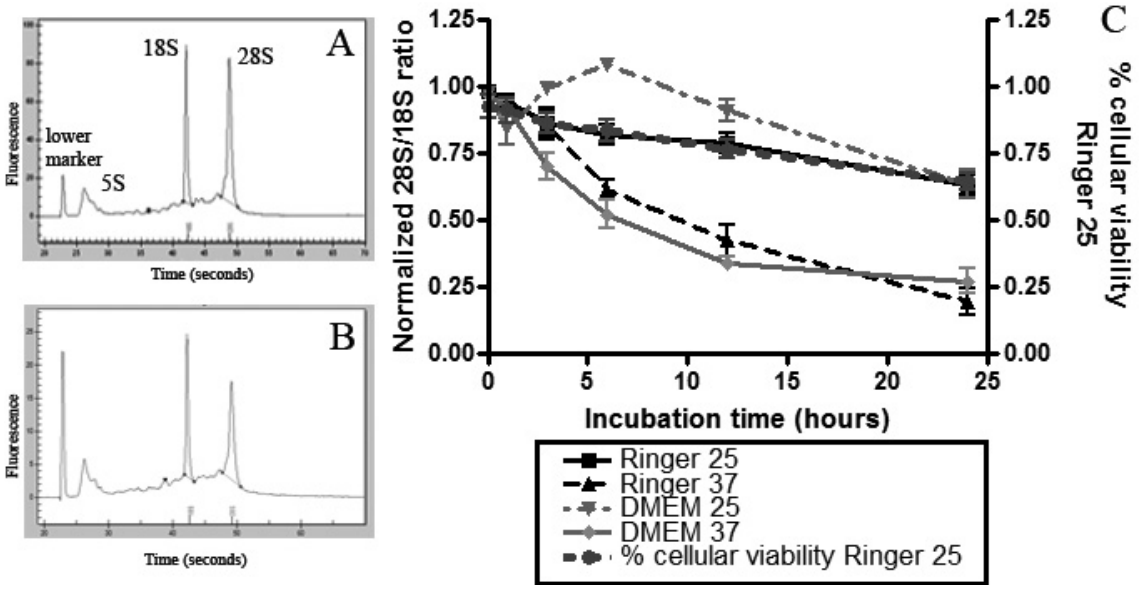

Fig. 1 A) and **B)**: Representative electropherograms showing three peaks corresponding to 28S, 18S and 5S rRNA species under different study conditions. A) RNA from cerebral cortex just removed from skull B) RNA from vibratome slices just sectioned. **C)** Normalized 28S/18S ratios along time in all conditions studied. Data represent mean ± SEM (n=4). Cell viability percentage with respect to control samples at 25ºC in Ringer is also plotted in the

same graph (right axis). Note the strong correlation between RNA integrity and cell viability at 25ºC following Ringer incubation (Pearson coefficient r=0.98).

A significant decrease by up to 50% in the mRNA levels of all genes studied was found in slices just sectioned on the vibratome with respect to those of the cortical tissue just removed from brain. The fold-changes in GAPDH, β-actin and Mast2 mRNA levels along time with respect to control slices (just sectioned on the vibratome) at different temperatures and different incubation media are shown in figure 2.

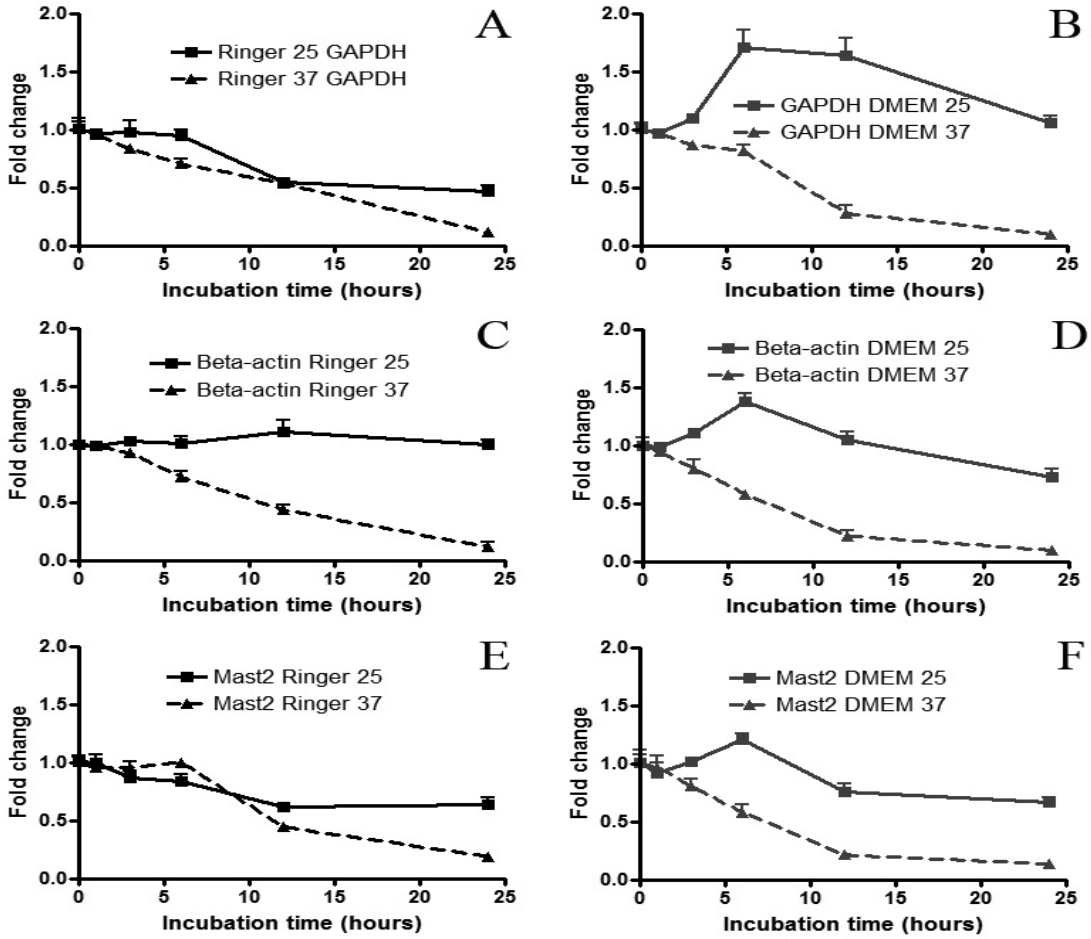

Fig. 2 Fold changes in mRNA levels in the genes studied along time at different temperatures and different media. A, C and E in Ringer solution and B, D and E in DMEM solution. The results are normalized with respect to those of the sections just obtained with the vibratome (mean ± SEM). One-way ANOVA followed by the Student-Newman-Keuls (SNK) test was used for comparing fold changes at different times of incubation. (n = 4).

At 25ºC we did not find significant changes in mRNA levels in any genes in the first hour of incubation either in Ringer solution or DMEM culture media with

respect to the control group. At this temperature, using DMEM we observed a progressive increase in GAPDH, β-actin and Mast2 mRNA levels from 1 to 6 hours (fig. 2); these decreased progressively from 6 to 24 hours of incubation. Incubation in Ringer solution did not elicit significant modifications in β-actin mRNA from 1-24 hours. However, GAPDH and Mast2 mRNA levels remained constant from 1 to 6 hours of incubation, after which they decreased rapidly and significantly (One way ANOVA $p<0.01$, $n=4$) with respect to the levels observed in the 6 first hours of incubation. Mast2 mRNA levels showed the best correlation (Pearson coefficient $r = 0.92$) with cell viability along the time of incubation in Ringer solution at 25ºC.

In contrast, at 37ºC, significant decreases in GADPH, and β-actin and Mast2 mRNA levels were observed along the whole incubation time, with statistically significant differences (ANOVA $p<0.05$, $n=4$) over time in both Ringer solution and the DMEM incubation media (fig 2).

4. DISCUSSION

The three genes studied showed similar mRNA levels in an optimal "working window" up to 6 hours of incubation after slicing. In contrast, Mast2 seems to be the gene whose mRNA levels better fitted cell viability in a wider "working window" including the first 12 hours of incubation. The significant decrease (ANOVA $p<0.01$, $n=4$) observed in both RNA integrity and mRNA levels of the genes studied from just obtained slices with respect to those from cerebral cortex just removed from the skull provides an estimate of the cortical damage in the RNA or mRNA expression (about 50%) induced by procedures associated with vibratome slicing, including the mechanical stress of the blade through the tissue, carbogen bubbling, temperature change, etc.

The assessment of RNA integrity is a critical first step in obtaining meaningful gene expression data. Working with low-quality RNA may strongly compromise the experimental results of downstream applications [3]. Checking RNA integrity seems to be especially important in studies such as the one reported here, which analyzed changes in mRNA over time. The data reported in the present work show that temperature is the main factor affecting RNA integrity and mRNA levels, in agreement with the results reported for liver [2]. Our data show that after twelve hours of incubation at 37ºC brain slices display half the RNA integrity of those incubated at 25ºC, which correlates with a decrease in the reference mRNA levels studied. This confirms that assays comparing mRNA levels must take into account RNA integrity.

Incubations at 25ºC revealed similar RNA degradation levels up to 6 hours, which matched the plateaus observed in housekeeping mRNA levels following incubation in Ringer solution. Interestingly, incubation experiments in DMEM revealed that RNA integrity improved from 1 to 6 hours, in agreement with a significant increase in mRNA of ß-actin, Mast2 and GAPDH from 1 to 6 hours. This suggests that the use of a complex incubation medium such as DMEM is required to allow new RNA synthesis, complicating the comparison of results at different times. However, increases in RNA integrity and mRNA levels did not appear until the first hour in DMEM incubation, which indicates that during the first hour gene expression in the tissue was still recovering from the sectioning stress. Since this fact cannot be detected in the Ringer incubation experiments we believe that the

temporal working window should be restricted from 1 to 6 hours. Accordingly, the use of Ringer solution at 25ºC provides a more stable model to perform experimental procedures, such as OGD (oxygen glucose deprivation), than when DMEM is used at 25ºC.

Our results demonstrate the importance in the choice of the reference genes depending on the temporal working window. Thus, the rapid decrease in GAPDH and Mast2 mRNA levels after six hours of incubation contrasts with the maintenance of β-actin mRNA levels up to 24 hours, since this could bias the analysis of data, depending on the reference gene used. In this regard, GAPDH, Mast2 and β-actin mRNA measurements seem to be useful as reference genes for *in vitro* experiments lasting no longer than six hours, while outstanding differences may appear in a broader working window, depending on the reference gene used. Taking into account the correlation between cell viability and mRNA levels, Mast2 seems to be the most accurate gene for experimental procedures requiring slice incubation for 12 hours, but data concerning RNA integrity and mRNA levels suggest that experimental procedures involving mRNA measures should be performed in the first 6 hours after sectioning. On the other hand, the progressive decrease in gene expression observed at 37ºC provides additional evidence against the suitability of using this temperature in studies involving mRNA expression.

In sum, the present study demonstrates that the incubation of brain slices at 25ºC leads to a plateau in RNA integrity and the mRNA levels of housekeeping genes that occurs between 1 and 6 hours that allows experiments related to gene expression dynamics to be performed more suitably than those carried out at 37ºC. Incubation of brain slices at 25ºC using balanced salt solutions, such as Ringer or complex media as DMEM, allows differences to be detected in housekeeping mRNA levels that are not detected at 37ºC due to the constant degradation of RNA. Additionally, we show that housekeeping mRNAs may be modified in dynamic experiments and may compromise studies on gene expression.

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EARLY MODIFICATIONS IN NMDA RECEPTOR SUBUNIT mRNA LEVELS IN AN OXYGEN AND GLUCOSE DEPRIVATION MODEL USING RAT HIPPOCAMPAL BRAIN SLICES.

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ABSTRACT

Glutamatergic NMDA receptors (NMDAR) are considered to play a key role in ischemia-induced damage. Long-term (hours) changes in their expression upon ischemia have been shown. Here we report short-term changes in the mRNA levels of the major hippocampal NMDAR subunits (NR1, NR2A and NR2B), as well as c-fos, in an *ex vivo* ischemia model using hippocampal slices. This effect can be also observed in a calcium free incubation solution. Striking early decreases in the NMDAR subunit mRNA levels were observed after 30 minutes of oxygen and glucose deprivation (OGD) as well as a partial recovery when the tissues were returned to the balanced salt solution (reperfusion-like period) for 3 hours. Since OGD-induced damage has been reported to be a consequence of the increase in OGD-related glutamate release, we also analyzed NMDAR mRNA levels following increased glutamate levels in hippocampal sections in which no significant effects on NMDAR subunit mRNA levels were detected. Furthermore, we describe that the presence of MK-801 (a selective NMDAR antagonist), CNQX (a selective AMPA/Kainate receptor antagonist) or their combined action in the incubation solution is able to induce a significant decrease in NMDAR expression but in these conditions the OGD does not induce further decreases in mRNA levels. We suggest that the mechanisms triggered during OGD to downregulate mRNA levels of NMDAR subunits could be the same than those induced by glutamate receptor antagonists.

Keywords: hippocampus, OGD, N-Methyl-D-Aspartate, glutamate, real-time PCR

Abbreviations:

OGD, oxygen-glucose deprivation

NMDA, N-methyl-D-aspartate

AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione

1. INTRODUCTION

Isolated brain slice preparations were first developed for biochemical studies in the early 1960s (McIlwain, 1961). Later, Yamamoto (Yamamoto and McIlwain, 1966) employed this technique to obtain the first electrophysiological recordings from brain slices. Since then, brain slice preparations have been widely used as a model for ex vivo studies on neurotransmitters and receptors. Since the model provides the advantages of highly controlled *in vitro* environments and preserves much of the cellular complexity and interactions inherent to the *in vivo* brain (Sick and LaManna, 1995), it should be able to provide relevant insight about gene expression. The main problem of using brain slices is the short temporal availability of the slices for experimental procedures (<24 h), which limits ex vivo assays aimed at studying gene expression to acute experimental assays. This time of analysis is, in turn, limited to a temporal working window where gene transcription is preserved when brain slices are incubated in a balanced salt solution such as Ringer's solution (Dos Anjos *et al.*, 2008), particularly if results are to be compared with those obtained from electrophysiological recordings, where this type of balanced salt solution is commonly used.

The complexity of the response of the brain, mainly the highly vulnerable hippocampus (Kirino *et al.*, 1985), to the ischemia-reperfusion damage reported in *in vivo* studies, has led to the development of a model in which brain slices are subjected to oxygen and glucose deprivation (OGD) in order to reduce the factors involved in the *in vivo* ischemia (Moro *et al.*, 1998). Ischemia-reperfusion experiments *in vivo* have been reported to elicit important modifications in the expression of a number of genes, mainly those related to the glutamate receptors and, in particular, glutamate NMDA receptors (NMDARs), which are considered to play a key role in the pathogenesis of ischemia (Lee *et al.*, 1999; Liu *et al.*, 2007). This subfamily of ionotropic glutamate receptors are overactivated by glutamate during ischemia leading to intracellular calcium increase and finally to neuronal cell death (excitotoxicity) (Arundine *et al.*, 2003), and their blockade has been found to be neuroprotective in brain ischemia models (Arias *et al.*, 1999). Thus, *in vivo* studies have been able to detect changes in NMDAR gene expression several hours after the ischemic insult (Hsu *et al.*, 1998, Won *et al.*, 2001) but no early changes or slightly reduced levels in NMDAR gene expression have been observed following the challenge (Zhang *et al.*, 1997). To date, there are no data concerning the regulation

of NMDAR genes following OGD insult. The increase in glutamate levels elicited by OGD (Fujimoto *et al.*, 2004) has been reported to induce a excitotoxic effect responsible for at least part of the damage due to OGD (Brongholi *et al.*, 2006), but it is not clear how glutamate activity is involved in NMDAR gene expression. In this report we study for the first time the major hippocampal NMDAR subunits NR1, NR2A and NR2B (Al-Hallaq *et al.*, 2007) mRNA levels in the model involving hippocampal slices subjected to OGD and reperfusion. We also analyze how the mRNA levels of NMDAR are influenced by the presence of NMDAR and AMPA/Kainate receptor antagonists to gain greater insight into the mechanism of action of glutamate through the NMDAR expression.

2. EXPERIMENTAL PROCEDURES

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.

2.1. Preparation and incubation of slices

Two-month-old Sprague-Dawley rats were killed by decapitation and the forebrain was carefully removed and placed on a Petri dish over ice, after which the rostral, caudal and lateral portions to the hippocampus were removed with a blade. The resulting portion was then cut sagitally along the interhemispheric fissure with the blade and each part was firmly attached to the vibratome stage with instant glue to allow sagittal sectioning of the hippocampus from the lateral to the medial part. The plate with the tissues was introduced into a cold (4 ºC) cutting solution (CS) bubbled with carbogen (95% O2 and 5% CO2), containing 120 mM NaCl, 2 mM KCl, 0.5 mM CaCl2, 26 mM NaHCO3, 10 mM MgSO4 , 1.18 mM KH2PO4, 11 mM glucose and 20 mM sucrose pH 7.4. Each hippocampus was sliced (350 μm thick) and then transferred into a Petri dish with preincubation solution (PS) at 25ºC, similar to the cutting solution but without sucrose, and bubbled with 95% O2 and 5% CO2 for at least 30 min before the slices were transferred to the incubation solution (IS) -120 mM NaCl, 2 mM KCl, 2 mM CaCl2, 26 mM NaHCO3, 1.19 mM MgSO4, 1.18 mM KH2PO4 and 11 mM glucose, pH 7.4 at 25ºC and bubbled with 95% O2 and 5% CO2. The sections were transferred from this incubation solution to wells with 2 ml of the solutions with the different experimental conditions (tables 1A and 1B). Two different experimental procedures were carried out: A) oxygen and glucose deprivation (OGD) as well as the further return to the condition previous to the OGD —which is usually named reperfusion-like to compare to the reperfusion following ischemia that happens *in vivo*, in this procedure specific antagonists for NMDARs (MK-801) and AMPA-Kainate receptors (CNQX) were used to analyze the effect of these receptors on NMDAR mRNA levels; and B) assays for testing the effect of glutamate (4μM) on the hippocampal slices at times equivalent to those used in the OGD assays.

To test the effect of calcium on the mRNA levels in the OGD assays, we repeated the assays in a similar incubation solution than indicated above but CaCl2 was replaced by 200 μM EGTA, a calcium chelator. Osmolarity in all the solutions was 300 ± 5 mOsm.

After the appropriate incubation times and under the chosen experimental conditions, samples were collected from the incubation solution to determine LDH (Lactate dehydrogenase) activity and glutamate release, after which slices were collected and immediately frozen in dry ice.

Table1A.

Experimental condition

Time	1	1b	OGD	R	M1	M-OGD	MR	C1	C-OGD	CR	Procedure reference
0 minute	CS				CS			CS			**Sacrifice and sectioning**
15 minute	PS				PS+M			PS+C			**Start of preincubation**
45 minute	IS				IS+M			IS+C			**Start of incubation**
75 minute	IS		OGDS		IS+M	OGDS+M		IS+C	OGDS+C		**Start of OGD**
105 minute	FT	IS	FT	IS	FT	FT	IS+M	FT	FT	IS+C	**End of OGD or back to the**
285 minute		FT		FT			FT			FT	**condition previous to OGD**

Table1B.

Time	1	1b	2	3	4	**Procedure reference**
0 minute	CS					**Sacrifice and sectioning**
15 minute	PS					**Start of preincubation**
45 minute	IS					**Start of incubation**
75 minute	IS		IS+G			**Glutamate addition**
105 minute	FT	IS	FT	IS	IS+G	**Back to conditions previous to glutamate addition**
285 minute		FT		FT	FT	

Tables 1A and 1B. The tables indicate the different experimental conditions. C: 30 µM CNQX; M: 10 µM MK-801, G: 4 µM glutamate, FT: Freezing of the tissues on dry ice. CS: Cutting solution, IS: Incubation solution, PS: Preincubation solution, OGD: incubation solution without glucose and bubbled with 95% N2 and 5% CO2 to simulate an ischemic period. Solution composition is explained in the text. The procedure references are indicated clarify the different steps that were performed along time. Conditions 1 and 1b are similar but 1 was frozen at the same time as OGD while 1b was frozen 3 hours after finishing OGD. Since 1 and 1b did not present significant changes in any of the genes, they are not represented in figures 2 and 3.

2.2. Quantification of glutamate released

The amount of glutamate released to the incubation solution in the different conditions were estimated with the Amplex red glutamic acid/glutamate oxidase assay kit (Molecular Probes, Eugene, OR, USA), according to the manufacturer's instructions. In this assay, glutamate is oxidized by glutamate oxidase to produce α-ketoglutarate, NH_3 and H_2O_2. Alanine and glutamate pyruvate transaminase regenerate glutamate by transamination of α-ketoglutarate, resulting in multiple cycles of the initial reaction and the amplification of H_2O_2 production. H_2O_2 reacts with the Amplex red reagent, which is catalyzed by horseradish peroxidase to generate a highly fluorescent product, resorufin. After each treatment, 50 µl of the incubation solution in each well was collected and mixed with 50 µl of a reaction buffer containing Amplex red, glutamate oxidase, glutamate pyruvate transaminase, alanine, and horseradish peroxidase. After 30 min of incubation at 37ºC, the fluorescence of the reaction mixture emitted at 590 nm was measured with a fluorescence microplate reader (Synergy HT, Bio-Tek) using an excitation wavelength of 530-560 nm. The glutamate concentration in each sample was determined by using a standard glutamate curve.

2.3. LDH activity assay

LDH is a widely used marker of tissue cell death that is released from damaged cells to the incubation solution. LDH activity was measured spectrophotometrically at 492 nm by following the reduction of NAD+ by the LDH-catalyzed conversion of lactate to pyruvate, and further transfer to H+ from NADH/H+ to the tetrazolium salt which is reduced to formazan, using a HT Synergy microplate reader (Bio-Tek). Data are expressed as OD and show LDH release as percentages of the control group values (Experimental condition 1 described in table 1).

2.4. Real time PCR

RNA extraction

Total RNA was extracted using the Tripure™ Isolation Reagent (Roche), according to the manufacturer's instructions. This procedure allows the isolation of total RNA, DNA and protein fractions from a single sample. The contaminating DNA in the RNA samples was removed by incubation with DNase (Sigma) 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, USA). Isolated RNA was finally frozen at −80°C until further processing.

Reverse transcription

The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used. The 20 µl transcription mix for each sample contained 0.6 µg total RNA (10 µl), 2 µl 10x RT buffer, 0.8 µl dNTP 25x (100 mM), 2 µl 10x RT Random Primers, 4.2 µl DEPC water and 1 µl MultiScribeTM Reverse Transcriptase (50 units/µl).

Reactions were performed for 10 min at 25°C, 2 h at 37°C and terminated with 5 s at 85°C. Subsequently, the reaction mixture was maintained at -20°C until used for PCR amplification.

Quantitative real-time PCR

Real-time PCR quantification of the different mRNAs analyzed was performed in triplicate using gene-specific primers and SYBR® Green. Oligonucleotide primers were designed using Primer Express 2.0 software (Applied Biosystems). The primer sequences and the GenBank Accession Numbers are given below.

As an internal control for normalization, PCR reactions were performed concurrently with the amplification of a reference gene, 18S ribosomic RNA (rRNA).

Primers used:

NR1 forward: 5'-cagccgtgaacgtgtggag; NR1 reverse: 5'-tgctctaccactctttctatcc (NM 017010 GenBank accession number).

NR2A forward: 5'-cagtgatgtgtatatttcagagcatgtta; NR2A reverse: 5'-acactcgtctattgctgcaggaa (NM 012573 GenBank accession number).

NR2B forward: 5'-tccgtctttcttatgtggatatgc; NR2B reverse: 5'-cctctaggcggacagattaagg (NM 012574 GenBank accession number).

c-fos forward: 5´-acccctcgccgagcttt; c-fos reverse: 5´-cctcgtagtccgcgttgaa (NM 022197 GenBank accession number).

18S rRNA forward 5'-gattaagtccctgccctttgta; 18S rRNA reverse: 5'-gatccgagggcctcactaaac (V01270 GenBank accession number).

Beta-actin forward: 5´-ccctggctcctagcaccat; beta-actin reverse: 5´-tagagccaccaatccacacag (NM 031144 GenBank accession number).

Real time-PCR was performed on an ABI PRISM 7000 real-time thermal cycler using the SYBR® Green PCR Master Mix kit (Applied Biosystems, Foster City,

CA) and the following thermal cycler settings: 1 cycle of 2 min at 50ºC, 1 cycle of 10 min at 95ºC, 40 cycles of 15 s at 95ºC and 1 min at 60ºC. Cycle thresholds for both genes were selected immediately above the baseline and within the linear range on log scaling. The reaction mixture (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). The relative change in the mRNA expression of the subunits studied following the experimental conditions was determined by the equation:

Fold change = $2^{-\Delta Ct}$, ΔCt = (Ct target–Ct 18S rRNA) (Livak and Schmittgen, 2001). The Ct value is the cycle number at which the fluorescence signal crosses the threshold.

2.5. Statistical Analysis

Two animals were killed for each assay and at least two hippocampal 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 a total of 10 animals were used. The n thus considered increases the potency of the analysis to detect significant differences.

All results are expressed as means ± SEM of the fold-change values of at least two different experiments for each PCR assay. Statistical significance among the mRNA levels of the different experimental groups was 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 (Banda *et al.*, 2008).

3. RESULTS

3.1. Reference genes

The levels of 18S rRNA and beta-actin were tested as putative reference genes for mRNA expression studies in ex vivo hippocampal slices along the same temporal working window used for cerebral cortical slices where beta-actin and GAPDH mRNA maintained constant levels along time (Dos Anjos *et al.*, 2008). We compared beta-actin mRNA and 18S rRNA levels under experimental conditions including 30 min of OGD followed by 3 hours of reperfusion.

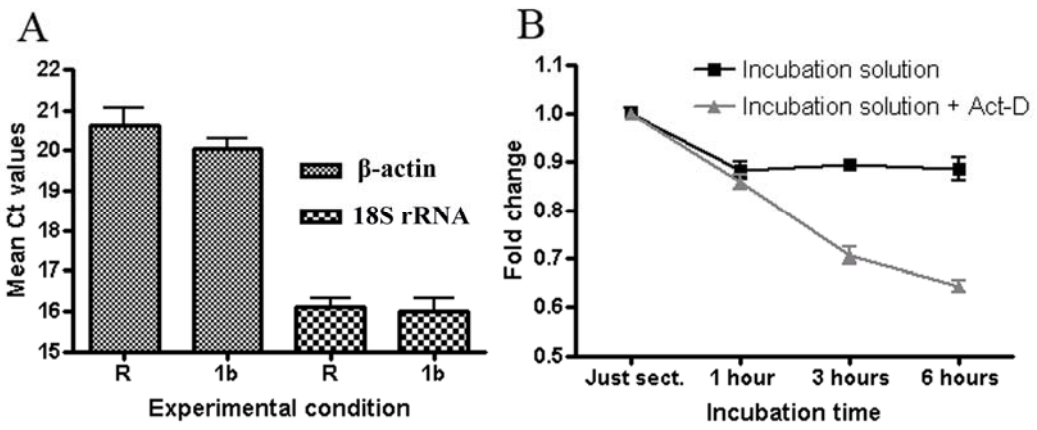

Figure 1.A: Ct values for 18S rRNA and ß-actin obtained from hippocampal slices along 5 hours of incubation after sectioning (condition 1b) and from hippocampal slices following OGD and the reperfusion-like period (condition R). **B:** Effect of actinomycin-D (8 µg/ml) on the expression of beta-actin along the time of the slice in the incubation solution. Fold-change values are referred to the just sectioned slices.

The results are displayed in figure 1A. No significant differences were observed between the control and experimental conditions using any of these genes, but the 18S rRNA levels presented less variability than those of beta-actin (lower coefficient of variation). Quantitative PCR was also performed during the temporal working window in the presence of actinomycin D, a mRNA transcription inhibitor, to check whether mRNA levels persisted in the slices without new transcription. Figure 1B shows similar mRNA levels during the first hour, followed by a decrease of up to 60% in the ensuing hours.

3.2. Extracellular lactate dehydrogenase and glutamate levels

The glutamate and LDH levels following the OGD procedure are shown in tables 2 and 3 respectively. Glutamate levels remained unchanged or slightly reduced for at least 5 hours of incubation. However, an outstanding increase in glutamate (about 20-fold) was observed during OGD. The levels of glutamate increased progressively along the reperfusion-like time, reaching more than three times the concentration attained at the end of the OGD after three hours (cumulative effect). The presence of the NMDAR antagonist MK-801 during OGD reduced the OGD-induced glutamate release and also the glutamate release observed for the reperfusion-like time. In contrast, the presence of the AMPA/kainate receptor antagonist CNQX increased glutamate release two-fold during OGD but the glutamate levels observed in the reperfusion-like time were similar to those observed in their relative control samples (C1 condition).

Condition	**Glu release (nM)**
1	34.23 ± 16.36
OGD	609.56 ± 98.86 (*)
R	2094.43 ± 451.94 (*)
1b	18.34 ± 7.07
M1	13.67 ± 17.02
M-OGD	217.53 ± 10.38 (*)
MR	206.13 ± 144.9(*)(#)
C1	43.67 ± 9.29
C-OGD	118.20 ± 11.13 (*)
CR	102.77 ± 98.73 (#)

Table 2: Glutamate release values (Mean ± S.E.M) obtained in the different conditions indicated in table 1A. (*) $p<0.05$ vs 1, (#) $p<0.05$ MR and CR vs R; One way ANOVA-SNK (n=5).

Significant changes in LDH levels were only observed during the reperfusion-like time and the presence of MK-801 throughout the whole experiment maintained similar LDH levels to those observed when no OGD was carried out (table 3). The release of LDH in the presence of CNQX in the incubation solution was significantly lower than that observed in the presence of MK-801 but higher than in the absence of glutamate antagonists (table 3).

Condition	% of control samples
1	100 ± 9.36
OGD	93.89 ± 9.19
R	443.84 ± 21.74 (*)
1b	208.86 ± 1.79 (*)
M1	84.65 ± 12.85
M-OGD	99.98 ± 14.21
MR	204.8 ± 3.21 (*)(#)
C1	96.96 ± 4.93
C-OGD	120.84 ± 21.87
CR	290.13 ± 33.35 (*)(#)

Table 3: LDH release values obtained in the different conditions indicated in table 1a. Values are expressed as percentage of the samples of condition 1 (Mean ± S.E.M). (*) $p<0.05$ vs 1, (#) $p<0.05$ MR and CR vs R; One way ANOVA-SNK (n=5).

3.3. Real time PCR studies

The levels of mRNAs from the NMDAR subunits NR1, NR2A and NR2B, as well as c-fos in the different conditions (control condition (1), OGD period (OGD) and reperfusion-like period (R)) are shown in figure 2. Significant decreases in the mRNA levels in all the NMDAR subunits studied as well as in the c-fos mRNA were observed after the OGD condition and also when the assays were performed in a calcium free incubation solution (data not shown). The mRNA levels of NMDAR subunits obtained following the reperfusion like-time were lower than those of the control condition and higher than those of the OGD conditions and no significant differences with respect to control or OGD conditions were found, suggesting a partial recovery in mRNA levels during the reperfusion-like time for NMDAR subunits (figure 2). The presence of MK-801 or CNQX in the incubation media induced significant decreases in the mRNA levels in all the NMDAR subunits NR1, NR2A and NR2B, but not in c-fos (1 versus M1 and C1 in figure 2). The combined action of both antagonists in the incubation media resulted in a similar response compared to that observed for each of them independently. In contrast, there was no additional decrease during OGD in the presence of MK-801 or CNQX (M-OGD versus M1 and OGD, and C-OGD versus C1 and OGD in figure 2).

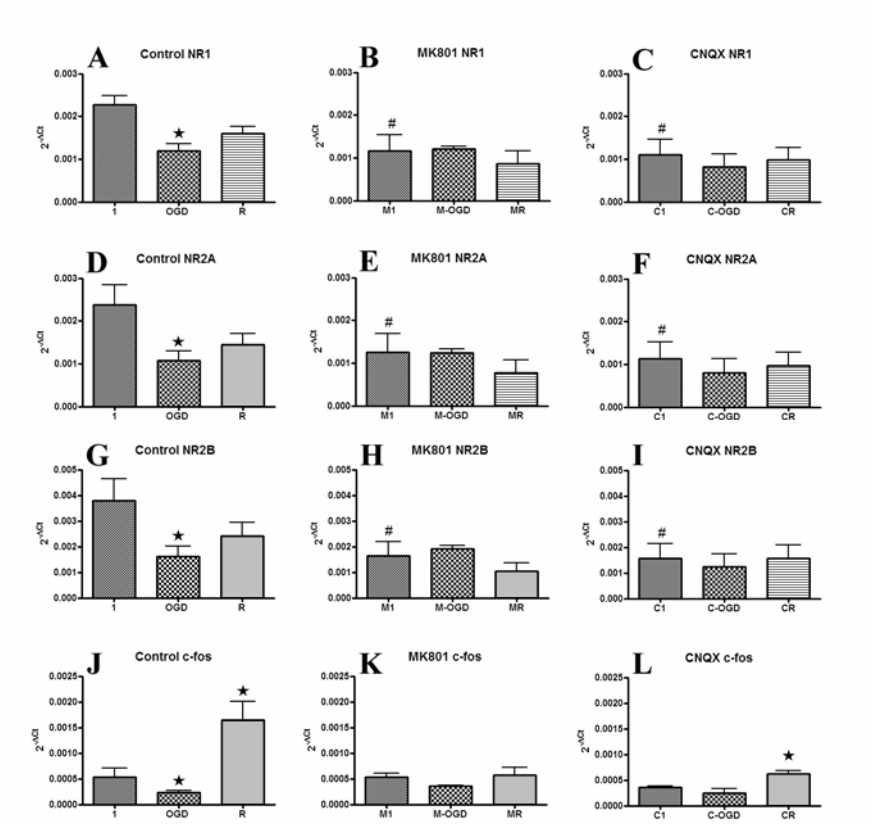

Figure 2. A, D, G, J) NMDAR NR1, NR2A and NR2B subunits and c-fos mRNA levels following oxygen and glucose deprivation (OGD) and reperfusion-like period. **B, E, H, K)** NMDAR NR1, NR2A and NR2B subunits and c-fos mRNA levels following oxygen and glucose deprivation (OGD) and reperfusion-like condition in the presence of the NMDAR antagonist MK-801. **C, F, I, L)** NMDAR NR1, NR2A and NR2B subunits and c-fos mRNA levels following oxygen and glucose deprivation (OGD) and reperfusion-like condition in the presence of the AMPA/Kainate antagonist CNQX. Different conditions correspond to those explained in table 1A. Significant differences ($p<0.05$) with respect to control conditions (1,M1,C1) are indicated by *; significant differences between M1 and C1 with 1 for each gene are represented by #. One-way ANOVA - SNK analysis (n=5).

The NMDAR and c-fos mRNA levels in hippocampal slices incubated in the presence of 4 μM glutamate (a glutamate slightly higher concentration but in the range to that observed following OGD), and reported to elicit significant effects on neuronal excitability (Herman and Jahr., 2007) are shown in figure 3. We failed to detect modifications in expression in the NMDAR subunit mRNA levels after 30 minutes or 3 hours of incubation in the presence of glutamate (bars 1, 2, 3, 4 in fig 3A, B and C). However, significant increases in c-fos mRNA levels were observed after 3 hours of incubation with glutamate for 30 minutes and then the incubation solution was renewed without glutamate (bars 3 and 4 in figure 3).

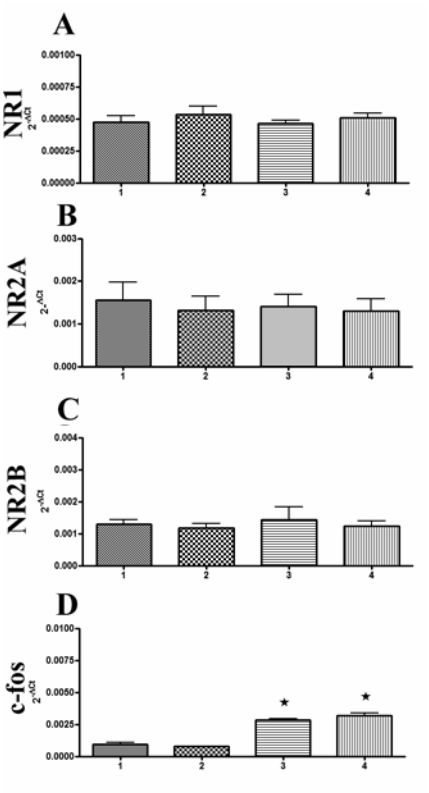

Figure 3. A, B, C, D) NMDAR NR1, NR2A and NR2B subunits and c-fos mRNA levels following 4 μM glutamate incubation. Different conditions correspond to those explained in table 1B. Significant differences ($p<0.05$) with respect to 1 are indicated by *. One-way ANOVA - SNK analysis (n=5).

4.-DISCUSSION

We have previously described an optimal temporal working window from 1 to 6 hours for analyzing mRNA levels in brain cortical slices by quantifying beta-actin, GAPDH and MAST-2 mRNAs (Dos-Anjos *et al.*, 2008). Here we performed a similar study in hippocampal slices and a similar optimal working window was observed for beta-actin, GAPDH, MAST-2 and 18S rRNA mRNAs (only data from 18S rRNA and beta-actin are shown), confirming that this temporal window is suitable for mRNA expression studies and that housekeeping genes used present constant mRNA levels along this period. Although no significant differences can be observed among the different experimental conditions (1, OGD and R) in any of the reference genes studied, the "relative variability" shows that 18S rRNA expression exhibits the lower coefficient of variation than the remaining genes studied, indicating that 18S rRNA is more accurate for use as a reference gene in the conditions studied.

The occurrence of mRNA transcription in hippocampal slices has been reported previously for iNOS (inducible nitric oxide synthase), where an up-regulation following OGD was observed in a similar hippocampal slice model (Cárdenas *et al.*, 2000). Our data showing increases in c-fos mRNA levels 5 hours after sectioning (bar R in figure 2) indicate that the synthesis of mRNA is still possible 5 hours after sections have been obtained in a balanced salt solution such as those normally used for electrophysiological studies. Furthermore, the effect of incubation with actinomycin-D indicates a decrease in mRNA levels after 1 hour, providing further support to the data indicating that new mRNA is synthesized in hippocampal slices *in vitro*. These data confirm the existence of a temporal working window suitable for gene expression studies that extends at least between 1 and 5 hours in the hippocampus and probably longer: at least 6 hours, like that reported for cortical brain slices. The relevance of assays in this incubation solution lies in the possibility of analyzing quantitative changes at mRNA levels in brain slices combined with the widely used electrophysiological and biochemical studies carried out in ex vivo assays.

Our data indicate that the NMDAR subunits studied present an early down-regulation at mRNA levels following OGD. The c-fos gene is induced by hypoxia both *in vivo* and in cell cultures (Haxhiu *et al.*, 1995; Greenberg *et al.*, 1999; Yu *et al.*, 2009) and in our OGD assays we found significantly increased mRNA levels

following the reperfusion-like period. This confirms that cells suffered hypoxia during the OGD assays and also that the decreases in the NMDA receptor subunit mRNAs observed were specific for at least these genes and not a generalized down regulation affecting the whole genome.

Delayed compensatory mechanisms in gene response *in vivo* against increased glutamate signaling through NMDAR (Won *et al.*, 2001). These authors have found diminished immunoreactivity for NR1 subunit 12-24 after ischemia in CA1 hippocampal region. Our data in slices indicate that the down-regulation of NMDAR genes seems to be an early response, in contrast with the reports obtained in *in vivo* studies, where no decreases in mRNA levels of NMDA receptor subunits have been found until 12 hours after ischemic challenge (Zhang *et al.*, 1997). Our results also indicate that the use of the OGD model in slices allows to detect changes at mRNA levels of a variety of genes before than those observed in *in vivo* ischemia. Moreover, the possibility of modifying the conditions during the OGD incubation, as for example controlling the extracellular calcium levels here reported, adds valuable information to that observed in *in vivo* models. The strong OGD-induced release of glutamate has been proposed to be responsible for neuronal injury (Fujimoto *et al.*, 2004). However, it is not clear how glutamate might modify the NMDAR mRNA levels. Thus, the data in figure 3 (A, B and C) show that in hippocampal slices 4 μM glutamate (similar amounts to those elicited in OGD) is not able to induce changes in the expression of any of the main NMDAR subunit genes. In contrast, c-fos mRNA expression levels show a different response (Fig 3 D) since the presence of 4 μM glutamate for 30 minutes was sufficient to induce a significant increase in c-fos mRNA levels 3 hours later. This results indicate that glutamate receptor activation is able to induce a significant up-regulation of c-fos mRNA levels, in agreement with data from cultured neurons (Rogers *et al.*, 2004). Accordingly, glutamate receptor activation is able to induce different effects, depending on the gene studied.

Considering a possible role of calcium currents through NMDAR and AMPA channels as possible modifiers in the changes observed in mRNA levels, it could be speculated that calcium current blockade through the NMDAR channel could be responsible for the decrease in NMDAR mRNA levels observed in the presence of the antagonist MK-801. In this regard, a calcium influx via the NMDAR has been reported to induce immediate early gene transcription through a MAP kinase/ERK-

dependent mechanism (Xia *et al.*, 1996). Additionally, the blockade of AMPA receptors by CNQX reduces the sodium current through this channel. This decreases the calcium current through NMDAR, since the entry of Na+ through AMPA receptors mediates the depolarization that releases the Mg^{2+} from NMDARs, allowing these receptors to be activated by glutamate (Neveu *et al.*, 1993; Burnashev *et al.*, 1998; Constantine-Paton *et al.*, 1998). Thus, the presence of CNQX induces an indirect calcium NMDAR channel blockade, which would be in agreement with the decrease in NMDAR mRNA levels observed when AMPA receptors are blocked.

To test this hypothesis we have repeated our experiments in a calcium free incubation solution to prevent the extracellular calcium entrance through AMPA and NMDA receptors under ischemic-like conditions. We observed a similar response for NMDA receptor subunits with significantly decreased mRNA levels after OGD and also in the presence of MK-801 and CNQX antagonists. These results discard extracellular calcium entrance through ionotropic glutamate receptors as responsible for the changes observed in mRNA levels.

It must therefore be concluded that none of the currents considered independently can explain the responses observed after blocking NMDAR, AMPAR or both. Thus, any of the ionic currents mediated by these receptors seems to be able of inducing decreases in the NMDAR subunits comparable to those observed following OGD. However, when OGD is performed in the presence of these antagonists no effect can be observed in the mRNA levels. We suggest that during OGD, a blockade of NMDAR or AMPAR could occur as a protective mechanism for glutamatergic overexcitation. This blockade would induce a decrease in the mRNA levels of the NMDA subunit genes and this would explain why the use of antagonists blocking the NMDAR or AMPAR result in similar effects.

We should also consider the action of glutamate on metabotropic receptors. In this regard, it has been reported that glutamate ionotropic activity modulates metabotropic glutamate activity (Alagarsamy *et al.*, 2005) and also that metabotropic glutamate receptors are able to modulate NMDA and AMPA gene expression (at mRNA and protein level) in neocortical interneurons (Lindemeyer *et al.*, 2006). Since the blocking of ionotropic receptors is able to modify the effect on NMDAR gene expression it is not possible to discard the existence of some

relationship(s) between the imbalance of glutamatergic ionotropic activity and the metabotropic glutamatergic receptors that modify NMDAR mRNA levels, and hence the effect of glutamate on gene expression could be very complex.

The effect of OGD *ex vivo* is probably multifactorial, in a similar way as suggested for ischemia in *in vivo* studies (Quandt *et al.*, 1987; Clemens *et al.*, 1991). As analyzed above, the data on glutamate activation show that this compound itself cannot induce the outstanding decreases observed in NMDAR mRNA levels induced by OGD, although -interestingly- the presence of ionotropic antagonists is able to inhibit the effect of OGD, or possibly these agents and OGD undergo the same pathway that could be important to the expression of these genes. Thus, it is clear that glutamatergic ionotropic activity plays a role in modifying early NMDAR subunit mRNA levels in hippocampal slices and our results indicates that mechanisms other than high levels of extracellular glutamate are also involved in eliciting the effects of OGD on the mRNA expression levels of these genes.

In conclusion, our data support the suitability of hippocampal slices as a useful model for studying early mRNA levels, allowing specific mechanisms of the control of gene expression to be dissected. Our data also suggest that the OGD-induced down-regulation of NMDAR mRNA levels does not only depend on the release of glutamate and that mechanisms triggered during OGD responsible for downregulating mRNA levels of NMDAR subunits could be also induced by glutamate receptor antagonists.

Acknowledgments

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IV. DISCUSIÓN GENERAL

4.1. Trabajo 1: La isquemia global transitoria promueve distinta regulación de los receptores de NMDA dependiendo de la estructura cerebral estudiada.

Nuestros resultados muestran importantes descensos en los niveles de ARNm de las subunidades NR1, NR2A y NR2B (mayoritarias en cerebro anterior) tras isquemia/reperfusión (I/R). Estos datos indican que existe *down-regulation* en las subunidades del receptor de NMDA en todas las estructuras cerebrales estudiadas: corteza cerebral, caudado-putamen y áreas hipocampales. Este descenso en la expresión génica podría ser explicado como un mecanismo compensatorio desarrollado por las células nerviosas para contrarrestar la sobreactivación de los receptores de NMDA durante la isquemia debido al aumento del glutamato extracelular (Won *et al.*, 2001).

Esta disminución en los niveles de ARNm es más evidente en algunas estructuras. Así, en el hipocampo y estriado se encuentran los niveles de ARN mensajero más bajos tras la I/R, mientras que la corteza cerebral se ve significativamente menos afectada por este mecanismo de regulación. El estudio de las proporciones de ARNm de las subunidades del receptor de NMDA (NR1:NR2A:NR2B) en ratas *sham* y ratas sometidas a isquemia/reperfusión indica también diferencias entre las estructuras estudiadas, de modo que la proporción 1:1:2 (animales *sham*) se modifica a 2:1:4 (animales I/R) en hipocampo, mientras que no se observan cambios significativos en el patrón de expresión en caudado-putamen y corteza cerebral.

Estos datos muestran que en el hipocampo, 48 horas después del daño isquémico existe la mitad de ARNm de la subunidad NR2A con respecto a los ARNm de NR1 y NR2B, lo que sugiere que el control de la regulación de la expresión de las subunidades que componen el receptor de NMDA varía con la estructura cerebral, lo que podría permitir la formación de distintos NMDAR funcionales. La subunidad NR2A juega un papel muy importante en los fenómenos de excitotoxicidad mediada por receptores de NMDA, y aunque existe cierta controversia, se ha sugerido que puede tener un papel tanto neuroprotector como promotor del daño neuronal (Liu *et al.*, 2007; Morikawa *et al.*, 1998).

En nuestro estudio, aunque se ha señalado la existencia de distinta vulnerabilidad en las regiones CA1, CA3 y DG y hemos observado diferente daño en estas regiones, todas estas áreas presentan niveles similares de ARNm de la subunidad NR2A por lo que no podemos afirmar que esta subunidad, ni ninguna de las otras estudiadas por sí mismas tenga un papel relevante en la diferente vulnerabilidad de estas áreas al daño isquémico. Se puede concluir que la regulación de la expresión del ARNm de las subunidades que componen el receptor de NMDA tras el daño isquémico depende de la estructura cerebral estudiada, y no parece que haya una relación directa con la vulnerabilidad de la misma.

4.2. Trabajo 2: Modificaciones producidas por la isquemia global transitoria en la expresión de los receptores de AMPA e inflamación.

Este trabajo nos ha permitido comprobar que los niveles de ARNm de las subunidades GluR1 y GluR2 del receptor de AMPA (AMPAR) también presentan valores significativamente más bajos en este modelo de isquemia cerebral transitoria seguida de reperfusión. Al igual que en el caso de los receptores de NMDA se encontraron diferencias importantes entre las distintas estructuras estudiadas. Aunque la corteza cerebral presentó una reducción significativa en los niveles de ARNm, el caudado-putamen y las áreas hipocampales presentaron niveles de ARNm mucho más reducidos. Se demuestra, por tanto, que los niveles de expresión de ARNm de los genes de los receptores de NMDA y AMPA se modifican de la misma forma por el proceso de isquemia/reperfusión.

Actualmente no existen datos concluyentes para explicar si la expresión de los AMPAR podría estar relacionada con la vulnerabilidad neuronal. Una de las hipótesis existentes es la “hipótesis GluR2” (Pellegrini-Giampietro *et al.*, 1997), que relaciona el descenso en los niveles de la subunidad GluR2 tras la isquemia cerebral, con la mayor vulnerabilidad al daño isquémico que presenta el área CA1 (Kirino, 1982; Pulsinelli, 1985) con respecto a otras áreas cerebrales. Esta *down-regulation* de la expresión génica de la subunidad GluR2 en el área CA1 da lugar a la formación de AMPAR permeables al calcio, lo cual contribuiría al daño excitotóxico debido al glutamato extracelular, y finalmente a la muerte neuronal. Nuestros datos corroboran esta hipótesis en el hipocampo, ya que el área CA1 presenta descensos muy significativos de la subunidad GluR2 (tanto en los niveles de ARNm como de proteína), mientras que el área CA3 menos vulnerable (Opitz *et*

al., 2000), a pesar de presentar descensos importantes en los niveles de ARNm de la subunidad GluR2, no muestra diferencias significativas en los niveles de proteína para esta subunidad entre animales *sham* e isquémicos. Sin embargo, los resultados obtenidos en las otras estructuras estudiadas (córteza cerebral y caudado-putamen) no se ajustan a esta hipótesis, ya que la corteza cerebral, una estructura resistente en comparación con otras áreas cerebrales como el estriado (Hermann *et al.*, 2001) o el área CA1, también presenta un descenso significativo en los niveles de la subunidad GluR2. Nuestros datos sugieren que la subunidad GluR2 no es la causa subyacente de la vulnerabilidad neuronal de las distintas áreas estudiadas, sin embargo no podemos descartar que juegue un papel en los mecanismos de muerte celular.

Otra de las hipótesis que se ha sugerido como posible causa de la muerte neuronal retrasada tras la isquemia implica el mecanismo de bloqueo traduccional (DeGracia *et al.*, 2008). Nuestros resultados indican una *down-regulation* en los niveles de ARNm y proteína en todas las áreas hipocampales estudiadas para las subunidades GluR1 y GluR2. Sin embargo, en el área CA3 los niveles proteicos de la subunidad GluR2 no presentan descensos significativos, lo cual podría indicar una recuperación de los mecanismos de traducción en esta área de acuerdo con la hipótesis de bloqueo traduccional. Esto sugiere que existen diferencias en el control traduccional o post-traduccional de los niveles de estas proteínas en las distintas áreas hipocampales, que pudieran jugar un papel en la vulnerabilidad diferencial observada. No obstante, a partir de nuestros datos, la hipótesis del bloqueo traduccional no puede ser aplicada en el caso de estructuras relativamente resistentes como la corteza cerebral. Ésta presenta un menor daño tisular y sin embargo persiste el bloqueo traduccional, y se observan descensos significativos de la subunidad GluR2 (y también de GluR1) tras 48 horas de reperfusión.

Otro aspecto que se debe considerar son los procesos de inflamación, que juegan un papel muy importante en la fisiopatología de los procesos isquémicos (Rodríguez-Yáñez y Castillo, 2008; Wang *et al.*, 2007). La isquemia global promueve una respuesta inflamatoria aguda y prolongada que se caracteriza por la producción de citoquinas inflamatorias, infiltración de leucocitos y monocitos desde el torrente sanguíneo hacia el cerebro, y la activación de células gliales residentes del sistema nervioso central (Kriz, 2006). Las células microgliales en particular tienen una función fundamental en la respuesta al daño isquémico, y tras su

activación se incrementa la expresión de la integrina CD11b/CD18 (Mac-1), la cual se ha sugerido como marcador del proceso inflamatorio (Clausen *et al.*, 2008; Gonzalez-Scarano y Baltuch, 1999). Todas las estructuras estudiadas presentaron niveles muy altos de este marcador tras 48 horas de reperfusión, lo que indica que existe una respuesta inflamatoria importante en estas estructuras usando como referencia este marcador.

En resumen, el descenso generalizado en los niveles de expresión de los receptores de AMPA y NMDA tras la isquemia cerebral y reperfusión en todas las estructuras estudiadas indica la existencia de un mecanismo de *down-regulation* generalizado para ambos subtipos de receptores ionotrópicos. No obstante, este bloqueo traduccional no explica la vulnerabilidad diferencial observada. Por otra parte, la proporción en la expresión de las subunidades GluR1 y GluR2 tampoco se correlaciona con la vulnerabilidad de las áreas cerebrales estudiadas, al menos para este tiempo de estudio, y sólo el área CA3 se ajusta a la hipótesis GluR2. Por último, el uso del marcador CD11b no permite observar ningún tipo de correlación entre áreas con distinta vulnerabilidad y los niveles de inflamación que indica este marcador.

4.3. Trabajo 3: Análisis de la expresión génica en modelo *ex vivo* de secciones cerebrales: influencia de la temperatura y el medio de incubación.

La determinación de la integridad del ARN es un paso crítico previo al análisis de la expresión génica (Fleige y Pfaffl, 2006), y su estudio en un modelo *ex vivo* que requiere incubaciones de secciones cerebrales a lo largo del tiempo parece de capital importancia. Nuestros estudios muestran que la temperatura es un factor crítico que afecta tanto a la integridad del ARN como a los niveles de ARNm, indicando que en la corteza cerebral se producen resultados similares a los publicados por otros autores en hígado (Almeida *et al.*, 2004). La degradación del ARN total y en particular del ARNm observada en nuestros ensayos de incubación de secciones cerebrales a una temperatura de 37ºC indica que a esta temperatura no se pueden realizar estudios de expresión génica en este modelo experimental. Sin embargo, los estudios realizados a 25ºC mostraron que a esta temperatura se mantiene la integridad del ARN y existe estabilidad en los niveles de ARNm en los tres genes analizados (β-actina, GAPDH y Mast2) durante unas horas permitiendo

así una ventana de trabajo. Estos resultados confirman la importancia de la determinación de la integridad del ARN, previa a los estudios de expresión génica.

Los tres genes analizados mostraron niveles de ARNm estables en una ventana de trabajo óptima que se extiende hasta las 6 horas desde la obtención de las secciones de tejido. La expresión del ARNm del gen que codifica para Mast2 fue la que mejor se ajustó a la curva de viabilidad celular observada durante las primeras 12 horas. En nuestra opinión, los valores significativamente más bajos en la integridad del ARN y en los niveles de ARNm entre las muestras de tejido de corteza cerebral obtenidas por disección inmediatamente después de la extracción del encéfalo y las secciones obtenidas en vibratomo son debidas al daño inducido por procedimientos asociados fundamentalmente al estrés mecánico producido por el seccionamiento, o el burbujeo con carbógeno. De hecho, el aislamiento de las secciones en la solución para disminuir el efecto mecánico del burbujeo redujo notablemente el daño celular. Es probable que otros factores como el choque térmico puedan contribuir a la degradación del ARN o modificar los niveles de expresión del ARNm en los primeros momentos de obtención de las muestras.

El uso de una solución tamponada de sales (solución Ringer) a 25ºC mantuvo los niveles de integridad de ARN durante las 6 primeras horas, así como niveles similares de ARNm en todos los genes estudiados. En contraste, el uso de un medio de cultivo complejo (DMEM) muestra una mejora en la integridad del ARN, así como un aumento en los niveles de ARNm. Esta modificación de los niveles de ARNm durante la ventana de trabajo, cuando se utiliza DMEM hace difícil la interpretación de los resultados cuando se quiere analizar los cambios de expresión génica en el tiempo. Por tanto, el uso de solución Ringer a 25ºC proporciona un modelo estable durante una ventana de trabajo de unas 6 horas para desarrollar procedimientos experimentales que requieran cuantificación de ARNm usando secciones cerebrales, por ejemplo, ensayos de privación de oxígeno y glucosa (OGD).

Nuestros resultados muestran la importancia de una elección cuidadosa de los genes de referencia. Así, el rápido descenso en los niveles de ARNm a partir de las 6 horas de incubación para GAPDH y Mast2, contrasta con el mantenimiento de los niveles de β-actina hasta las 24 horas. Hay que señalar, no obstante que los tres

genes pueden ser adecuados para este tipo de experimentos *in vitro* en una ventana de trabajo que incluye las 6 primeras horas.

En resumen, este estudio demuestra que la incubación de secciones cerebrales corticales a 25ºC en soluciones de sales tamponadas de tipo Ringer mantiene la integridad del ARN y los niveles de ARNm de estos genes, durante las 6 primeras horas desde el sacrificio del animal. No obstante, en todos los ensayos que hemos realizado posteriormente y que se describen más adelante, se ha esperado una hora para permitir una estabilización del tejido, como es habitual en los estudios de electrofisiología, si bien no hemos observado diferencias en los niveles de ARNm entre las secciones recién obtenidas en vibratomo y las que llevan una hora de estabilización.

4.4. Trabajo 4: Modificaciones tempranas en los niveles de expresión de ARNm de las subunidades del receptor de NMDA en secciones hipocampales sometidas a privación de oxígeno y glucosa.

Los resultados obtenidos del trabajo anterior permitieron definir una ventana de trabajo en la que es posible realizar estudios de expresión de niveles de ARNm de genes *housekeeping* en corteza cerebral en modelos experimentales *in vitro*. En este trabajo se demuestra que este intervalo temporal también es adecuado cuando se utilizan secciones hipocampales y se utiliza el modelo para analizar el efecto de la OGD en la expresión de las subunidades NR1, NR2A y NR2B de los receptores NMDA. Además se realiza un análisis de la variabilidad de los niveles de ARNm obtenido para diferentes genes *housekeeping* que a pesar de no presentar diferencias significativas en la ventana de trabajo presentan diferentes coeficientes de variación (CV). La comparación entre los CV (Banda *et al.*, 2008) permite seleccionar genes de referencia que proporcionan un análisis más preciso. En este sentido, se decidió utilizar el ARNr 18S como gen de referencia por tener el CV más bajo de todos los estudiados (GAPDH, ARNr 18S, Mast2 y β-actina).

En este trabajo también se demuestra que en las secciones hipocampales aún existe síntesis de ARNm cuando se incuban en una solución tamponada de sales, ya que la incubación en presencia de actinomicina-D (inhibidor de la transcripción) durante seis horas produjo un descenso significativo de los niveles de ARNm (figura 2) a diferencia de lo que ocurre cuando no se añade este compuesto.

Además, los incrementos en los niveles de ARNm para el gen c-fos tras 5 horas de incubación observados en nuestros estudios muestran la capacidad de síntesis de nuevo ARNm en las secciones hipocampales. Estos datos corroboran la existencia de síntesis de ARNm en un modelo similar en el que estudian la expresión de iNOS mediante RT-PCR (Cárdenas *et al.*, 2000).

Nuestros ensayos confirman, en este modelo, la existencia de un aumento importante en los niveles de ARNm del gen c-fos durante el periodo de reperfusión que sigue a la OGD. Esto había sido descrito previamente en modelos de cultivo celular y de isquemia/hipoxia *in vivo* (Greenberg *et al.*, 1999; Haxhiu *et al.*, 1995; Yu *et al.*, 2009). Estos datos confirman que el modelo de secciones hipocampales presenta una capacidad de respuesta del gen c-fos a la hipoxia similar a la observada en otros modelos y contribuyen a la validación de este modelo en experimentos de OGD.

El efecto de la OGD *ex vivo* es probablemente multifactorial, al igual que lo ya sugerido para isquemia *in vivo* (Clemens *et al.*, 1991; Gao *et al.*, 2006). Esto incluye gran número de variables que pueden actuar sobre la expresión de los genes, particularmente aquellos que codifican para las subunidades del receptor de NMDA. En este trabajo se analizan algunos de estos factores.

Los descensos significativos en los niveles de ARNm de las subunidades que componen los receptores de NMDA, indican que la OGD desencadena un mecanismo de *down-regulation* de la expresión génica, similar al que hemos descrito anteriormente en los estudios *in vivo*, que podría contrarrestar la sobreactivación de los receptores de NMDA. Se ha señalado que esta sobreactivación es promovida por el aumento del glutamato extracelular que ocurre en los experimentos de cultivos organotípicos (Fujimoto *et al.*, 2004) durante el periodo de OGD y en los experimentos de isquemia *in vivo* (Gascón *et al.*, 2008). Este efecto específico a través de los receptores de NMDA es confirmado por la adición experimental de NMDA al medio, que promueve una *down-regulation* de los receptores de NMDA en cultivos hipocampales (Gascón *et al.*, 2005).

No se conoce con claridad cómo afecta el glutamato extracelular a la expresión de las subunidades que conforman el receptor de NMDA. Nuestros resultados indican que la acción exclusiva del glutamato en el medio (4 μM) no es

capaz de promover los cambios en los niveles de ARNm en las subunidades de los receptores de NMDA originados por la OGD, aunque es capaz de inducir un aumento en la expresión del gen c-fos durante la reperfusión, que podría ser mediado por la activación de receptores de glutamato como se ha descrito en cultivos neuronales (Rogers *et al.*, 2004).

Una de las hipótesis que se han planteado en la literatura es que el efecto excitotóxico de la isquemia se debe a la entrada de calcio a través de los receptores de NMDA y AMPA (Arundine y Tymiansky, 2003; Martin *et al.*, 1998) y nuestra hipótesis es que este aumento en los niveles de calcio podría ser un factor clave en la modificación de la expresión de los receptores de NMDA observada. En este sentido, se ha descrito que la entrada de calcio promovida por los receptores de NMDA induce la transcripción de genes de expresión temprana a través de un mecanismo dependiente de MAP kinasas (Xia *et al.*, 1996). Para comprobar esta hipótesis realizamos experimentos de OGD en una solución de incubación sin calcio para evitar la entrada de calcio extracelular en la célula a través de los receptores de NMDA y AMPA. La respuesta observada fue muy similar a lo descrito previamente, con descensos significativos en los niveles de ARNm de las subunidades del receptor de NMDA tras la OGD, y también cuando los ensayos se realizaron en presencia de los antagonistas MK-801 y CNQX. Estos resultados indican que la entrada de calcio a través de los receptores de glutamato ionotrópicos no es responsable de los cambios en los niveles de ARNm observados.

Cuando los ensayos de OGD se realizaron en presencia de antagonistas de los receptores de NMDA no se observó disminución significativa de los niveles de ARNm de las subunidades de los receptores de NMDA con respecto a las muestras en presencia de antagonistas y no sometidas a OGD. Esto sugiere que el bloqueo de los receptores de NMDA y AMPA durante la OGD podría actuar como un mecanismo protector de la sobreexcitación de los receptores glutamatérgicos ionotrópicos. Sin embargo no está claro qué mecanismo pondrían en marcha estos canales para realizar una disminución en la transcripción dado que no parece ser mediado por la entrada de calcio extracelular.

Se ha señalado que la estimulación de los receptores glutamatérgicos metabotrópicos del grupo I produce una alteración de la expresión génica mediada por NMDA (por ejemplo del factor de transcripción CREB) en cultivos primarios de

interneuronas corticales (Lindemeyer *et al.*, 2006), por lo que hay tener en cuenta que los receptores metabotrópicos de glutamato jueguen un papel en los cambios en los niveles de ARNm de los receptores de NMDA y AMPA.

En resumen, nuestros datos indican que el modelo de secciones hipocampales utilizado es una herramienta útil para el análisis de la expresión génica de los receptores de NMDA, y probablemente de otros genes modificados por la OGD. Los resultados obtenidos indican un bloqueo, al menos parcial, de la transcripción de las subunidades de los receptores de NMDA que no puede ser inducido solamente por la sobreactivación de los receptores glutamatérgicos mediada por glutamato. No obstante, los mecanismos implicados en este bloqueo podrían ser los mismos que los activados por la presencia de antagonistas de los receptores glutamatérgicos ionotrópicos.

4.5. Consideraciones finales

En esta memoria se han incorporado varios trabajos que han tenido como finalidad el estudio, a través de distintos modelos y aproximaciones, de las modificaciones en la expresión de los receptores glutamatérgicos NMDA y AMPA tras la isquemia cerebral. Nuestros resultados indican que existe una importante disminución de los niveles de ARNm en todas las estructuras cerebrales estudiadas, a pesar de que estos descensos dependen del área del encéfalo estudiada.

La respuesta observada en los receptores de NMDA y AMPA parece tener un papel importante en los mecanismos que conducen a la muerte neuronal tras procesos isquémicos, sin embargo no hemos podido observar una relación causa-efecto directa entre la expresión de las subunidades que los componen, y la distinta susceptibilidad o vulnerabilidad diferencial al daño isquémico.

Los datos obtenidos de los estudios de OGD *in vitro* nos han permitido establecer un modelo útil para el estudio de los niveles de expresión (ARNm) de los receptores de glutamato, así como para profundizar en el conocimiento de los mecanismos que subyacen a los cambios de expresión observados en los receptores de NMDA tras isquemia experimental.

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V. RESUMEN

La isquemia cerebral es una de las principales causas de muerte y discapacidad en los países industrializados y ocasiona un gran costo socioeconómico a la sociedad. Uno de los factores importantes que afectan la muerte neuronal promovida tras el daño isquémico 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 activa vías intracelulares que finalmente promueven la muerte neuronal.

En esta memoria se describe la expresión de los receptores ionotrópicos de glutamato de NMDA y AMPA a través de distintos modelos experimentales, lo cual contribuye al conocimiento de los mecanismos implicados en el daño cerebral y la vulnerabilidad regional promovidos por isquemia/reperfusión (I/R) y OGD (privación de oxígeno y glucosa). Los ensayos I/R *in vivo* se llevaron a cabo usando un modelo de isquemia global transitoria en rata. El estudio de OGD se realizó en ensayos *ex vivo* usando secciones hipocampales. Para ello se ha cuantificado la expresión de las distintas subunidades que componen los receptores de NMDA (NR1, NR2A y NR2B) y AMPA (GluR1 y GluR2) en distintas estructuras cerebrales de cerebro de rata, a nivel de ARNm y de proteína, a través de las técnicas de PCR en tiempo real y western blot respectivamente. Asimismo, se han cuantificado los niveles del marcador de microglia activada CD11b/CD18 (Mac-1) mediante técnicas de PCR en tiempo real e inmunohistoquímica como medida de la respuesta inflamatoria observada tras I/R *in vivo*.

Los ensayos *in vivo* mostraron un descenso significativo en los niveles de ARNm de las subunidades NR1, NR2A y NR2B del receptor de NMDA, así como de las subunidades GluR1 y GluR2 del receptor de AMPA en todas las estructuras cerebrales estudiadas (corteza cerebral, caudado-putamen y áreas hipocampales) después de 48 horas de reperfusión tras el proceso isquémico. Sin embargo, el nivel de descenso es distinto entre las diferentes áreas cerebrales estudiadas, mostrando principalmente niveles de ARNm más altos la corteza cerebral que las áreas hipocampales. Se observaron también descensos significativos en los niveles de proteína a través de los ensayos de western blot. En contraposición a los resultados anteriores, se obtuvieron incrementos significativos en los niveles de ARNm y proteína del marcador CD11b/CD18 en estas condiciones (Dos-Anjos *et al.*, 2009a, b).

Los ensayos *ex vivo* para cuantificar niveles de ARNm fueron caracterizados inicialmente analizando el efecto del tiempo, la temperatura de incubación y distintos medios de incubación sobre la expresión de genes de referencia (GAPDH, Mast2 y β-actina). Esto permitió definir una ventana de trabajo de 6 horas tras el sacrificio del animal, en la cual los niveles de ARNm se mantienen estables en una solución tamponada de sales de Ringer a una temperatura de 25ºC. El resto de condiciones no dieron lugar a modelos estables (Dos-Anjos *et al.*, 2008). Usando este modelo, las secciones hipocampales fueron sometidas durante 30 minutos a condiciones de OGD, lo cual dio lugar a un descenso significativo en los niveles de ARNm de las subunidades NR1, NR2A y NR2B del receptor de NMDA, así como del factor de transcripción *c-fos*. Después de 3 horas de reperfusión se observaron aumentos de los niveles de ARNm, especialmente para el gen *c-fos*. Se obtuvieron resultados similares en ausencia de calcio extracelular, lo cual descarta que este ión sea el factor desencadenante de los cambios observados en los niveles de ARNm tras la OGD. Los niveles de ARNm de las subunidades del receptor de NMDA no se modificaron en ensayos en presencia de glutamato, a concentraciones similares a las que se producen durante la OGD, lo que indica que el aumento de glutamato no es la única causa responsable de los descensos encontrados tras la OGD. La presencia de antagonistas de los receptores de NMDA y de AMPA/kainato en el medio de incubación originaron descensos similares en los niveles de ARNm de las subunidades del receptor de NMDA a los que ocurren durante la OGD, sin embargo no se observaron descensos adicionales durante la OGD (Dos-Anjos *et al.*, 2009c, en prensa).

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VI. CONCLUSIONES

Los estudios presentados en este trabajo han dado lugar a las siguientes conclusiones:

1.- Este estudio muestra que 48 horas después de la isquemia cerebral se produce una disminución muy significativa en los niveles de ARNm de las subunidades NR1, NR2A y NR2B del receptor de NMDA. Aunque la regulación de los niveles de ARNm de los receptores de NMDA depende de la estructura cerebral estudiada, no parece existir una relación directa entre la expresión de estas subunidades del receptor de NMDA con la vulnerabilidad neuronal al daño isquémico.

2.- De manera análoga se observa que 48 horas después del proceso isquémico se produce una disminución de los niveles de ARNm y proteína de las subunidades GluR1 y GluR2 del receptor de AMPA en todas las áreas cerebrales estudiadas, excepto para la subunidad GluR2 en el área hipocampal CA3. Estos resultados sólo apoyan la hipótesis del bloqueo traduccional y la hipótesis GluR2 en el área CA3, lo que indica que las hipótesis actuales sólo explican resultados parciales y parecen necesarios nuevos datos experimentales para explicar la vulnerabilidad diferencial a la isquemia.

3.- El marcador CD11b/CD18 indica que existe una respuesta inflamatoria importante tras isquemia/reperfusión en todas las áreas cerebrales estudiadas, tanto a nivel de ARNm como de proteína. La vulnerabilidad de las distintas áreas cerebrales estudiadas parece independiente de los niveles de inflamación obtenidos utilizando el marcador CD11b/CD18 48 horas tras la isquemia cerebral.

4.- El estudio de los genes de referencia en los ensayos de secciones de corteza cerebral indicó que la incubación de secciones cerebrales a 25ºC da lugar a niveles estables tanto en la integridad del ARN como en los niveles de ARN mensajero en una ventana de trabajo de unas seis horas tras el sacrificio del animal. El modelo es inviable a 37ºC para estudios de cuantificación de ARNm dado el excesivo nivel de degradación de ARN.

5.- La incubación de secciones cerebrales en una solución tamponada de sales (tipo Ringer) en esta ventana de trabajo permite obtener niveles de expresión estables de los ARNm de genes *housekeeping* a diferencia del uso del medio de cultivo complejo

DMEM, que muestra variaciones en los niveles de ARNm a lo largo del tiempo de incubación.

6.- La respuesta observada en el modelo de secciones hipocampales reproduce los cambios de expresión génica (a nivel de ARNm) descritos en ensayos organotípicos, cultivos celulares o ensayos *in vivo* lo que sugiere que este modelo es válido para estudiar las modificaciones inducidas por OGD en los mecanismos implicados en el control de la expresión génica.

7.- Los datos obtenidos en el modelo de secciones hipocampales indican que la OGD da lugar a un bloqueo transcripcional, al menos parcial, de los genes de las subunidades NR1, NR2A y NR2B de los receptores de NMDA y que éste no depende solamente de la sobreactivación de receptores de NMDA mediada por el glutamato extracelular. Los mecanismos implicados en este bloqueo podrían ser los mismos que los activados por la presencia de antagonistas de los receptores glutamatérgicos ionotrópicos.

VII. SUMMARY

Brain ischemia is one of the major causes of death and disability in industrialized countries and causes a huge socioeconomic burden to society. One of the main factors affecting neuronal death promoted by ischemic damage is the overactivation of glutamate receptors as a consequence of the extracellular raise of glutamate levels produced during the ischemic insult. This overexcitation leads to increased intracellular calcium levels that activate specific pathways that eventually elicit delayed neuronal death.

This study presents data on the NMDA and AMPA glutamatergic receptor expression obtained using different experimental models to contribute to the knowledge of the mechanisms involved in the brain damage and differential vulnerability elicited by ischemia-reperfusion and OGD (Oxygen Glucose Deprivation). Ischemia-reperfusion *in vivo* assays were performed using a transient global cerebral ischemia model *in vivo*. The effect of OGD was studied in *ex vivo* assays using hippocampal slices. The expression of the NMDA (NR1, NR2A and NR2B subunits) and AMPA (GluR1 and GluR2 subunits) receptors in different rat brain structures was studied by measuring mRNA and protein levels using real time PCR and Western blot techniques respectively. Additionally, the activated microglia marker CD11b/CD18 (Mac-1) has been quantified using real time PCR and immunocytochemistry as a measurement of the inflammation process following ischemia-reperfusion *in vivo*.

In vivo assays showed significant lower NMDAR NR1, NR2A and NR2B as well as AMPA GluR1 and GluR2 subunit mRNA levels in every brain structure studied (cerebral cortex, hippocampus and caudate-putamen) after a 48 hour reperfusion following the ischemic insult. However, the strength of this response showed significant differences between the areas studied, revealing higher mRNA levels in the cerebral cortex than in the hippocampal areas. Significant decreases in the protein levels were also observed by Western blot. In contrast with these results, high CD11b/CD18 mRNA and protein levels were observed indicating the inflammatory response following the ischemic challenge in these conditions (Dos-Anjos *et al.*, 2009a, 2009b).

Ex vivo assays for measuring mRNA were firstly characterized analyzing the effect of time, temperature and different incubation media, which made possible to define a 6 hour work window after killing the animal where the levels of mRNA are

stable in an oxygenated Ringer balanced salt solution at 25ºC. Other conditions and incubation media assayed resulted in non stable results (Dos-Anjos *et al.*, 2008). Using this model, hippocampal sections were subjected to 30 minutes OGD which resulted in significantly lower mRNA levels for both NMDAR NR1, NR2B and NR2B subunits and the c-fos transcription factor. A slight increase in the mRNA levels, specially for c-fos, was observed after a 3 hour reperfusion-like period with respect to the mRNA levels observed in OGD. Similar results were observed in the absence of extracellular calcium, discarding the role of this ion as a trigger for the OGD dependent transcriptional decrease. The NMDAR subunit mRNA levels were not modified in assays in the presence of glutamate concentrations similar to those elicited by OGD, indicating that glutamate itself is not responsible for the OGD dependent decreased mRNA levels. The presence of NMDA and AMPA/kainate antagonists in the incubation medium resulted in a decrease of the NMDA subunit mRNA levels similar to that observed during OGD but we could not observe additional decreases as a consequence of performing OGD in the presence of this antagonists (Dos-Anjos *et al.*, accepted in 2009)

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VIII. CONCLUSIONS

This study has led to the following conclusions:

1.- The study of the mRNA levels of NMDA receptor subunits indicates that the regulation of NMDAR subunits mRNA levels depends on the brain structure studied, although it seems not to have a direct relationship with neuronal vulnerability to ischemic damage.

2.- Gene expression studies on GluR1 and GluR2 subunits of AMPAR showed that 48 hours after ischemic insult an important down-regulation occurs in cerebral cortex, caudate-putamen and hippocampus. CA3 hippocampal area shows a unique regulation with respect to the other brain structures studied. Nevertheless, none of the suggested hypothesis (GluR2 hypothesis and translational arrest) explain the regional vulnerability differences found in the other brain areas (except for CA3 hippocampal area). Further research is needed to explain differential vulnerability to brain ischemia.

3.- There is an important inflammatory component after ischemia in all brain structures studied, as measured by CD11b/CD18 marker, at both mRNA and protein levels. The regional ischemic vulnerability seems to be independent from the inflammation levels obtained, at least 48 hours after the ischemic insult.

4.- The study of reference genes in the assays using cortical cerebral slices showed that the incubation of brain slices at 25ºC results in stability of RNA integrity and mRNA levels in a working window of around six hours after animal sacrifice. This model is not suitable using 37ºC as incubation temperature because of the excessive RNA degradation.

5.- The incubation of brain slices in a balanced salt solution (Ringer) during this working window allows to obtain constant mRNA expression levels of housekeeping genes, in contrast to the use of complex incubation media (DMEM), which shows changes in mRNA expression levels.

6.- The response found in the OGD model using hippocampal slices reproduce the gene expression changes reported in organotypic assays, cell culture and *in vivo* assays. This suggests that this model is suitable for studying the OGD-induced modifications in the gene expression control mechanisms.

7.- Data from hippocampal slices show that the OGD assays induce a significant decrease in the mRNA levels of NR1, NR2A and NR2B NMDAR subunits. This down-regulation could be explained as a transcriptional arrest —at least a partial one— which does not only depend on the overactivation of NMDAR promoted by extracellular glutamate and calcium entrance. The mechanisms involved in this arrest could be the same as those activated by the ionotropic glutamate receptor antagonists.

Anexo (Carta de aceptación del trabajo 4)

Date: Sep 08, 2009
To: "Arsenio Fernandez-Lopez" arsenio.fernandez@unileon.es
From: "Neuroscience, Editorial" neuroscience@journal-office.com
Subject: Acceptance of NSC-09-1048R1
Ms. No.: NSC-09-1048R1
Title: EARLY MODIFICATIONS IN NMDA RECEPTOR SUBUNIT mRNA LEVELS IN AN OXYGEN AND GLUCOSE DEPRIVATION MODEL USING RAT HIPPOCAMPAL BRAIN SLICES.

Dear Dr. Fernandez-Lopez,

We are pleased to inform you that your manuscript referenced above has been accepted for publication in Neuroscience. We hope that the review has been a positive experience and that your manuscript has been improved by the process.

Many thanks for submitting your fine paper to Neuroscience. We look forward to receiving additional papers from your laboratory in the future.

Kind regards,

Dr. Ole Petter Ottersen
Chief Editor
Neuroscience