UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

DOCTORADO EN CIENCIAS BIOMEDICAS

CAMBIOS EN LA NEUROTRANSMISION GLUTAMATERGICA EN EL HIPOCAMPO DE RATA DURANTE LA INHIBICION METABOLICA. RELEVANCIA PARA EL DAÑO NEURONAL ASOCIADO A ALGUNAS ENFERMEDADES NEUROLOGICAS.

TESIS

QUE PARA OBTENER EL TITULO DE DOCTOR EN CIENCIAS

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Estar preparado es importante,
saber esperar lo es aún más,
pero aprovechar el momento adecuado
es la clave de la vida.

Arthur Schnitzler
(1862-1931)
Dramaturgo austriaco

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II-ABSTRACT

Neuronal death associated with cerebral ischemia, hypoglycemia and some neurodegenerative diseases is related to the failure of glycolytic metabolism and increased release of excitatory amino acids (EAA). The extracellular concentration of EAA is highly regulated through Na^+-dependent transporters to prevent its neurotoxic action. Prolonged activation of glutamate receptors leads to neuronal death through a mechanism known as excitotoxicity. Alterations in glutamate transporters and NMDA receptors have been associated with ischemia, hypoglycemia and neurodegenerative chronic diseases, suggesting altered glutamatergic neurotransmission. However, the relationship between glycolytic failure, altered glutamatergic neurotransmission and neuronal death, has not been elucidated. Here we have investigated the participation of glycolytic metabolism in glutamate release, glutamate transport and NMDA glutamate receptors levels in two *in vivo* experimental models used in rats. The first model consisted in the acute inhibition of glycolysis induced by the administration of iodoacetate (IOA), an inhibitor of glyceraldehyde 3-phosphate dehydrogenase (G3PDH), in the hippocampus. Results indicate that IOA induces the release of EAA. The early component of the release is inhibited by riluzole, a voltage-dependent sodium channel blocker, and by the volume sensitive channels blocker (VSOAC), tamoxifen. Both the early and late components are blocked by the glutamate transport inhibitors, L-trans-pyrrolidine-2,4-dicarboxylate (PDC) and DL-threo-beta-benzyloxyaspartate (DL-TBOA); and by the VSOAC blocker 4,4´-dinitrostilben-2,2´-disulfonic acid (DNDS). Iodoacetate administration induces neuronal death in the hippocampus. Riluzole, DL-TBOA and tamoxifen did not prevent IOA-induced neuronal death, while PDC and DNDS did.

In the second model, sustained glycolysis impairment was induced by the intraperitonal administration of iodoacetate during three days. Acccording to previous studies this treatment facilitates EAA-mediated neuronal damage. In the present study, we observed that IOA treatment induced a decrease in glutamate uptake and in the content of the GLT-1 glutamate transporter, while it promoted an up regulation and phosphorylation of the NR2B subunit of NMDA receptors. These

changes might render neurons more vulnerable to excitotoxic neuronal damage. Results are relevant to the unsderstanding of the mechanisms leading to cell death associated to neurological disorders involving excitotoxicity and metabolic impairment.

III- RESUMEN

La muerte neuronal asociada a la isquemia cerebral, la hipoglicemia y a algunas enfermedades neurodegenerativas, está relacionada con la falla en el metabolismo glicolítico y la acumulación extracelular de aminoácidos excitadores (AAE). La concentración extracelular de AAE se regula a través de sus transportadores evitando el daño neuronal excitotóxico. Cambios en la expresión de los transportadores de glutamato y en los receptores glutamatérgicos de tipo NMDA se han asociado a la isquemia, la hipoglicemia y a algunas enfermedades neurodegenerativas, sugiriendo alteraciones en la neurotransmisión glutamatérgica. Sin embargo, la relación entre la falla glicolítica, la alteración en la neurotransmisión glutamatérgica y la muerte neuronal, no se ha dilucidado. En esta tesis investigamos la participación del metabolismo glicolítico en la liberación y en la captura de aminoácidos excitadores, así como en el contenido de las distintas subunidades del receptor a NMDA en dos modelos experimentales. El primer modelo consistió en la inhibición aguda de la glicólisis por la administración de yodoacetato (IOA), un inhibidor de la enzima gliceraldehido 3 fosfato deshidrogenasa (G3PDH), en el hipocampo de rata. Los resultados indican que este tratamiento induce la liberación de AAE. El componente temprano de la liberación se inhibe por riluzol, un bloqueador de los canales de sodio dependientes de voltaje, y por el bloqueador de los canales aniónicos sensibles a volumen (CASV), tamoxifen; sin embargo esto no previene la muerte neuronal. Sólo aquellos bloqueadores que inhiben ambos componentes de la liberación, como los inhibidores del transporte de glutamato, L-trans-pirrolidin-2,4-dicarboxilato (PDC) y principalmente el bloqueador de los CASV, el ácido 4,4'-dinitrostilben-2,2'-disulfonico (DNDS), previenen el daño neuronal.

El segundo modelo consistió en un tratamiento sistémico con IOA durante tres días en la rata. Estudios previos indican que este tratamiento facilita el daño neuronal mediado por AAE en el hipocampo de la rata. De acuerdo a los resultados este tratamiento induce una disminución en la captura de glutamato, un decremento en el contenido de proteína del transportador de glutamato GLT-1, y un aumento en los niveles de proteína y de la fosforilación de la subunidad NR2B

de los receptores a NMDA. Los resultados sugieren que la falla glicolítica sostenida promueve cambios en los niveles de proteína de transportadores y receptores a glutamato volviendo a las neuronas más vulnerables a la excitotoxicidad. Los resultados son relevantes para entender los mecanismos que llevan a la muerte neuronal asociada a la isquemia y a la hipoglicémia cerebral, así como a algunas enfermedades neurológicas vinculadas con la excitotoxicidad y la falla metabólica.

IV- INTRODUCCIÓN

Metabolismo energético cerebral

La percepción y comprensión del medio que nos rodea radica en el procesamiento coordinado de los estímulos externos por un conjunto complejo de circuitos celulares conocido como sistema nervioso. Es relativamente fácil describir la anatomía del sistema nervioso pero resulta difícil entender la fisiología de un órgano del cuerpo humano con millones de años de evolución, y a la fecha son más las preguntas que las respuestas acerca de su funcionamiento. En la práctica, el cerebro funciona como una sofisticadísima supercomputadora. Con un peso promedio en el adulto de 1 400 gr, el cerebro humano representa tan sólo el 2% del peso total del cuerpo; sin embargo, el gasto energético que requiere es particularmente alto en relación a su masa. Posee alrededor de 100 mil millones de neuronas. En particular, 40.2 millones de neuronas en el hipocampo (West MJ 1990) y 23 mil millones de neuronas en la corteza (Pakkenberg and Gundersen, 1997). En ésta última región cerebral, se estima que las neuronas pueden tener hasta 10^{12} sinápsis (Tang et al., 2001), que en un sólo segundo son capaces de procesar hasta 200 mil millones de bitios de información. Esta alta capacidad de procesamiento promueve un alto consumo de oxígeno a una tasa de 1.5 µmol/min/g tejido, equivalente a casi el 20% del consumo total del organismo, y el 25% del total de la glucosa oxidada en el cuerpo (Hyder et al., 2006; Shulman et al., 2004; Voutsinos-Porche et al., 2003). En general, se conoce que el cerebro adulto en reposo consume del 40-60% del ATP total producido (Hyder et al., 2006; Shulman et al., 2004). Se ha sugerido que algunas de las razones de tan alta demanda energética se deben a que cerca del 95% de las sinapsis en el cerebro son glutamatérgicas (excitadoras) y gabaérgicas (inhibidoras) (Abeles, 1991; Braitenberg and Schüz, 1998), las cuales exigen un alto consumo de energía (Patel et al., 2005b; Waldvogel et al., 2000). La activación de receptores a glutamato y GABA promoverá el movimiento de iones cargados tanto positiva como negativamente, creando un desequilibrio a ambos lados de la membrana celular. El mantenimiento del equilibrio electroquímico es un proceso que requiere de un alto suministro de energía en forma de ATP consumido por parte de las

ATPasas Na^+/K^+ e intercambiadores iónicos (Hyder et al., 2006; Patel et al., 2005a; Shulman et al., 2004; Voutsinos-Porche et al., 2003). Se estima que aproximadamente el 75% de la energía que consume el sistema nervioso central (SNC) se relaciona con el señalamiento ligado a la activación de receptores neuronales (Attwell and Laughlin, 2001). El otro 25% restante, es útil en la síntesis y degradación de proteínas, reciclamiento de nucleótidos y de fosfolipidos, transporte axonal y fuga de protones de la mitocondria (Attwell and Laughlin, 2001).

La habilidad del SNC para utilizar reservas alternativas de energía es muy restringida. Aunque el SNC en ciertas condiciones, como el ayuno prolongado, puede metabolizar cuerpos cetónicos no es capaz de metabolizar las grasas. Adicionalmente, el cerebro posee reservas muy limitadas de glicógeno (0.1 g/100 g tejido fresco, comparado con 1 g en músculo y 6-10 g en hígado), que en condiciones de ausencia de glucosa pueden sostener por solo algunos minutos el funcionamiento cerebral. Por lo anterior, el cerebro es un órgano que requiere del suministro ininterrumpido de glucosa y de oxígeno, para suplir sus necesidades energéticas basales. La interrupción en su aporte da lugar al decremento de la síntesis de ATP favoreciendo el daño neuronal.

A nivel cerebral, la glucosa interviene en múltiples vías. Es el principal sustrato para la producción de energía por su oxidación a través de la vía glicolítica y el ciclo de krebs. Se relaciona con la biosíntesis de neurotransmisores y aminoácidos vía intermediarios del ciclo de krebs; interviene en la síntesis del glucógeno; mediante su oxidación a través de la vía de las pentosas genera ribosa 5 fosfato y NADPH, necesarios para la síntesis de nucleótidos y de lípidos, respectivamente (Tabernero et al., 1996). En la mayoría de las células de mamífero el transporte de glucosa y de otras hexosas ocurre a través de un proceso saturable de difusión facilitada que depende principalmente de la participación de proteínas de transporte de la familia SLC2 (GLUTs 1-12) y por el transportador de mio-inositol (HMIT) (Uldry and Thorens, 2004). Dada la heterogeneidad celular que presenta el SNC, no es sorprendente que la mayoría de los miembros de la familia de los transportadores de glucosa se expresen en

las células que lo conforman (astroglia, microglia, oligodendrocitos, neuronas, células endoteliales). Sin embargo, muchos de los transportadores presentes en el SNC son muy ineficaces para el transporte de glucosa debido a su baja afinidad (GLUT 5, 6, 11 y HMIT). Algunos, tienen una localización y concentración limitada en el cerebro (GLUT 2 y 4); mientras que para otros su localización y su capacidad de transporte no se ha determinado todavia (GLUT 8 y 10) (comunicación personal Simpson et al; 2007). Los transportadores predominantes en el cerebro son GLUT1 y GLUT3 (McEwen and Reagan, 2004; Vannucci et al., 1997a). El GLUT1 fue el primer transportador de glucosa que se clonó (Mueckler et al., 1985). Se han detectado dos diferentes variantes de peso molecular (45 y 55 kDa) en el SNC de mamífero debido a su patrón de glicosilación, sin embargo, esto no influye en su estructura protéica o en su mecanismo cinético de captura (Birnbaum et al., 1986). La forma de GLUT1 de 55 kDa, parecida a la de los eritrocitos de mamíferos, se encuentra exclusivamente en las membranas luminar y abluminal de las células endoteliales que forman la barrera hematoencefálica (BHE) (comunicación personal Simpson et al, 2007). Por su parte, la forma de 45 kDa se encuentra en todas las células glíales, en membranas apicales y basolaterales del plexo coroideo y en el canal del epéndimo. En condiciones fisiológicas hay muy poca expresión de la forma GLUT1 de 45 kDa en neuronas, aunque su expresión puede incrementarse como respuesta al estrés del medio ambiente o en cultivo de neuronas (Gerhart et al., 1994; Lee and Bondy, 1993). El número de transportadores GLUT1 en las membranas de las células endoteliales de la BHE es mucho mayor que en los astrocitos (400 pmol/mg prot, y 5.8-7.3 pmol/mg prot, respectivamente) (Simpson et al., 2001; Vannucci et al., 1997b).

El transportador GLUT3 se clonó de una línea celular de músculo esquelético (Nagamatsu et al., 1992). En el cerebro se ha localizado casi exclusivamente en neuronas (Gerhart et al., 1992; Maher et al., 1992; Nagamatsu et al., 1993). Cabe hacer mención que algunos tipos celulares que presentan altas tasas de metabolismo de la glucosa, como plaquetas, placenta, esperma y ciertos tipos de gliomas humanos, exhiben la expresión de GLUT3 (para revisión ver

Uldry and Thorens, 2004). En las neuronas, el GLUT3 se encuentra a una concentración de 9.5 pmol/mg prot (Maher et al., 1996).

La glucosa captada por los transportadores del SNC se distribuye en seis compartimentos: el plasma, las células endoteliales, la lamina basal, los astrocitos, el espacio intersticial y las neuronas (comunicación personal Simpson et al; 2007). La concentración de glucosa en la sangre es del orden de 5-8 mM y la del cerebro de 1.5-2.5 mM en humanos y roedores adultos (Erecinska et al., 2004). La glucosa se captura del plasma al cerebro por el GLUT1 de 55 kDa presente en las células endoteliales que tiene una km= 3 mM. Adicionalmente, el GLUT1 tiene una Km= 20-30 mM para el eflujo de la glucosa hacia el plasma. La baja afinidad del GLUT1 para la glucosa intracelular permite incrementar la concentración de éste azúcar en el lumen de las células endoteliales y mantener un adecuado suplemento a los astrocitos (comunicación personal Simpson et al; 2007).

Los astrocitos capturan la glucosa por medio del GLUT1 de 45 kDa (km= 8 mM), mientras que en las neuronas la captura de este azúcar es a través del transportador GLUT3 (km= 2.8 mM). Esto puede explicar en parte porque la capacidad de transporte de glucosa en membranas aisladas de neuronas es 9 veces más grande que en las membranas de astrocitos (Maher et al., 1996). Esto permite que las neuronas acumulen la glucosa a una concentración de 1.2 mM, comparado con 0.9 mM de los astrocitos (comunicación personal Simpson et al; 2007). Esta característica es particularmente importante, ya que como se mencionó anteriormente cerca del 75% de la energía que consume el SNC se relaciona con la transmisión sináptica (para revisión ver Attwell and Laughlin, 2001), y se propone que la vía glicolítica esta íntimamente relacionada con su aporte (para revisión ver Attwell and Gibb, 2005).

Intracelularmente la oxidación de una molécula de glucosa hasta CO_2 y H_2O involucra la participación de dos vías metabólicas que se encuentran interconectadas, la vía glicolítica o anaeróbica y la vía oxidativa o aeróbica.

Metabolismo glicolítico

Hace millones de años los organismos primitivos se originaron en un ambiente con una atmósfera carente de O_2, por tanto la glicólisis se considera como la vía metabólica más primitiva, que está presente en todas las formas de vida actuales. De hecho, la glicólisis es la única vía metabólica en los animales que produce ATP en ausencia de oxígeno. La glucólisis o glicólisis (del griego *glukus* = dulce y *lusis* = ruptura) fue descubierta en el año 1941 por Fritz Lipmann y Herman Kalckar. La vía glicolítica es la primera parte del metabolismo energético y consta de una secuencia de diez reacciones enzimáticas en la que se oxida la glucosa produciendo dos moléculas de piruvato, 2 ATP y 2 NADH (Fig. 1). Las dos enzimas paso limitante en la oxidación de la glucosa son la hexocinasa y la fosfofructocinasa. Bajo condiciones normales la tasa de oxidación de la glucosa en el cerebro, no se regula por su transporte sino por su fosforilación a glucosa 6-fosfato catalizada por la acción de la hexocinasa (km= 40 µM) (Qutub and Hunt, 2005). La concentración de hexocinasa en el cerebro se correlaciona con la utilización de la glucosa local (Wilson, 1980; Wilson, 2003). En un modelo *in vitro* de BHE se demostró que la localización y la concentración de la hexocinasa puede alterar la densidad de los transportadores de glucosa GLUT1 (McAllister et al., 2001). Adicionalmente, se ha demostrado que su actividad pero no su concentración, parece ser el paso limitante en el metabolismo de la glucosa cerebral. En condiciones de demanda energética se incrementa la actividad de la hexocinasa para iniciar la glicólisis. La hexocinasa se inhibe alostericamente por su producto, glucosa-6-fosfato, ATP, y por su unión a membranas mitocondriales (Wilson, 2003).

La fosfofructocinasa se inhibe por ATP, Mg^{2+} y citrato y se estimula por K^+, PO_4^{3-}, 5´-AMP, ADP y fructosa 2,6-bifosfato (Dunaway, 1983). En condiciones basales, la concentración de ATP y citrato en el cerebro inhiben la actividad de la fosfofructocinasa. Esta cinasa es una enzima oligomérica, las formas activas más pequeñas son tetrámeros formados por la unión al azar de las subunidades disponibles. Existen diferentes subtipos de subunidades codificados a partir de su propio gen que se han encontrado en conejos, ratas y humanos (Mhaskar and Dunaway, 1995). El subtipo C es el que se encuentra abundantemente expresado

en cerebro. La habilidad del tejido para utilizar la glucosa depende de la expresión y la composición de subunidades que conforman a la cinasa (Dunaway, 1983). A diferencia del subtipo M presente en el músculo, el subtipo C tiene una baja afinidad para la fructosa 6 fosfato, y menor respuesta a la inhibición por ATP (Kasten and Dunaway, 1993).

Las dos moléculas de NADH generadas durante la vía glicolítica se transportan del citosol a la mitocondria por medio de la lanzadera de malato-aspartato. Esta última, es la ruta más importante para la transferencia de equivalentes reductores en el SNC (Palmieri et al., 2001). La actividad de éste sistema es mayor en neuronas que en astrocitos debido a que se encuentra estrechamente relacionado con la síntesis de neurotransmisores (Palaiologos et al., 1988). Una vez en la mitocondria los NADH ceden sus electrones al complejo I de la cadena respitaroria y producen 2.5 moléculas de ATP (Fig. 1).

La reacción global de la glicólisis es:

$$\text{Glucosa} + 2\ NAD^+ + 2\ ADP + 2\ P_i \rightarrow 2\ NADH + 2\ \text{piruvato} + 2\ ATP + 2\ H_2O + 4\ H^+$$

El piruvato formado durante la glicólisis se transporta a la mitocondria a través de un sistema de proteínas acarreadoras. Una vez en la mitocondria el piruvato se descarboxila formando acetil CoA mediante la acción de un complejo enzimático conocido como piruvato deshidrogenasa localizado en la matriz mitocondrial (Fig. 1). El complejo esta compuesto por tres subunidades (E1, E2 y E3) que requiere diversos sustratos y cofactores: piruvato, NAD^+, pirofosfato de tiamina, la coenzima A, FAD^+ y ácido lipóico (Reed, 2001). Su actividad se regula por fosforilación, calcio, Mg^{2+} y ATP.

Metabolismo oxidativo

El metabolismo oxidativo de la glucosa se lleva a cabo en la matriz mitocondrial. La acetil CoA formada sufre una secuencia de reacciones conocidas como ciclo de Krebs o ciclo de los ácidos tricarboxílicos. El ciclo de Krebs fue descubierto en 1937 por Hans Krebs, y está conformado por una serie de ocho reacciones enzimáticas que generan 1 GTP, 3 NADH, 1 FADH2 como los

principales equivalentes reductores. En el ciclo de los ácidos tricarboxílicos se reducen el NAD^+ y FAD^+ que deben de reoxidarse inmediatamente en la mitocondria, puesto que una disminución en su concentración bloquearía los procesos catabólicos. La reoxidación tiene lugar gracias al O_2 y la participación de una serie de proteínas que forman cuatro complejos proteicos (I-IV) localizados en la membrana mitocondrial interna y que se les conoce como cadena respitaroria (Fig. 1). Los complejos protéicos son acarreadores de electrones que funcionan como un par redox. El complejo I (deshidrogenasa de NADH) se compone de 42-43 polipeptidos diferentes. Tiene una forma de “L”, la cual el brazo más largo forma parte de una proteína integral de membrana y el brazo corto se extiende dentro de la matriz con una parte hidrofílica que contiene un grupo flavín mononucleótido (FMN) y un centro activo que contiene un NADH (Navarro and Boveris, 2007). Como una parte importante de la función del complejo I, la ubiquinona (lípido soluble con cadena isoprenoide) se difunde lateralmente en cada una de las capas de fosfolípidos de la membrana interna y acarrea los electrones entre las proteínas de membrana. Se estima que el complejo I contiene aproximadamente 4 mol de ubiquinona/mol de FMN (Hinchliffe and Sazanov, 2005). El complejo II (deshidrogenasa de succinato) es una proteína que forma parte del ciclo de krebs y que también funciona como un componente de la cadena respiratoria. El complejo tiene unido un grupo flavín adenin dinucleótido (FAD) y centros hierro-azufre en el dominio catalítico útiles en la transferencia de los electrones a la ubiquinona y grupos hemo b en el dominio de membrana hidrofóbico (Cecchini, 2003). El complejo III (citocromo bc_1) esta compuesto de 9-10 polipéptidos, de los cuales, tres de ellos se asocian con centros redox. Estos centros son b_{562}, b_{566} y c_1 hemo y un grupo de [2Fe-2S] (Hatefi, 1985). Adicionalmente, dos ubisemiquinonas se unen a dos dominios del complejo III (Crofts, 2004). Durante la transferencia de electrones del complejo III al IV interviene el citocromo C. Este último es una proteína periférica localizada en el espacio intermembranal que transfiere los electrones del complejo III al Cu_A del complejo IV. El complejo IV (oxidasa de citocromo c) reduce el O_2 a H_2O a partir de cuatro electrones del citocromo c, consumiendo 4 H^+ de la matriz mitocondrial.

El flujo de electrones a través de los cuatro complejos en el que se involucran los donadores (NADH, succinato, FADH), y el aceptor (O_2), se genera mediante el potencial de oxidación. Los electrones se mueven hacia los componentes que tienen una energía de oxidación positiva. La diferencia en el potencial redox de los acarreadores de electrones definen las reacciones que son exergónicas y que proveen la energía libre requerida para el bombeo de H^+ dentro del espacio intermembranal. Los complejos I, III y IV funcionan como bombas de H^+ que actúan en serie con respecto al flujo de electrones y en paralelo con el circuito de H^+. El bombeo de H^+ se genera por la energía libre de las reacciones de oxidación acopladas, que involucra el movimiento de H^+ de la parte negativa de la mitocondria (la matriz) a la parte positiva (espacio intermembranal). Experimentos recientes han sugerido que las proteínas acarreadoras de la cadena respitaroria se ensamblan mediante interacción proteína-proteína acortando las distancias intermoleculares y haciendo más efectiva la transferencia de electrones (Navarro and Boveris, 2007). De hecho, se estima que una sola mitocondria puede contener hasta 10 000 interacciones entre proteínas.

La síntesis de ATP se genera por el flujo de protones a través del complejo ATPsintetasa (complejo V) (Fig. 1). La ATPsintetasa mitocondrial es una ATPasa de tipo F que esta formada por dos subunidades: F_1, una proteína de membrana externa, y F_0, proteína integral de membrana. La F_1 se compone de 5 subunidades (α, β, γ, δ, y Є) que forman el dominio catalítico. El movimiento de las subunidades de la ATPsintetasa es vital para su funcionamiento, y se conoce que puede rotar a 50-100 veces/s. La rotación se produce en la F_0 siguiendo el flujo electroquímico de H^+ (Walker et al., 1995). Se estima que se requieren aproximadamente 3-4 H^+ para dar lugar a 1 molécula de ATP (Chance, 1977; Mitchell and Moyle, 1965). El proceso de síntesis de ATP es termodinámicamente posible debido a que la transferencia de electrones genera energía suficiente, 34 kJ/mol por par de electrones, que se conserva en el gradiente de protones para dirigir la formación de 1 mol ATP (se requieren 32 kJ/mol ATP). Al proceso de síntesis de ATP se le conoce como teoría quimiosmótica y fue sugerida por primera vez en el año 1961 por Peter Mitchell. Por su parte, al proceso en el cual

se forma ATP a través de la transferencia de electrones se le denomina fosforilación oxidativa (porque el oxígeno capta los protones formando H_2O) y fue descubierta en el año 1948 por Eugene Kennedy y Albert Lehninger (Fig. 1).

La oxidación completa de una molécula de glucosa hasta CO_2 + H_2O genera de 30-32 moléculas de ATP.

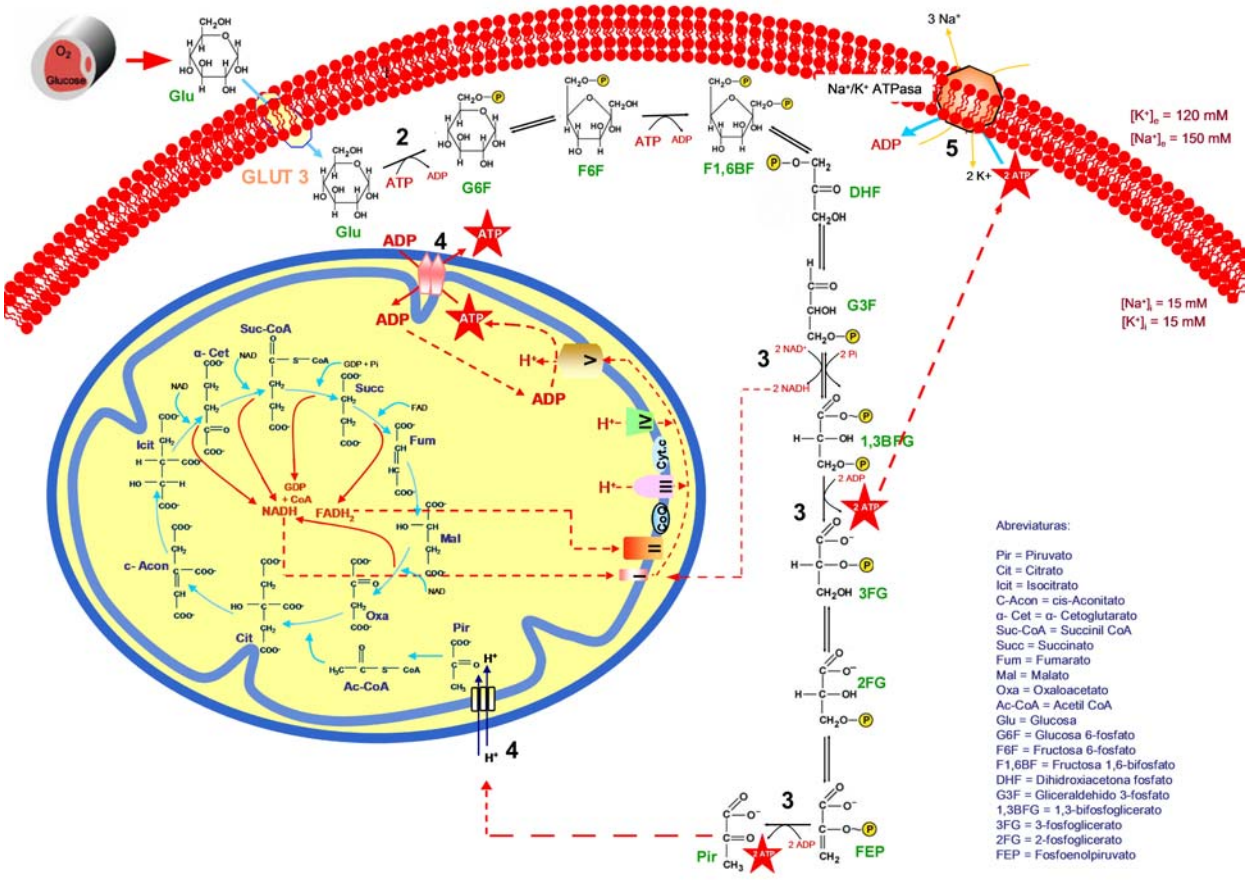


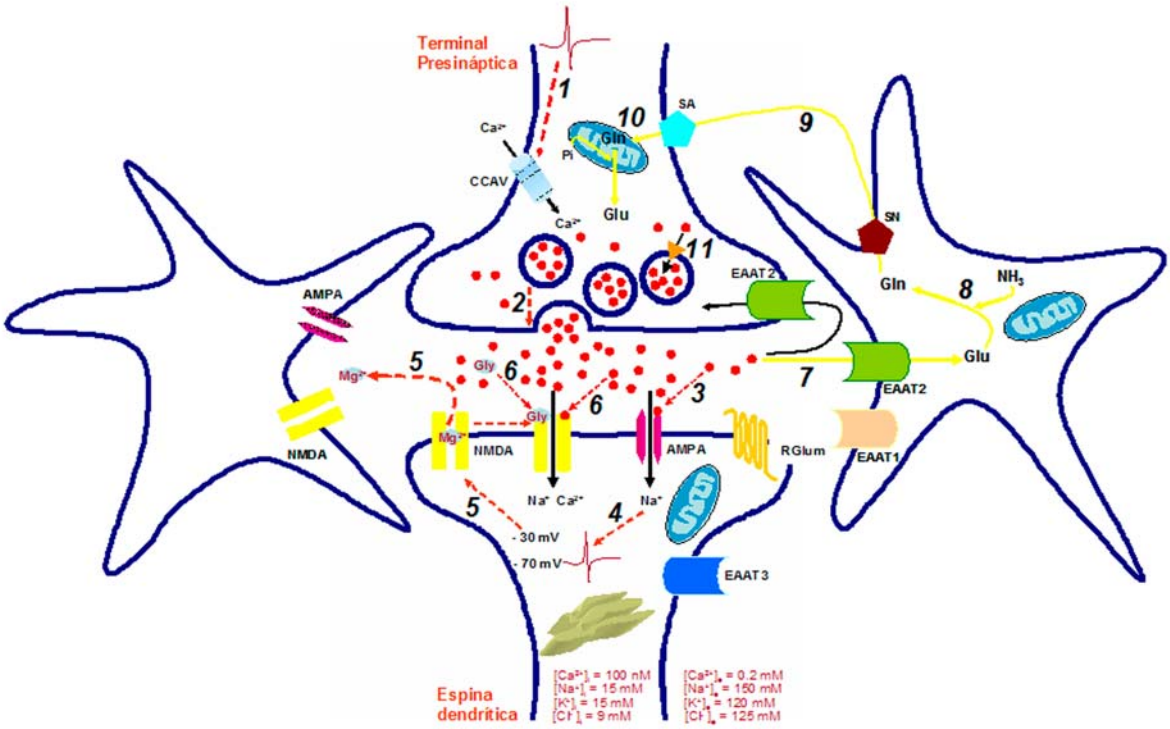
Fig. 1. Metabolismo energético cerebral. La glucosa proveniente de la circulación sanguínea se captura por un sistema de transportadores localizados en la membrana de neuronas y astrocitos (GLUT3 y GLUT5 respectivamente) (1). La primera fase de la oxidación de la glucosa se lleva a cabo a través de la vía glicolítica (2). El metabolismo glicolítico (etapa anaeróbica) constituye una serie de 10 reacciones sucesivas en que la ganancia energética final radica en la formación de 2 NADH y 2 ATP (3). En adición, el producto final de la vía glicolítica (piruvato) se transporta a la mitocondria en donde a través de la fosforilación oxidativa (etapa aeróbica) se oxida hasta formar 30 ATP, 1GTP, 3NADH, 1FADH2 (4). En particular, se propone que el ATP proveniente del metabolismo glicolítico esta involucrado en la función de la ATPasa Na+/K+ localizada en la membrana celular (5). De esta forma el desbalance en el equilibrio electroquímico generado por la activación de receptores y canales durante la neurotransmisión glutamatérgica esta relacionado con la función del metabolismo glicolítico.

Neurotransmisión glutamatérgica

El glutamato es un aminoácido con carga negativa, no esencial en la dieta, forma parte de proteínas, su síntesis esta involucrada con el metabolismo de la glucosa y aminoácidos. Se estima que entre el 80-90% de las sinapsis en el cerebro son glutamatérgicas (Braitenberg and Schüz, 1998). Las neuronas glutamatérgicas constituyen del 80%-88% del contenido total de glutamato del cerebro (Ottersen et al., 1992; Storm-Mathisen et al., 1983). El glutamato es el neurotransmisor excitador más abundante en el sistema nervioso de mamíferos (Collingridge and Lester, 1989; Fonnum, 1984). Se encuentra a una concentración de hasta 5-15 µmol/g tejido (Erecinska and Silver, 1990; Perry et al., 1987). En el espacio sináptico y en condiciones de reposo, su concentración puede variar de 0.6 - 5 µM dependiendo del método de cuantificación empleado. La capacidad que tiene el glutamato para excitar a neuronas del sistema nervioso central (SNC) de mamíferos se demostró hace más de 40 años a través de la administración iontoforética de glutamato o sus análogos en la médula espinal (Curtis and Watkins, 1960). Por otra parte, la administración oral o intraperitoneal a dosis altas en ratas neonatas y ratones genera neurodegeneración a nivel de la retina (células ganglionares) y en diversas estructuras periventriculares del cerebro, incluyendo el núcleo arcuato del hipotálamo (Olney, 1969; Olney, 1971; Olney and Ho, 1970; Olney and Sharpe, 1969). El glutamato es una molécula excitadora que promueve la sobrevivencia neuronal; sin embargo, el incremento descontrolado de su concentración en el espacio extracelular lo convierte en una molécula generadora de daño neuronal y muerte.

Cuando el glutamato se libera de una terminal presináptica ocurre la activación de sus receptores en milisegundos (Fig. 2). La activación de los receptores glutamatérgicos y de cualquier receptor, es un proceso estocástico en donde el receptor puede activarse y desactivarse sucesiva y azarosamente. Se propone que el glutamato, en primer lugar activa a los receptores sensibles al ácido alfa-amino-3-hidroxi-5-metil-4-isoxazolepropiónico (AMPA) localizados a nivel postsináptico, generando el paso de iones sodio y la despolarización parcial de la membrana (Fig. 2). Se estima que un cambio parcial en el voltaje de la membrana de 40 mV genera que el ión magnesio, que se encuentra bloqueando

normalmente al receptor a NMDA, se libere de su sitio de unión (Spruston et al., 1995; Vargas-Caballero and Robinson, 2003). Es solo bajo estas condiciones que el receptor a NMDA puede ser activado por la unión de glutamato y su co-agonista glicina (Fig. 2). La activación de ambos receptores producirá por un lado la entrada de calcio y por el otro la activación de los canales de calcio y sodio dependientes de voltaje promoviendo la transmisión del impulso nervioso entre las neuronas (Fig. 2).



Fíg.2. Neurotransmisión glutamatérgica. El arribo del potencial acción a la terminal presináptica genera la despolarización de la membrana y la subsecuente activación de CCAV (1). El incremento de calcio en microdominios selectivos de la terminal presinaptica genera la fusión de vesículas conteniendo el neurotransmisor (Glutamato, círculos rojos) (2). En primera instancia, la difusión de Glu en el espacio sináptico activa a receptores AMPA localizados en la región postsináptica (3). El influjo de sodio a través de estos últimos promueve la despolarización parcial de la membrana de 40 mV (4), generando que el ión Mg^{2+}, que se encuentra bloqueando el canal del receptor a NMDA se desprenda de su sitio de unión (5). En estas condiciones es posible que la activación del receptor a NMDA por Glu y Gly promueva el influjo de sodio y calcio (6). El glutamato en el espacio sináptico se captura por un sistema de proteínas de transporte, dependientes del gradiente electroquímico de sodio, localizadas en la membrana plasmática de neuronas y principalmente de astrocitos. De manera general, el Glu se captura por los astrocitos a través de EAAT2 (7). En el citoplasma el Glu se transforma a Gln por la acción enzimática de la glutamina sintetasa (8). La Gln sale del astrocito a través del sistema de transporte N (SN) y se captura por las neuronas a traves del sistema A (SA) (9). En las mitocondrias la Gln se convierte a Glu por la acción de la glutaminasa activada por fosfato (10). El Glu sintetizado se captura en las vesículas presinápticas mediante un sistema de transporte dependiente de gradiente de protones (11). Al ciclo metabólico mediante el cual el Glu se captura y se transforma a Gln en astrocitos, y la gln se transforma a Glu en neuronas (líneas amarillas), se le conoce como ciclo Gln-Glu, y constituye uno de los principales mecanismos en la síntesis de Glu presináptico. NMDA, N-methil-D-aspartato; Mg^{2+}, magnesio; AMPA, ácido alfa-amino-3-hidroxi-5-methil-4-isoxazolepropiónico; Glu, Glutamato; Gly, Glicina; CCAV, Canales de calcio activados por voltaje; EAAT 1-3 Transportador de aminoacidos excitadores 1-3; Gln, Glutamina; RGlum, Receptor de glutamato metabotrópico.

Transportadores de glutamato

La transmisión glutamatérgica en el cerebro tiene una duración promedio de 1-2 ms, lo que incrementa la concentración extracelular de glutamato hasta cerca de 1 mM (Clements et al., 1992). Dado que el aumento extracelular de glutamato puede promover bajo ciertas condiciones patológicas el daño neuronal excitotóxico, como se discutirá más adelante, el proceso de neurotransmisión glutamatérgica, así como el de cualquier neurotransmisión, es un evento sumamente controlado (Fig. 2). En el control de la neurotransmisión glutamatérgica participan diversas proteínas y tipos celulares. Entre las proteínas más importantes se encuentran los transportadores de glutamato localizados en neuronas y principalmente en las células glíales (Grewer and Rauen, 2005; Sonders et al., 2005) (Fig. 2). Diversos experimentos han demostrado que el bloqueo de los transportadores de glutamato genera el incremento en la amplitud y en las corrientes postsinápticas excitadoras (Barbour et al., 1994; Turecek and Trussell, 2000). Esto sugiere que los transportadores no solo participan en el mantenimiento de los niveles basales de glutamato en el espacio sináptico, sino que probablemente están influyendo en la excitabilidad neuronal. Dado que el glutamato extracelular puede aumentar hasta 1 mM durante su liberación sináptica (Clements et al., 1992), se ha propuesto que los transportadores controlan la concentración sináptica de este aminoácido mediante su unión, más que por su transporte. Esto se propone por dos razones, 1) el tiempo en el que ocurre el ciclo de captura de los transportadores es mucho más largo (70 ms) comparado con los 1-2 ms en los que ocurre la neurotransmisión glutamatérgica (Clements et al., 1992; Grewer et al., 2000; Otis and Jahr, 1998; Otis and Kavanaugh, 2000), y 2) en la sinapsis existen hasta 10 000 transportadores/μm^2 capaces de unir glutamato (Lehre and Danbolt, 1998).

Tipos de transportadores

A principios de los años noventa, la caracterización molecular de los transportadores de glutamato experimentó un avance considerable debido a que sus tres primeros subtipos se clonaron. Inicialmente, los transportadores GLAST y

GLT-1 se clonaron de cerebro de rata (Pines et al., 1992; Storck et al., 1992), mientras que el transportador EAAC1 fue clonado de intestino delgado de conejo (Kanai and Hediger, 1992). Un par de años más tarde los tres tipos de transportadores conocidos a la fecha, se clonaron a partir de tejido cerebral de humano (Arriza et al., 1994) y su nomenclatura se estandarizó como EAAT (transportador de aminoácidos excitadores, por sus siglas en Inglés), siendo para cada uno GLAST/EAAT1, GLT-1/EAAT2 y EAAC1/EAAT3. A la fecha se han clonado dos subtipos más, el EAAT4 (Fairman et al., 1995) y el EAAT5 (Arriza et al., 1997). En particular, el transportador GLT1/EAAT2 presenta variantes específicas en su secuencia de aminoácidos que probablemente le otorguen características funcionales particulares (Grewer and Rauen, 2005). El patrón de expresión celular de los transportadores es específico para cada subtipo. Los transportadores GLAST/EAAT1 y GLT1/EAAT2 se expresan principalmente en glía, aunque se ha reportado una variante del transportador GLT1/EAAT2 en neuronas (Berger et al., 2005; Chen et al., 2004; Schmitt et al., 2002; Suchak et al., 2003). El transportador EAAC1/EAAT3 se expresa en neuronas, mientras que el EAAT4 es específico del cerebelo, así como el EAAT5 es específico de retina. Durante el desarrollo del sistema nervioso el patrón de expresión de los transportadores se regula de manera selectiva. Mientras que por un lado, el transportador EAAC1 es el más abundante durante la etapa embrionaria y hasta el tercer día post-natal, por el otro, a partir del cuarto día post-natal y hasta la etapa adulta, se presenta un incremento en la expresión de GLAST y GLT-1 (Furuta et al., 1997; Rothstein et al., 1994; Schluter et al., 2002; Velaz-Faircloth et al., 1996). Esto demuestra que los transportadores se expresan diferencialmente durante el desarrollo del sistema nervioso influyendo probablemente en la excitabilidad y función neuronal.

Estructura y mecanismo de acción

Tuvieron que pasar cerca de 15 años a partir de la clonación del primer transportador de glutamato para conocer la estructura tridimensional del GLT-1 presente en la bacteria termófila *Pyrococcus horikoshii* (Yernool et al., 2004). La

estructura tridimensional de esta proteína está de acuerdo con la sugerida por análisis bioquímicos y biofísicos, y con el modelo propuesto por los grupos de Kanner y Lolkema (Grunewald and Kanner, 2000; Slotboom et al., 1999). Ahora está claro que el transportador de glutamato GLT-1, se ensambla como un trímero de subunidades idénticas, conformada por dos asas reentrantes y un dominio carboxilo terminal. Al parecer las asas reentrantes forman dos compuertas que se abren alternativamente para que el glutamato pueda transportarse al interior de la célula (Fig. 3 A-B). Un posible acercamiento al mecanismo de transporte de glutamato y los iones involucrados se muestra y describe en la Figura 3 C.

Los transportadores de glutamato dependen del gradiente electroquímico del Na^+ generado por el funcionamiento de las ATPasas Na^+/K^+ membranales. De acuerdo al modelo de (Watzke et al., 2001), durante la captura de glutamato intervienen varios iones. Previamente a la unión del glutamato el transportador tiene unido un ión K^+. Antes de que se inicie la captura, el ión potasio se desprende de su sitio y la captura se inicia por la unión de un ión Na^+ y posteriormente la de un ión H^+. El glutamato se une en este momento seguido de un segundo ión de Na^+. Se sabe que se requiere de la unión de tres iones Na^+ para que se lleve a cabo la captura a través de un mecanismo de simporte, sin embargo; aún no está claro si el tercer ión Na^+ se une al transportador antes o después de unirse el glutamato (Grewer and Rauen, 2005). La translocación del glutamato al interior de la célula, ya sea una neurona o célula glíal, induce el heterotransporte de un ion K^+ que se cree se une al transportador después de que éste ha transportado 3 Na^+, 1 H^+ y 1 glutamato. El transporte iónico durante la captura de glutamato es de naturaleza electrogénica, ya que se mueven dos cargas positivas dentro de la célula por cada ciclo completo de transporte (Grewer and Rauen, 2005). Se ha descrito la activación de una corriente entrante de Cl^- asociada a la captura de glutamato en algunos tipos de transportadores; sin embargo, a la fecha no se conoce con certeza si el canal permeable a Cl^- forma parte de la proteína del transportador o simplemente es un canal independiente (Wadiche et al., 1995) (Fig. 3). Lo que sí está claro es que todos los transportadores exhiben cierto influjo de cloro a través de su canal, sin embargo la

permeabilidad a este es superior en los subtipos EAAT4 y EAAT5 (Bergles et al., 2002; Palmer et al., 2003; Wadiche et al., 1995).

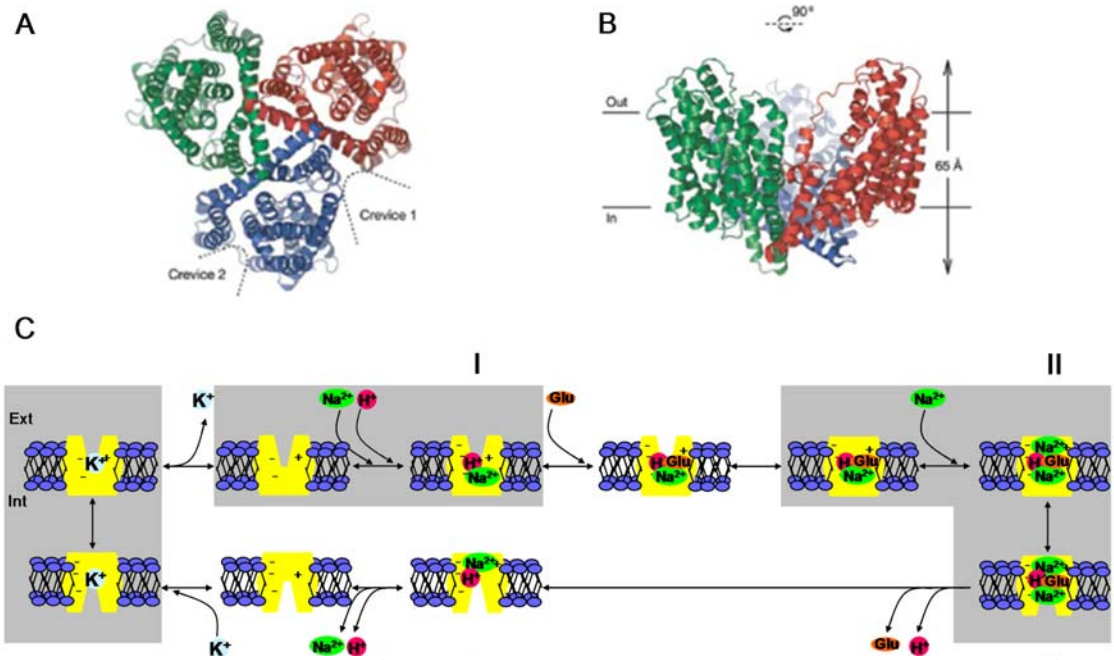


Fig. 3. Estructura y mecanismo de acción del transportador de glutamato GLT-1. Estructura tridimensional por análisis cristalográfico del transportador de Glu mostrando la conformación en trímeros (A) y su distribución en la membrana (B) de acuerdo al análisis de Yernool et al., 2004. C) El mecanismo cinético del transporte de Glu incluye 4 estados de conductancia iónica (expresados como I y II) que transcurren en un tiempo de 14 ms de acuerdo al modelo propuesto por Grewer y Rauen, 2004. Se propone que se requiere de la previa unión de un ión H^+ y de un Na^+ para que el Glu se una a residuos específicos (estado I, porcion extracelular). Se piensa que un segundo ión Na+ interviene en el proceso final de captura (estado II extracelular). El desprendiemiento de Glu, H^+ y Na^+ a nivel intracelular y la previa unión de un ión K^+ conforman el estado I y II intracelular. Las etapas de reacción electrogénica que ocurren durante la captura de Glu se presentan en gris.

Ciclo glutamina-glutamato

Experimentos pioneros empleando los isótopos ^{14}C y ^{15}N en el estudio de flujos metabólicos y su compartamentalización demostraron la participación de la interacción neurona-glía en el ciclo glutamina-glutamato (Benjamin and Quastel, 1975). El concepto de ciclo glutamina-glutamato se estableció firmemente por el descubrimiento de las enzimas que participan en dicha ruta metabólica, como la glutamina sintetasa y la piruvato carboxilasa, localizadas en la glía (Martinez-Hernandez et al., 1977; Norenberg and Martinez-Hernandez, 1979). La primera medición cuantitativa del ciclo glutamina-glutamato *in vivo* se realizó en los años 1990s empleando espectroscopía de resonancia magnética (Sibson et al., 1997). Los estudios demostraron que durante la oxidación de la glucosa neuronal y el

ciclo glutamina-glutamato existía una relación de 1:1 entre el consumo de glucosa y la producción de glutamina. Lo anterior está en relación con algunos estudios *in vitro* que demostraron que durante la captura de glutamato por la glía se cotransporta Na^+ lo cual estimula la glicólisis glíal y la producción de lactato en las mismas células (Magistretti et al., 1999; Pellerin and Magistretti, 1994). Una vez en la glía, el glutamato se transforma a glutamina, en un proceso dependiente de ATP y Mg^{2+}, por la acción de la glutamina sintetasa (Fig. 2). La glutamina sale de la glía a través del sistema de transporte N y regresa a las neuronas por el sistema de transporte A (Broer and Brookes, 2001). La glutamina entra a la mitocondria neuronal y se transforma a glutamato por la acción de la glutaminasa activada por fosfato. De esta manera, el glutamato se encuentra nuevamente disponible para ser capturado en las vesículas y liberado mediante un estímulo despolarizante. Sin embargo, el glutamato (en astrocitos) pueden ser metabolizado oxidativamente y generar α-cetoglutarato (Hertz et al., 1999; Yudkoff et al., 1993). La posa de glutamato oxidado debe de reemplazarse, lo cual ocurre a través de la actividad de la enzima piruvato carboxilasa. Al ciclo mediante el cual el glutamato se transforma a glutamina en la glía, y la glutamina se transforma a glutamato en las neuronas se le conoce como ciclo glutamina-glutamato (Fig. 2) y constituye una de las vías metabólicas más importantes para el reciclamiento del glutamato en la terminal presináptica de neuronas (Erecinska and Silver, 1990).

En neuronas gabaérgicas el ciclo glutamina-glutamato también está presente y es de gran importancia para generar la posa citoplásmica de GABA (Sonnewald et al., 1993). Para la síntesis de GABA en neuronas el requisito indispensable es que el glutamato se convierta a glutamina en los astrocitos mediante la acción de la glutamina sintetasa. Posteriormente, la glutamina se transforma a glutamato y el glutamato a GABA por la acción de la glutaminasa activada por fosfato y la glutamato descarboxilasa, respectivamente. Similar al glutamato en astrocitos, el GABA puede ser metabolizado en el ciclo de krebs mediante la acción de la transaminasa de GABA y la deshidrogenasa de semialdehído succínico. A éste último ciclo se le conoce como lanzadera de GABA

del ciclo de krebs, y representa el 10% de la actividad basal en neuronas (Schousboe and Waagepetersen, 2007).

Durante las reacciones producidas por la glutaminasa en las neuronas glutamatérgicas hay formación de amonio, que debe de excretarse debido a sus acciones toxicas. En el cerebro la concentración de amonio tiene valores <0.25 mmol/kg peso seco, mientras que en el plasma la concentración puede estar en <0.1 mM (Marcaggi and Coles, 2001). El amonio formado se transporta a las células gliales para inducir la síntesis de glutamina (Waniewski, 1992). Se estima que por cada molécula de glucosa oxidada, 0.87 moléculas de glutamato se transforman a glutamina (Sibson et al., 1998). Por tanto, la formación de una molécula de glutamina corresponde a la transferencia de 0.87 moléculas de amonio de neuronas a astrocitos. El ciclo glutamina-glutamato en el cerebro constituye probablemente el mayor flujo intracelular de amonio (Marcaggi and Coles, 2001).

Relevancia de los transportadores de glutamato en la generación de la muerte neuronal isquémia

Diversos reportes sugieren que la alteración en el transporte de glutamato juega un papel muy importante en el daño neuronal isquémico. Esta evidencia se revisa en el artículo: Camacho A and Mássieu L. Role of glutamate transporters in the clearance and release of glutamate during ischemia and its relation to neuronal death. Arch Med Res. 2006. 37(1):11-18.

En contraste, el papel de los transportadores de glutamato en la muerte neuronal asociada a la hipoglicemia no se ha estudiado y parte del presente trabajo de tesis se dedicó a este tema en un modelo farmacológico que mimetiza una condición de hipoglicemia aguda (ver más adelante).

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REVIEW ARTICLE

Role of Glutamate Transporters in the Clearance and Release of Glutamate during Ischemia and its Relation to Neuronal Death

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Glutamate neurotransmitter action on postsynaptic receptors is terminated by its clearance from the synaptic cleft by transporter proteins located in neurons and glial cells. Failure of glutamate removal can lead to neuronal death due to its well-known neurotoxic properties. Glutamate transporters are dependent on external Na+, and thus on the activity of Na+/K+ ATPases, which maintain the Na+ concentration gradient. When the energy brain requirements are not fulfilled by the appropriate blood supply of glucose and oxygen, the Na+ gradient collapses leading to impaired glutamate and aspartate removal, or even to the release of these amino acids through the reverse operation of their transporters. Such a scenario would be associated with brain ischemia and hypoglycemia due to the prompt decline in ATP levels. In addition, some evidence suggests that downregulation of glutamate transporters after the ischemic period, or the dysfunction induced by oxidation, contributes to the accumulation of extracellular glutamate and neuronal death. Neuronal damage is associated with excitotoxicity, a type of cell death triggered by the overactivation of glutamate receptors and the loss of calcium homeostasis. Throughout this review we will discuss recent evidence suggesting that failure of glutamate transport during ischemia contributes to the elevation of extracellular glutamate and to the induction of excitotoxicity. We will also discuss the contribution of glial vs. neuronal glutamate transporters in ischemic damage, and the involvement of the different glutamate transporter subtypes. We will focus on experimental data from rodent models, because many of the studies on glutamate transport and ischemic damage have been performed in these animal species.

Key Words: Glutamate transporters, Energy metabolism, Excitotoxicity, Ischemia, Neuronal death.

Introduction

The correct processing and integration of external signals leads to the generation of adequate responses and interactions with the surrounding medium. This process involves a complex system of biochemical signals regulated by the central nervous system. Optimal brain functioning requires the continuous supply of glucose and oxygen from blood (1). When the large energy demand of brain metabolism is not completely satisfied due to decreased or complete interruption of blood flow, a pathological condition known as brain ischemia arises. According to statistical data, brain ischemia represents the third cause of death in industrialized countries. From the 5.5 million deaths registered worldwide during 1999, 10% were related to cerebrovascular disorders (2). The high costs of medical care required by surviving individuals, from which a large percentage are permanently disabled, is a major cause of economic losses (3).

According to recent statistics from the Instituto Nacional de Estadística Geografia e Informatica (INEGI), cerebrovascular disease represents the sixth cause of death in Mexico with 26,583 deaths recorded during 2002 (4).

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In most cases decreased cerebral blood flow results from the presence of an embolus or local thrombosis, causing damage to the brain region irrigated by the occluded artery.

Cerebral ischemia resulting from the complete interruption of cerebral blood flow, for example, during cardiac arrest, is less common.

Diverse *in vivo* animal models of cerebral ischemia have been widely used to investigate the mechanisms involved in the generation of cell death. Although the physiopathology of brain ischemia is similar among the different animal species including man, there are some features of cerebral structure, function and vascular anatomy particular to each one of them (5). For example, it is estimated that glucose and oxygen metabolism, as well as blood flow, is threefold higher in the rat than in the human, and the dimensions, regional distribution and temporal evolution of the infarcted area will vary among the different animal species (5,6).

Global cerebral ischemia can be induced in the rat by the temporary occlusion of both carotid and vertebral arteries, leading to the complete interruption of cerebral blood supply. Focal ischemia is commonly generated by the occlusion of the middle cerebral artery irrigating a particular brain area (7).

When cerebral blood flow decreases to <20 mL/100 g tissue/minute, two areas of neuronal damage are generated: 1) the ischemic core where blood flow is completely interrupted and neurons are committed to die because of the lack of glucose and oxygen; and 2) the penumbral zone, adjacent to the ischemic core, which is potentially rescued from death due to collateral irrigation (1,8). Cell death induced during these conditions is related to the increase in the extracellular levels of the excitatory amino acids (EAA), aspartate and glutamate (9,10).

It is well accepted that the dramatic increase in EAA levels is responsible for the excitotoxic death of neurons in the penumbral zone (7). Excitotoxicity is the type of neuronal death induced by overstimulation of glutamate receptors, particularly the N-methyl-D-aspartate (NMDA) receptor subtype (11,12). This type of receptor is largely permeable to calcium leading to a massive influx of these ions into the cell. Calcium ions will activate diverse enzymatic routes involved in neuronal death such as lipases, proteases, phosphatases and nucleases (Figure 1).

Increased formation of reactive oxygen species due to the activation of nitric oxide synthase and mitochondrial calcium overload will also contribute to the disruption of cellular homeostasis and cell death (Figure 1) (12,13). Despite the well-accepted excitotoxic hypothesis of ischemic neuronal damage, the use of glutamate receptor antagonists for the treatment of ischemic patients has failed due to a variety of factors, including impairment of excitatory neurotransmission, the presence of toxic side

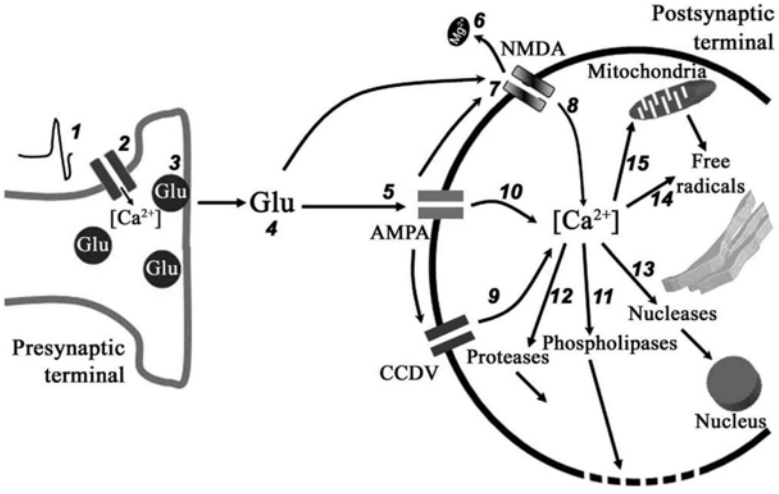

Figure 1. Excitotoxic death induced by glutamate. The arrival of the action potential to the presynaptic terminal (1) depolarizes the plasma membrane and activates voltage-dependent calcium channels (2). Calcium influx induces the fusion of presynaptic vesicles docking in the fusion zone (3) promoting glutamate release and increasing its concentration in the synaptic cleft (4). Glutamate will activate AMPA receptors on postsynaptic neurons (5), inducing sodium influx and depolarization of the plasma membrane. In this condition the magnesium ion that normally blocks the NMDA receptors is extruded (6), and the receptor activated by the binding of glutamate and its co-agonist glycine (7). The activation of NMDA receptor induces sodium and calcium influx (8). Voltage-dependent calcium channels (CCDV) will also activate (9). In some circumstances calcium can also enter the cell through AMPA receptors (10). Calcium influx will induce cytoplasmic calcium overload and activation of diverse enzymes such as phospholipases (11), proteases (12) and nucleases, which will drive the breakdown of phospholipids, proteins and nucleases (13). In addition, cytoplasmic calcium overload will lead to free radical production (14), oxidative stress, and impairment of mitochondrial metabolism, which will lead to energy failure (15). As a result, neuronal death will take place.

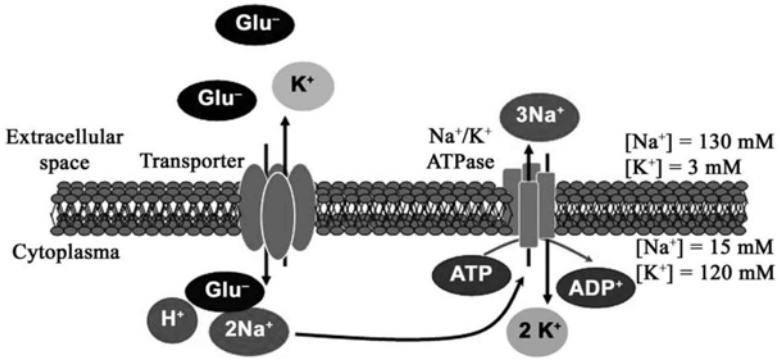

Figure 2. Extracellular glutamate uptake. The increase in the extracellular concentration of glutamate during glutamatergic neurotransmission is highly regulated by transporter proteins located at the plasma membrane of neurons and glia. Uptake is dependent on the sodium electrochemical gradient that is maintained through the activity of Na+/K+ ATPases. A large amount of the ATP generated in neurons and astrocytes is used to reestablish the sodium gradient after glutamate uptake.

effects and the induction of hypothermia, which itself contributes to neuronal survival (14,15).

Glutamate Transporters and the Control of Extracellular Glutamate

The extracellular concentration of glutamate after its synaptic release is highly regulated by Na+-dependent high affinity transporters located both in neurons and astrocytes (16–18). To date, five glutamate transporters have been identified. The glutamate-aspartate transporter (GLAST) is a glial transporter highly distributed throughout the brain, mainly in Bergman glial cells in cerebellum, with less expression in forebrain and spinal cord (19,20).

Glutamate transporter 1 (GLT1) is an astroglial transporter highly distributed in the entire forebrain (20–22). Recently, a mRNA splice variant of GLT1 (GLT1b) has been described (23,24). At difference from the originally reported form, this splice variant lacks an 11-amino acid segment in its carboxy-terminal end, which possibly confers particular functional properties to this transporter (22). This variant is present both in neurons and glia. Excitatory amino acid carrier 1 is a neuronal transporter abundantly expressed in the hippocampus, striatum and cerebellum (15,23). Human homologues of each one of these transporters have been identified and are designated as EAAT1 (GLAST), EAAT2 (GLT1) and EAAT3 (EAAC1). EAAT4 is a neuronal transporter present in Purkinje cell dendrites (21,25), and EAAT5 is present in photoreceptors and bipolar cells in the retina (26,27). During development of the rat nervous system, expression of glutamate transporters is selectively regulated.

While EAAC1 is abundantly expressed from the embryonic stage to the third postnatal day, the expression of GLAST and GLT1 increases substantially thereafter, from the fourth day of birth to adulthood (25,28,29).

Activity of glutamate transporters depends on the Na+ electrochemical gradient generated by the membrane Na+/K+ ATPases. One glutamate molecule is transported to the cytoplasm together with two sodium ions, while one potassium ion is extruded to the extracellular medium (30,31). Recovery of the Na+ electrochemical gradient after the uptake of extracellular amino acids occurs at the expense of the ATP consumed by the membrane Na+/K+ ATPases (Figure 2) (1). Therefore, the maintenance of energy levels is essential for the control of glutamatergic transmission. During energy failure, such as that occurring during cerebral ischemia or acute glycolytic inhibition, ionic gradients will disrupt and the regulation of glutamate uptake lost, leading to increased excitatory amino acid levels (10,32). In addition, studies from our group have shown that blockade of glutamate uptake leads to neuronal damage during impairment of energy metabolism, suggesting that altered glutamate clearance is harmful to neurons during energy-limiting conditions (33–36).

Routes of Glutamate Release during Brain Ischemia

Early studies suggested two main pathways involved in the elevation of excitatory amino acid extracellular levels during ischemia: one calcium dependent occurring at the onset of the ischemic episode, and one delayed, which is independent of calcium (37). Both pathways are energy-dependent.

Recent studies indicate that glutamate release during ischemia shows at least three components, an early one that is calcium-dependent and related to synaptic exocytosis (37,38), a second component related to the activation of volume-sensitive channels (39,40), and a third one associated with the reversed activation of EAA transporters (41). Glutamate release through these pathways will occur at different stages of the ischemic episode. Synaptic release

is an energy-dependent process (42), and therefore declines during energy failure.

During the ischemic episode ATP hydrolysis will be enhanced increasing the intracellular levels of adenosine (43), which in turn inhibits calcium entry to the synaptic terminal (44) decreasing further glutamate release. Thus, glutamate release from synaptic terminals will occur immediately after the onset of the ischemic period while there is sufficient ATP to drive the exocytotic pathway.

Afterwards, shortage of ATP will alter the functioning of the Na+/Ca+ exchangers and the Na+/K+ ATPases leading to the collapse of the ionic gradients.

Accumulation of intracellular Na+ will drive the extrusion of osmotically active compounds, such as glutamate and aspartate for cell volume regulation (39,45).

It will also facilitate the reverse action of Na+-dependent EAA transporters, extruding glutamate to the extracellular space (41,46). Recent data from our laboratory suggest that acute inhibition of glycolytic metabolism *in vivo* promotes the release of EAA at least by two pathways, reversed activation of glutamate transporters and volume-sensitive channels (unpublished data). Along the following pages recent advances in the knowledge of the role of EAA transporters on the regulation of extracellular glutamate and the induction of ischemic neuronal death will be discussed.

Failure of Glutamate Transport during Brain Ischemia

As mentioned above, one of the most accepted hypotheses suggests that neuronal death associated with brain ischemia is of excitotoxic nature. A large amount of glutamate released during ischemia might result from altered glutamate transport triggered by different mechanisms: 1) decreased uptake; 2) reversed activation; or 3) decreased expression of protein transporters.

The generation of free radicals during ischemia is well documented (47,48). Glutamate transporters are vulnerable to oxidation due to the presence of SH-based redox regulatory sites (49), resulting in decreased uptake (50–52). Although there is no direct evidence that free radicals generated during ischemia *in vivo* can inhibit glutamate uptake, a recent study showed that glutamate receptor stimulation in neuronal-glia co-cultures of spinal cord results in enhanced production of free radicals and decreased glutamate uptake in glia (53). These observations suggest that free radical generation after overactivation of glutamate receptors can inhibit glutamate uptake in sites distant from the place they have been produced (53). Free radicals will promote the oxidation of diverse residues in the transporter protein (47,48), particularly Cys186 and Cys375 (52). Apparently, these residues are highly conserved among the different EAA transporter subtypes.

Deficient functioning of glutamate transporters is also correlated with increased lactic acidosis (54,55). The physiological concentration of glucose in blood is on the order of 4−6 mM, and due to the glycolytic nature of the nervous system, most of this glucose is oxidized. Completely oxygenized blood contains approximately 8 mM O_2 and 6 molecules of oxygen are needed in order to oxidize one molecule of glucose. The exceeding molecules of glucose cannot be metabolized through the aerobic oxidative pathway but they are oxidized through anaerobic glycolysis leading to the production of lactic acid. When cerebral blood flow decreases to levels <50 mL/100 g tissue/minute, the rate of anaerobic glycolysis is increased in order to carry ATP production, leading to the accumulation of lactic acid. Although lactate can reduce glutamate uptake (54,55), the mechanism involved is unknown.

The most direct evidence favoring decreased glutamate uptake during the ischemic period *in vivo* is the recent observation by Bruhn et al. (56).

Perfusing [^{3}H]-aspartate through a microdialysis membrane in the CA1 hippocampal region, these authors observed a 68% decrease in the uptake of this amino acid during the ischemic period. Similarly, results from our laboratory have shown that glycolysis inhibition *in vivo* promotes a significant decrease in glutamate uptake (Camacho et al., submitted).

Altogether, the aforementioned studies suggest that augmented extracellular levels of excitatory amino acids during ischemia result, at least in part, from decreased uptake (Figure 3).

On the other hand, several authors suggest that augmented glutamate results from the reversed action of glutamate/aspartate transporters after the collapse of the Na+ gradient (Figure 3) (57). It has been observed that an increase in the intracellular concentration of Na+ from 15 mM to 30 mM is sufficient to cause the inverse functioning of glutamate transporters (46,58). This result indicates that the complete collapse of the ionic gradients is not necessary for the inverse operation of glutamate transporters, but a partial perturbation of the electrochemical equilibrium would be sufficient to trigger this process.

The contribution of specific transporter subtypes (neuronal or glial) to the release of excitatory amino acids during ischemia has not been elucidated. It has been reported that the elimination of the excitatory afferents to the CA1 region, before the induction of the ischemic period, attenuates glutamate release and neuronal damage, suggesting that amino acid efflux originates at least in part from glutamate transporters located in presynaptic terminals (59,60). On the other hand, a study on hippocampal slices suggested that glutamate release is preferentially neuronal because incubation with dihydrokainate (a relative selective blocker of glial GLT1 transporters) does not inhibit the increase in glutamate extracellular levels observed during glucose and oxygen deprivation (61). Moreover, neuronal rather than glial transporters might

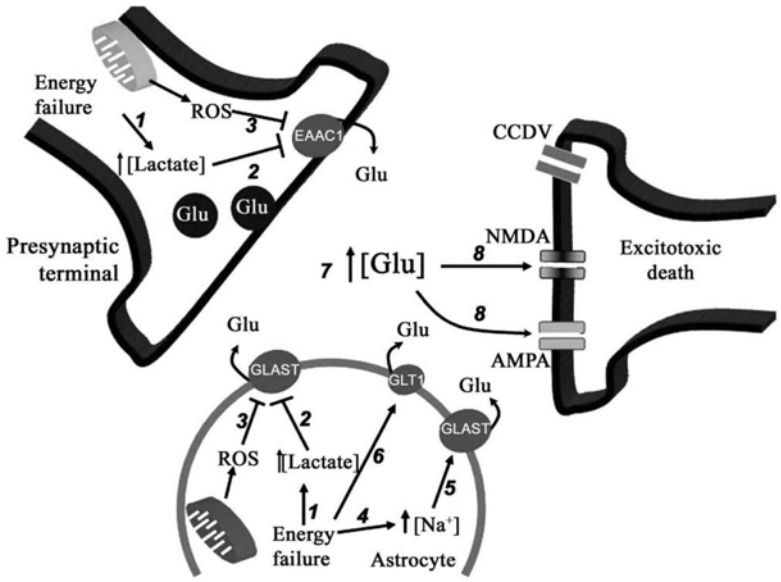

Figure 3. Possible alterations in the activity and the expression of glutamate transporters during cerebral ischemia. The lack of glucose and oxygen during cerebral ischemia leads to energy failure and increased intracellular lactate (1), which inhibits glutamate uptake both in neurons and glial cells (2). Free radical production induced after glutamate receptor activation might induce the oxidation of cysteine residues on glutamate transporter, inhibiting uptake (3). Additionally, energy failure promotes the increase in intracellular sodium (4), and in turn the inverse functioning of glutamate transporters (5). Through a still unknown mechanism, downregulation of glutamate transporters at the plasma membrane occurs after the ischemic insult (6). These alterations might be involved in the elevation of extracellular glutamate (7) and the development of neuronal death associated with ischemia (8).

mediate glutamate release because the cytosolic concentration of glutamate in neurons is around 10 mM or more whereas in glia it is ~3 mM (31). However, the number of glutamate transporters present in glial cells is higher than that in neurons, and neuronal transporters are mainly located on postsynaptic regions (64). The presence of GLT1 transporter in synaptic terminals in the hippocampus was recently confirmed (65). However, its role in glutamate clearance or release during ischemia remains to be elucidated. Besides reversed transport, synaptic release from nerve terminals might contribute to glutamate efflux during the ischemic episode (62,63).

Glutamate Transporters during Ischemia: Release or Glutamate Removal?

The studies described in the sections above suggest that glutamate transporters can have a dual role during ischemia: on one hand they would eliminate glutamate from the synaptic cleft after its synaptic release and prevent excitotoxic cell death; and on the other, they could operate in the inverse direction extruding glutamate to the extracellular space, contributing to neuronal damage. Thanks to the rapid advance in molecular biology tools, it has been possible to eliminate the genes encoding for the different glutamate transporter proteins in mice (knockout mice) and study its consequences on animal behavior and degenerative processes. Heterozygote mice lacking one allele of the GLT1 transporter show enhanced brain edema after ischemia (66). Similarly, the administration of antisense oligonucleotides of GLT1 transporter increases infarct volume and mortality after cerebral ischemia (67). These observations agree with those of a recent study showing the presence of neuronal death in the hippocampus, epileptic seizures, and increased mortality in knockout mice of GLT1 transporter (68). All these observations favor the role of GLT1 in the clearance of EAA.

On the other hand, downregulation of GLT1 mRNA and protein has been observed after ischemia even before neuronal death is detected, suggesting that a reduction in the number of GLT1 transporters might be involved in the neurodegenerative process (Figure 3) (69–72). Accordingly, we have recently observed a decrease in GLT1 protein levels as well as a reduction in glutamate uptake during sustained glycolysis inhibition in the rat hippocampus *in vivo* (Camacho et al., submitted). These results are in agreement with data from human brain showing decreased expression of GLAST and EAAT4 transporters in neonatal human cerebellum after hypoxia/ischemia (73). Authors suggested a role of these transporter subtypes in the

vulnerability of cerebellar Purkinje cells to the hypoxic/ischemic lesion. In contrast, Ikematsu et al. (74) argue that the decreased content of GLT1, observed in postmortem cerebral tissue of individuals dying from asphyxia, was mainly the consequence of postmortem changes in the analyzed samples. The aforementioned observations support the main role of glutamate transporters in the clearance of this amino acid during the ischemic period, and therefore in the prevention of excitotoxic cell death. However, several recent studies support the alternative hypothesis suggesting that glutamate transporters are responsible for glutamate release during ischemia. The main observation supporting this hypothesis is that administration of glutamate uptake blockers before the induction of the ischemic insult significantly reduces the release of excitatory amino acids (38–41). However, the consequences of this manipulation on infarct volume or cell survival were not studied.

A recent study greatly contributes to elucidate the role of glutamate transporters in EAA release and neuronal death during ischemia (75). This study shows that in wild-type mice expressing the GLT1 transporter, glutamate is efficiently removed from the extracellular medium during a short (5 min) period of ischemia, and neuronal death of CA1 pyramidal neurons is prevented. In contrast, when the ischemic period is extended to 20 min, glutamate is efficiently removed during the first minutes, but afterwards it is no longer cleared but even augmented, leading to neuronal death. Such effects are not observed in knockout animals lacking the GLT1 transporter (75). These observations lead to the conclusion that during the first minutes of ischemia, when energy levels are not exhausted, glutamate transporters operate normally eliminating released glutamate. However, when energy metabolism is severely altered, glutamate transporters operate in the reverse direction extruding glutamate to the extracellular space and contributing to cell death.

In summary, there are three mechanisms that might be mainly responsible for glutamate transport failure during cerebral ischemia: decreased uptake (probably due to oxidation of transporter proteins); inverse activity (resulting from the collapse of ionic gradients); and decreased expression (due to downregulation of transporter proteins) (Figure 3). The contribution of glutamate transporters either to the clearance or the release of glutamate, and therefore to the induction of neuronal death will be highly influenced by the duration of the ischemic period.

Sufficient evidence supports the participation of GLT1 transporter in glutamate removal during both physiological and ischemic conditions. However, the role of GLAST and neuronal transporters is still unknown. This is due in part to the lack of selective inhibitors of the different transporter subtypes. The understanding of glutamate uptake regulation during energy deficiency will be helpful to the knowledge of the mechanisms involved in ischemic neuronal damage, and in the design of new drugs with therapeutic potential.

Acknowledgments

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Receptores glutamatérgicos

Los receptores glutamatérgicos se dividen en dos grandes grupos: ionotrópicos y metabotrópicos. Los primeros se encuentran formando un canal iónico y permiten el paso de iones cuando se activan por su agonista. Los segundos se acoplan a proteínas G, que una vez activadas desencadenan señales intracelulares de segundos mensajeros, influyendo en la actividad o fisiología de la célula.

Receptores ionotrópicos

Los receptores ionotrópicos se dividen en tres subtipos de acuerdo al agonista por el cual se activan: N-metil-D-aspartato (NMDA), AMPA (ácido alfa-amino-3-hidroxi-5-metil-4-isoxazolepropiónico) y ácido kaínico (Kew and Kemp, 2005). Los receptores de las tres familias son de conformación heteromérica, conteniendo cada una más de un tipo de subunidad. A pesar de que la secuencia homóloga de aminoácidos entre los tres subtipos es de un 20-30%, comparten ciertas características en común.

Receptores a NMDA

Los receptores NMDA presentan características particulares que los distinguen de los otros tipos de receptores sensibles a ligandos. En primer lugar, sus canales iónicos están sujetos al bloqueo por el ión magnesio dependiente de voltaje. Segundo, los receptores son altamente permeables al ión calcio. Tercero, la activación inicial del receptor requiere de la presencia no sólo de glutamato sino de su co-agonista la glicina. Cuarto, su actividad esta regulada por H^{+}, Zn^{2+} y poliaminas.

Los receptores NMDA están conformados por las subunidades, NR1, NR2A-D y NR3A y B, cada un de ellas provienen de genes diferentes. Para el caso de la subunidad NR1 se han descrito ocho diferentes tipos formados por el corte y empalme alternativo del RNAm y una variante truncada no funcional (McBain and Mayer, 1994). Los subtipos que forman la subunidad NR2 contienen entre 40-55% de homología en su identidad de secuencia, y un 27% con la

subunidad NR1. Las subunidades NR3 presentan un 50% de homología en su identidad de secuencia, y comparten un 27% con NR1 y con NR2. La subunidad NR2 está codificada por cuatro genes diferentes NR2A-D. Para que un receptor sea funcional requiere la asociación de varias subunidades, siendo fundamental la presencia de al menos una NR1 y al menos una o más subunidades NR2, (como: NR1/NR2A, NR1/NR2B, o NR1/NR2A/NR2B) o NR1 en combinación tanto con las subunidades NR2 como NR3 (como: NR1/NR2A/NR3A). De hecho, el requerimiento mínimo para que un receptor sea funcional es un tetrámero compuesto de dos subunidades NR1 y dos NR2. El patrón de expresión de las subunidades NR2 y NR3 se restringe a cierto tipo de células y a un determinado tiempo de desarrollo (Kew et al., 1998; Monyer et al., 1992; Watanabe et al., 1994). Durante la etapa embrionaria y desarrollo temprano, el cerebro de los roedores y del humano, presenta principalmente la subunidad NR2B, mientras que la NR2D está presente en diencéfalo y cerebro medio. En los días postnatales, la expresión de la NR2A se establece en la mayoría de las regiones cerebrales, y la NR2C aparece posteriormente y de manera prominente en el cerebelo (Cull-Candy and Leszkiewicz, 2004). En el adulto hay un decremento en la expresión de la subunidad NR2B durante el tiempo de vida, lo cual se asocia con un incremento en la expresión de la subunidad NR2A (Cull-Candy and Leszkiewicz, 2004). En cambio, el patrón de expresión de la subunidad NR1, misma que es fundamental para que el receptor funcione, se mantiene desde el desarrollo embrionario y hasta el animal adulto (Ishii et al., 1993; Kutsuwada et al., 1992; Monyer et al., 1992). La subunidad NR3 también presenta un patrón de expresión espacio-temporal, ya que mientras la NR3A se expresa principalmente durante el desarrollo embrionario y llega a persistir en el adulto, la NR3B está confinada a motoneuronas somáticas del tallo y médula espinal (Nishi et al., 2001).

Las subunidades individuales del receptor a NMDA contienen un dominio N terminal extracelular (parte del cual forma la S1), un dominio C terminal intracelular, y un asa extracelular entre el M3 y el M4 que forma el S2 (Fig 4). El dominio que delimita el canal esta formado por un asa reentrante (M2) que se embebe en la membrana de fosfolípidos. La compuerta del canal parece estar

cerca de estos límites (Cull-Candy and Leszkiewicz, 2004). La selectividad del canal para el bloqueo por el ión magnesio y para la permeabilidad al calcio depende del residuo de asparagina localizado dentro del asa reentrante. Este sitio es homólogo al sitio denominado Q/R (glutamina-arginina) presente en las subunidades no-NMDA el cual controla la permeabilidad al calcio de los receptores a AMPA y kainato. El modelo propuesto mediante análisis bioquímicos, está de acuerdo con el modelo de los dominios de interacción con el ligando demostrado mediante la estructura cristalográfica (Armstrong et al., 1998). Como se muestra en la Figura 4, el glutamato se une al espacio que forman las regiones S1 y S2 de la subunidad NR2, mientras que la glicina interactúa con el que forman la S1 y S2 de la subunidad NR1.

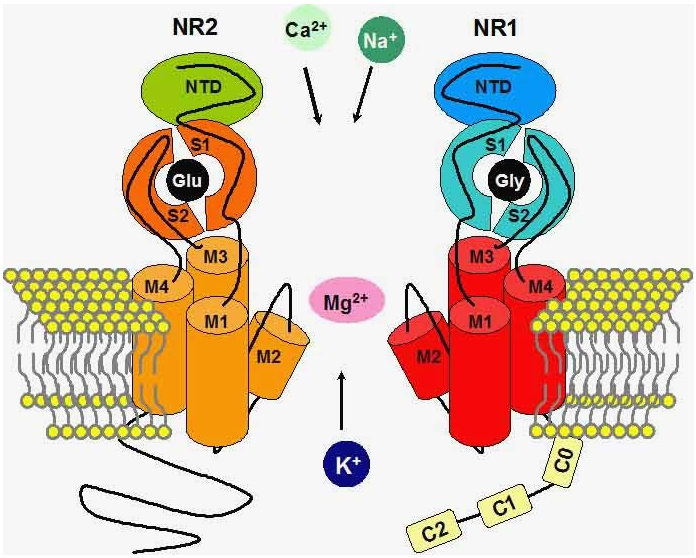

Fig. 4. Estructura de las subunidades NR2 y NR1 del receptor a NMDA. Las subunidades NR2 y NR1 se componen de diversas regiones que se encuentran conservadas. La región extracelular incluye el dominio N terminal (NTD) que contiene sitios moduladores que unen Zn^{2+} o ifenprodil. Los dominios S1 y S2 forman el sitio de unión para el Glu en la subunidad NR2 y para la Gly en la subunidad NR1. La región M2 que circunda el canal forma un poro re-entrante que conecta a la membrana con el espacio extracelular. El canal iónico es permeable a Na^{+}, K^{+} y Ca^{2+}. El magnesio extracelular causa un bloqueo sensible al voltaje.

La proporción diferente de subunidades NR1/NR2 que conforman a este receptor, le confiere una cinética característica de activación por glutamato. Tomando en consideración que la NR1 posee 8 diferentes isoformas de corte y empalme alternativo, más 4 y 2 genes que codifican las subunidades NR2 y NR3 respectivamente, se calcula que puedan ensamblarse hasta 384 distintos tipos de receptores a NMDA. En particular, la asociación NR1/NR2A presenta una

constante de activación de tiempo menor (50 ms), a la que se manifiesta con la asociación de NR1/NR2B (300 ms), esta última es muy similar a la que presenta la combinación NR1/NR2C (280 ms); siendo la combinación NR1/NR2D la que presenta la cinética más lenta (1.7 s) (Cull-Candy and Leszkiewicz, 2004). Estas características son de gran importancia en la excitabilidad neuronal y en el desarrollo de daño neuronal en diversas patologías, como se discutirá más adelante.

Receptores AMPA

Los receptores AMPA están compuestos de una familia de cuatro subunidades (GluR1-4) codificadas por genes separados y que se ensamblan formando tetrámeros funcionales (Rosenmund et al., 1998). Los receptores AMPA, semejante a los receptores a NMDA, tienen una composición heteromérica. En especial la subunidad GluR2 juega un papel crucial, ya que determina la permeabilidad al calcio. Receptores AMPA que no contienen a la subunidad GluR2 son permeables al calcio (Bowie and Mayer, 1995). El residuo que determina la impermeabilidad al calcio es el aminoácido arginina (R) localizado en el poro del asa, del dominio M2 de las subunidades GluR1, 3 y 4, y que corresponde a una glutamina en la subunidad GluR2 (Rosenmund et al., 1998; Seeburg and Hartner, 2003). Dado que los receptores AMPA permiten el paso de sodio como ión principal, su activación participa en la transmisión rápida de las sinapsis glutamatérgica, despolarizando a la célula e induciendo la activación de diversos canales sensibles a voltaje.

Receptores Kainato

Los receptores kainato están compuestos de dos familias de subunidades relacionadas, GluR5-7, KA1 y 2. Este subtipo está conformado de manera tetramérica en combinación homomérica o heteromérica. Los receptores KA1 y KA2 se combinan en ensamble heteromérico con miembros de la familia GluR5-7 y forman receptores funcionales (Bleakman et al., 2002). Las subunidades GluR5-

7 forman receptores homoméricos funcionales que se combinan con KA1 y KA2 formando receptores heteroméricos con distintas propiedades farmacológicas (Alt et al., 2004). Similar a las subunidades de los receptores a NMDA, las subunidades GluR5-7 se forman por corte y enpalme alternativo y forman nuevos receptores con heterogeneidad diferente. El patrón de expresión de cada subunidad es específico para las distintas regiones cerebrales: en el hipocampo la subunidad GluR6 se encuentra localizada a nivel pre y postsináptico en la mayoría de las fibras musgosas de la región CA3 (Contractor et al., 2000). La subunidad GluR5 se expresa predominantemente en interneuronas de hipocampo en donde media un efecto de facilitación de la liberación de glutamato por los astrocitos. GluR5 se expresa en el lóbulo temporal, y está muy relacionada con la excitación y la epileptogénesis en la amígdala (Rogawski et al., 2003). Además de las neuronas principales de CA3, se ha identificado la presencia de receptores postsinápticos de kainato en neuronas de la amígdala lateral, en interneuronas del hipocampo, en neuronas del asta dorsal de la médula espinal, en algunas células bipolares de la retina, en el corteza cerebral y en el cerebelo (Rodriguez-Moreno, 2006).

Además de la participación de los receptores tipo kainato en la despolarización de la membrana plasmática, algunos experimentos han propuesto que estos receptores también pueden ser de tipo metabotrópico acoplados a la activación de proteínas G y localizarse en terminales presinápticas. De esta forma, la activación de los receptores promoverá la activación de la PKC y la inhibición de la corriente de K^+ dependiente de Ca^{2+}. La consecuencia final de su activación es la repolarización de la membrana celular y la subsiguiente inhibición de la transmisión sináptica. De hecho la inhibición de la corriente asociada a la activación de los receptores a kainato incrementa drásticamente la excitabilidad de la membrana y la liberación de transmisores (Lerma, 2006).

Receptores metabotrópicos

Los receptores metabotrópicos representan un nuevo grupo que comprende al menos ocho diferentes subtipos, divididos de acuerdo a su secuencia,

farmacología y al mecanismo de transducción de señales al que se encuentran acoplados. En general, su estructura posee un largo extremo extracelular bilobular que representa el dominio N-terminal, el cual se ha propuesto como el sitio al que se une el glutamato de acuerdo a estudios de mutagénesis y cristalografía de rayos X. El dominio N-terminal se une mediante una región rica en cisteínas al dominio de siete segmentos transmembranales, el cual interviene en la activación de las proteinas G. El carboxilo terminal es intracelular y está involucrado en la regulación de la actividad del receptor y del tráfico del mismo, mediante su asociación con varias proteínas incluyendo la calmodulina y proteínas estructurales como las de la familia Homer y PICKI (Kew and Kemp, 2005). Existen tres grupos de receptores metabotrópicos: grupo I que incluye a los subtipos $Glum_1$ y $Glum_5$ y que está acoplado positivamente a la fosfolipasa C, el grupo II que incluye a $Glum_2$ y $Glum_3$; y el grupo III incluyendo a $Glum_4$, $Glum_6$, $Glum_7$ y $Glum_8$, estos dos últimos grupos inhiben la actividad de la adenilato ciclasa (Swanson et al., 2005). Los subtipos del grupo I se expresan en la mayoría de las sinapsis glutamatérgicas a nivel postsináptico. La función de este grupo está vinculada a la plasticidad sináptica, incluyendo la potenciación y la depresión a largo plazo y se relaciona con desórdenes de ansiedad. En el grupo II se encuentran los subtipos que se localizan tanto a nivel presináptico ($Glum_2$ y $Glum_3$) como postsináptico ($Glum_3$). La distribución del $Glum_2$ es abundante a nivel de hipocampo y amígdala. Funcionalmente el $Glum_2$ está relacionado con la depresión a largo plazo. Por su parte el subtipo $Glum_3$ se distribuye en hipocampo y en tálamo, y se encuentra asociado a la liberación de neurotrofinas de las células glíales, ambos subtipos también intervienen en los desórdenes de ansiedad. Los subtipos del grupo III poseen la mayor diversidad en cuanto a función y localización se refiere. Los subtipos $Glum_4$ y $Glum_7$ se localizan tanto pre como postsinápticamente. El subtipo $Glum_4$ a nivel presináptico está relacionado con la plasticidad sináptica y aprendizaje en las fibras paralelas del cerebelo. Por su parte el $Glum_7$ a nivel presináptico se localiza en las zonas activas de la sinapsis, proponiéndolo como un autoreceptor con función inhibidora. El $Glum_6$ es un subtipo especial ya que su expresión está confinada a las células bipolares de

la retina, por lo que su función es importante en los procesos de visión. Finalmente el subtipo $Glum_8$ esta principalmente localizado a nivel presináptico en las sinapsis glutamatérgicas y en algunos otros tipos. Se distribuye abundantemente en el hipocampo y amígdala, por lo que su función está relacionada con la ruta perforante lateral.

Muerte neuronal excitotóxica

El término excitotoxicidad, fue acuñado hace más de 30 años a partir de algunos experimentos pioneros del grupo de Olney y colaboradores (Olney, 1971; Olney and Ho, 1970; Olney and Sharpe, 1969). Durante este tiempo aún no se conocía que el glutamato tenía un papel dual dentro del SNC, como un neurotransmisor que promueve la sobrevivencia neuronal, y como una neurotoxina, ya que su administración intracerebral a ratas adultas o bien intraperitoneal a ratas recién nacidas, genera muerte neuronal (Olney, 1969; Olney and Ho, 1970).

A partir de esos años se definió a la excitotoxicidad como la propiedad que tiene el glutamato para generar muerte neuronal a través de la sobreactivación de sus receptores de tipo NMDA, AMPA y KA (Olney, 1969; Olney and Sharpe, 1969). Debido a que los receptores de tipo NMDA son principalmente permeables a calcio, una vez que son activados por su agonista, la activación prolongada de éstos genera la entrada masiva del ión al citoplasma y la sobrecarga del mismo. Intracelularmente el calcio activará diversas rutas de muerte neuronal, como la activación de fosfolipasas que degradan fosfolípidos y liberarán ácido araquidónico activo (Lazarewicz et al., 1990). El metabolismo del ácido araquidónico por oxidasas genera radicales libres, que desencadenan la degradación de lipídos de membrana (Chan and Fishman, 1982). El calcio también activará proteasas, como la calpaina I que degradará a proteínas estructurales como fodrina, espectrina, tubulina, y MAP2 causando alteración o rompimiento del citoesqueleto (Siman and Noszek, 1988). La activación de fosfatasas por calcio, aunado a la formación de diacilglicerol, activa a proteínas cinasas sensibles a Ca^{2+}, generando la fosforilación de diversas proteínas. La activación de nucleasas, romperá el DNA

genómico. El calcio inclusive inducirá daño mitocondrial, generando un desbalance en la homeostasis neuronal hasta producir la muerte (Choi, 1988; Choi and Rothman, 1990; Schinder et al., 1996) (Fig. 5).

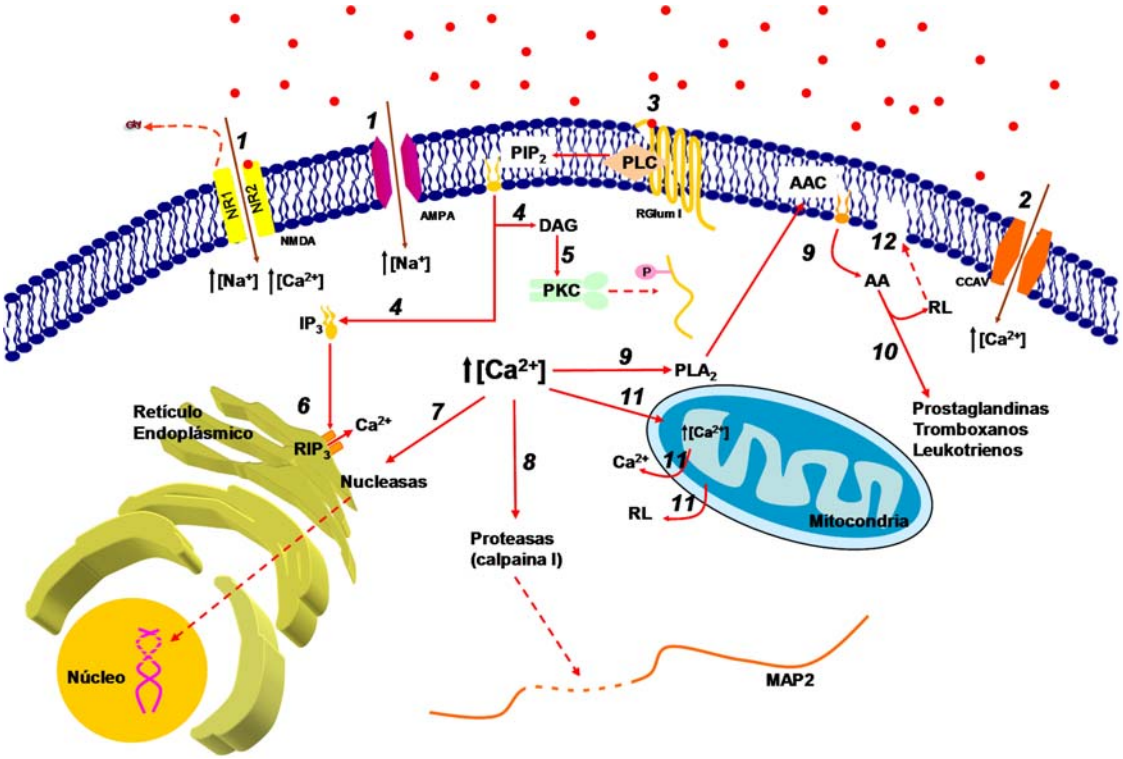

Fig. 5. Daño celular excitotóxico por Glu. La acumulación descontrolada de Glu en el espacio sináptico (círculos rojos), genera la sobreactivación de los receptores glutamatérgicos localizados en la membrana postsináptica. Por el mecanismo descrito en la Fig. 2, la activación de los receptores AMPA y NMDA promueven la entrada de sodio y calcio (1). La sobreactivación de mas receptores promoverá la despolarización de la membrana y la activación de canales adicionales dependientes de voltaje, como los CCAV, exacerbando el incremento citoplasmático de calcio (2). Una tercera vía vinculada a la sobrecarga de calcio en el citoplasma esta ligada a la activación de RGlum I (3). La unión del Glu a este receptor promueve la activación de PLC y la subsecuente hidrólisis de PIP2, formando IP3 y DAG (4). Mientras que DAG activa a PKC promoviendo la fosforilación de proteínas (5), IP3 se une a RIP3 del retículo endoplasmático induciendo la salida de calcio (6). La sobrecarga de calcio activara diversas rutas de daño celular: nucleasas que degradaran el DNA nuclear (7); proteasas que degradaran a proteínas del citoesqueleto y membrana celular (8); fosfolipasas (PLA2) que harán blanco en fosfolípidos de la membrana celular liberando AA (9); el metabolismo de AA por la acción de ciclooxigenasas y lipooxigenasas promovera la formacion de RL (10); una segunda vía de formación de RL se debe a la sobrecarga de calcio en la mitocondria y a la salida de este mismo al citoplasma (11); los RL promueven la peroxidación de lípidos de la membrana (12). PLA2, Fosfolipasa A2; AA, Ácido araquidónico; AAC, Ácido araquidonil alkil, p-colina; RL, Radicales libres; RIP3, Receptor a inositol trifosfato; IP3, Inositol trifosfato; DAG, Diacil glicerol; PIP2, Inositol 1,4 bifosfato; PLC, Fosfolipasa C; RGlum I, Receptor a glutamato metabotropico tipo I.

Papel del glutamato en el daño neuronal asociado a algunas enfermedades neurodegenerativas crónicas y enfermedades neurológicas agudas

Aunque el glutamato está asociado a procesos de memoria y aprendizaje entre otros procesos fisiológicos, a lo largo de los años se le ha vinculado con el desarrollo de enfermedades neurodegenerativas crónicas y desórdenes

neurológicos agudos. Se ha propuesto que un mecanismo excitotóxico forma parte de la muerte neuronal en estas condiciones.

Enfermedades neurodegenerativas crónicas

Como enfermedades neurodegenerativas crónicas podemos citar a la enfermedad de Alzheimer, Huntington, la epilepsia, la esclerosis múltiple, esclerosis lateral amiotrófica y la demencia asociada al SIDA.

La enfermedad de Alzheimer, es un desórden neurodegenerativo caracterizado por la pérdida irreversible de la memoria, culminando con la demencia completa del individuo que la padece. La enfermedad constituye cerca del 60-65% de los casos de demencia en la población adulta. La formación de placas neuríticas, que están constituidas por agregados de la proteína β-amiloide y filamentos helicoidales apareados, representa el signo característico de la patología del Alzheimer. Se han propuesto varias hipótesis para explicar la base patogénica del Alzheimer, entre ellas se incluye a la excitotoxicidad por glutamato, y al estrés oxidativo con la subsiguiente peroxidación de lípidos inducida por agregados del péptido β-amiloide. Neuroquímicamente el daño cognitivo en el Alzheimer está vinculado a la muerte de las neuronas colinérgicas de la corteza cerebral. La presencia de demencia también se ha relacionado con la muerte neuronal producida por glutamato (Sonkusare et al., 2005). Se ha demostrado un incremento en los niveles de glutamato en el líquido cefalorraquideo de pacientes con Alzheimer (Csernansky et al., 1996; Hoyer and Nitsch, 1989; Jimenez-Jimenez et al., 1998), y se ha asociado un componente excitotóxico a la neurodegeneración en esta enfermedad (Choi, 1988; Csernansky et al., 1996; Schwarcz and Meldrum, 1985). Se sugiere que la participación del mecanismo excitotóxico de muerte en la enfermedad de Alzheimer está relacionado con alteraciones en la neurotransmisión glutamatérgica como son, el decremento en la captura de glutamato, la disminución en la expresión de sus transportadores detectada en cerebros *postmortem* de pacientes (Arzberger et al., 1997; Ferrarese et al., 2000; Heath and Shaw, 2002; Zoia et al., 2004), y cambios en la expresión de los

receptores glutamatérgicos a NMDA (Arning et al., 2005; Arzberger et al., 1997; Bi and Sze, 2002; Mishizen-Eberz et al., 2004).

La enfermedad de Huntington es de naturaleza hereditaria originada por una mutación en el primer exón del gen de la huntingtina localizado en el brazo corto del cromosoma 4. La expresión normal de este gene codifica para la proteína conocida como huntingtina, que en su extremo N-terminal presenta un fragmento polimórfico que contiene un número variable (entre 3-30) de residuos del aminoácido glutamina. La manifestación fenotípica de la enfermedad de Huntington es el movimiento involuntario constante de manos, brazos, tronco e incluso cara, alteraciones en la marcha y pérdida de la capacidad para realizar movimientos finos. En etapas avanzadas se presenta la aparición de movimientos involuntarios llamados coreicos, por su semejanza con posturas de danzantes. Evidencia experimental sugiere que la muerte neuronal excitotóxica mediada por la activación de receptores a glutamato, así como una deficiencia en el metabolismo energético, son dos componentes involucrados en la patogénesis de dicha enfermedad. De manera similar a la enfermedad de Alzheimer, se ha propuesto que un aumento en la excitotoxicidad glutamatérgica puede jugar un papel importante en su etiología (Ferrante et al., 1993; Storey and Beal, 1993; Storey et al., 1992). Modelos experimentales han demostrado que la administración intraestriatal del agonista endógeno de los receptores glutamatérgicos, ácido quinolínico (QUIN), o la administración de excitotoxinas reproduce en la rata características histopatológicas y conductuales similares a las de pacientes con la enfermedad de Huntington (Beal et al., 1986). La progresión de la enfermedad se retrasa por la administración de antagonistas de los receptores glutamatérgicos (Beister et al., 2004; Zeron et al., 2002), sugiriendo la participación de un mecanismo excitotóxico en el daño. Además, algunos estudios sugieren que los niveles de QUIN, están aumentados en etapas tempranas en el cerebro de los pacientes con la enfermedad de Huntington (Guidetti et al., 2004). Por otra parte, algunas evidencias señalan que existen alteraciones en el transporte de glutamato tanto en los pacientes (Arzberger et al., 1997; Cross et al., 1986), como en los modelos transgénicos de la enfermedad (Behrens et al., 2002;

Lievens et al., 2001; Shin et al., 2005), sugiriendo deficiencias en la remoción de glutamato del espacio extracelular. Sin embargo, la concentración extracelular de glutamato es muy similar en animales transgénicos R6/2 presimtomáticos y ratones silvestres (Gianfriddo et al., 2004), por lo que aún no está del todo clara la relación entre el incremento de glutamato y los síntomás en el animal. Lo que sí está claro es que bajo condiciones despolarizantes inducidas por concentraciones altas de KCl, la acumulación de glutamato en el estriado es mayor en los ratones transgénicos R6/1 que en los silvestres (Nicniocaill et al., 2001). Se ha encontrado también que en animales transgénicos que contienen la huntingtina mutada, la estimulación de los receptores a NMDA induce una respuesta con mayor amplitud de corriente, lo cual posiblemente se asocie al incremento de la subunidad NR2B (Ravenscroft and Brotchie, 2000). Alteraciones en la expresión de las subunidades del receptor a NMDA y de su RNAm pueden estar asociadas a la enfermedad (Arning et al., 2005; Arzberger et al., 1997). En particular, estudios *in vitro* en células de los ratones transgénicos YAC 72 sugieren que son más vulnerables a la muerte excitotóxica mediada por NMDA (Zeron et al., 2004). Sin embargo, en estudios *in vivo* no se ha dado evidencia de que los ratones transgénicos de huntingtina sean vulnerables a la muerte excitotóxica (Hansson et al., 1999). Así, el papel de las alteraciones en el transporte de glutamato y en la expresión de las subunidades del receptor a NMDA en el desarrollo de la enfermedad de Huntington, no se ha dilucidado.

La epilepsia es un desórden neurológico en el cual la función normal del cerebro se interrumpe como consecuencia de la intensa actividad de disparo de un grupo o conjunto específico de neuronas. Se ha sugerido que el glutamato es el principal neurotransmisor involucrado en la patogénesis de esta enfermedad (Meldrum, 1994). Cerebros de pacientes epilépticos presentan un incremento de los niveles extracelulares de glutamato y glicina previos a la generación de una crisis epiléptica (Meldrum, 1994; Sherwin, 1999), que correlaciona muy bien con la generación de muerte neuronal en el foco epiléptico.

La esclerosis múltiple es un desorden inflamatorio, desmielenizante del SNC de etiología desconocida. Las principales características patológicas de la

enfermedad son la presencia de linfocitos en el cerebro y médula espinal. El papel del glutamato en este desorden es desconocido; sin embargo, algunos trabajos han sugerido un incremento en la concentración de glutamato en el líquido cefalorraquídeo cerebro espinal de pacientes con esclerosis múltiple, aunque en otros estudios esto no se ha comprobado (Groom et al., 2003). Dado que el empleo de antagonistas de los receptores glutamatérgicos, en especial los del tipo AMPA, previenen la muerte de neuronas motoras y oligodendrocitos en esta enfermedad, se ha propuesto al glutamato como un agente causal asociado a este padecimiento. Adicionalmente, se ha vinculado al glutamato y a la activación de los receptores AMPA, como el causante de enfermedades inflamatorias como la encefalitis de Rasmussen y el síndrome neurodegenerativo paraneoplástico (Groom et al., 2003).

La esclerosis lateral amiotrófica es una enfermedad neurodegenerativa crónica que involucra la muerte progresiva de motoneuronas de la médula espinal y corteza cerebral (Cleveland and Rothstein, 2001). La muerte selectiva de las neuronas se ha asociado con la activación de los receptores AMPA. En esta enfermedad se ha descrito una reducción de la expresión del transportador GLT1 en la médula espinal de las células glíales, lo que la vincula con un mecanismo de daño excitotóxico (Rothstein, 1995a; Rothstein, 1995b). Sin embargo, aún no esta claro si un incremento en los niveles extracelulares de glutamato en el líquido cefalorraquídeo puede ser el causante de la muerte neuronal. Esto se sugiere ya que existen reportes que apoyan esta alternativa, aunque otros la descartan (Kostera-Pruszczyk et al., 2002; Niebroj-Dobosz and Janik, 1999; Plaitakis and Caroscio, 1987; Rothstein et al., 1990; Shaw et al., 1994).

La demencia asociada al SIDA se manifiesta con la muerte neuronal de regiones subcorticales de pacientes adultos y una progresiva encefalopatía de infantes y niños. En los pacientes adultos se presenta una disfunción en el desarrollo cognitivo y en la conducta. Esto se asocia con una atrofia cortical y una pérdida desproporcionada del volumen de los ganglios basales (Aylward et al., 1993). La neuropatología de la infección por HIV-1 incluye la presencia de macrófagos derivados de la sangre infectada con el virus y microglía reactiva. Se

ha propuesto que los productos inflamatorios de la microglía activa están implicados en el daño neuronal excitotóxico (Giulian et al., 1990). Adicionalmente, monocitos infectados por HIV-1 liberan TNF-$\propto$ que bloquea la captura de glutamato en los astrocitos. Esto correlaciona con un incremento en los niveles cerebrales de glutamato y con la atrofia cerebral de pacientes (Ferrarese et al., 2001). La participación de la activación de receptores a glutamato en la muerte neuronal asociada al SIDA está aún más fundamentada por un estudio reciente en donde se demostró el incremento en los niveles cerebrales del QUIN en pacientes seropositivos a la enfermedad (Valle et al., 2004).

Enfermedades neurológicas agudas

Las enfermedades neurológicas agudas son aquellos en donde la muerte neuronal se manifiesta durante un periodo corto de tiempo que puede abarcar de algunas horas hasta un par de días. Entre ellas se puede mencionar a la isquemia cerebral, al trauma craneoencefálico y a la hipoglicemia aguda. En éstas se ha propuesto que la muerte neuronal ocurre por un mecanismo excitotóxico.

Dado que el metabolismo cerebral requiere de un aporte ininterrumpido de oxígeno y glucosa para suplir sus necesidades energéticas basales, durante el decremento en el flujo sanguíneo que ocurre en la isquemia cerebral, o bien durante un accidente vascular cerebral o trauma craneoencefálico, se generan dos zonas de daño neuronal: 1) el centro isquémico o de muerte, donde se presenta la más severa reducción del flujo sanguíneo y las neuronas mueren irremediablemente; y 2) la zona de penumbra, adyacente al centro isquémico y en la cual existe irrigación colateral, lo que la convierte en una zona potencialmente rescatable de la muerte neuronal. Se ha sugerido que el desarrollo de la muerte neuronal bajo estas condiciones, se debe a un incremento de los niveles extracelulares de AAE (ver más adelante) (Benveniste et al., 1984; Takagi et al., 1993; Yi and Hazell, 2006). De manera similar, durante la hipoglicemia aguda inducida por insulina, así como durante la inhibición farmacológica de la vía glicolítica en el cerebro, se produce un incremento de los niveles extracelulares de AAE (Massieu et al., 2000; Sandberg et al., 1986; Sandberg et al., 1985). Se han

propuesto dos mecanismos moleculares para explicar la acumulación de AAE durante eventos de isquemia e hipoglucemia, 1) el decremento del contenido de proteína de los transportadores de glutamato (Chen et al., 2005; Kim et al., 2006; Rao et al., 2001a; Yeh et al., 2005), y 2) el funcionamiento deficiente de los mismos (Allen et al., 2004; Mitani and Tanaka, 2003; Yeh et al., 2005). Diversos grupos de trabajo han propuesto que tales alteraciones preceden a la muerte neuronal (Raghavendra Rao et al., 2000; Rao et al., 2001b). La acumulación extracelular de AAE es la responsable de inducir muerte neuronal de tipo excitotóxica en la zona de penumbra ya que la administración de antagonistas de los receptores glutamatérgicos previene el daño neuronal (Akins and Atkinson, 2002; Wang and Shuaib, 2005). Por otra parte, la muerte neuronal inducida por la hipoglicemia aguda puede también prevenirse por la administración de antagonistas del receptor a NMDA (Wieloch, 1985). Adicionalmente, se han descrito alteraciones en el patrón de expresión de algunas subunidades que forman el receptor a NMDA durante eventos de isquemia e hipoglicemia (Gascon et al., 2005; Quintana et al., 2006; Sutcu et al., 2005; Zhang et al., 1997), lo cual posiblemente está relacionado con la alteración en la neurotransmisión glutamatérgica y con el incremento en la susceptibilidad a la muerte neuronal mediada por la acumulación de glutamato.

Metabolismo glicolítico cerebral y muerte neuronal

La demanda energética que requiere el cerebro para funcionar correctamente es muy alta, por lo que alteraciones en la producción de ATP, pueden tener consecuencias graves en la sobrevivencia neuronal. A pesar de que la vía glicolítica aporta solo del 4.6-7.7% del ATP total, su correcto funcionamiento es esencial en el desarrollo óptimo de la neurotransmisión glutamatérgica (Hyder et al., 2006; Magistretti et al., 1999; Pellerin and Magistretti, 1994; Sibson et al., 1997). Experimentos *in vitro* en cultivos de astrocitos indican que la inhibición farmacológica del metabolismo glicolítico inducida por yodoacetato (IOA, un inhibidor de la enzima gliceraldehído 3-fosfato deshidrogenasa (G3PDH), induce un incremento de los niveles extracelulares de AAE, que es precedido por el

decremento agudo en los niveles de ATP (Gemba et al., 1994; Massieu et al., 2000; Ogata et al., 1995). Asimismo, la administración de IOA directamente en el hipocampo de la rata induce una disminución en los niveles de ATP y un incremento en la concentración extracelular de AAE (Massieu et al., 2000; Massieu et al., 2003). La deficiencia energética aguda puede producirse durante un evento isquémico, hipoglucémico o como consecuencia de la inhibición farmacológica del metabolismo glicolítico o mitocondrial (Greene and Greenamyre, 1995; Hyder et al., 2006; Massieu et al., 2000; Massieu et al., 2003; Swanson et al., 1997a; Zeevalk et al., 1998). La pérdida de ATP en estas circunstancias dará lugar al funcionamiento deficiente de las ATPasas y por tanto al colapso de los gradientes iónicos. En consecuencia, la actividad de los transportadores de glutamato puede disminuir, o incluso siguiendo el gradiente electroquímico del sodio, funcionar en dirección opuesta, incrementando las concentraciones de AAE en el espacio extracelular (Longuemare et al., 1999; Nicholls and Attwell, 1990; Phillis et al., 2000; Rossi et al., 2000). Adicionalmente a este mecanismo se ha sugerido la participación de rutas alternativas que den lugar a la liberación de glutamato, como la activación de canales aniónicos sensibles a volumen, la liberación exocitótica dependiente de calcio y la liberación de AAE inducida por la activación de hemicanales en los astrocitos (Dawson et al., 2000; Seki et al., 1999; Ye et al., 2003). Sin embargo, el papel que juega cada una de estas rutas en la liberación de AAE durante la inhibición glicolítica y su papel en la muerte neuronal no se había explorado a la fecha y constituye uno de los objetivos de estudio de la presente tesis.

Si bien es cierto que el incremento en los niveles extracelulares de AAE produce muerte neuronal de tipo excitotóxica por la sobreactivación de los receptores glutamatérgicos (Choi, 1988), evidencia experimental señala que la inhibición farmacológica de los transportadores para AAE *in vivo* no induce muerte neuronal, a pesar de la acumulación extracelular de AAE (Massieu et al., 1995; Massieu and Tapia, 1997). Lo anterior sugiere la participación de factores adicionales involucrados en la susceptibilidad al daño neuronal por AAE. Estudios *in vitro* han propuesto que la alteración del estado energético durante la inhibición

del metabolismo mitocondrial, o bien por la exposición a un medio sin glucosa, exacerban la muerte neuronal inducida por glutamato (Greene and Greenamyre, 1995; Novelli et al., 1988; Pang and Geddes, 1997; Zeevalk and Nicklas, 1990). De la misma manera, experimentos *in vivo* han demostrado que la inhibición metabólica inducida por la administración de inhibidores del metabolismo mitocondrial o glicolítico, facilita el daño excitotóxico en el hipocampo y el estriado de la rata (Greene and Greenamyre, 1995; Massieu et al., 2000; Sanchez-Carbente and Massieu, 1999). Los mecanismos responsables de dicha facilitación no se conocen, pero es posible que la alteración de los niveles energéticos desencadene mecanismos moleculares adicionales a la salida de AAE que contribuyan al daño excitotóxico. En este sentido, es posible que una alteración parcial, pero sostenida, del metabolismo energético no sea suficiente para inducir un cambio en la dirección del funcionamiento del transportador de glutamato, pero sí podría promover cambios persistentes en otros sistemas que contribuyan a la cascada excitotóxica. Por ejemplo, se han descrito alteraciones en los sistemas de recaptura de AAE y de sus receptores en cerebros *postmortem* de pacientes con las enfermedades de Alzheimer y Huntington, las cuales podrían promover la sobreactivación de receptores a glutamato, el incremento en la concentración de calcio citoplasmático y el daño neuronal excitotóxico. Por otra parte, alteraciones crónicas del metabolismo glicolítico se han descrito en pacientes con la enfermedad de Alzheimer y de Parkinson, así como durante el proceso de envejecimiento (Joo et al., 1999; Mazzola and Sirover, 2001; Mazzola and Sirover, 2002; Mazzola and Sirover, 2003; Planel et al., 2004; Roberts and Chih, 1995). De hecho, en la enfermedad de Alzheimer y de Parkinson se presenta un decremento de la actividad de la enzima G3PDH (Kish et al., 1998; Mazzola and Sirover, 2001; Mazzola and Sirover, 2003), mientras que durante el envejecimiento se observan reducciones tanto en el metabolismo glicolítico, como en los niveles de sustratos energéticos cerebrales (Hoyer, 1985; Roberts and Chih, 1995).

V. PLANTEAMIENTO DEL PROBLEMA

En esta tesis estudiamos la participación del metabolismo glicolítico en la regulación de los niveles extracelulares de AAE, y en la muerte neuronal inducida por glutamato en dos modelos experimentales, uno agudo y uno crónico.

Como hemos señalado, la liberación de AAE durante la isquemia cerebral involucra la participación de diversas rutas; sin embargo, muy poca atención se ha dado a los mecanismos involucrados en la liberación de AAE inducida durante la hipoglicemia aguda *in vivo*. Estudios recientes de nuestro laboratorio, han demostrado que la inhibición farmacológica aguda del metabolismo glicolítico inducida por la administración de IOA en el hipocampo de la rata, induce un incremento de los niveles extracelulares de glutamato y principalmente de aspartato, lo que se traduce en muerte neuronal (Massieu et al., 2000). La liberación de los AAE puede ser la consecuencia del funcionamiento inverso de sus transportadores, ya que este mecanismo podría favorecerse debido a que los niveles de ATP disminuyen cerca del 50% en estas condiciones (Massieu et al., 2003)**.** Sin embargo, otras rutas como la exocitosis y la liberación inducida por hinchamiento celular también podrían estar participando.

Con base en lo anterior, en un modelo <u>in vivo</u> estudiamos las posibles rutas de liberación de AAE asociadas a la inhibición aguda del metabolismo glicolítico, así como su participación en la muerte neuronal en el hipocampo de rata.

Por otra parte, nuestro grupo de trabajo ha demostrado que la inhibición sostenida de la glicólisis no produce muerte neuronal *per se,* pero sí facilita los efectos tóxicos del glutamato administrado localmente en el hipocampo de la rata (Massieu et al., 2000). Trabajos previos indican que este tratamiento induce un decremento de sólo el 20% en los niveles de ATP en el hipocampo (Massieu et al., 2003), el cual posiblemente no sea suficiente para inducir la operación inversa de los transportadores de glutamato. Alternativamente la alteración moderada pero sostenida de la vía glicolítica podría producir alteraciones en la captura de glutamato y/o en sus receptores, facilitando así la inducción de la muerte neuronal excitotóxica.

Con base en lo anterior, en el segundo modelo experimental se determinó, el efecto de la inhibición sistémica sostenida de la vía glicolítica sobre el transporte de glutamato y los niveles de proteína de las subunidades del receptor a NMDA.

VI. HIPÓTESIS

I. La liberación de AAE inducida durante la falla aguda del metabolismo glicolítico estará mediada por la operación inversa de los transportadores de glutamato y generará muerte neuronal. Posiblemente participen también la liberación exocitótica y la relacionada con la activación de los canales de cloro dependientes de volumen.

II. La inhibición moderada y sostenida de la glicólisis dará lugar a alteraciones en los sistemas de recaptura de AAE y en el contenido de las distintas subunidades del receptor a NMDA.

VII. OBJETIVOS

Objetivo 1

Identificar las rutas involucradas en la liberación de glutamato y aspartato durante la administración de IOA en el hipocampo de la rata y su participación en el daño neuronal.

Para cumplir este objetivo se determinó:

- El efecto de inhibidores de: 1) la liberación exocitótica dependiente de calcio; 2) de los canales aniónicos sensibles a volumen (CASV), y 3) de los transportadores de glutamato, sobre la liberación de AAE inducida por IOA en el hipocampo de la rata.
- El efecto de los tratamientos descritos en el párrafo anterior sobre el volumen de la lesión inducida por IOA en el hipocampo de la rata.

Objetivo 2

Determinar si la inhibición sostenida de la glicólisis altera los sistemas de recaptura de AAE y el contenido de las diferentes subunidades del receptor a NMDA.

Para cumplir este objetivo se determinó:

- La actividad enzimática de la G3PDH en el hipocampo de la rata antes, durante y después del tratamiento con IOA
- Los niveles extracelulares de aspartato y glutamato antes, durante y después del tratamiento con IOA
- La captura de [^{3}H]-glutamato en rebanadas de hipocampo de ratas control y tratadas con IOA
- El contenido de proteína de los transportadores de AAE, GLT-1, GLAST y EAAC1 antes, durante y después del tratamiento con IOA
- El contenido de proteína de las subunidades NR2A, NR2B y NR1 del receptor a NMDA antes, durante y después del tratamiento con IOA

JUSTIFICACIÓN

El presente trabajo de tesis tiene como finalidad estudiar las posibles rutas relacionadas en la generación de la muerte neuronal durante la inhibición de la vía glicolítica. Este estudio es muy importante para poder conocer las alteraciones y posibles rutas de señalización que participan en la generación de muerte neuronal durante el trauma craneoencefálico, isquemia cerebral e hipoglicemia.

VIII. MODELO EXPERIMENTAL 1

Inhibición aguda del metabolismo glicolítico por yodoacetato (IOA)

MATERIALES Y MÉTODOS

- Identificación de las rutas de liberación de AAE durante la inhibición glicolítica

El efecto de la inhibición glicolítica por la administración aguda de IOA sobre los niveles extracelulares de AAE se estudió a través de microdiálisis. Se utilizaron ratas macho de la cepa Wistar (250-320 g) que fueron colocadas en un aparato estereotáxico para la realización de la cirugía. Los experimentos de microdiálisis se llevaron a cabo en la región CA1 del hipocampo siguiendo las siguientes coordenadas de acuerdo al atlas de Paxinos y Watson (1986), AP= -3.6, L= +2.0 y V= -2.0. La microdiálisis se inició perfundiendo medio Ringer-Krebs normal que contiene (en mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.18, KH_2PO_4 1.2, $NaHCO_3$ 25, glucosa 10 y calcio 2.5, a un flujo de 2 µl/min durante 1.5 horas para lograr la estabilización de la membrana de diálisis. Se trabajó con cuatro grupos experimentales, en el primero se perfundió IOA, en el segundo grupo IOA más el bloqueador de la liberación exocitótica dependiente de calcio, Riluzol; en el tercero IOA más uno de los bloqueadores de los canales aniónicos activados por volumen, ácido 4,4'-dinitrostilbene-2,2'-disulfonico (DNDS), ácido 5-nitro-2-(3 fenilpropilamino benzoico (NPPB), tamoxifen o floretina. En el último grupo se co-perfundió IOA más uno de los bloqueadores de los transportadores de AAE: DL-β-benziloxiaspartato (DL-TBOA) o ácido L-trans-2,4-Pirrolidin dicarboxilato (PDC). Después de la estabilización de la membrana de diálisis, en todos los grupos experimentales se colectaron 10 fracciones de 25 µl cada una. En el caso del primer grupo experimental las colectas se dividieron como sigue: 3 fracciones basales perfundiendo medio Ringer-Krebs, 4 fracciones perfundiendo IOA y en las últimas cuatro fracciones se perfundió nuevamente el medio Ringer-Krebs. Para el segundo, tercero y cuarto grupo experimental se perfundió sólo una fracción con medio Ringer-Krebs para obtener los valores basales de aminoácidos extracelulares. En las dos siguientes fracciones se perfundíó uno de los inhibidores a estudiar: Riluzol (1 mM), DNDS (10 mM), NPPB (1 mM), tamoxifen

(250 µM), floretina (1 mM), TBOA (100 y 500 µM) o PDC (1 y 5 mM). Durante las cuatro siguientes fracciones se co-perfundió IOA (1 mM) y cada uno de los diferentes inhibidores, y en las últimas 3 fracciones se perfundió nuevamente el medio Ringer-Krebs.

- Liberación de AAE inducida por despolarización con alto potasio después del bloqueo de sus transportadores

Con el fin de descartar el agotamiento de las pozas intracelulares de AAE después de la perfusión de IOA más los inhibidores del transporte de glutamato, en una serie de experimentos se estudió el efecto de la despolarización con KCl sobre los niveles de glutamato y aspartato, después de la perfusión de los fármacos mencionados.

- Análisis de los niveles basales de AAE en presencia de los bloqueadores de los CASV

La participación de los canales aniónicos sensibles a volumen sobre los niveles basales de AAE se estudió a través de la perfusión de los bloqueadores, DNDS (10 mM) y NPPB (1 mM) en el hipocampo de la rata. Para ello, se perfundieron tres fracciones basales con medio Ringer-Krebs, 4 fracciones con DNDS o NPPB, y en las últimas cuatro fracciones se perfundió nuevamente el medio Ringer-Krebs

- Análisis del contenido de aminoácidos extracelulares

El contenido de aminoácidos en las fracciones de la microdiálisis se determinó por HPLC, utilizando una técnica modificada a la descrita por (Antoine et al., 1999). Las colectas de 25 µl se derivatizaron con el mismo volumen de o-phtalaldehído y después de tres minutos de incubación se inyectaron 10 µl en el sistema HPLC (Waters). Se utilizó una columna ODS (25 cm X 4 mm de diámetro interno, Supelco, Inc). Se usó una fase móvil conteniendo metanol:acetonitrilo:isopropanol:amortiguador de fosfatos (60 mM,

pH=6.65)/amortiguador de fosfatos (60 mM, pH=6.65). Se utilizó un gradiente a porcentajes de 10 y 90%, respectivamente. Las muestras se analizaron a un flujo de 1 ml/min durante 33 minutos. La concentración de aminoácidos se calculó comparando cada muestra con cromatogramas obtenidos de muestras tratadas de manera idéntica y conteniendo concentraciones conocidas de aminoácidos (10 pmolas de aminoácido/µl de muestra = µM).

- *Obtención y procesamiento de las muestras de hipocampo*

Se hizo la tinción de Nissl para realizar el análisis histológico de los cerebros de ratas tratadas y determinar el volumen de lesión en la región CA1 del hipocampo. Para ello, 24 h después de cada experimento, las ratas se anestesiaron con 63 mg de pentobarbital administrado por vía intraperitoneal y se perfundieron intracardiacamente con 250 ml de solución salina al 0.9% y 250 ml de paraformaldehido al 4% en amortiguador de fosfatos 0.1 M, pH = 7.3. Los cerebros se disectaron y se postfijaron por 24 h en solución de paraformaldehído al 4% en amortiguador de fosfatos 0.1 M, pH=7.3; se transfirieron a gradientes de sacarosa 10, 20 y 30%. Se realizaron cortes coronales de 40 µm a nivel del hipocampo en un criostato y se tiñeron con violeta de cresilo.

- *Volumen de lesión*

Las cortes coronales teñidos con violeta de cresilo se analizaron bajo el microscopio óptico a través de un programa de análisis de imágenes (NIH Macintosh Image 1.6) para determinar el volumen de lesión producido por la perfusión de las diferentes drogas. Para ello se analizaron todas las secciones cerebrales en donde el daño neuronal fue evidente. El tejido se consideró lesionado por la presencia de núcleos picnóticos intensamente teñidos y la ausencia de células morfológicamente viables. El volumen de lesión fue calculado multiplicando el promedio del área lesionada por corte, por la distancia entre el primero y el último corte en donde se observó la lesión.

RESULTADOS

Efecto de la inhibición aguda del metabolismo glicolítico sobre la liberación de AAE y la muerte neuronal en el hipocampo de la rata

Los resultados descritos en esta sección se publicaron en:

- Montiel T, Camacho A, Estrada-Sanchez AM, Mássieu L. Differential effects of the substrate inhibitor L-trans-pyrrolidine-2,4-dicarboxylate (PDC) and the non-substrate inhibitor DL-threo-beta-benzyloxyaspartate (DL-TBOA) of glutamate transporters on neuronal damage and extracellular amino acid levels in rat brain in vivo. Neuroscience, 2005, 133: 667-678.

- Camacho A, Montiel T and Mássieu L. The anion channel blocker, 4,4'-dinitrostilbene-2,2'-disulfonic acid prevents neuronal death and excitatory amino acid release during glycolysis inhibition in the hippocampus in vivo. Neuroscience., 2006, 142: 1005-1017.

Efecto del IOA sobre los niveles basales de AAE

En experimentos previos, demostramos que la perfusión de IOA (5 mM) a través de una membrana de microdiálisis en la región CA1 del hipocampo, incrementa los niveles extracelulares de glutamato y principalmente de aspartato (Massieu et al., 2000); sin embargo, el mecanismo de salida de dichos aminoácidos no se había determinado. En este trabajo perfundimos IOA en la región CA1 del hipocampo para investigar la ruta o rutas de liberación de dichos aminoácidos.

Table 1. Niveles basales (μM) de glutamina, taurina, alanina y glicina en el hipocampo y sus cambios inducidos por el tratamiento con IOA.

Aminoácido	Niveles basales	Pico de concentración durante el tratamiento con IOA	Niveles después del tratamiento con IOA
Glutamina	43.06 ± 1.15	48.56 ± 2.33	44.65 ± 4.91
Taurina	15.82 ± 1.55	12.58 ± 1.94	17.34 ± 1.62
Alanina	12.06± 0.61	12.16 ± 1.03	12.41 ± 0.54
Glicina	10.09 ± 0.64	13.25 ± 1.43	15.53 ± 2.43

Niveles de aminoácidos fueron determinados en los dialisados por HPLC como se describió en los Materiales y Métodos. Niveles en el pico de concentración representan la concentración máxima de aminoácidos observada durante el tratamiento con IOA. Los datos son el promedio ± SEM de 5-7 animales.

Los resultados señalan que la perfusión de IOA (1 mM), en la región CA1 del hipocampo, incrementa los niveles extracelulares de AAE (aspartato 150% y glutamato 100%) con respecto a sus valores basales (Fig. 1). Por su parte, los niveles extracelulares de glutamina, taurina, alanina y glicina no presentaron alteraciones (Tabla 1). Esto sugiere que el metabolismo glicolítico participa en el control de la concentración extracelular de AAE.

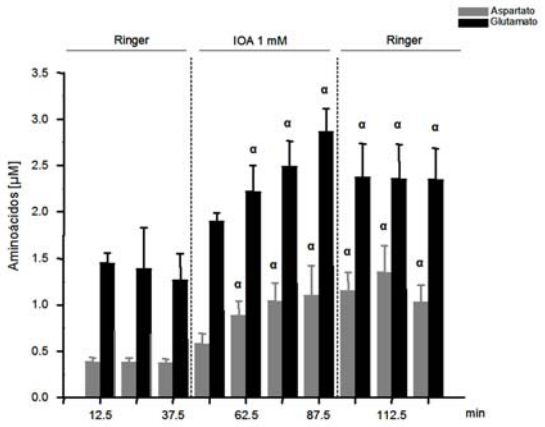

Fig. 1. La perfusión intrahipocampal de IOA incrementa los niveles extracelulares de aminoácidos excitadores (AAE: aspartato y glutamato). Las ratas fueron colocadas en un aparato estereotáxico y la microdiálisis se llevó a cabo como se describió. Los niveles de aminoácidos extracelulares se analizaron en un sistema de HPLC. Los datos representan el promedio ± ES de cinco experimentos independientes. $^{\alpha}$ $p < 0.025$ vs Ringer *(Datos publicados en Camacho et al, Neuroscience. 2006. 142: 1005-1017).*

Ya que una de las hipótesis mayormente fundamentada propone que la acumulación extracelular de AAE durante la falla energética debida a la isquemia

se debe al funcionamiento inverso de los transportadores (Dawson et al., 2000; Hamann et al., 2002; Phillis et al., 2000; Rossi et al., 2000; Saransaari and Oja, 1999; Seki et al., 1999), analizamos si esta ruta de liberación está participando en nuestro modelo experimental como se ha reportado en estudios in vitro (Gemba et al., 1994; Ogata et al., 1995). Para realizar estos experimentos, se emplearon dos bloqueadores de los transportadores para AAE, el PDC y el DL-TBOA. El bloqueador PDC es un inhibidor sustrato que inhibe la captura de aminoácidos excitadores interactuando con el sitio de unión extracelular del glutamato e intercambiándose por el glutamato intracelular, induciendo así la salida de este aminoácido (Volterra et al., 1996). Por tanto, en el espacio extracelular el PDC compite por la unión del glutamato previniendo su captura. Por su parte, el DL-TBOA es un inhibidor no sustrato y se une extracelularmente al transportador bloqueando su interacción con el glutamato (Shimamoto et al., 1998). A diferencia del PDC, el DL-TBOA incrementa los niveles extracelulares de AAE por inhibición de su recaptura y no por inducir la salida del glutamato intracelular.

Efecto de dos bloqueadores del transporte de glutamato, el L-trans-pirrolidin-2,4-dicarboxilato (PDC) y el DL-Treo-beta-benziloxiaspartato (DL-TBOA) sobre los niveles basales de aminoácidos y la muerte neuronal

Antes de analizar el efecto del PDC y el DL-TBOA sobre la liberación de AAE inducida por IOA se estudió el efecto de ambos inhibidores sobre los niveles basales de AAE y la sobrevivencia neuronal.

La Fig. 2 A y B, muestra que tanto el PDC como el DL-TBOA aumentan los niveles basales de AAE. El DL-TBOA promueve un incremento mayor de aminoácidos que el PDC. Además, el DL-TBOA promueve una lesión de mayor magnitud que el PDC (Fig. 2 C).

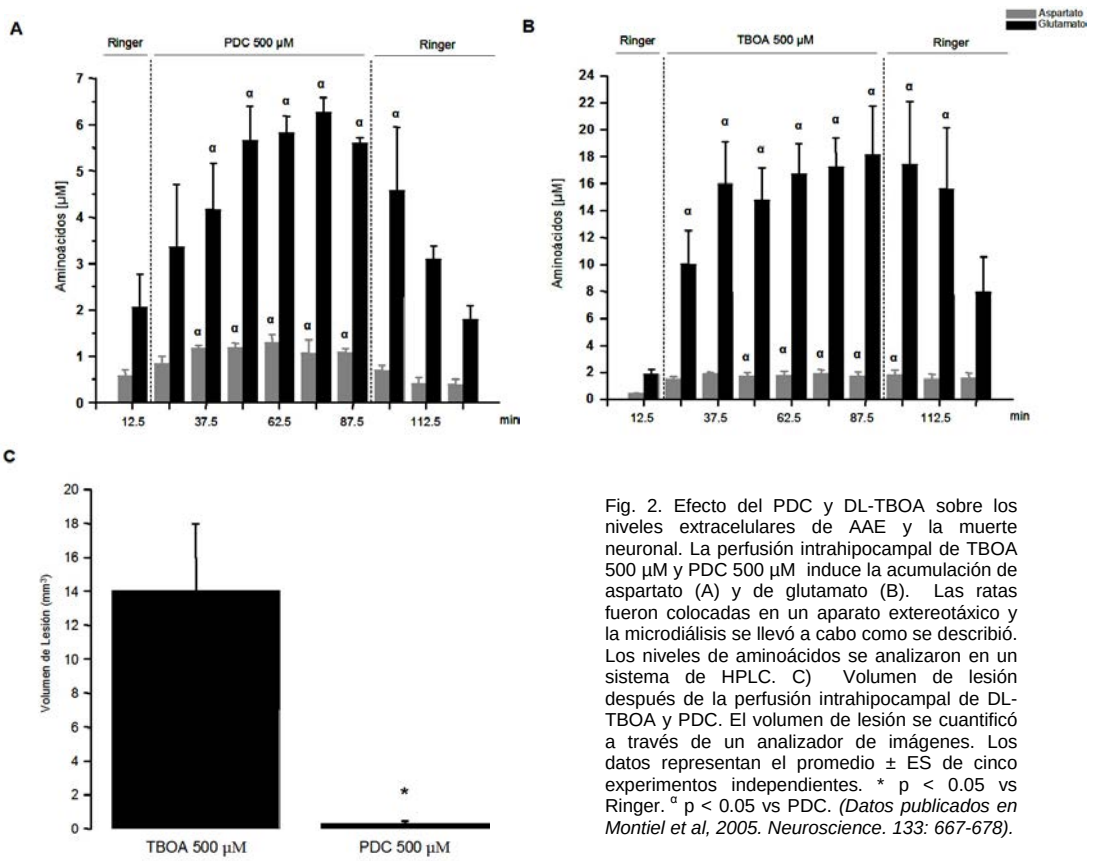

Fig. 2. Efecto del PDC y DL-TBOA sobre los niveles extracelulares de AAE y la muerte neuronal. La perfusión intrahipocampal de TBOA 500 µM y PDC 500 µM induce la acumulación de aspartato (A) y de glutamato (B). Las ratas fueron colocadas en un aparato extereotáxico y la microdiálisis se llevó a cabo como se describió. Los niveles de aminoácidos se analizaron en un sistema de HPLC. C) Volumen de lesión después de la perfusión intrahipocampal de DL-TBOA y PDC. El volumen de lesión se cuantificó a través de un analizador de imágenes. Los datos representan el promedio ± ES de cinco experimentos independientes. * $p < 0.05$ vs Ringer. $^{\alpha}$ $p < 0.05$ vs PDC. *(Datos publicados en Montiel et al, 2005. Neuroscience. 133: 667-678).*

Papel de los transportadores de glutamato sobre la liberación de AEE inducida por IOA

La administración de PDC (1 mM) bloqueó parcialmente la liberación de glutamato inducida por IOA, pero no la de aspartato (Fig. 3 A). Ya que experimentos previos han demostrado que la membrana de microdiálisis tiene una recuperación de los aminoácidos aproximadamente del 10% (Massieu et al., 1995; Massieu and Tapia, 1997), y la IC_{50} del PDC para inhibir la captura de glutamato es del orden de 20-30 µM (Anderson et al., 2001; Bonde et al., 2003; Shimamoto et al., 1998; Volterra et al., 1996), se incrementó la concentración de PDC a 5 mM para asegurar un bloqueo total de los transportadores a glutamato. La Fig. 3 B muestra que a esta concentración el PDC 5 mM bloquea totalmente la acumulación extracelular de glutamato y parcialmente la de aspartato.

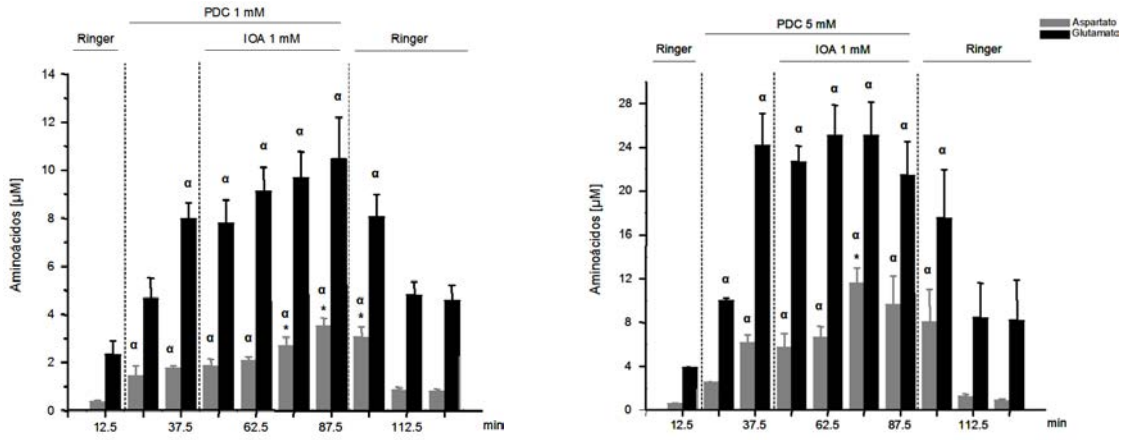

Fig.3. La perfusión intrahipocampal de PDC (5 mM) bloquea la acumulación de glutamato inducida por la inhibición glicolítica. Las ratas fueron colocadas en un aparato estereotáxico y la microdiálisis se llevó a cabo como se describió. Los niveles de aminoácidos extracelulares se analizaron en un sistema de HPLC. Los datos representan el promedio ± ES de seis experimentos independientes. $^{\alpha}$ p < 0.05 vs Ringer, * p < 0.05 vs PDC (min. 37.5). . *(Datos publicados en Camacho et al, Neuroscience. 2006. 142: 1005-1017)*.

De manera similar el bloqueador de los transportadores DL-TBOA (100 µM) presenta una inhibición parcial de la salida de AAE, mientras que a 500 µM se inhibe totalmente la liberación inducida por IOA (Fig. 4 A, B). Sin embargo, a esta concentración se genera un alto porcentaje de muerte neuronal (Fig. 2 C).

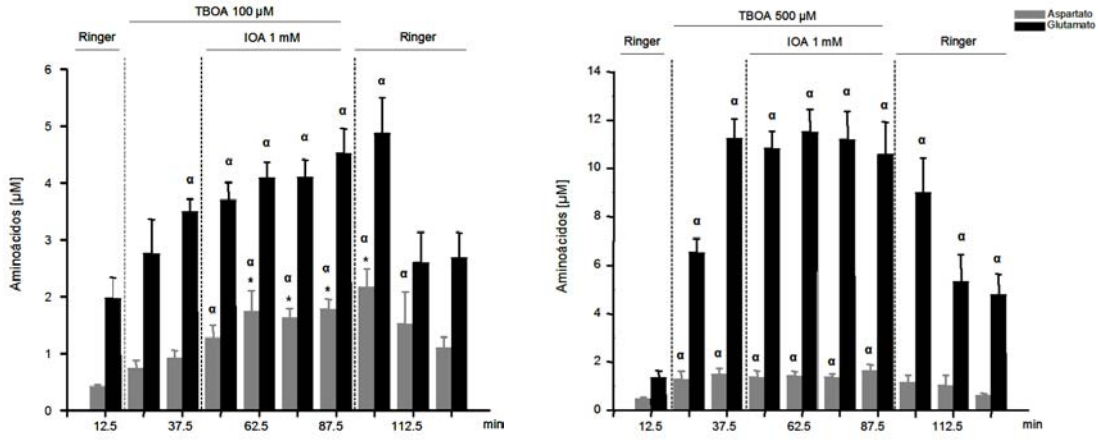

Fig. 4. La perfusión intrahipocampal de DL-TBOA (500 µM) bloquea la acumulación de AAE inducida por el IOA. Las ratas fueron colocadas en un aparato estereotáxico y la microdiálisis se llevó a cabo como se describió. DL-TBOA 100 µM (A) o DL-TBOA 500 µM se prefundió intrahipocampalmente y los niveles de aminoácidos extracelulares se analizaron en un sistema de HPLC. Los datos representan el promedio ± ES de seis experimentos independientes. $^{\alpha}$ p < 0.05 vs Ringer, * p < 0.05 vs DL-TBOA (min. 37.5). *(Datos publicados en Camacho et al, Neuroscience. 2006. 142: 1005-1017).*

Los resultados señalan que el DL-TBOA bloquea eficientemente la salida de aminoácidos inducida por la inhibición del metabolismo glicolítico *in vivo*. Sin embargo, es posible que tanto el DL-TBOA como el PDC funcionen como

inhibidores sustrato de los transportadores y se intercambian por el glutamato intracelular (Anderson et al., 2001; Volterra et al., 1996), vaciando la poza de aminoácidos intracelulares disponible de ser liberada de neuronas y glía por la acción del IOA. Para descartar esta alternativa, se realizó microdiálisis siguiendo el mismo protocolo experimental descrito en las Figs. 3 y 4, y al término de éste, se administró una solución despolarizante con KCl para inducir la liberación de AAE por despolarización. Los resultados confirman que el bloqueo de los transportadores de AAE con DL-TBOA 500 µM y PDC 5 mM, no agotan la poza de aminoácidos disponibles de ser liberada por despolarización (Fig. 5 C, D). Esto sugiere que la inhibición del efecto del IOA en presencia de DL-TBOA o PDC, no se debe al agotamiento de los niveles intracelulares del neurotransmisor sino al bloqueo del funcionamiento inverso de sus transportadores.

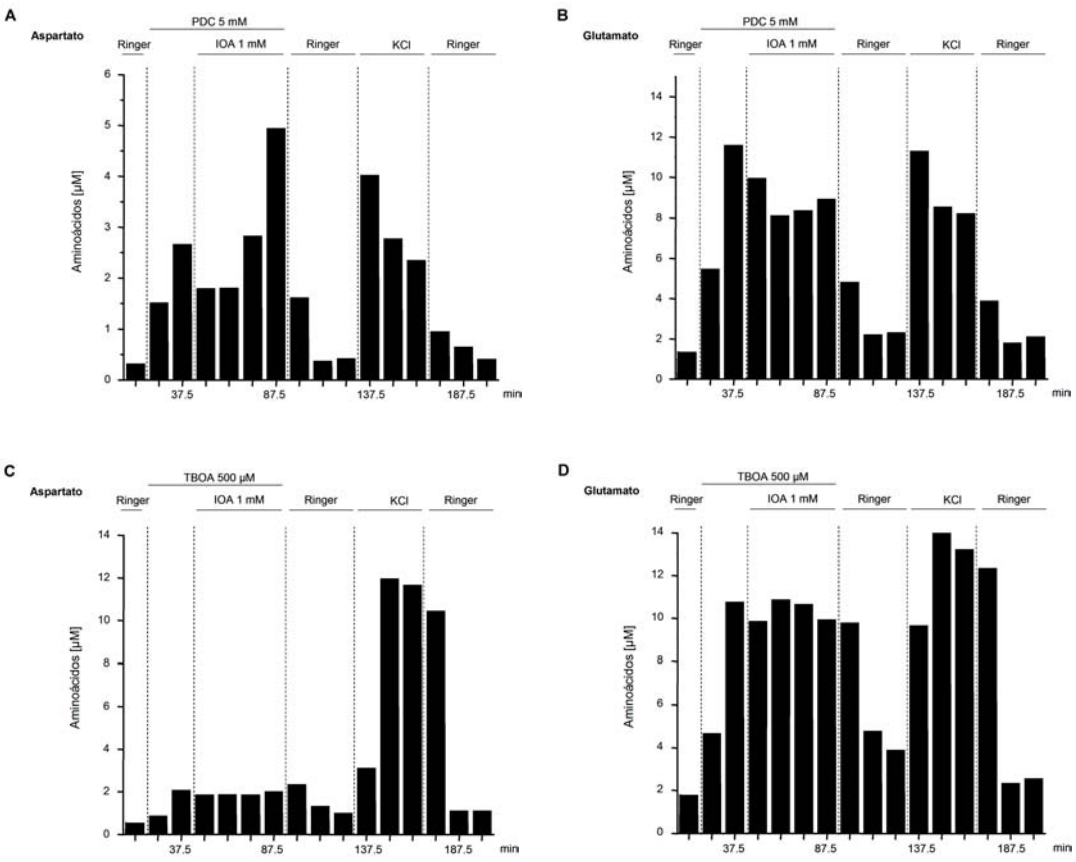

Fig. 5. La perfusión intrahipocampal de PDC y DL-TBOA no agota la concentración de AAE disponible de ser liberada por IOA. Las ratas fueron colocadas en un aparato estereotáxico y la microdiálisis se llevó a cabo como se describió. (A y B) perfusion de PDC 5 mM, (C y D) perfusión de DL-TBOA 500 µM. Los niveles de aminoácidos extracelulares se analizaron en un sistema de HPLC. Las curvas representan un experimento representativo. *(Datos publicados en Camacho et al, Neuroscience. 2006. 142: 1005-1017).*

Papel de la exocitosis sobre la liberación de AEE inducida por IOA

Se estudió la participación de la liberación exocitótica dependiente de calcio en la salida de AAE inducida por IOA. Para esto se empleó un inhibidor de los canales de sodio dependientes de voltaje, el riluzol. La perfusión intrahipocampal de riluzol inhibió la liberación de AAE durante los primeros minutos después de la administración de IOA (Fig. 6).

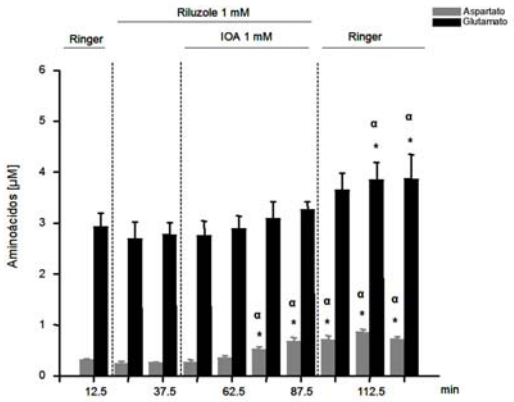

Fig. 6. El bloqueo de los canales de sodio sensibles a voltaje inhibe la salida temprana de AAE inducida por IOA. Las ratas fueron colocadas en un aparato estereotáxico y la microdiálisis se llevó a cabo como se describió. Los niveles de aminoácidos extracelulares se analizaron en un sistema de HPLC. Los datos representan el promedio ± ES de cuatro experimentos independientes. $^{\alpha}$ $p < 0.05$ vs Ringer, * $p < 0.05$ vs Riluzol. (Datos publicados *en Camacho et al, Neuroscience. 2006. 142: 1005-1017).*

Efecto de los canales aniónicos sensibles a volumen (CASV) sobre la acumulación de AAE inducida por IOA

Una tercera vía de liberación que participa en la salida de AAE durante la isquemia involucra la activación de CASV (Seki et al., 1999). Para estudiar esta alternativa en nuestro modelo experimental, empleamos cuatro inhibidores de dichos canales ampliamente usados en el análisis de liberación de aminoácidos durante hinchamiento celular: NPPB, tamoxifen, floretina y DNDS (Abdullaev et al., 2006; Cabantchik and Greger, 1992; Evanko et al., 2004; Haskew-Layton et al., 2005; Phillis et al., 1998; Zhang et al., 1994). Los resultados demuestran que la perfusión de los inhibidores NPPB y floretina no previene la liberación de AAE durante la inhibición glicolítica, sino por el contrario exacerba su liberación (Fig. 7 B, D). Por su parte, la inhibición de los CASV con tamoxifen inhibe el componente inicial de la liberación de aminoácidos (Fig. 7 C). La perfusión de DNDS antes del IOA inhibe los componentes inicial y tardío de la liberación de AAE. (Fig. 7 A). Sin

embargo, la perfusión de DNDS después del IOA no previene la acumulación extracelular de aminoácidos (Fig. 8 A).

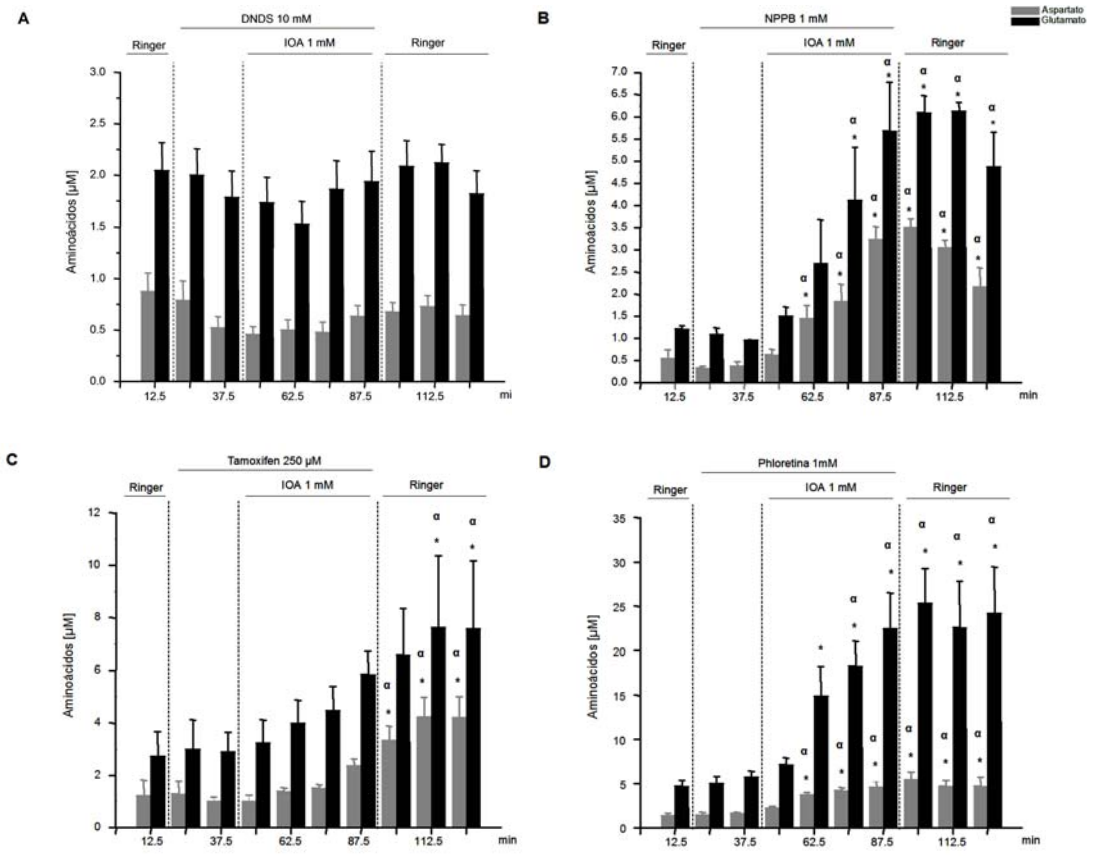

Fig. 7. Efecto de los bloqueadores de los CASV, DNDS (A), NPPB (B), tamoxifen (C) y floretina (D) sobre la liberación de AAE inducida por IOA. Se perfundió DNDS (10 mM), NPPB (1 mM), tamoxifen (250 µM) o floretina (1 mM) antes de la administración de IOA y los niveles de aminoácidos extracelulares se analizaron en un sistema de HPLC. Los datos representan el promedio ± ES de cuatro a seis experimentos independientes. $^{\alpha}$ $p < 0.05$ vs valores basales, * $p < 0.05$ vs 37.5 min. *(Datos publicados en Camacho et al, Neuroscience. 2006. 142: 1005-1017).*

Adicional, al efecto del DNDS sobre la liberación de AAE inducida por el IOA, la perfusión de este compuesto (10 mM) promueve una disminución de los niveles basales de glutamato (Fig. 8 B). Por el contrario, el NPPB no alteró los niveles basales de AAE (Fig. 8 C).

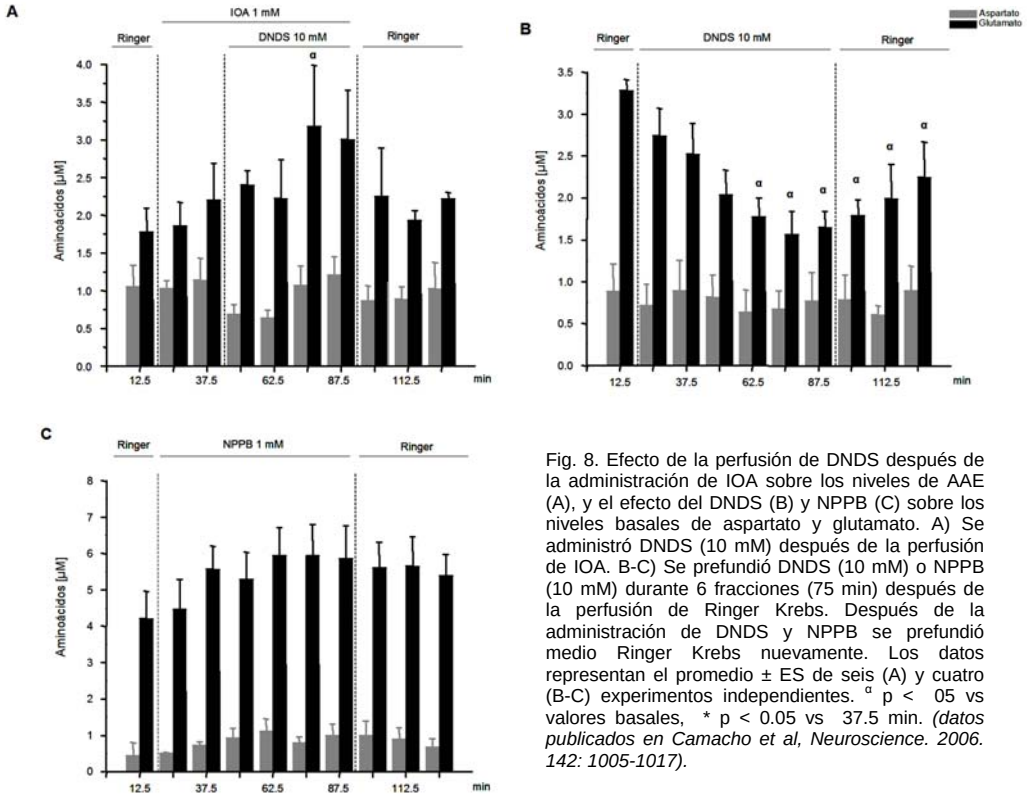

Fig. 8. Efecto de la perfusión de DNDS después de la administración de IOA sobre los niveles de AAE (A), y el efecto del DNDS (B) y NPPB (C) sobre los niveles basales de aspartato y glutamato. A) Se administró DNDS (10 mM) después de la perfusión de IOA. B-C) Se prefundió DNDS (10 mM) o NPPB (10 mM) durante 6 fracciones (75 min) después de la perfusión de Ringer Krebs. Después de la administración de DNDS y NPPB se prefundió medio Ringer Krebs nuevamente. Los datos representan el promedio ± ES de seis (A) y cuatro (B-C) experimentos independientes. $^{\alpha}$ $p <$ 05 vs valores basales, * $p < 0.05$ vs 37.5 min. *(datos publicados en Camacho et al, Neuroscience. 2006. 142: 1005-1017).*

Relación entre la liberación de AAE inducida por IOA y la muerte neuronal

Una de las hipótesis más fundamentadas en la literatura propone que la muerte neuronal inducida durante la isquemia y la hipoglicemia se produce por un mecanismo excitotóxico (Benveniste et al., 1984; Simon et al., 1984; Wieloch, 1985). Diversos grupos de investigación han empleado a los bloqueadores de los transportadores para AAE, como una herramienta farmacológica para inhibir la liberación de aminoácidos durante la isquemia (Dawson et al., 2000; Phillis et al., 2000; Seki et al., 1999). Sin embargo, no existen datos que demuestren que esta estrategia prevenga la muerte neuronal en modelos *in vivo*. En la siguiente serie de experimentos analizamos el efecto de los distintos fármacos empleados en los experimentos de liberación, sobre el tamaño de las lesiones producidas por el IOA (Fig. 9). Nuestros resultados demuestran que la administración de PDC (5 mM) y de DNDS (10 mM) previene la muerte neuronal 24 h después de la administración de IOA (Fig. 9 D, H y J). La administración de DNDS después del IOA no previene

la muerte neuronal (Fig. 9 I y J). Ninguno de los otros fármacos redujo el tamaño de las lesiones.

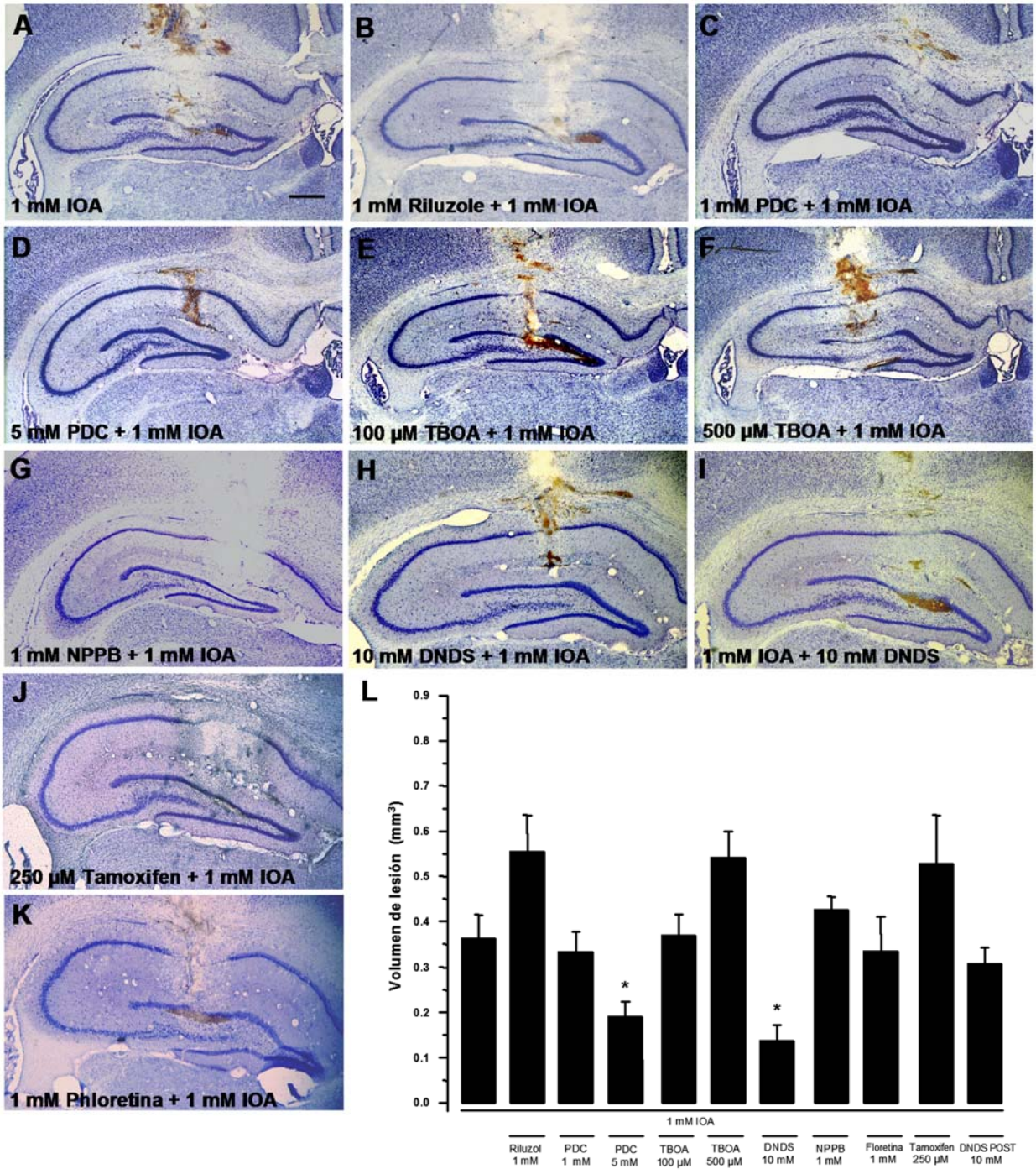

Fig. 9. Efecto de los diferentes compuestos estudiados sobre el daño inducido por la administración de IOA en el hipocampo. A-K) Micrografias de cortes de cerebro animales representativos indicando el daño neuronal en el hipocampo evaluado 24 h después de la administración de IOA (A) y su prevención por PDC 5 mM (D) y DNDS 1O mM (H). Cuando el DNDS se perfundió después del IOA no hubo prevención contra la muerte neuronal (I). Escala de la barra = 500 µm. L) La gráfica muestra el volumen (mm^3) de las lesiones en el hipocampo inducidas por el IOA en la presencia o ausencia de los diferentes inhibidores empleados. El volumen de lesión se cuantificó 24 h después de la microdiálisis por medio de un programa de análisis de imágenes. Los datos representan el promedio ± ES de cinco experimentos independientes. * $p < 0.05$ vs IOA. *(Datos publicados en Camacho et al, Neuroscience. 2006. 142: 1005-1017).*

IX. MODELO EXPERIMENTAL 2

Inhibición sostenida de la glicólisis

MATERIALES Y MÉTODOS

- Actividad enzimática de la G3PDH

Ratas macho de la cepa Wistar (250-300 g) se trataron intraperitonealmente con una dosis de amortiguador de fosfatos (10 mM) en las ratas control, o de IOA (15 mg/kg/ml) en las ratas tratadas durante uno, dos o tres días consecutivos. Dos horas después de cada administración y 24 h después de la última, las ratas fueron sacrificadas por decapitación y se extrajo la región del hipocampo. El tejido se homogenizó en amortiguador Tris-HCl 0.1 M, pH = 8.5 (1:10 peso:volumen). La actividad enzimática fue analizada utilizando 200 µg de proteína en una mezcla de reacción de 1 ml que contenía (en mM): 1.7 arsenato de sodio, 20 fluoruro de sodio, 1 NAD, 1 gliceraldehído 3-fosfato y 5 KH_2PO_4 de acuerdo a (Ikemoto et al., 2003; Mejia-Toiber et al., 2006). La actividad fue calculada por la formación de NADH a 340 nm en un espectrofotómetro. La actividad se reportó como nmol/mg prot/ min

- Implantación de cánula guía

Siete días antes del tratamiento las ratas fueron anestesiadas con una mezcla de gas de halotano 4-5% en 95% O_2/5% CO_2 y se colocaron en un aparato estereotáxico para la realización de la cirugía. La implantación de la cánula guía se llevó a cabo en el hemisferio izquierdo de la rata, abarcando la región CA1 del hipocampo, siguiendo las siguientes coordenadas de acuerdo al atlas de Paxinos y Watson (1986), AP= -3.6, L= +2.0 y V= -2.0. La cánula guía se fijó al cráneo con cemento de acrílico dental.

- Microdiálisis

Siete días posteriores a la implantación de la cánula guía se iniciaron los experimentos de microdiálisis en la región CA1 del hipocampo. La microdiálisis se llevó a cabo durante 5 días: en el primer día se administró intraperitonealmente

vehículo (amortiguador de fosfatos 10 mM) con el fin de obtener los valores basales de aminoácidos extracelulares, durante los 3 días siguientes se sometió a las ratas al tratamiento sistémico con IOA (una dosis de 15 mg/kg/ml por día) y el quinto día post-tratamiento, se administró nuevamente el vehículo. La microdiálisis se realizó perfundiendo medio Ringer-Krebs como se describió anteriormente, a un flujo de 2 µl/min durante 1.5 horas para lograr la estabilización de la membrana de diálisis; pasando este periodo de tiempo, se colectaron 3 fracciones de 25 µl para su posterior análisis. Paralelamente se realizó microdiálisis en ratas control, tratadas intraperitonealmente con vehículo y sometidas al mismo protocolo experimental. Después de cada experimento se verificó la localización de la membrana de microdiálisis en la región CA1 por análisis histológico de secciones coronales teñidas con Nissl.

- Análisis del contenido de proteína de las subunidades de los receptores a NMDA y de los transportadores de glutamato por la técnica de western blot

Se empleó western blot para analizar los cambios en los niveles de proteína de las subunidades NR1, NR2A y NR2B del receptor a NMDA, así como también, de los transportadores para AAE dependientes de sodio GLAST, GLT-1 y EAAC1, antes, durante y después de la inhibición crónica de la glicólisis. Para ello, se emplearon ratas Wistar (250-300 g) tratadas intraperitonealmente con vehículo o con IOA (15 mg/Kg) durante tres días consecutivos. Para el análisis de la subunidad NR2B y del transportador de glutamato GLT-1, se decapitaron las ratas a 2, 24 y 72 horas después de la última administración de IOA y el hipocampo se extrajo en amortiguador de fosfatos 25 mM, EDTA 2 mM, sin detergente. Para las subunidades y transportadores de glutamato restantes, se analizó su contenido de proteína a las 2 y 24 horas posteriores al tratamiento bajo las mismas condiciones. La electroforesis se realizó en geles de poliacrilamida del 7.5% en condiciones no reductoras y posteriormente se transfirieron a membrana inmmobilon-P. La concentración de anticuerpo primario para cada proteína analizada fue como sigue: NR1, NR2A y NR2B (1:500), GLAST y GLT-1 (1:5000), EAAC1 (1:200). La membrana fue revelada utilizando un kit de quimioluminisencia (ECL_{TM}). El análisis

densitométrico de las placas se determinó empleando un analizador Bio Rad, Laser-Pix (Sottware versión 4.0.0.13). Los datos se corrigieron por el contenido de actina utilizado como control de carga.

- Inmunoprecipitación

Para los experimentos de inmunoprecipitación se siguió la metodología descrita por (Arias et al., 2002) con algunas modificaciones. Las muestras fueron homogenizadas en amortiguador de lisis (Tris-HCl 50 mM, NaCl 150 mM, Nonidet P40 1%, SDS 0.5%, ortovanadato de sodio 10 mM, fluoruro de Sodio 25mM, inhibidores de proteasas, pH 7.5). Todos los procedimientos se realizaron a 4°C. Se centrifugó a 1500g por 5 minutos para retirar núcleos y restos celulares grandes. Se midió la concentración de proteínas mediante ensayo de Lowry. La subunidad NR2B se inmunoprecipitó a partir de 2 mg de proteína con 12 µl de anticuerpo monoclonal específico para NR2B en 0.5ml de amortiguador de lisis, se incubó toda la noche y se agregó 30 µl de proteína G-agarosa, después de 2 horas se centrifugó por 2 minutos a 1500 g. El sedimento se lavó con 300µl de amortiguador de lisis y se centrifugó nuevamente a 1500 g, se lavó 2 veces más con amortiguador de alta concentración de sales (Tris-HCl 50 mM, NaCl 150 mM, Nonidet P40 0.1%, pH 7.5) y para retirar las sales se realizó un lavado con Tris-HCl 50mM pH 7.5, Nonidet P40 0.1%. Después de estos lavados se centrifugó a 12000 g durante 3 min, se retiró el sobrenadante y el sedimento fue resuspendido en 30µl de amortiguador Laemmli con 10% de β-mercapto-etanol. Se hirvieron las muestras durante 5 min y se centrifugó a temperatura ambiente a 2000g durante 3 min. Los sobrenadantes fueron analizados en un gel SDS-poliacrilamida al 7.5% y trasferidas a una membrana de PVDF. Para analizar la fosforilación la membrana se incubó con anticuerpos contra Tirosina 1472. Las bandas fueron reveladas en placas fotográficas utilizando anticuerpos secundarios acoplados a peroxidasa y un substrato quimioluminiscente.

- Inmunohistoquímica

Se realizó inmunohistoquímica para las subunidades NR1 y NR2B del receptor a NMDA en cortes de cerebro de las ratas tratadas intraperitonealmente con vehículo o con IOA. Después de dos, veinticuatro, setenta y dos horas y ocho días después de la última administración de IOA, las ratas fueron anestesiadas con 100 U de pentobarbital por vía intraperitoneal y se perfundieron intracardiacamente con 250 ml de solución salina 0.9%, y 250 ml de paraformaldehído al 4%, ácido pícrico al 0.4% en amortiguador de fosfatos 0.1 M, pH=7.3. Los cortes cerebrales se incubaron con anticuerpo primario (1:500) y secundario (1:300) contra la subunidad NR1 y NR2B y se revelaron por diaminobenzidina. Los cambios en el contenido de las subunidades NR1 y NR2B en el hipocampo, fueron cuantificados por análisis densitométrico de al menos cuatro secciones coronales de una misma rata con un sistema analizador de imágenes (NIH Macintosh. Imagen 1.6). El control negativo en cada experimento se realizó omitiendo la incubación con anticuerpo primario con el fin de evidenciar la señal específica para ambas subunidades.

- Captura de ^{3}H-D-aspartato en rebanadas de hipocampo

La captura de aspartato en cerebro de ratas tratadas con IOA se realizó en rebanadas de hipocampo. Veinticuantro horas después del tratamiento con IOA, las ratas se sacrificaron por decapitación, el hipocampo se extrajo y se cortaron rebanadas de hipocampo de 250 µm con el uso de un rebanador. La colecta de las rebanadas se realizó en un medio Ringer-Krebs previamente oxigenado conteniendo en mM: NaCl 118, KCl 4.5, $MgSO_4$ 2.5, NaH_2PO_4 4.0, $CaCl_2$ 2.5, $NaHCO_3$ 25, y glucosa 10, at 4°C. Las rebanadas se oxigenaron durante 20 minutos en un baño de agitación a 37°C. Para los ensayos de captura las rebanadas se incubaron en medio Ringer-Krebs conteniendo [^{3}H]-D-aspartato (1 µCi) y aspartato no marcado (5-1000 µM) a 37°C, durante 5 minutos en oxigenación constante. La captura inespecífica de [^{3}H]-D-aspartato se determinó sustituyendo el NaCl por cloruro de colina. Bajo estas condiciones la captura de [^{3}H]-D-aspartato fue sólo de 5-10%.

Para determinar la captura de [^{3}H]-D-aspartato dependiente del transportador GLT-1, las rebanadas de hipocampo de ratas tratadas con IOA y controles se incubaron con el ácido dihidrokaínico (DHK), un inhibidor selectivo del transportador GLT-1 (Kawahara et al., 2002),1 h antes de iniciar la captura. El hipocampo se extrajo como se describió previamente y se preincubo durante 1 h a 37 ºC con DHK (1mM) de acuerdo a lo reportado previamente (Shin et al., 2005). Las rebanadas se incubaron con [^{3}H]-D-aspartato y diferentes concentraciones de aspartato no marcado (50-750 µM) durante 5 min. La captura específica del transportador GLT-1 se calculó como la diferencia entre la captura observada en presencia y/o ausencia de DHK.

Después del experimento las rebanadas se digirieron en 500 µl de NaOH 0.2 N durante 24 h. La radiactividad se cuantificó después de la adición de 500 µl de ácido acético y de 10 ml de tritosol (Fricke, 1975), en un contador de centelleo líquido. Los resultados se corrigieron por el contenido de proteína determinado por el método de Lowry (Lowry et al., 1951). Se realizó el análisis de Linewaver-Burke para determinar la Km y la Vmax.

- Análisis estadístico

Para el análisis estadístico de los datos se empleó ANOVA de una vía seguido de la prueba de Fisher de comparación múltiple.

RESULTADOS

Efecto de la inhibición sostenida del metabolismo glicolítico sobre el transporte de glutamato y los niveles de las distintas subunidades del receptor a NMDA

Los resultados descritos en esta sección se publicaron en:

- Camacho A, Montiel T and Mássieu L . Sustained metabolic inhibition induces an increase in the content and phosphorylation of the NR2B subunit of NMDA receptors and a decrease in glutamate transport in the rat hippocampus in vivo. Neuroscience 2007, 147: 873-886.

Efecto del tratamiento sistémico de IOA sobre la actividad de la G3PDH en el hipocampo de la rata

Se investigó si la administración de IOA a ratas durante 3 días por vía intraperitoneal, inhibe el metabolismo glicolítico cerebral. Para ello, se extrajo el tejido del hipocampo y se midió la actividad enzimática de la G3PDH a diferentes tiempos después del tratamiento (Fig. 10). La administración de IOA indujo una disminución progresiva de la actividad enzimática de la G3PDH en el hipocampo. El mayor porcentaje de inhibición enzimática fue a las 2 y 24 horas después de la última administración del inhibidor 48 y 44%, respectivamente (Fig. 10).

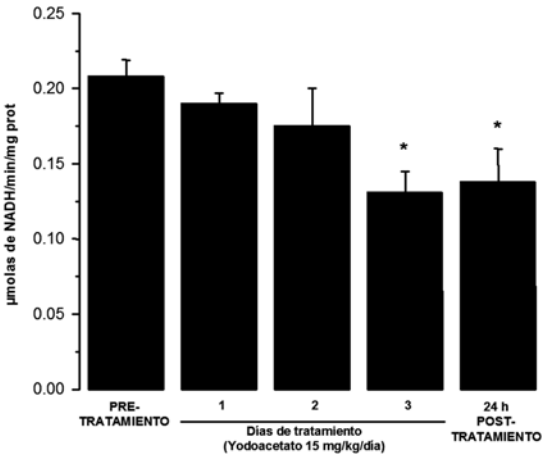

Fig. 10. Inhibición de la actividad enzimática de la G3PDH en el hipocampo durante la administración crónica de IOA. Las ratas se trataron intraperitonealmente con IOA (15 mg/kg) y se sacrificaron a los diferentes tiempos. El análisis de la actividad enzimática se llevó a cabo cuantificando la formación de NADH a 340 nm. Los datos representan el promedio ± ES de cinco experimentos independientes. * p < 0.002 respecto al valor pretratamiento. Datos publicados en Neuroscience, 2007, 147:873-886.

La inhibición sostenida del metabolismo glicolítico incrementa los niveles extracelulares de aspartato y glutamato

Ya que existe una estrecha relación entre el metabolismo glicolítico y el control de los niveles extracelulares de AAE (Hyder et al., 2006; Voutsinos-Porche et al., 2003), a continuación se investigó si la inhibición sostenida de la glicólisis altera los niveles basales de AAE en el hipocampo. El análisis de los dialisados colectados de la región CA1 mostró que no hay alteraciones en la concentración extracelular de alanina, GABA, glutamina, glicina y taurina, antes, durante y después del tratamiento con IOA (Tabla 2). Los niveles extracelulares de estos aminoácidos en una rata tratada con IOA y en una rata control tratada con vehículo son muy similares (datos no mostrados).

Tabla 2. Niveles basales (μM) de glutamina, taurina, alanina, glicina y GABA en el hipocampo y la ausencia de cambio durante y después del tratamiento con IOA

Aminoácidos	Niveles basales	Pico de concentración durante el tratamiento con IOA	Niveles de aminoácidos 24 h después del tratamiento
Glutamina	58.55 ± 4.32	64.14 ± 7.14	61.98 ± 6.69
Taurina	33.31 ± 5.05	32.95 ± 2.67	34.48 ± 4.47
Alanina	18.41 ± 1.93	24.16 ± 3.39	18.56 ± 1.88
Glicina	41.62 ± 5.18	59.31 ± 7.57	62.76 ± 13.9
GABA	0.326 ± 0.03	0.369 ± 0.09	0.291 ± 0.03

Los niveles de aminoácidos se determinaron en los dialisados por HPLC como se describió en Materiales y Métodos. El pico de concentración representa la concentración de aminoácidos máxima observada durante el tratamiento con IOA. Los datos representan el promedio ± SEM de 5-7 animales.

En contraste, el tratamiento con IOA incrementó progresivamente los niveles extracelulares de glutamato, con respecto a sus valores basales antes del tratamiento (día 1) (Fig. 11). Por su parte, se observa un incremento extracelular de aspartato sólo después de 24 horas de la última administración del inhibidor (Fig. 11). Es importante resaltar que a pesar de la inhibición de la G3PDH y del incremento significativo en los niveles extracelulares de aspartato y glutamato, no se presenta muerte neuronal en la región CA1 del hipocampo (Datos no mostrados), como se ha reportado previamente (Massieu et al., 2000)

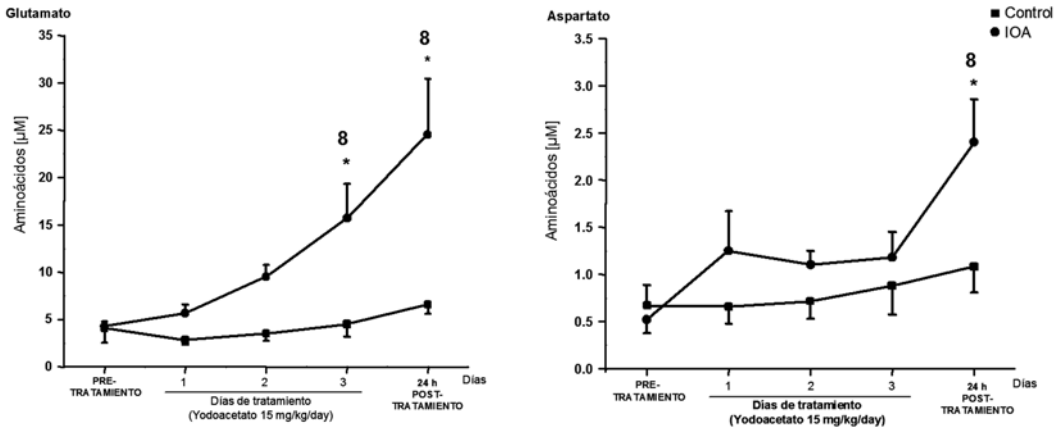

Fig. 11. Niveles extracelulares de aminoácidos antes, durante y después del tratamiento con IOA. Las ratas fueron tratadas intraperitonealmente con IOA (15 mg/Kg), y las controles con buffer de fosfatos (10 mM), durante tres días consecutivos. La microdiálisis se llevó a cabo como se describió en la metodología. Los niveles de aminoácidos extracelulares se analizaron en un sistema de HPLC. Los datos representan el promedio ± ES de siete experimentos independientes. * p< 0.01 respecto valores basales, ∞ p< 0.01 respecto valor pretratamiento. Datos publicados en Neuroscience, 2007. 147: 873-886.

El tratamiento con IOA induce la disminución en el contenido de proteína del transportador GLT-1

La acumulación extracelular de AAE durante la inhibición sostenida del metabolismo glicolítico podría explicarse por dos mecanismos: 1) el decremento en los niveles de proteína de los transportadores de AAE (EAAC1, GLAST y GLT1) y 2) alteraciones en la capacidad de captura del glutamato. El análisis del contenido de proteína por western blot mostró un decremento en el transportador GLT-1, 24 h después del tratamiento con IOA (Fig. 12). Los niveles basales se recuperan 72 hrs después del estrés metabólico. Por su parte, el contenido de proteína de los transportadores EAAC1 y GLAST no presenta alteraciones significativas (Fig. 12). Los resultados sugieren que la acumulación de AAE durante la inhibición prolongada del metabolismo glicolítico está relacionada con un decremento en el contenido de proteína del transportador GLT-1. Este resultado fue corroborado con el análisis de captura de [^{3}H]-D- aspartato en rebanadas de hipocampo de ratas tratadas con IOA, 24 h después de haber terminado el tratamiento. La captura de [^{3}H]-D- aspartato fue altamente dependiente de sodio ya que se obtuvo menos del 10% de la radiactividad en las rebanadas de hipocampo en la ausencia de sodio (Fig. 13 A). El análisis cinético indica un decremento en la Vmax en las ratas tratadas con IOA (Controles Vmax = 7.041 ± 0.967 nmoles/mg prot/min, tratadas Vmax = 2.887 ± 0.3141). También se

encontró un incremento en la afinidad que no resultó ser estadísticamente significativo (Km = 766.2 ± 194 µM, y Km = 240 ± 69 µM en las ratas control y tratadas, respectivamente) (Fig. 13 B).

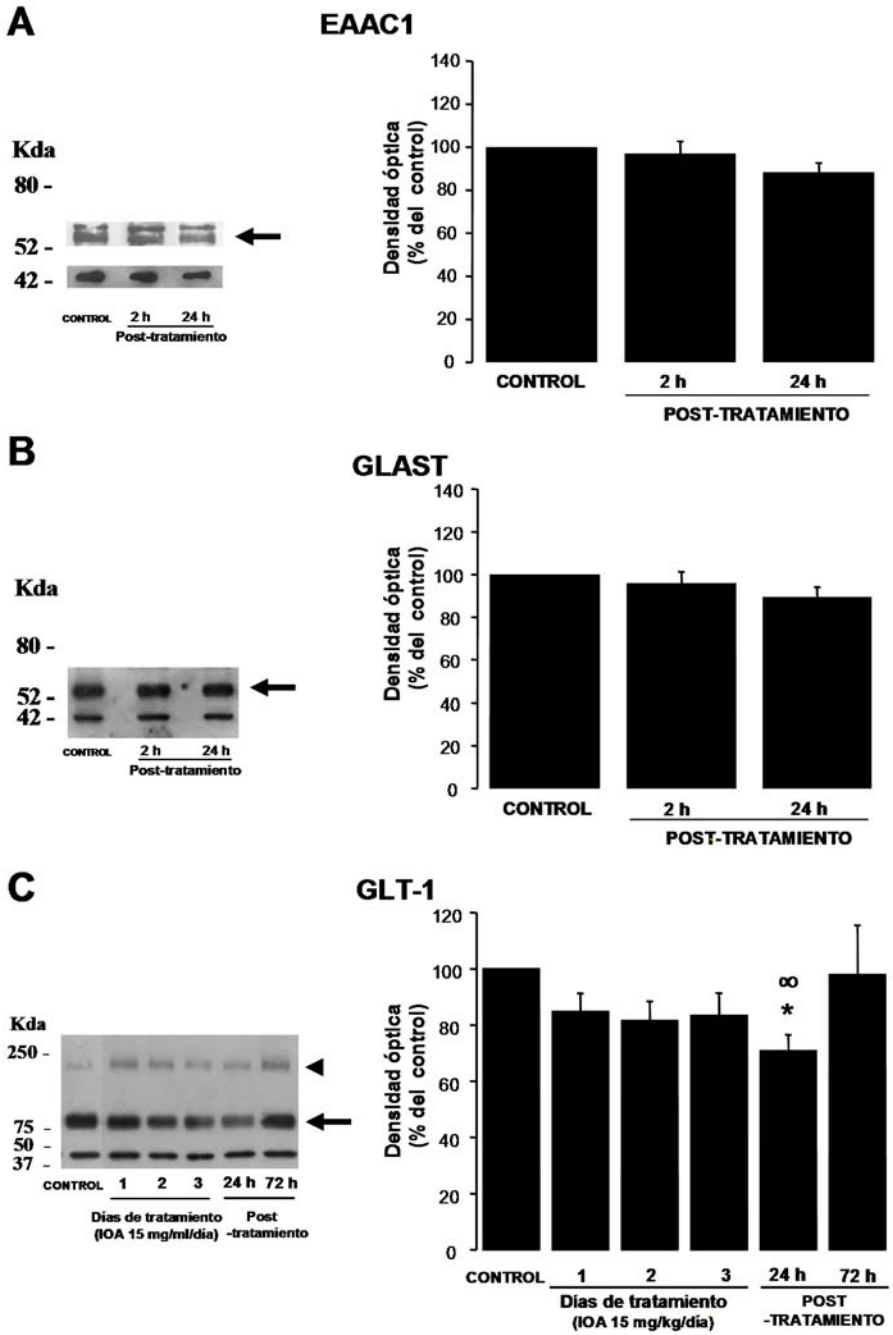

Fig.12. Efecto de la inhibición de la G3PDH sobre los niveles de proteína de los transportadores de glutamato. Las ratas recibieron una inyección de IOA o amortiguador de fosfatos durante tres días consecutivos. Dos y veinte cuatro horas después de la última inyección, (A, B), dos horas después de cada inyección y veinte cuatro y setenta y dos horas después de la última inyección (C), el hipocampo se extrajo y se homogenizó para el análisis de inmunoblot como se describió en materiales y métodos. Panel izquierdo muestra inmunoblots de animales representativos. Las flechas corresponden a la banda del transportador. La banda superior inferior indica los niveles de actina utilizados como control de carga. En C la cabeza de flecha y la flecha indican la conformación de multímeros y monómeros, respectivamente del GLT-1. Las barras representan el promedio ± ES de cinco experimentos independientes. * $p < 0.01$ vs control, $^{\alpha}$ $p < 0.01$ vs 72 h. Datos publicados en Neuroscience, 2007. 147: 873-886.

Por último evaluamos si la reducción de la captura de [^{3}H]-D- aspartato en ratas tratadas con IOA podría deberse a la disminución del GLT-1. Para ello la captura se realizó en la presencia del inhibidor del transportador GLT-1, el DHK (Kawahara et al., 2002; Shin et al., 2005). Los resultados indican que 24 h después del tratamiento con IOA la captura de [^{3}H]-D- aspartato sensible a DHK es menor en estos animales con respecto al control (Fig. 13 C). Estos resultados sugieren que la inhibición glicolítica promueve la acumulación extracelular de AAE debido a un decremento del transportador GLT-1 en la membrana.

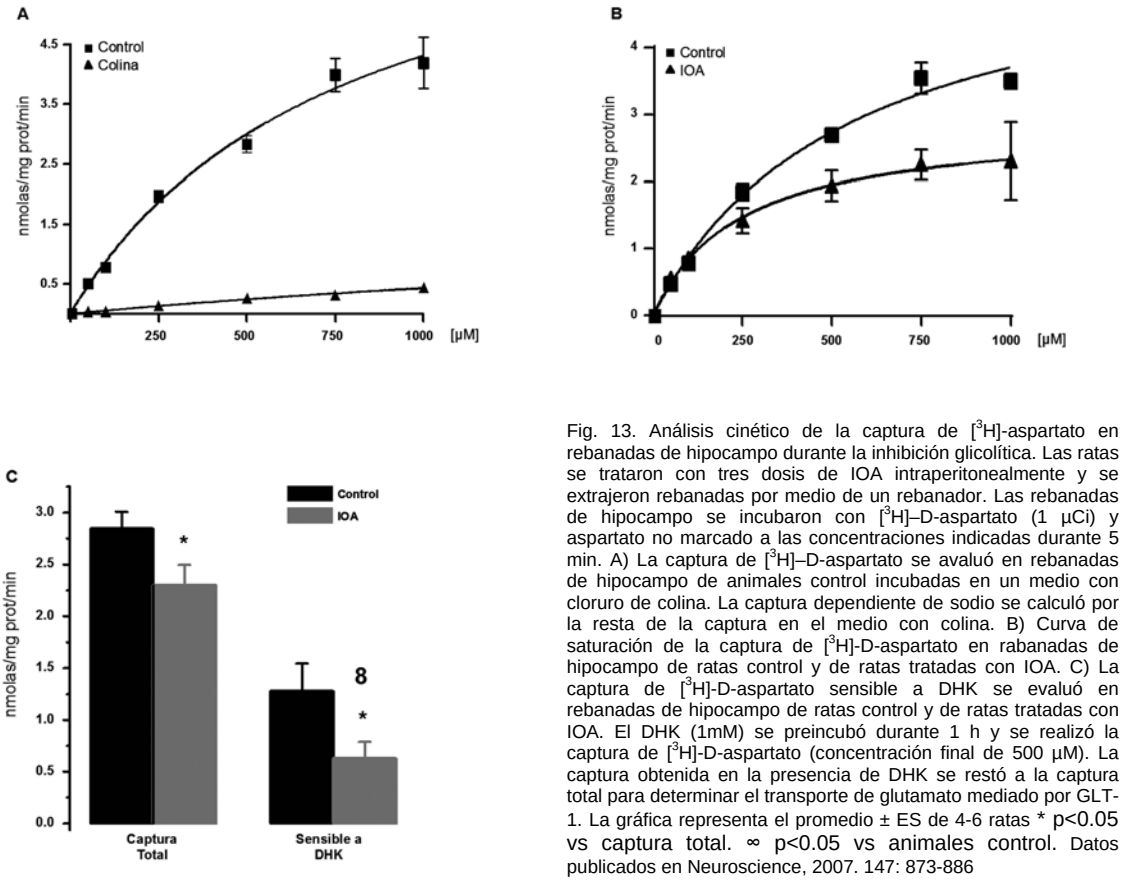

Fig. 13. Análisis cinético de la captura de [^{3}H]-aspartato en rebanadas de hipocampo durante la inhibición glicolítica. Las ratas se trataron con tres dosis de IOA intraperitonealmente y se extrajeron rebanadas por medio de un rebanador. Las rebanadas de hipocampo se incubaron con [^{3}H]–D-aspartato (1 µCi) y aspartato no marcado a las concentraciones indicadas durante 5 min. A) La captura de [^{3}H]–D-aspartato se avaluó en rebanadas de hipocampo de animales control incubadas en un medio con cloruro de colina. La captura dependiente de sodio se calculó por la resta de la captura en el medio con colina. B) Curva de saturación de la captura de [^{3}H]-D-aspartato en rabanadas de hipocampo de ratas control y de ratas tratadas con IOA. C) La captura de [^{3}H]-D-aspartato sensible a DHK se evaluó en rebanadas de hipocampo de ratas control y de ratas tratadas con IOA. El DHK (1mM) se preincubó durante 1 h y se realizó la captura de [^{3}H]-D-aspartato (concentración final de 500 µM). La captura obtenida en la presencia de DHK se restó a la captura total para determinar el transporte de glutamato mediado por GLT-1. La gráfica representa el promedio ± ES de 4-6 ratas * $p<0.05$ vs captura total. ∞ $p<0.05$ vs animales control. Datos publicados en Neuroscience, 2007. 147: 873-886

Efecto del tratamiento sistémico de IOA sobre los niveles de proteína de las subunidades del receptor a NMDA y la fosforilación de la subunidad NR2B

Estudios previos sugieren que la inhibición sostenida del metabolismo energético no genera muerte neuronal, pero si facilita los efectos tóxicos del glutamato cuando éste se administra directamente en el estriado o en el hipocampo de ratas (Massieu et al., 2000; Massieu et al., 2003). Es posible que éste efecto facilitador se deba a una alteración a nivel de las subunidades que conforman al receptor a NMDA. Estudios previos han sugerido que después de un evento isquémico o durante el desarrollo de enfermedades neurodegenerativas los niveles de las subunidades que conforman al receptor a NMDA se alteran (Arzberger et al., 1997; Behrens et al., 2002; Bruhn et al., 2000; Heurteaux et al., 1994; Hsu et al., 1998; Kang et al., 2001; Zhang et al., 1997; Zoia et al., 2005). Con base en esta información se estudió si la inhibición del metabolismo glicolítico induce alteraciones en los niveles de proteína de las subunidades NR1, NR2A y NR2B del receptor a NMDA. La Fig. 14 muestra que la administración intraperitoneal de IOA, no genera cambios aparentes en los niveles de proteína de las subunidades NR1 y NR2A 2 y 24 hrs después del tratamiento. El efecto más importante se presenta a nivel de la subunidad NR2B, observándose un incremento gradual a partir de las 2 horas después del tratamiento y que es significativo 24 horas. Sin embargo, el incremento es sólo transitorio ya que a las 72 h los niveles de proteína se recuperan a valores control (Fig. 14).

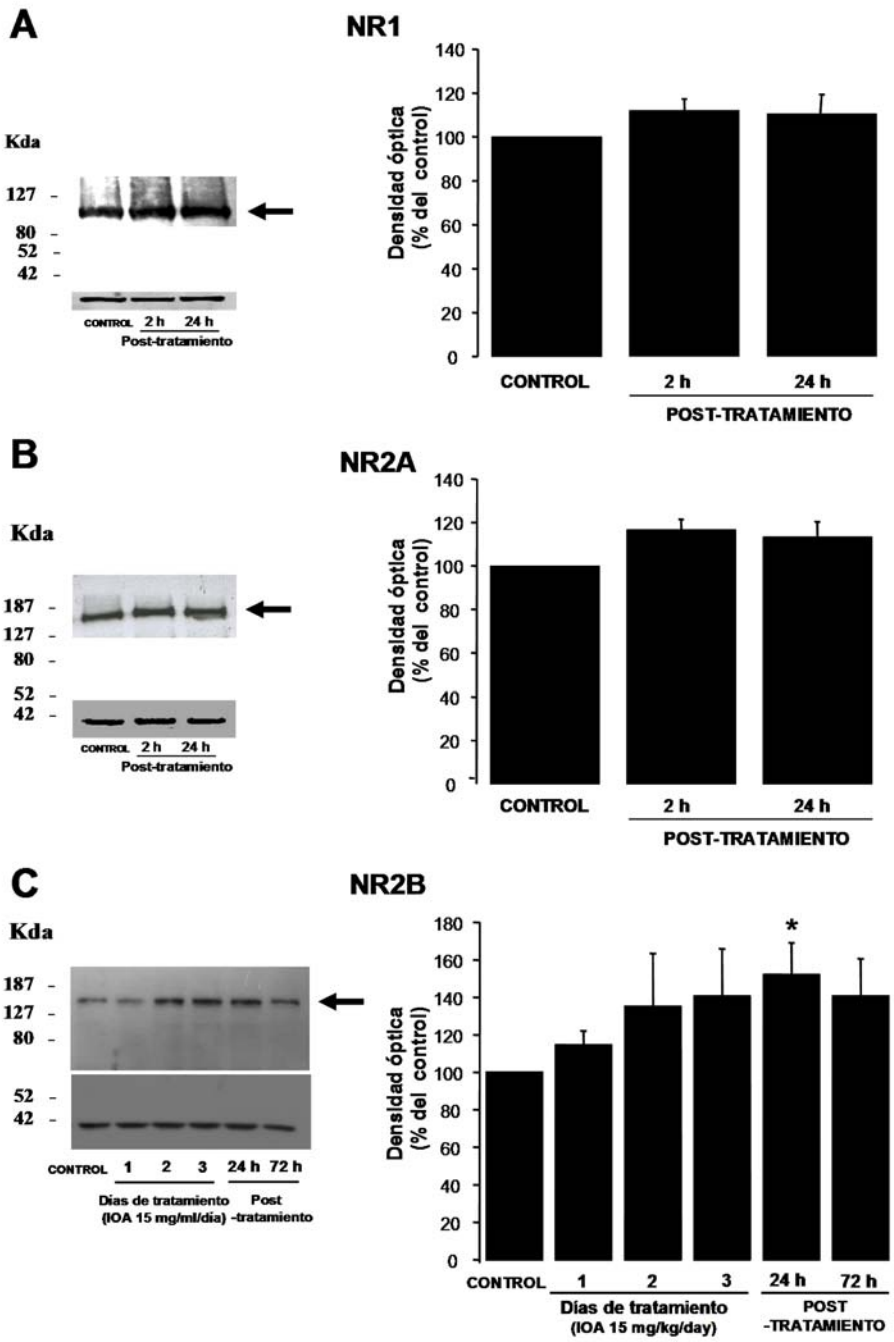

Fig.14. La inhibición de la glicólisis incrementa el nivel de proteína de la subunidad NR2B en el hipocampo. Las ratas se trataron como se describió en la Fig. 12, y el hipocampo se extrajo y se homogenizó para el análisis de western blot como se describió en Materiales y Métodos. A-C) muestran werstern blot de las subunidades NR1, NR2A y NR2B respectivamente, de experimentos representativos. La gráfica indica el análisis desitométrico de las diferentes subunidades del receptor a NMDA. Las flechas indican la banda que corresponde a cada subunidad. Las barras representan el promedio ± ES de cinco experimentos independientes. * $p < 0.05$ vs control. Datos publicados en Neuroscience, 2007. 147: 873-886.

El resultado de western blot se corroboró por inmunohistoquímica. Los resultados muestran un incremento progresivo en la inmunoreactividad desde las 2 h y hasta las 72 h posteriores a la administración de IOA (Fig. 15). En la región CA1 el cambio ocurre a nivel de las dendritas apicales (cabezas de flecha). El incremento en la inmunoreactividad para NR2B también se observa en la región CA3 y en el giro dentado, aunque el incremento en estas zonas es máximo desde las 2 h posteriores a la inhibición glicolítica, permaneciendo después de 24 y 72 h (Fig. 15). Cabe señalar que los cambios fueron transitorios ya que la inmunoreactividad para NR2B regresa a sus niveles basales 8 días después del tratamiento. En correlación con el análisis de western blot, no se observaron cambios en la marca inmunoreactiva para la subunidad NR1 en ratas tratadas con IOA (datos no mostrados), sugiriendo un posible incremento de receptores formados por las subunidades NR1/NR2B en lugar de NR1/NRA. La relevancia de está observación se discutirá más adelante.

En la parte final de la tesis analizamos si el incremento en la expresión de la subunidad NR2B durante la inhibición metabólica sostenida correlaciona con cambios en la fosforilación. Los resultados muestran que 24 h después del tratamiento con IOA se presenta un incremento significativo del 80% en la fosforilación de la tirosina 1472 de la subunidad NR2B en el hipocampo de la rata (Fig. 16).

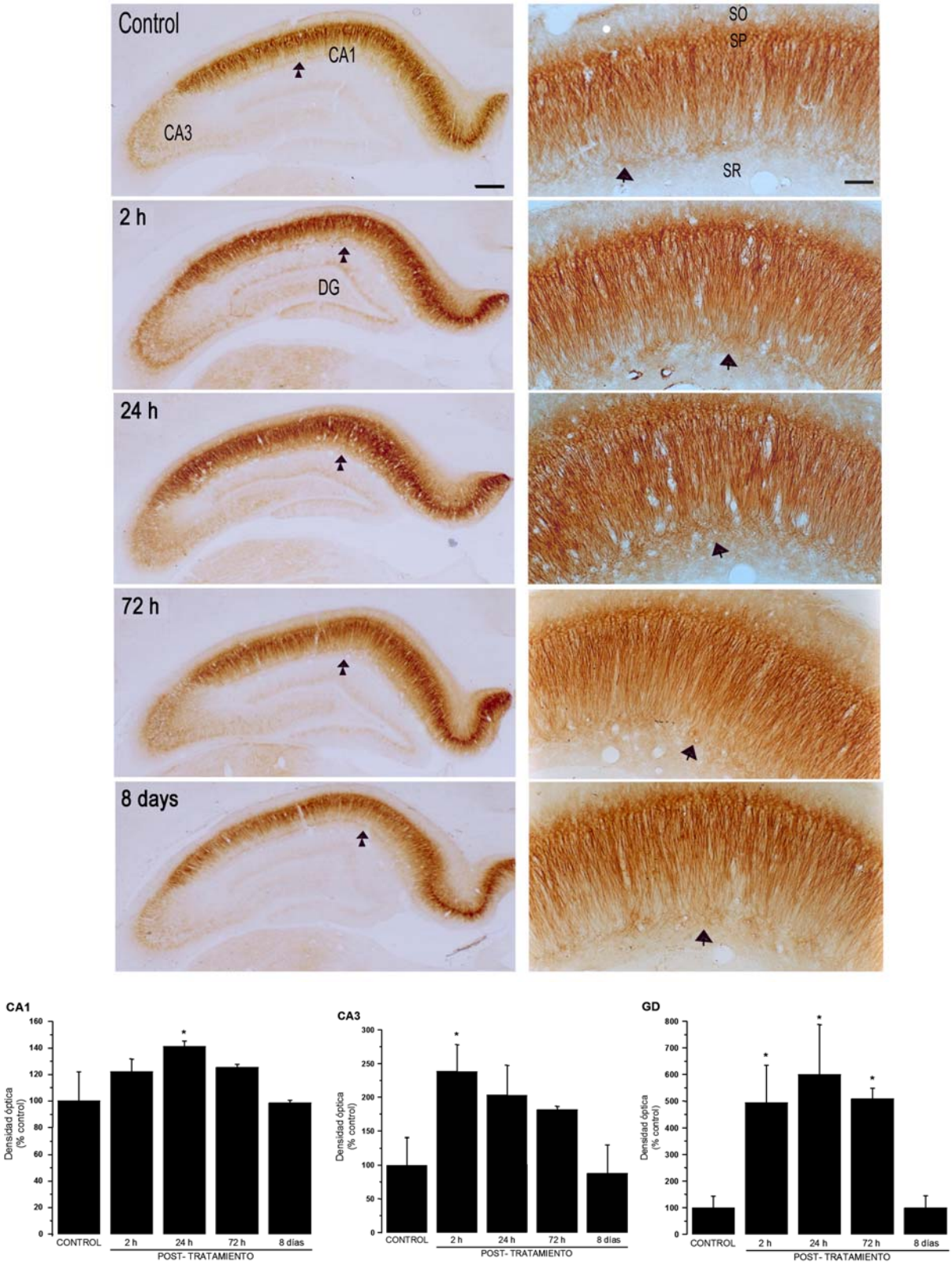

Fig.15. Inmunohistoquíca de la subunidad NR2B en el hipocampo después de la administración crónica de IOA. Las ratas fueron tratadas intraperitonealmente con IOA (15 mg/kg), y las controles con amortiguador de fosfatos (10 mM), durante tres días consecutivos, como se describió. El análisis de las secciones se realizó por medio de en un analizador de imágenes. Note que el incremento en la inmunoreactividad para NR2B es transitorio y regresa a sus niveles básales 8 días después del tratamiento. Barra = 200 µm. Panel derecho muestra una magnificación de la región CA1. Note que el incremento en la marca inmunohistoquímica se presenta a nivel de las dendritas apicales de la capa piramidal (flechas). Barra = 100 µm. Abajo se muestra el análisis densitométrico de la inmunoreactividad de la subunidad NR2B en cada región hippocampal. El incremento en la inmunoreactividad es transitorio y regresa a sus niveles basales 8 días después del tratamiento. Las gráficas representan el promedio ± ES de cinco experimentos independientes. * $p < 0.05$ vs control. DG, Giro dentado. Datos publicados en Neuroscience. 2007. 147: 873-886.

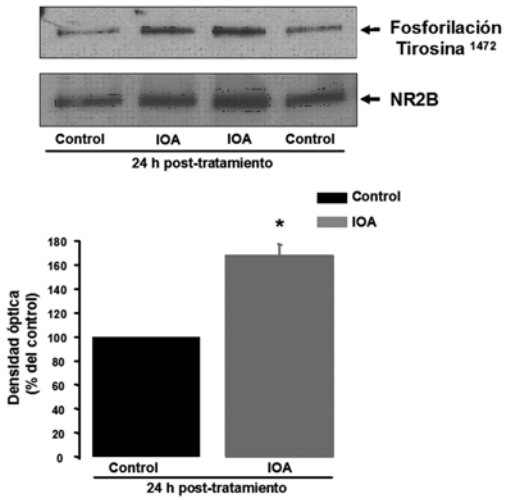

Fig.16. La inhibición sostenida de la glicólisis promueve la fosforilación de la tirosina 1472 de la NR2B. La inmunoprecipitación y el western blot de la subunidad NR2B se realizó en tejido de hipocampo de ratas tratadas con IOA y sacrificadas 24 h posteriores a la última administración como se describió en Materiales y Métodos. La gráfica indica el análisis densitométrico de la banda correspondiente a la tirosina 1472 fosforilada de la subunidad NR2B respecto al contenido total de NR2B. Las gráficas representan el promedio ± ES de cinco experimentos independientes. * $p < 0.0001$ vs control. Datos publicados en

X. DISCUSIÓN GENERAL

La función óptima del sistema nervioso central depende principalmente del metabolismo oxidativo de la glucosa. Se estima que aproximadamente el 20% de la glucosa total que se ingiere se consume para mantener el funcionamiento cerebral (Hyder et al., 2006; Shulman et al., 2004). Esta característica peculiar tiene relación con que el mayor porcentaje de sinápsis en el cerebro son glutamatérgicas y la vía glicolítica está íntimamente ligada a su control (Hyder et al., 2006; Shulman et al., 2004). Particularmente, la relevancia funcional de la G3PDH en la etiología de las enfermedades neurodegenerativas se ha sugerido debido a que ésta se encuentra asociada a las marañas neurofibrilares, proteínas tau, huntingtina y alfa-sinucleina presentes en pacientes con la enfermedad de Alzheimer, Huntington y Parkinson, respectivamente (Anderson et al., 2001; Bae et al., 2006; Burke et al., 1996; Tsuchiya et al., 2005; Wang and Shuaib, 2005). Se ha demostrado el decremento en la actividad enzimática de la G3PDH en fibroblastos de pacientes que padecen la enfermedad de Alzheimer y de Huntington (Mazzola and Sirover, 2001; Sonkusare et al., 2005; Spokes, 1980), o en algunas enfermedades que contienen proteínas con repetidos de poliglutamina (Burke et al., 1996). Es posible que alteraciones en el metabolismo glicolítico participen en la acumulación extracelular de AAE asociada a condiciones patológicas como la isquemia o el trauma cerebral y algunas enfermedades neurodegenerativas.

En esta tesis nos enfocamos a investigar las consecuencias de la inhibición del metabolismo glicolítico sobre la neurotransmisión glutamatérgica en dos modelos experimentales. En el primero investigamos las rutas de liberación de AAE y su participación en la inducción de muerte neuronal, inducida por la inhibición aguda del metabolismo glicolítico *in vivo*. Este primer modelo está relacionado con la inducción de muerte neuronal en condiciones de falla energética aguda como las presentes durante la isquemia o la hipoglicemia cerebral. En el segundo modelo se analizó si la inhibición sostenida de la vía glicolítica mediante la administración intraperitoneal de IOA induce alteraciones en los sistemas de recaptura del glutamato y/o en sus receptores. Este segundo modelo tiene como finalidad estudiar las alteraciones en la neurotransmisión

glutamatérgica presente en situaciones de inhibición metabólica moderada y sostenida similar a la que podrían ocurrir durante el desarrollo de enfermedades neurodegenerativas tipo Alzheimer, Huntington, Parkinson y durante el envejecimiento, e inclusive en la zona de penumbra de un foco isquémico.

Inhibición aguda del metabolismo glicolítico inducida por la perfusión de IOA en el hipocampo de la rata

Diversos estudios sustentan la participación del metabolismo glicolítico en la regulación de la concentración extracelular de glutamato (Gemba et al., 1994; Hyder et al., 2006; Ogata et al., 1995; Voutsinos-Porche et al., 2003). Los mecanismos responsables de la acumulación de AAE durante la inhibición de la glicólisis *in vivo* no se habían explorado antes. Tampoco se había estudiado el papel de dicha liberación en la muerte neuronal inducida en estas condiciones. Los resultados descritos en el presente trabajo demuestran que la inhibición aguda de la glicólisis *in vivo* induce un incremento de los niveles extracelulares de glutamato, y principalmente de aspartato a través de tres vías, una temprana asociada a la activación de canales de sodio dependientes de voltaje, y dos tardías relacionadas con la activación de los canales sensibles a DNDS, y al funcionamiento inverso de los transportadores de glutamato.

El componente inicial de la liberación se inhibe por el bloqueador de los canales de sodio dependientes de voltaje, riluzol (1 mM). Este resultado se parece mucho al obtenido en un modelo de isquemia en la rata (Drejer et al., 1985; Nelson et al., 2003), y sugiere que durante los primeros minutos se presenta la liberación exocitótica de AAE posiblemente debido a que aún existe ATP suficiente para que se lleve a cabo este proceso. Datos experimentales demuestran que en estas condiciones la liberación exocitótica de AAE se bloquea con el transcurso del tiempo (Nicholls and Attwell, 1990). En estudios *in vitro* se ha identificado una segunda ruta de liberación durante la inhibición glicolítica, constituida por el funcionamiento inverso de los transportadores de glutamato (Gemba et al., 1994; Ogata et al., 1995). Para que ocurra un cambio de dirección en el funcionamiento del transportador se requiere una alteración del gradiente iónico membranal

debido a una disminución en la función de la ATPasa de Na^+/K^+ causada por el decremento en los niveles de ATP. Se sugiere que un incremento en la concentración de sodio intracelular de 15 a 30 mM es suficiente para inducir un cambio en la dirección del transportador (Gemba et al., 1994; Longuemare et al., 1999). Es posible que en nuestras condiciones experimentales ocurra el funcionamiento inverso de los transportadores, ya que la inyección estereotáxica de IOA en la región CA1 del hipocampo causa un decremento del 50% en los niveles de ATP (Massieu et al., 2003). El incremento en los niveles extracelulares de glutamato se inhibe totalmente cuando se bloquean previamente sus transportadores con DL-TBOA (500 µM) o PDC (5 mM). Sin embargo, la perfusión de TBOA no previno la muerte neuronal, sino que por el contrario exacerbó el proceso neurodegenerativo. De acuerdo a nuestros resultados este efecto puede explicarse de dos maneras: 1) el DL-TBOA tiene afinidad por los receptores a NMDA (Shimamoto et al., 1998) activándolos e induciendo muerte neuronal y, 2) el DL-TBOA induce el incremento en la concentración extracelular no solo de glutamato sino también de glicina (Montiel et al., 2005) y posiblemente pueda promover la activación de los receptores a NMDA. Diversos estudios *in vitro* han confirmado la naturaleza tóxica del TBOA (Bonde et al., 2005; Guiramand et al., 2005; Selkirk et al., 2005).

A pesar de que el PDC a una concentración de 5 mM indujo un incremento mayor de los niveles basales de AAE que el DL-TBOA 500 µM, éste protege eficientemente contra la muerte neuronal inducida por IOA. Este efecto puede estar relacionado con efectos adicionales del PDC al bloqueo de los transportadores de glutamato tales como el mantenimiento de los niveles de glutatión intracelular (GSH) (Martin et al., 2005), modulación presináptica de receptores a glutamato metabotrópicos (Dudel and Schramm, 2003), o favoreciendo el mantenimiento de la concentración de ATP intracelular (Martin et al., 2005).

Nuestros resultados muestran que existe una tercera ruta de liberación de AAE durante la inhibición glicolítica, que involucra la activación de canales sensibles al bloqueador DNDS. La perfusión intrahipocampal de DNDS 10 mM no

sólo previene completamente la acumulación de AAE durante la inhibición glicolítica, sino también el daño neuronal. Su efecto neuroprotector correlaciona con la disminución de los niveles basales de AAE. Es posible que el DNDS bloquee la liberación de glutamato basal mediante la inhibición del complejo exocitótico de membrana, sinexina (Liu and Chander, 1995), o por el bloqueo de los receptores purinérgicos tipo $P2X_7$ (Bultmann and Starke, 1994). Se ha sugerido que ambos contribuyen a la liberación basal de glutamato (Bultmann and Starke, 1994; Liu and Chander, 1995). Adicionalmente, un reporte reciente señala que la liberación tónica de glutamato se inhibe por el bloqueador de los canales sensibles a volumen DIDS en rebanadas de hipocampo (Cavelier and Attwell, 2005). Ya que el bloqueador DIDS es un compuesto estructuralmente relacionado con DNDS, es posible que compartan mecanismos de acción similares. Aunque se ha reportado que el bloqueador DNDS inhibe a los CASV (Cabantchik and Greger, 1992; Seki et al., 1999), en nuestro caso dichos canales no parecen participar en la generación de la muerte neuronal, ya que inhibidores de amplio espectro de tales canales, como NPPB (1 mM) y floretina (1 mM) no previnieron ni la liberación de AAE, ni la muerte neuronal inducida por IOA. Sin embargo, la participación parcial de estos canales fue demostrada al perfundir un tercer bloqueador, el tamoxifen (250 µM), que previno ligeramente la acumulación de AAE durante los primeros minutos de la inhibición glicolítica. Sin embargo a pesar de ello, esto no fue suficiente para proteger en contra del daño neuronal. Por lo anterior se propone que sólo la inhibición de ambos componentes temprano y tardío de la liberación de AAE por el PDC y particularmente por el DNDS, previenen del daño neuronal. La protección por el DNDS podría involucrar mecanismos adicionales al bloqueo de la liberación de AAE, ya que los antagonistas de los receptores a glutamato tipo NMDA y no-NMDA, previenen solo el 50 % del daño celular (datos no mostrados), sugiriendo que mecanismos adicionales a la excitotoxicidad podrían participar en la muerte. Mecanismos relacionados con la reducción de la salida de glutatión de la célula (Wallin et al., 2003), o de las especies reactivas de oxígeno de la mitocondria hacia el citoplasma, como se ha sugerido para su análogo DIDS, podrían estar participando en el efecto protector del DNDS (Vanden Hoek et al., 1998).

Experimentos de nuestro laboratorio (Hernandez-Fonseca and Mássieu, 2005) y de algunos otros han demostrado la participación de estres oxidativo y la formación de ROS en la muerte inducida por IOA (Hernandez-Fonseca and Massieu, 2005; Malcolm et al., 2000; Matthews et al., 1997; Uto et al., 1995). Estudios más detallados son necesarios para conocer los mecanismos a través de los cuales el DNDS previene la muerte neuronal. Un resumen de los resultados del modelo de inhibición aguda de la glicólisis se presenta en la Fig. 17.

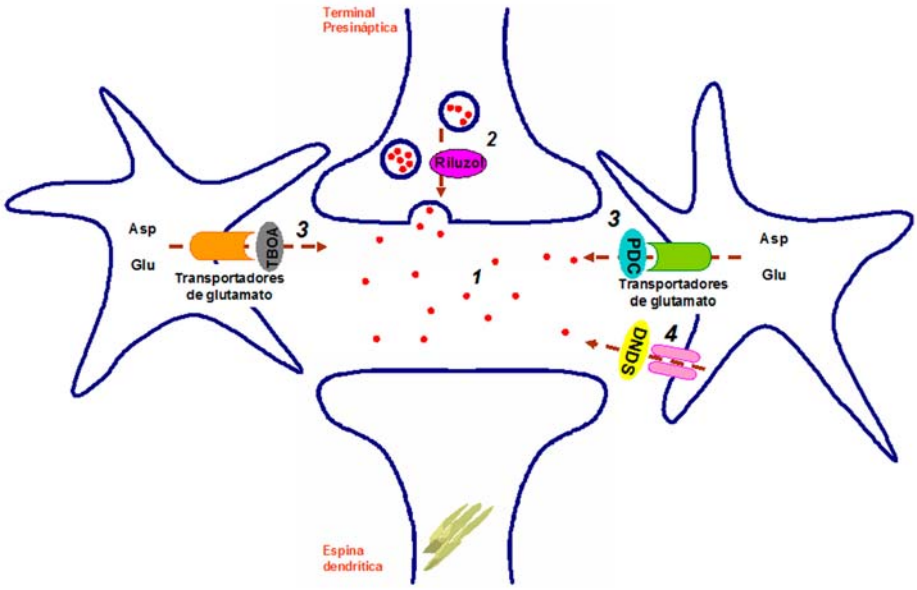

Fig.17. Rutas relacionadas con la acumulación de AAE durante la inhibición aguda de la glicólisis. La inhibición de la glicólisis por IOA promueve la acumulación extracelular de AAE (1). Los AAE se acumulan por la activación de tres vías: la liberación exocitótica de aminoácidos que se bloquea por Riluzol (2); el funcionamiento inverso de los transportadores que se inhibe por DL-TBOA y PDC (3); y la activación de los canales sensibles a DNDS (4). Adicionalmente, los canales sensibles a DNDS participan en el mantenimiento de los niveles basales de AAE (1), aunque se desconoce si éste mecanismo se encuentra en las neuronas o en la glía. El DNDS y el PDC previenen la muerte neuronal.

Inhibición sostenida de la glicólisis por la administración sistémica de IOA

Dada la importante participación del metabolismo glicolítico durante la neurotransmisión glutamatérgica, alteraciones parciales pero crónicas en esta vía metabólica pueden dar lugar a daño neuronal irreversible o facilitar el daño producido por agentes tóxicos como el glutamato. Un decremento en el metabolismo de la glucosa está relacionado con procesos de envejecimiento (Eberling et al., 1995; Moeller et al., 1996) y con enfermedades neurodegenerativas crónicas (Mielke et al., 1998; Planel et al., 2004; Slosman et

al., 2001; Stein et al., 1998). Adicionalmente, se ha reportado la reducción de la actividad de la enzima G3PDH en cerebro y fibroblastos de pacientes con las enfermedades de Alzheimer y Huntington (Kish et al., 1998; Mazzola and Sirover, 2001). En particular, la alteración moderada, pero progresiva en los niveles energéticos puede predisponer a las neuronas a la excitotoxicidad (Garcia and Massieu, 2003; Massieu et al., 2001; Massieu et al., 2000; Zeevalk and Nicklas, 1990). Se ha propuesto que la muerte asociada con enfermedades neurodegenerativas tales como la enfermedad de Alzheimer y Huntington tiene un componente excitotóxico (Csernansky et al., 1996; Schwarcz et al., 1983; Young et al., 1988), posiblemente relacionado con alteraciones en el contenido de proteína de los transportadores o receptores a glutamato (Arzberger et al., 1997; Behrens et al., 2002; Zhang et al., 1997; Zoia et al., 2004; Zoia et al., 2005). En el segundo modelo experimental estudiamos si la inhibición parcial pero sostenida del metabolismo glicolítico promueve alteraciones en los sistemas de recaptura de AAE y/o en la expresión de los receptores a glutamato. Datos experimentales de nuestro laboratorio habían demostrado que la administración intraperitoneal de IOA (15 mg/kg) durante tres días consecutivos, exacerba la muerte neuronal producida por la inyección intrahipocampal de glutamato (Massieu et al., 2000). Nuestros datos indican que bajo estas condiciones hay un decremento del 48 % de la actividad enzimática de la G3PDH en esa misma región. Está inhibición parcial podría explicar la reducción del 20% en los niveles de ATP en el hipocampo previamente descrita (Massieu et al., 2003). Asimismo, la actividad de la enzima no recupera sus valores basales aún después de suspendido el tratamiento con IOA. Aunque el recambio de la enzima en el cerebro no se conoce, datos experimentales indican que la síntesis de la G3PDH en células hepáticas ocurre cada 72 horas, por lo que podría ser que el decremento de la actividad enzimática de la G3PDH, a las 2 y 24 horas posteriores a la administración de IOA, sea el producto de una inhibición continua e irreversible, sugiriendo que esta no alcanza a recambiarse durante el tiempo del tratamiento.

Nuestros resultados muestran un incremento progresivo de los niveles extracelulares de aspartato y principalmente de glutamato, durante la

administración intraperitoneal de IOA. El incremento mayor de AAE se presenta 24 h después de suspender el tratamiento, lo que correlaciona muy bien con el mayor porcentaje de inhibición de la G3PDH. Estudios *in vitro* han demostrado que la inhibición de la glicólisis por IOA promueve la reducción de la captura de [^{3}H]-glutamato en astrocitos y en neuronas (Gemba et al., 1994; Jabaudon et al., 2000; Longuemare et al., 1999). En este sentido, analizamos si la acumulación de AAE estaba relacionada con la reducción de su capacidad de captura o con un decremento en el contenido de proteína de sus transportadores. De acuerdo al análisis cinético, la captura de [^{3}H]-D-aspartato en rebanadas de hipocampo de ratas tratadas con IOA presenta un decremento de la Vmax sugiriendo una disminución en el número de transportadores membranales respecto a sus controles. Los datos obtenidos en nuestros experimentos muestran valores de *km* más altos a los reportados en otras preparaciones *in vitro* tales como sinaptosomás, cultivo de astrocitos y diversos sistemas de expresión como células PC12 (Danbolt, 2001; Garlin et al., 1995; Kanai and Hediger, 1992; Robinson, 1998), Aunque, valores de *km* más altos se han reportado en rebanadas de cerebro (Heron et al., 1995; Kuwahara et al., 1992), éstas aún son menores a los descritos en la presente tesis. Esta discrepancia puede estar relacionada con la dificultad de observar sistemas de captura de alta afinidad en una preparación que presenta distintos tipos celulares, los cuales dificultan la difusión y captura de [^{3}H]-D-aspartato. La consecuencia de ello ocasionaría un incremento de los valores de *km* debido a que la concentración de D-aspartato requerida en el medio de incubación para saturar a los transportadores podría ser mucho mayor que en un sistema homogéneo como los cultivos celulares o sinaptosomás (Garthwaite and Garthwaite, 1985; Robinson, 2006). Con base a los valores de *km* obtenidos, la captura de D-aspartato podría corresponder a un sistema de baja afinidad; sin embargo, la captura de baja afinidad es independiente de sodio (Anderson and Swanson, 2000; Benjamin and Quastel, 1976; Davies and Johnston, 1976; Kuwahara et al., 1992), y nuestros resultados demuestran que la captura es totalmente dependiente de sodio. Adicionalmente, ésta se inhibe por el bloqueador selectivo del transportador GLT-1, DHK, y por el bloqueador no selectivo de los

transportadores de alta afinidad, DL-TBOA, sugiriendo que la captura observada en las presentes condiciones es al menos en parte mediada por transportadores de glutamato sensibles a DHK y dependientes de sodio.

La captura de [^{3}H]-D-aspartato sensible a DHK en los animales tratados con IOA se redujo significativamente. Esto correlaciona con un decremento de proteína del transportador GLT-1, sugiriendo que el decremento en la captura durante la inhibición de la glicólisis *in vivo* puede estar relacionado con la reducción del número de transportadores de tipo GLT-1. En la regulación del número de transportadores de glutamato en las células participan dos mecanismos. El primero esta relacionado con la activación de la transcripción, traducción, ensamble y glicosilación. El segundo involucra el tráfico y la redistribución del transportador del citoplasma a la membrana plasmática (para revisión ver (Robinson, 2006). La mayoría de los datos disponibles acerca de la regulación de la síntesis de transportadores de glutamato proviene de experimentos *in vitro* analizando la expresión del transportador neuronal EAAC1 (Fournier et al., 2004; Robinson, 2006; Sheldon et al., 2006; Yang and Kilberg, 2002), y del glial GLAST (Bernabe et al., 2003; Gonzalez et al., 1999; Kim et al., 2003; Susarla et al., 2004). De acuerdo a algunos estudios el tiempo estimado para la síntesis y degradación del transportador ocurre en 8 horas (Yang and Kilberg, 2002), y la vida media en la membrana plasmática es de sólo 5 a 7 minutos (Fournier et al., 2004). Para el caso del transportador GLT-1 no se conoce cual es el tiempo total necesario para su síntesis y degradación, sin embargo, se estima sea muy parecido al EAAC1 (Robinson, 2006). En estudios previos se demostró que los astrocitos en cultivo no expresan normalmente el transportador GLT-1 (Schlag et al., 1998; Swanson et al., 1997b), a menos que se co-cultiven con neuronas (Schlag et al., 1998) o se incuben con el medio condicionado neuronal (Gegelashvili et al., 1997; Zelenaia et al., 2000). Esto sugiere que ciertas moléculas secretadas por neuronas promueven la expresión de GLT-1. Este mismo efecto puede reproducirse al incubar a los astrocitos con el factor de crecimiento derivado de la epidermis, con análogos del AMP cíclico y con el polipéptido que activa a la adenilato ciclasa de la glándula pituitaria (Figiel and Engele, 2000; Schlag et al., 1998; Swanson et al.,

1997b; Zelenaia et al., 2000). De manera general, la activación del promotor de GLT-1 en estas condiciones involucra dos diferentes vías: 1) la activación de receptores al factor de crecimiento inducen la actividad de la fosfatidil inositol 3 cinasa, la subsecuente activación de Akt y corriente abajo la del factor de transcripción NF-κB; y 2) los análogos de AMP cíclico inducen la activación de la PKA, la activación de la MAPK p42/p44 que promueve la activación de ERK y la fosforilación de CREB y ATF1 (Gegelashvili et al., 2000; Li et al., 2006; Rodriguez-Kern et al., 2003; Sitcheran et al., 2005; Zelenaia et al., 2000). Por el contrarío, el decremento en la actividad del promotor de GLT-1 se relaciona con la activación del receptor al factor de necrosis tumoral α, en un proceso que requiere del factor de transcripción N-myc (Kim et al., 2003; Su et al., 2003; Zelenaia et al., 2000). Sugerimos que el decremento de proteína del GLT-1 y de la *Vmax* observado en nuestros experimentos, puede estar relacionado con la inhibición y/ó activación de alguna de estas vías debido al incremento de glutamato extracelular, como se ha sugerido para el transportador GLAST en la glía de Bergmann (Gonzalez et al., 1999; Lopez-Bayghen and Ortega, 2004; Rosas et al., 2007). Asimismo, se ha observado que la activación de los receptores metabotrópicos de glutamato en astrocitos humanos promueve el decremento en los niveles de GLT-1 (Aronica et al., 2003). Sin embargo, es posible también que alteraciones en la degradación o el tráfico del transportador GLT-1 jueguen un papel importante. Son necesarios experimentos adicionales para conocer si el decremento en el contenido de proteína del GLT-1 correlaciona con la disminución de los niveles de RNA mensajero o sólo se debe a un cambio en la distribución celular del transportador por la activación de PKC.

Como mencionamos, se propone que un componente de la muerte neuronal asociada con las enfermedades neurodegenerativas es excitotóxico. Éste tipo de muerte resulta de la activación prolongada de los receptores glutamatérgicos (NMDA y no-NMDA) a causa del aumento de la concentración extracelular de AAE (Olney, 1969; Olney and Ho, 1970). En particular, los receptores a NMDA participan principalmente en esta cascada de daño, ya que son altamente permeables a calcio, respecto a los de kainato o AMPA (Arundine and Tymianski,

2003). Una de las características estructurales que determina que el receptor a NMDA presente una menor o mayor permeabilidad a calcio, es la composición de subunidades que lo forman. El patrón de expresión de las subunidades NR1 y NR2A en el hipocampo, muestra un incremento en los niveles de RNA mensajero y proteína desde el nacimiento hasta la etapa post-natal y que se mantiene en el adulto, mientras que los niveles de la subunidad NR2B decrecen en el adulto (Dumas, 2005; Guilarte and McGlothan, 1998; Luo et al., 1996; Monyer et al., 1994; Ritter et al., 2002; Wenzel et al., 1997). Esto correlaciona con un decaimiento en la amplitud de corrientes post-sinápticas excitadoras, debido a que la subunidad NR2B le confiere una cinética de desactivación al receptor mucha más larga (Cull-Candy and Leszkiewicz, 2004; Kirson and Yaari, 1996). Por tanto, la asociación diferencial entre las subunidades NR2 con la NR1 da lugar a receptores con un perfil farmacológico característico, en términos de afinidad por el agonista, sensibilidad al bloqueo por magnesio y respuesta a antagonistas (Cull-Candy and Leszkiewicz, 2004; Popescu, 2005; Vicini et al., 1998). Nuestros resultados indican que la inhibición del metabolismo glicolítico por IOA no altera significativamente el contenido de proteína de las subunidades NR1 y NR2A. Sin embargo, los niveles de la subunidad NR2B aumentan progresivamente durante el tratamiento con IOA. Este incremento es sólo transitorio y regresa a sus niveles basales 72 h después. El análisis inmunohistoquímico confirma el incremento de la subunidad NR2B en la región CA1 del hipocampo observado por western blot. Quizá aún más interesante resulta el hecho de que el incremento en la marca inmunohistoquímica para la NR2B se confina a nivel de las dendritas apicales de la región CA1. Este resultado es sumamente interesante ya que es precisamente en este sitio donde se encuentran los contactos sinápticos que provienen de la región CA3 (Nicoll and Schmitz, 2005). Adicionalmente, el análisis inmunohistoquimico demostró un incremento de la marca para la subunidad NR2B a nivel de CA3 y giro dentado. Proponemos que debido a la ausencia de cambios en el nivel de proteína de las subunidades NR1 y NR2A, y al incremento en la NR2B la proporción de los receptores conformados por las subunidades NR1/NR2B será mayor. Aunque nuestros datos experimentales no son suficientes

para confirmar esta hipótesis, sugerimos que la posible formación de este complejo puede incrementar la vulnerabilidad de las neuronas al glutamato, ya que los receptores que contienen a la subunidad NR2B permanecerán más tiempo abiertos, ocasionando un aumento mayor en la concentración de calcio en el citoplasma favoreciendo así la excitotoxicidad. Si bien es cierto que no conocemos cuál es la concentración de calcio intracelular que puede alcanzarse en nuestras condiciones experimentales, estudios previos de nuestro laboratorio han demostrado que la incubación de cultivos de neuronas de hipocampo en un medio libre de calcio o bien en un medio con BAPTA-AM, que es quelante de calcio intracelular, previenen el daño neuronal inducido por IOA; sugiriendo la participación del calcio en el proceso de muerte producido por la inhibición de la vía glicolítica (Hernandez-Fonseca and Massieu, 2005). Una regulación a la alta de la subunidad NR2B se ha reportado después de la isquemia en la rata (Besshoh et al., 2005; Kang et al., 2001; Quintana et al., 2006; Sutcu et al., 2005; Won et al., 2001). (Heurteaux et al., 1994), sugieren que el incremento de glutamato durante el periodo isquémico en la rata es el principal factor asociado con éste efecto. La expresión de la subunidad NR2B se regula por la actividad sináptica a través de la activación de cinasas CaM (que son estas proteinas), cinasas de tirosina y de la proteina CINAP (Corsi et al., 1998; Kalluri and Ticku, 1999; Wang et al., 2004). La subunidad NR2B es la fosfo-proteína predominante en la densidad postsináptica (Moon et al., 1994), y su tráfico a la membrana plasmática puede iniciarse por la fosforilación de tirosinas (Lavezzari et al., 2003; Prybylowski et al., 2005). Los resultados indican que la inhibición sostenida de la glicólisis promueve la fosforilación de la Tyr 1472 de la subunidad NR2B, lo cual podría promover su tráfico a la membrana plasmática. Si ésta hipótesis es correcta, la fosforilación de la Tyr 1472 promoverá la estabilidad de la subunidad NR2B en la membrana previniendo su internalización dependiente de clatrina (Lavezzari et al., 2003; Prybylowski et al., 2005). La fosforilación en tirosina de esta subunidad ha sido relacionada con la activación de la cinasa Fyn (Nakazawa et al., 2001), lo cual incrementa la actividad del receptor a NMDA (Xu et al., 2006). De hecho se ha demostrado la participación de la subunidad NR2B en la muerte

neuronal en modelos de deficiencia energética como la isquemia cerebral y enfermedades neurodegenerativas (Kundrotiene et al., 2004; Reyes et al., 1998; Wang and Shuaib, 2005). Son necesarios más experimentos para demostrar si el incremento de la subunidad NR2B exacerba la respuesta de los receptores NMDA al glutamato y promueve el incremento en la susceptibilidad a la muerte de las neuronas hipocampales**.** Un resumen de los resultados del modelo de inhibición sostenida de la glicólisis se presenta en la Fig. 18.

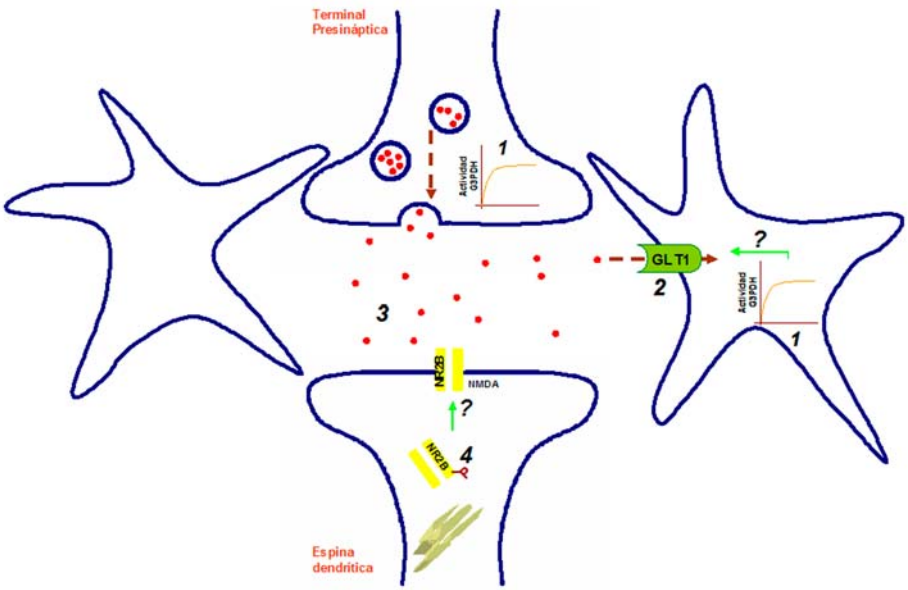

Fig.18. Esquema del modelo de inhibición sostenida de la glicólisis. La administración intraperitoneal de IOA decrece la actividad de la enzíma G3PDH (1). Por un mecanismo desconocido, la inhibición de la vía glicolítica promueve el decremento en el número de transportadores GLT-1 membranales (2). Esta alteración se asocia a la acumulación extracelular de AAE (3). Adicionalmente, el bloqueo del metabolismo glicolítico aumenta la proteína y la fosforilación de la subunidad NR2B del receptor a NMDA (4). Se desconoce si los receptores que expresan la NR2B se encuentren predominantemente en la membrana.

XI. CONCLUSIONES

Los resultados de esta tesis sugieren la participación del metabolismo glicolítico en el control de la concentración extracelular de AAE, y su relevancia en el desarrollo de la muerte neuronal *in vivo*. En ambos modelos experimentales se estableció que la inhibición de la glicólisis aguda o sostenida, incrementa los niveles extracelulares de AAE. El modelo agudo propone que no es suficiente con inhibir el componente temprano de liberación de AAE, sino que se requiere de la inhibición de ambos componentes, inicial y tardío para promover la sobrevivencia neuronal. Las rutas de liberación de AAE están relacionadas con la actividad de los transportadores de glutamato y de los canales sensibles a DNDS. Por su parte, los resultados obtenidos en el segundo modelo sugieren que la susceptibilidad neuronal a la muerte inducida por glutamato, podría explicarse por una acumulación de AAE debida al decremento en el número de transportadores de tipo GLT-1 y al incremento en el contenido de proteína y en la fosforilación en tirosina de la subunidad NR2B.

Los resultados de esta tesis son relevantes para comprender los mecanismos involucrados en la muerte neuronal asociada a condiciones patológicas en las que se presentan alteraciones en el metabolismo de la glucosa como ocurre en las enfermedades de Alzheimer y Huntington, y en estados patológicos como la isquemia y la hipoglicemia.

XII. PERSPECTIVAS

Los resultados del modelo agudo de inhibición glicolítica presentan evidencia histológica de que el mecanismo sensible al DNDS es el principalmente involucrado en la muerte neuronal. A la fecha, evidencias experimentales *in vitro* sugieren que este compuesto puede estar involucrado en la protección en contra de la generación de estrés oxidativo cerebral. En este sentido es importante conocer si dicho compuesto posee propiedades antioxidantes que pudieran explicar su efecto neuroprotector en contra de la muerte neuronal, además de su efecto sobre la liberación de AAE *in vivo*.

Por su parte, el segundo modelo indica que la inhibición glicolítica sostenida promueve la regulación a la baja del contenido de proteína del transportador GLT-1, y a la alta de la subunidad NR2B, lo cual correlaciona con el incremento de la fosforilación en tirosina de esta proteína. Aunque se conoce que la activación de proteínas cinasas participa en el tráfico y degradación de las subunidades del receptor NMDA, es necesario conocer la cascada de señalización vinculada a la regulación de las mismas. Adicionalmente, se requiere conocer si la activación de esta señal puede generar la muerte de las neuronas.

XIII. APÉNDICE

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DIFFERENTIAL EFFECTS OF THE SUBSTRATE INHIBITOR L-*TRANS*-PYRROLIDINE-2,4-DICARBOXYLATE (PDC) AND THE NON-SUBSTRATE INHIBITOR DL-THREO-β-BENZYLOXYASPARTATE (DL-TBOA) OF GLUTAMATE TRANSPORTERS ON NEURONAL DAMAGE AND EXTRACELLULAR AMINO ACID LEVELS IN RAT BRAIN *IN VIVO*

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Abstract—The extracellular concentration of glutamate is highly regulated by transporter proteins, due to its neurotoxic properties. Dysfunction or reverse activation of these transporters is related to the extracellular accumulation of excitatory amino acids and neuronal damage associated with ischemia and hypoglycemia. We have investigated by microdialysis the effects of the substrate and the non-substrate inhibitors of glutamate transporters, L-trans-2,4-pyrrolidine dicarboxylate (PDC) and DL-threo-β-benzyloxyaspartate (DL-TBOA), respectively, on the extracellular levels of amino acids in the rat hippocampus *in vivo*. In addition, we have studied the effect of both inhibitors on neuronal damage after direct administration into the hippocampus and striatum. Electroencephalographic activity was recorded after the intrahippocampal infusion of DL-TBOA or PDC. Microdialysis administration of 500 μM DL-TBOA into the hippocampus increased 3.4- and nine-fold the extracellular levels of aspartate and glutamate, respectively. Upon stereotaxic administration it induced neuronal damage dose-dependently in CA1 and dentate gyrus, and convulsive behavior. Electroencephalographic recording showed the appearance of limbic seizures in the hippocampus after DL-TBOA infusion. In the striatum it also induced dose-dependent neuronal damage. These effects were prevented by the i.p. administration of the glutamate receptor antagonists (+)-5-methyl-10,11-dihydroxy-5*H*-dibenzo(*a,d*)cyclohepten-5,10-iminemaleate and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*F*)-quinoxaline. In contrast to DL-TBOA, PDC (500 μM) induced a more discrete elevation of excitatory amino acids levels (2.6- and three-fold in aspartate and glutamate, respectively), no neuronal damage or behavioral changes, and no alterations in electroencephalographic activity. The differential results obtained with DL-TBOA and PDC might be attributed to their distinct effects on the extracellular concentration of amino acids. Results are relevant to the understanding of the role of glutamate transporters in amino acid removal or release and the induction of excitotoxic cell death. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: excitotoxicity, ischemia, glutamate transporters, neuronal damage.

The extracellular concentration of glutamate, the main excitatory neurotransmitter in the mammalian CNS, is highly regulated due to its neurotoxic action (Olney, 1971; Choi, 1987; for review see Bittigau and Ikonomidou, 1997). After its release from synaptic terminals, glutamate is efficiently removed from the synaptic cleft mainly by glial transporters terminating its synaptic action (Atwell et al., 1993; Danbolt, 2001). To date five glutamate transporters have been identified: GLAST (EAAT1) and GLT-1 (EAAT2) are present mainly in astrocytes, although they have been also observed in neurons (Rothstein et al., 1994; Chen et al., 2002, 2004). EAAC1 (EAAT3) is located in neurons (Kanai and Hediger, 1992; Rothstein et al., 1994), EAAT4 is present in Purkinje cell dendrites in cerebellum (Fairman et al., 1995), and EAAT5 is located in retina (Arriza et al., 1997). Glutamate transporters are sodium dependent and their correct functioning depends on the membrane Na^+ gradient and in turn on the activity of the Na^+/K^+ ATPase. During energy-limiting conditions such as ischemia and hypoglycemia, the Na^+ gradient collapses leading to the deficient functioning or even the inverse activation of glutamate transporters, extruding glutamate to the extracellular space (Szatkowski et al., 1990; Atwell et al., 1993; Rossi et al., 2000).

Previous studies have shown that inhibition of glutamate transporters by the substrate inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC) induces neuronal death in cortical cultured neurons which correlates with increased glutamate extracellular concentration (Velasco et al., 1996). Several studies have shown that glutamate release during PDC exposure results from heteroexchange rather than transport blockade (Blitzblau et al., 1996; Volterra et al., 1996). *In vivo* microdialysis perfusion of high concentrations of PDC (25–100 mM) into the rat hippocampus or the striatum, induces large increases in the extracellular concentration of glutamate and aspartate but only causes minimal damage (Massieu et al., 1995; Massieu and Tapia, 1997). In contrast, long-term infusions of PDC (3–14 days) or prolonged synthesis inhibition of glial glutamate transporters results in neuronal death (Rothstein et al., 1996; Liévens et al., 1997, 2000), suggesting that continuous

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Abbreviations: DL-TBOA, DL-threo-β-benzyloxyaspartate; MK-801, (+)-5-methyl-10,11-dihydroxy-5*H*-dibenzo(*a,d*)cyclohepten-5,10-iminemaleate; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*F*)-quinoxaline; PDC, L-*trans*-2,4-pyrrolidine dicarboxylate.

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dysfunction of these transporters is necessary in order to induce cell death *in vivo*.

Recently, it has been shown that the non-substrate glutamate transport inhibitor, DL-threo-β-benzyloxyaspartate (DL-TBOA), effectively blocks glutamate uptake in cultured neurons (Anderson et al., 2001; Waagepetersen et al., 2001). In cortical astrocytes it shows dose-dependent substrate activity, and induces significant release of [^{14}C]-aspartate, through heteroexchange at relatively high concentrations (100–1000 μM; Anderson et al., 2001). In hippocampal slices DL-TBOA increases the extracellular concentration of glutamate due to blockade of glutamate uptake (Jabaudon et al., 1999). On the other hand, low concentrations of DL-TBOA (10 μM) significantly reduce glutamate release induced during energy-depleting conditions in cortical cultured astrocytes (Anderson et al., 2001). Similarly, *in vivo* perfusion of DL-TBOA attenuates glutamate and aspartate release induced during cerebral ischemia (Phillis et al., 2000). However, during long-term incubation DL-TBOA induces neuronal cell death in cultured hippocampal slices (Bonde et al., 2003), although other study reports relative insensitivity of this preparation to the toxicity of glutamate uptake inhibitors (O'Shea et al., 2002). In the retina, DL-TBOA shows a rapid and potent neurotoxic effect (Izumi et al., 2002).

The study of the effects of glutamate transport blockers on the extracellular levels of glutamate and aspartate, might be useful to the understanding of the mechanisms involved in amino acid removal and release and its relation to excitotoxic cell death. It has been hypothesized that neuronal death associated with brain ischemia and hypoglycemia is of excitotoxic nature, due to the large increases in the extracellular concentration of glutamate and aspartate observed during these conditions (Benveniste et al., 1984; Sandberg et al., 1986). In the present study we investigated by microdialysis the effects of the substrate and the non-substrate inhibitors of glutamate transporters, PDC and DL-TBOA, respectively, on the extracellular concentration of amino acids in the hippocampus. The effect of direct administration of these glutamate uptake blockers on neuronal damage was also studied in the hippocampus and the striatum. Additionally, electroencephalographic activity and alterations in animal behavior were studied after intrahippocampal infusion of DL-TBOA or PDC. Results show important differential effects of PDC and DL-TBOA on the extracellular levels of amino acids that might explain their distinct neurotoxic and convulsive properties.

EXPERIMENTAL PROCEDURES

Microdialysis experiments

Males Wistar rats (250–320 g) were used throughout the study. They were handled according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) and the local Animal Care Committee approved all animal treatments. All efforts were made to minimize the number of animals used and their suffering. Microdialysis experiments were performed as previously described (Massieu et al., 2000). Briefly 2 mm long and 0.5 mm-diameter microdialysis probes were unilaterally implanted into the hippocampus of anesthetized animals (0.5–1.0% halothane) and fixed on a stereotaxic frame (coordinates AP −3.6 mm from bregma, L +2.0 mm from midline and V −4.0 mm from dura). Probes were continuously perfused with a Ringer Krebs medium containing (in mM): NaCl 118, KCl 4.5, $MgSO_4$ 1.18, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 10, at a flux rate of 2 μl/min via a microinjection pump. After a 90-min equilibration period one 25 μl (12.5 min) fraction was collected to determine basal amino acid concentrations. Immediately after, perfusion medium was changed for Ringer Krebs containing either 500 μM PDC or TBOA and six additional fractions were collected. Afterward, medium was changed for Ringer Krebs (wash), and three more fractions were collected. Animals were transcardially perfused 24 h after microdialysis perfusion and brains prepared for histological analysis as described. Dialysis fractions were kept at −80 °C until analyzed for amino acid content. Recovery of amino acids by microdialysis probes was assessed before the experiment as described previously (Massieu et al., 1995).

The concentration of PDC and DL-TBOA was chosen based on the previously determined dose-response curve for PDC (Massieu et al., 1995). According to these data 1 mM PDC significantly increases extracellular excitatory amino acids levels and induces no neuronal damage. In the present study lower dose (0.5 mM) was used because it approximates the lowest concentration of DL-TBOA (7.5 nmol) tested for intracerebral administrations that did not induce motor alterations.

Determination of amino acid content

Amino acid content in dialysates was determined by HPLC according to the methodology described by Antoine et al. (1999). Collected 25 μl aliquots were derivatized with the same volume of *o*-phthalaldehyde and 3 min later a 10 μl volume of this mixture was injected into a HPLC system (Waters 600, Milford, MA, USA) equipped with an ODS column (25 cm×4 mm internal diameter; Supelco, Inc., Bellefonte, PA, USA). The mobile phase consisted of phosphate buffer (60 mM pH=6.65) on one line, and 18% methanol, 22% acetonitrile, 14% isopropanol, 46% phosphate buffer on the other line. It was run at 1 ml/min in a linear gradient of 33 min duration from 10 to 90% solvent mixture. Amino acid concentration was calculated by comparison of the data obtained with a standard mixture of amino acids equally processed.

Administration of glutamate uptake blockers

For intracerebral injections animals were anesthetized with 2% halothane in a 95% O_2/5% CO_2 mixture and placed on a stereotaxic frame. A Hamilton needle was positioned either in the hippocampus (coordinates: AP −3.6 mm from bregma, L +2.0 mm from midline, and V −2.5 mm from dura, according to Paxinos and Watson, 1986), or the striatum (AP +0.7, L −2.8 and V −4.0), and 1 μl volume of PDC (300 nmol), DL-TBOA (7.5, 15 and 25 nmol) or saline (0.9%) was unilaterally injected at a rate of 0.5 μl/min with the aid of a microinjection pump (Harvard Apparatus; pump model 55, South Natick, MA, USA). Two minutes after the injection was completed the needle was withdrawn and the skin sutured. When the effect of the NMDA receptor antagonist, (+)-5-methyl-10,11-dihydroxy-5*H*-dibenzo(*a,d*)cyclohepten-5,10-iminemaleate (MK-801) was tested, it was intraperitoneally administered at 2 mg/kg 30 min before the intracerebral injection of glutamate uptake blockers. The non-NMDA receptor antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*F*)-quinoxaline (NBQX) was administered at 25 mg/kg 15 min before and immediately after the intracerebral injection of DL-TBOA. Twenty-four hours after the injection animals were anesthetized, transcardially perfused with 200 ml 0.9% saline followed by 200 ml 5% formaldehyde in 0.1 M phosphate buffer (pH 7.3). Brains were removed and left in fixative for additional 24 h. They were transferred successively to 20% and 30% sucrose (48 h each) and 40 μm coronal sections were cut in a cryostat and stained with Cresyl Violet. Lesion volume was calcu-

lated with the aid of an image analyzer (NIH Macintosh Image 1.6) as previously described (Massieu et al., 2001, 2003).

In previous studies we have used doses of 400 and 500 nmol PDC for intrastriatal and intrahippocampal administration. At these concentrations PDC produces lesions slightly larger but not statistically different to those induced by vehicle injection. Lesions observed in the present study using 300 nmol PDC are similar in size to those previously reported with 400 and 500 nmol PDC (Massieu et al., 1995, 2001, 2003). Doses of 150 and 50 nmol/μl DL-TBOA were initially tested, but due to the presence of intense behavioral alterations they were reduced to 25, 15 and 7.5 nmol/μl. The protocols of administration of MK-801 and NBQX were chosen based on previous studies showing they are effective against excitotoxic or ischemic neuronal damage (Massieu et al., 2000; Sheardown et al., 1990).

Electroencephalographic recording

Male Wistar rats (240–260 g) were anesthetized with 2% halothane in 95% O_2/5% CO_2 and stereotaxically implanted with 0.5 mm diameter stainless-steel electrode-cannulas in the right and left dorsal hippocampi (coordinates: AP −3.6, L 2.0, and V −2.0). A single stainless steel epidural electrode was implanted in the occipital cortex and a reference screw in the frontal skull. Electrodes were fixed to the skull with dental cement. After surgery the anesthesia was discontinued and rats were returned to individual cages and provided with food and water *ad libitum*. One week after surgery, EEG recordings were made using a Model 79E Grass polygraph (Quincy, MA, USA, filters 0.3–300 Hz) in the awake freely moving rats. Basal EEG was recorded for 30 min and then 25 nmol DL-TBOA or 300 nmol PDC, was injected into the left hippocampus in a 1 μl volume, using a microsyringe inserted into the electrode-cannula, at a rate of 0.5 μl/min. The EEG was recorded for 3–4 h, and in some rats it was recorded again for 60 min 24 h after the experiment. The EEG data were quantitatively analyzed by calculating the mean latencies to the onset of epileptic discharges, as well as their duration (measured from the beginning of the hypersynchronic activity to the end of the high-amplitude spike train) and their frequency of occurrence.

Materials

PDC, DL-TBOA, MK-801 and NBQX were purchased from Tocris (Ellisville, MO, USA). Some of the experiments were performed with DL-TBOA kindly donated by Dr. K. Shimamoto (Suntory Institute for Bioorganic Research, Osaka, Japan). Microdialysis probes (CMA/12) were obtained from CMA/Microdialysis BA (Stockholm, Sweden).

Statistics

The effects of PDC and DL-TBOA on glutamate and aspartate concentrations was analyzed by two-way ANOVA analysis (factor one was defined as inhibitors: PDC and DL-TBOA; and factor two as time: 0, 25, 50, 75, 100, 125 min). In addition, a separate one-way ANOVA analysis was used to compare the effect of each inhibitor relative to amino acid basal values. A post-hoc Fisher's multiple comparison test was used to compare data on each time point. Significant differences are indicated in graphs.

The effects of PDC and DL-TBOA on neuronal damage were analyzed by one-way ANOVA followed by a Fisher's least significant multiple comparison test. The protective effect of MK-801 and NBQX on neuronal damage induced by DL-TBOA were analyzed separately with its corresponding control (without antagonist), by one-way ANOVA followed by a Fisher's least significant multiple comparison test. In all cases data represent means± S.E.M. of the number of independent experiments indicated in figure legends.

RESULTS

Microdialysis experiments

The effect of PDC and DL-TBOA on the extracellular levels of glutamate and aspartate in the hippocampus *in vivo* was studied through microdialysis. Perfusion of 500 μM DL-TBOA increased 3.4-fold aspartate extracellular levels achieving concentrations up to 1.8 μM, while 500 μM PDC induced a 2.6-fold increase up to 1.3 μM aspartate (Fig. 1). According to the two-way ANOVA analysis significant differences were found in the effects of both inhibitors on aspartate levels ($F=10.67$, $P<0.0001$). In the case of glutamate, DL-TBOA induced a nine-fold increase in amino acid levels (up to 18 μM) while PDC produced only a 3.0-fold increase (up to 6.0 μM, Fig. 1). Statistical differences in the effects of both compounds were also found to be significant for glutamate ($F=52.27$, $P<0.0001$). The effects of PDC and DL-TBOA on glutamate and aspartate concentration relative to amino acid basal levels, were compared individually at each individual time-point. Significant differences were found for PDC during its peak effect (fractions 3–8). The effect of DL-TBOA on aspartate (all fractions) and glutamate (fractions 3–9) was also statistically significant. According to the results, amino acid concentrations returned to basal levels more rapidly after washing in the case of PDC as compared with DL-TBOA. We have no explanation for this differential effect, but it might be related to differences in the extracellular removal of these inhibitors from the extracellular space.

PDC perfusion had no effect on glutamine, alanine, taurine or glycine extracellular levels, while DL-TBOA significantly increased alanine levels (Table 1). The effect of DL-TBOA (500 μM) on glycine levels could not be determined due to the interference of DL-TBOA present in microdialysates on glycine HPLC detection. Therefore, in order to study the effect of DL-TBOA on the extracellular concentration of this amino acid, a 50 μM concentration was perfused through the dialysis probe in two animals. At this concentration of DL-TBOA, the peak corresponding to glycine by HPLC detection was clearly separated. Data showed increases of 164% in aspartate, 83% in glutamate and 91% in glycine levels at peak, relative to basal concentrations (not shown).

Twenty-four hours after microdialysis, rats were transcardially perfused and the brains removed and prepared for histological analysis and lesion volume determination. Rats perfused with 500 μM DL-TBOA showed neuronal damage throughout the CA1 region of the hippocampus and dentate gyrus, and a lesion volume of 13.9±1.99 mm^3 (data not shown, $n=5$). In contrast, in rats perfused with PDC neuronal damage was restricted to the region where the microdialysis probe was inserted (lesion volume= 0.260±0.048 mm^3, $n=4$). The two animals perfused with 50 μM DL-TBOA showed neuronal damage in the CA1 pyramidal cell layer and lesion volumes of 4.09 and 2.14 mm^3.

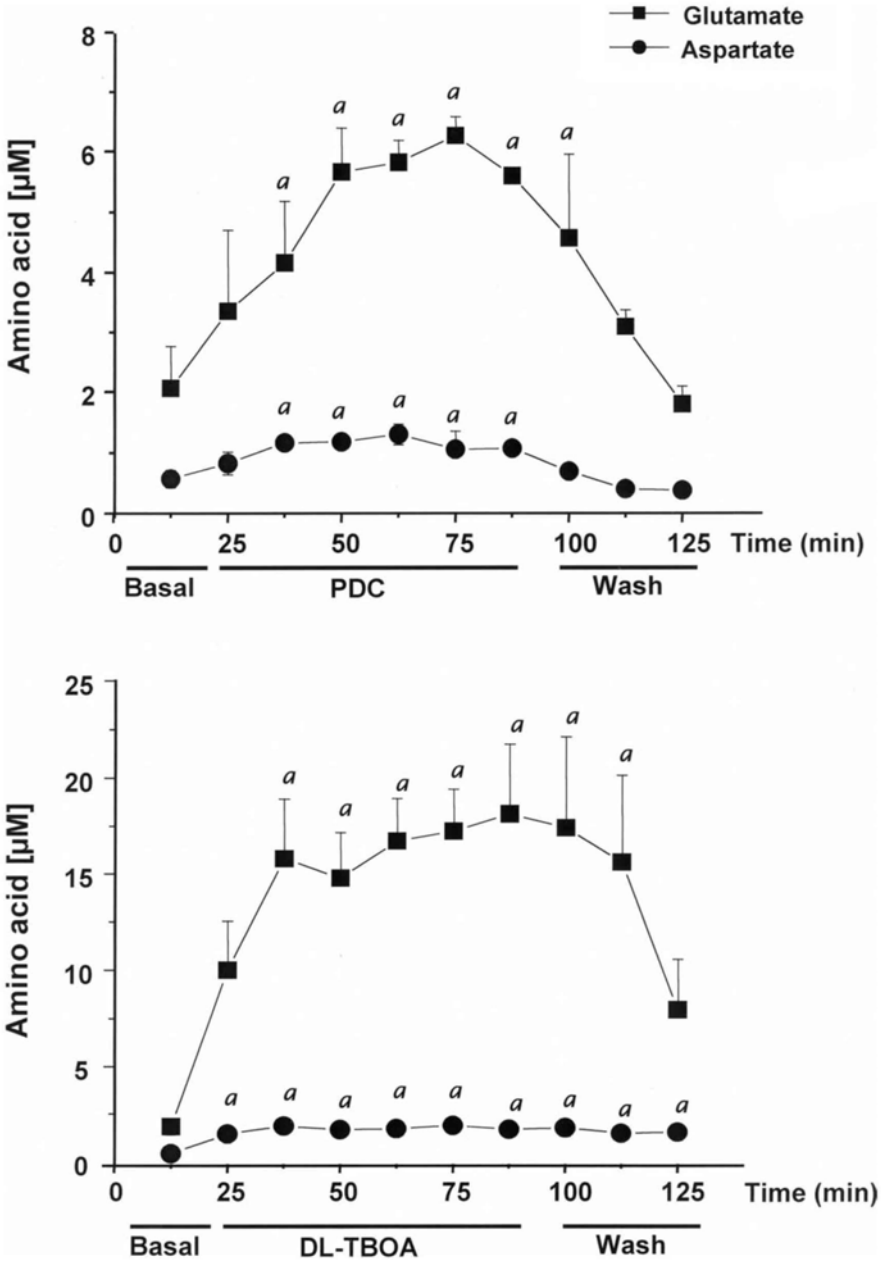

Fig. 1. Effects of PDC and DL-TBOA (500 μM) on the extracellular concentration of aspartate and glutamate monitored by microdialysis. Microdialysis experiments were performed as described in Experimental Procedures. The first fraction represents basal levels of amino acid in the extracellular space. PDC or DL-TBOA was perfused during the following six fractions, and afterward perfusion medium was changed for Ringer Krebs solution during three more fractions. Each fraction corresponds to 12.5 min. Data represent means±S.E.M. from three (PDC) and five (DL-TBOA) experiments. a $P<0.05$ relative to basal levels.

Table 1. Basal levels (μM) of glutamine, taurine, alanine and glycine and their changes induced by perfusion of PDC and DL-TBOA (500 μM) in the hippocampus through a microdialysis probe[a]

Amino acid	Basal levels	Levels at peak stimulation	Levels after washing
PDC			
Glutamine	44.6±2.1	48.5±3.0	50.2±2.2
Taurine	5.7±0.4	7.5±2.3	7.2±0.8
Alanine	17.4±3.3	20.0±1.6	19.8±1.1
Glycine	11.3±0.7	14.3±2.0	14.7±1.1
DL-TBOA			
Glutamine	47.5±6.6	57.8±3.5	57.7±4.2
Taurine	14.0±3.4	20.3±4.3	19.1±4.9
Alanine	16.1±1.7	36.0±4.9*	27.7±1.3*

[a] Amino acid levels were determined in collected microdialysis fractions by HPLC as described in Experimental Procedures. Peak stimulation represent the maximal peak of amino acid observed after PDC or DL-TBOA perfusion, and levels after washing represent amino acid concentration in the last collected fraction. Data are means±SEM from three to four animals. * $P<0.05$ relative to control.

Intracerebral administration of glutamate uptake inhibitors

The effect of the intracerebral administration of different concentrations of DL-TBOA was studied in the rat hippocampus and striatum, and was compared with that of PDC. In agreement with microdialysis experiments, administration of DL-TBOA induced neuronal damage 24 h later as assessed by histological analysis, even at low doses. In the hippocampus, 7.5 nmol DL-TBOA induced lesions of 14.2±3.5 mm^3 restricted to the CA1 region (Fig. 2A). This concentration of DL-TBOA is very similar to the total dose infused through the dialysis membrane, which is predicted to be around 6 nmol, assuming a similar membrane recovery for DL-TBOA to that for excitatory amino acids (close to 8%). Accordingly, lesions induced by 7.5 nmol DL-TBOA are of a similar size to those induced by microdialysis perfusion of 500 μM DL-TBOA.

As shown in Fig. 2D, injection of 15 nmol DL-TBOA induced damage mainly to neurons in the CA1 layer, sparing those in CA3 and CA4. At 25 nmol DL-TBOA, CA1 and dentate gyrus were severely damaged as evidenced by the presence of pyknotic cells through these cell layers (inset in Fig. 2F). Pretreatment with MK-801 completely prevented DL-TBOA-induced lesions at all concentrations tested (Fig. 2A, E, G). In these animals the presence of pyknotic cells was restricted to the region surrounding the needle track (insets in Fig. E, G). Treatment with NBQX reduced in 38% the size of the lesion induced by 7.5 nmol DL-TBOA. However, this effect was not statistically significant. In agreement with previous data (Sánchez-Carbente and Massieu, 1999; Massieu et al., 2000), administration of 300 nmol PDC induced no neuronal damage beyond that produced by the needle track, as observed after saline injection (Fig. 2A–C).

Similar to the hippocampus, DL-TBOA was highly toxic to the striatum (Fig. 3A) while rats injected with PDC showed no damage beyond the site of the needle insertion (Fig. 3C). Doses of 15 nmol TBOA induced lesions of 51 mm^3 (Fig. 3A), and 25 nmol induced lesions as large as 300 mm^3 extending throughout the striatum (Fig. 3A, D). Numerous pyknotic nuclei were observed in the region surrounding the injection site (Fig. 3H). DL-TBOA toxicity was completely prevented by pretreatment with MK-801 (Fig. 3A, E). In these animals cells located in the vicinity of the injection site showed a normal appearance such as those present in saline-injected animals (Fig. 3F, G). The size of the lesions induced by 25 nmol DL-TBOA was significantly reduced in 64% in animals treated with NBQX (Fig. 3A).

Behavioral observations

Administration of 50 nmol DL-TBOA (four animals) into the hippocampus of anesthetized animals induced severe behavioral changes which appeared between 30 and 90 min after the injection and were characterized by salivation, wet-dog shakes and limbic seizures. Two of these animals were killed 5 h later due to the presence of intense and continuous convulsions. The other two animals were killed at 24 h for histological analysis. Brains showed prominent lesions extending throughout the hippocampus, of 130 and 214 mm^3. At a concentration of 25 nmol none of these behaviors was present except in one from six animals that showed wild running, and according to histological analysis it exhibited a large lesion of 223 mm^3 extending through CA1 and dentate gyrus. No behavioral changes were observed in animals injected with 15 nmol DL-TBOA, and only one from eight animals injected with 7.5 nmol showed grooming and wet-dog shakes. None of the abovementioned behaviors were present in animals treated with MK-801 or NBQX, nor in animals injected with PDC.

Eight animals were injected with 25 nmol DL-TBOA into the striatum. Three of them showed head weaving, sporadic ipsilateral rotations along the longitudinal axis, chewing, grooming and tonic-clonic seizures. When pretreated with MK-801 or NBQX none of the rats showed any of these symptoms. No behavioral alterations were observed in animals intrastriatally injected with 15 nmol DL-TBOA or in animals injected with 300 nmol PDC.

Electroencephalographic observations

DL-TBOA (25 nmol) was injected into the hippocampus of free-moving animals ($n=4$) and electroencephalographic activity was recorded. All of the injected animals showed signs of hyperexcitability, wet-dog shakes, salivation, forelimb myoclonus and limbic seizures. At this dose, DL-TBOA induced intense epileptic EEG discharges characterized by initial hypersynchronic activity and trains of spike bursts. Discharges initiated in the injected hippocampus and propagated to the contralateral side. The spike-burst discharges were followed by trains of high-amplitude spike waves, which occurred simultaneously with wet-dog shakes and limbic seizures (Fig. 4). The mean latency to spike burst was 1.37±0.31 min, their mean duration was 36.7±8.4 s, and their frequency was two to three per 10 min period during the first hour. These EEG alterations were present for about 1.5 h and at 24 h the EGG was normal in all the animals. A dose of 7.5 nmol DL-TBOA was

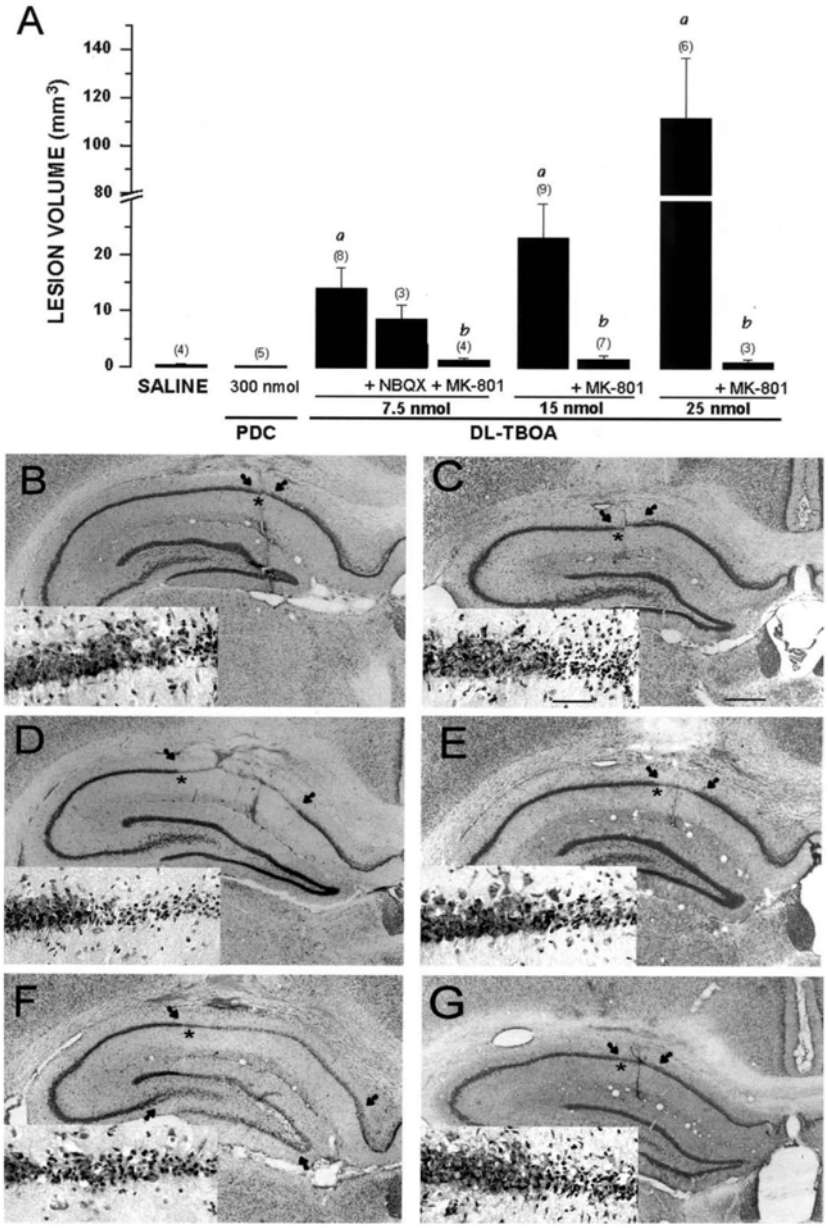

Fig. 2. (A) Lesion volume (mm^3) observed in rats injected with different concentrations of DL-TBOA (7.5, 15 and 25 nmol/μl), PDC (300 nmol/μl) or saline (0.9%) in the CA1 hippocampal region. Some groups of animals received an injection of 2 mg/kg MK-801 30 min before DL-TBOA administration, or NBQX (25 mg/kg) 15 min before and immediately after the intracerebral injection of DL-TBOA. Animals were prepared for histological analysis 24 h after the intrahippocampal injection and lesion volume was determined as described in Experimental Procedures. Data are expressed as means±S.E.M. of the number of animals indicated in parenthesis. (B–G) Photomicrographs of representative animals showing neuronal damage in the hippocampus after the administration of DL-TBOA at 15 nmol/μl (D) and 25 nmol/μl (F) and its protection by pretreatment with MK-801 (E and G). Administration of 300 nmol PDC (C) induced only a small lesion around the needle track similar to that produced by saline injection (B). The extension of the lesion is indicated by the arrows (scale bar=500 μm). Insets show magnifications of the zones indicated by (*). Scale bar=50 μm.

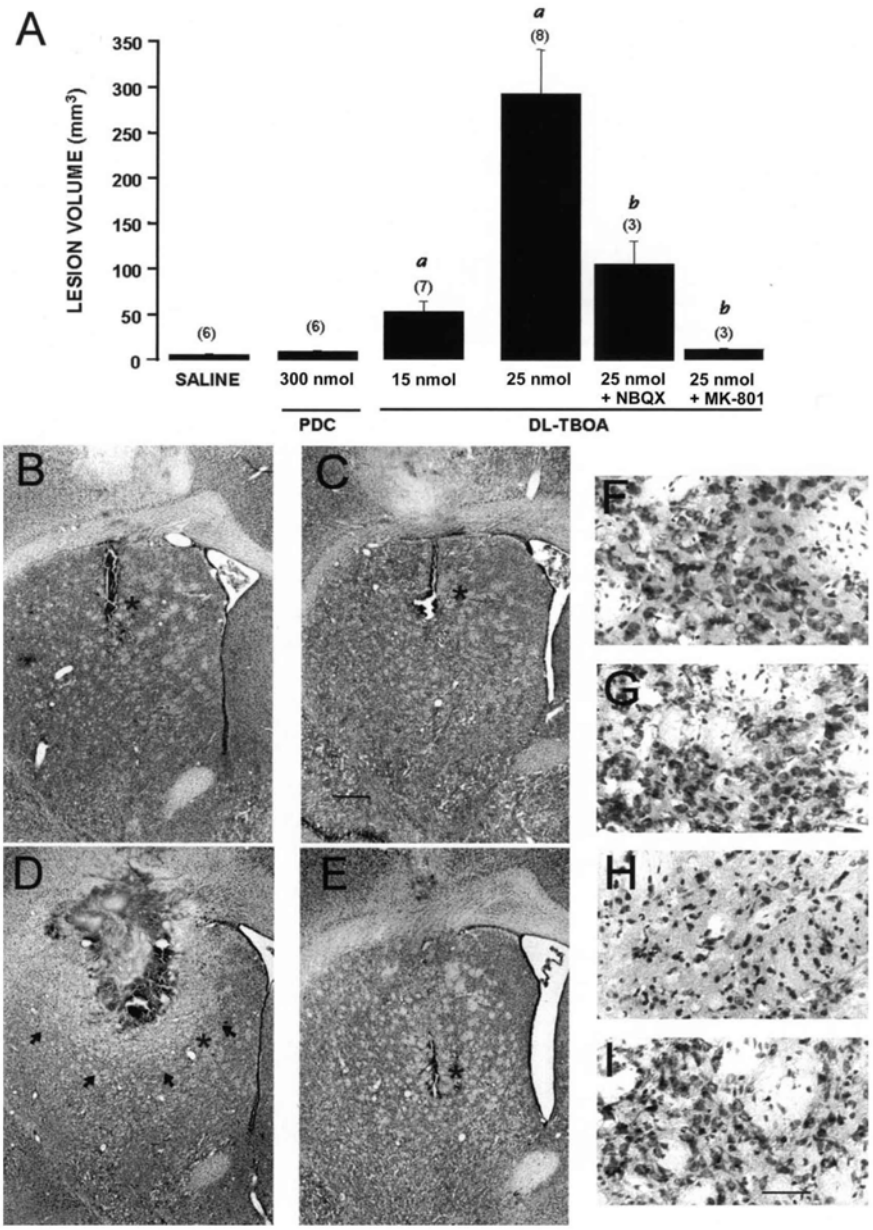

Fig. 3. (A) Lesion volume (mm^3) observed in rats intrastriatally injected with DL-TBOA (15 and 25 nmol/μl), PDC (300 nmol/μl) or saline (0.9%). A group of animals received an injection of 2 mg/kg MK-801 30 min before DL-TBOA (25 nmol/μl) administration, or NBQX (25 mg/kg) 15 min before and immediately after the intracerebral injection of DL-TBOA. Animals were prepared for histological analysis 24 h after the intrastriatal injection and lesion volume determined as described in Experimental Procedures. Data are expressed as means±S.E.M. of the number of animals indicated in parenthesis. (B–E) Photomicrographs of representative animals showing neuronal damage in the striatum after the administration of DL-TBOA at 25 nmol (D) and its protection by pretreatment with MK-801 (E). Administration of 300 nmol PDC (C) induced only a small lesion around the needle track very similar to that produced by vehicle injection (B). Scale bar=500 μm. The extension of the lesion is indicated by the arrows. F–I show magnifications of the zones indicated by (*). Scale bar=50 μm.

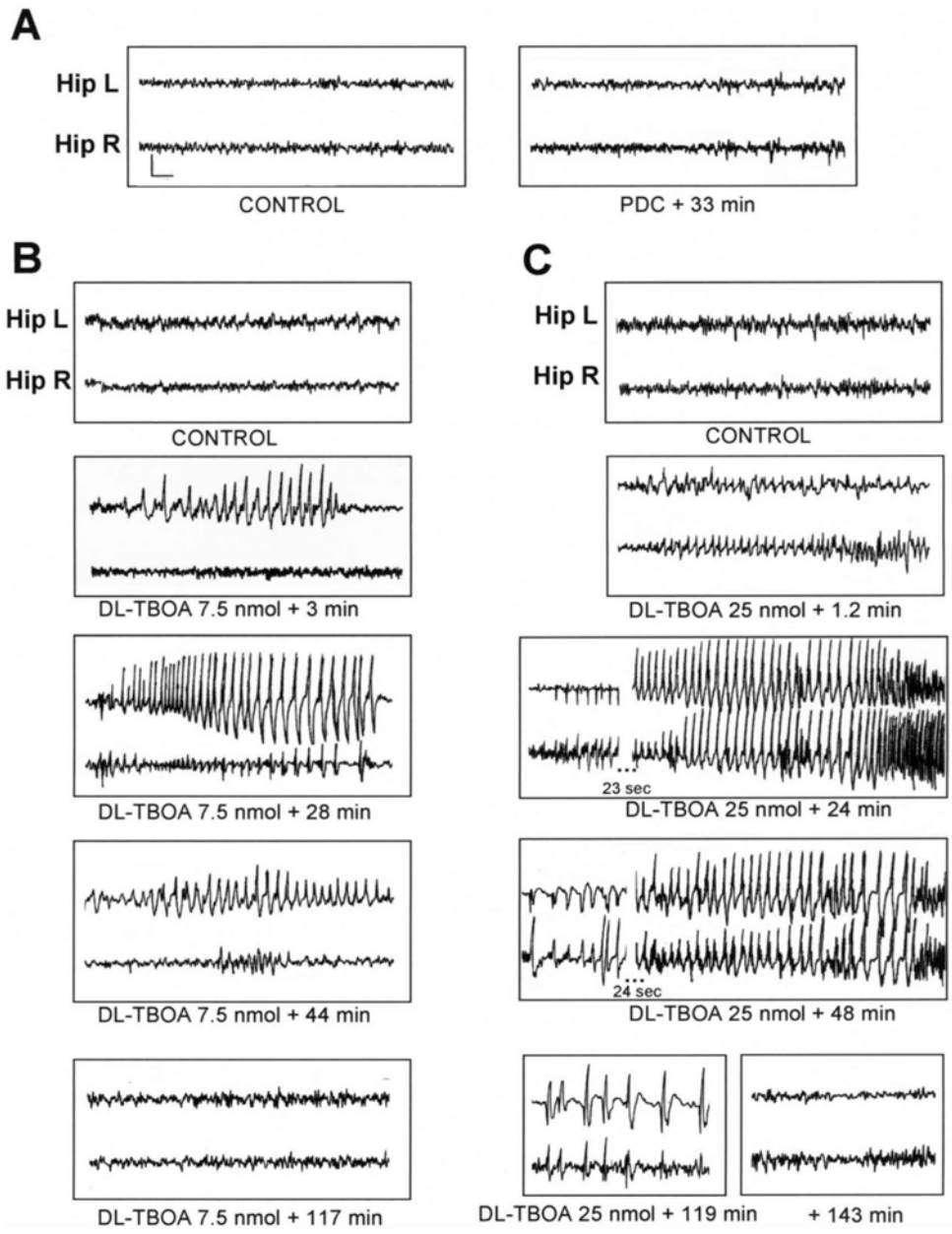

Fig. 4. EEG changes induced by DL-TBOA or PDC microinjection into the left hippocampus. (A) Basal EEG activity recorded before PDC administration (left panel), and lack of changes after 33 min PDC (300 nmol) administration (right panel). Hip L, left hippocampus; Hip R, right hippocampus. (B) Evolution of changes in EEG activity induced by intrahippocampal injection of 7.5 nmol DL-TBOA at the indicated times. Spike-burst discharges appeared 3 min after the injection and were followed by high amplitude spike waves. Normal EEG activity was recovered at 80 min. (C) Evolution of changes induced by intrahippocampal administration of 25 nmol DL-TBOA at the indicated times. Spike-burst discharges appeared 1.2 min after the injection and were followed by high amplitude spike waves, which propagated to the contralateral hippocampus. Discharges were of longer duration and were present for a longer period of time. In the animal shown normal EEG activity was recovered 140 min after the infusion. Scale bar=200 μV/s.

injected in two animals; they also showed EEG discharges of lower duration (29 s) and epileptic activity during the first hour (Fig. 4). In contrast to DL-TBOA, PDC (300 nmol) did not produce any alteration in the EEG, as previously reported (Obrenovitch et al., 1997).

DISCUSSION

Results show a dose-dependent toxic effect of DL-TBOA when administered into the rat hippocampus and striatum. Infusion of 500 μM DL-TBOA into the hippocampus resulted in notable increases in glutamate (nine-fold) and aspartate (3.4-fold) extracellular concentrations, and neuronal death in CA1 and dentate gyrus. PDC microdialysis infusion at the same concentration, similarly increased aspartate levels (2.6-fold increase) but was three times less potent in increasing glutamate (3.0-fold). In accordance to previous studies PDC did not cause a visible lesion beyond the site of the probe insertion (Massieu et al., 2000). These results suggest that neurotoxicity of glutamate transport inhibitors *in vivo* correlates with increased extracellular levels of excitatory amino acids. No changes were found in glutamine, alanine, glycine and taurine during perfusion of PDC. In previous studies we have observed rises in taurine levels in the striatum at higher PDC concentrations (1, 25 and 100 mM), while in the hippocampus PDC (25 mM) induces only a small nonsignificant increase in this amino acid (Massieu et al., 1995; Sánchez-Carbente and Massieu, 1999). These results agree with those reported by other authors describing augmented taurine levels in the hippocampus at 1 mM PDC but not at lower concentrations (Zuiderwijk et al., 1996; Kékesi et al., 2000).

In addition to glutamate and aspartate, DL-TBOA induced the increase in the extracellular concentration of glycine. Glycine content was not determined after 500 μM DL-TBOA perfusion due to the interference of DL-TBOA at this concentration with the fluorescent detection of glycine. However, perfusion of a 10-times lower concentration of DL-TBOA (50 μM) induced, in addition to excitatory amino acids, an increase in glycine extracellular levels and cell death in the CA1 region. In contrast, PDC had no effect on glycine levels. It is possible that the neurotoxic effect of DL-TBOA is influenced by glycine release due to the coagonistic action of this amino acid on the NMDA receptor subtype (Johnson and Ascher, 1987). The mechanism responsible of augmented extracellular glycine is uncertain and was not investigated in the present study. It might originate from the hydrolysis of extracellular glutathione released from astrocytes, to the dipeptide CysGly and then to free glycine and cysteine by ectopeptidases. This pathway has been suggested as the main source of cysteine for glutathione synthesis in neurons (Dringen, 2000; Dringen et al., 2000). Recent *in vitro* studies show that intracellular levels of glutathione decrease in the presence of glutamate transport blockers due to the lack of substrates for its synthesis and to oxidative stress (Chen and Swanson, 2003; Himi et al., 2003a,b; Ré et al., 2003). Blockade of glutamate carriers decreases cysteine uptake and intracellular cysteine concentration due to the substrate activity of this amino acid on glutamate transporters (Chen and Swanson, 2003; Himi et al., 2003). Accumulation of extracellular glycine parallel to cysteine (and glutamate) levels under these conditions is an interesting issue to be explored.

The larger effect of DL-TBOA on excitatory amino acid levels relative to PDC, might be related to the lower inhibition constant of this compound on glutamate uptake shown *in vitro*, especially on the GLT-1 transporter subtype (Anderson et al., 2001; Shimamoto et al., 1998; Waagepetersen et al., 2001). On the other hand, *in vitro* experiments in cultured cortical astrocytes have shown that DL-TBOA can slowly induce the release of ^{14}C-aspartate through heteroexchange (Anderson et al., 2001). It is ignored whether DL-TBOA can show a similar effect *in vivo*; therefore, the observed increase in excitatory amino acids levels might result as well from heteroexchange. On the other hand, it is possible that the differential effects of PDC and DL-TBOA on glutamate levels might be due to an inhibitory effect of the former on presynaptic glutamate release. It has recently been shown that such an effect can be mediated by glutamate influx through glutamate transporters located at presynaptic terminals in the crayfish neuromuscular junction (Dudel and Schram, 2003; Dudel, 2004). However, this possibility is not favored considering the lack of effect of PDC on K^+-induced excitatory amino acid release in the rat hippocampus *in vivo* (Zuiderwijk et al., 1996).

Direct administration of DL-TBOA into the rat striatum and hippocampus induced behavioral changes commonly associated with the administration of glutamate receptor agonists, such as NMDA and quinolinic acid (Schwarcz et al., 1983; Vezzani et al., 1986; Massieu and Tapia, 1994). Additionally, DL-TBOA intrahippocampal infusion induced epileptic activity dose-dependently, as monitored by the EEG. These alterations might result from increased excitability due to the activation of glutamate receptors by released glutamate. Results show complete protection by MK-801 against neuronal damage induced by DL-TBOA administration into the hippocampus at all concentrations tested, while the protective effect of NBQX was partial and not statistically significant. According to preliminary experiments (two animals) a third administration of NBQX improved its protective effect (to 50%, data not shown), suggesting involvement of non-NMDA receptors. Results suggest a major contribution of NMDA receptors, probably due to their relative abundance in the CA1 region (Young and Fagg, 1990). On the other hand, it was recently shown that L-TBOA exhibits a relatively high affinity for NMDA receptor binding in synaptic membranes (Shimamoto et al., 2000). Therefore, the presence of limbic seizures and neuronal damage induced by DL-TBOA might be influenced by direct activation of NMDA receptors, considering the high concentration of this compound expected to accumulate locally after its injection. However, in a previous study on hippocampal slices no activity of DL-TBOA on NMDA receptors was observed, and accumulation of extracellular glutamate was attributed to glutamate uptake blockade rather than release by heteroexchange (Jabau-

don et al., 1999). In accordance, a recent study suggested that the convulsive behavior induced in mice by DL-TBOA, results mainly from the accumulation of extracellular glutamate and not from direct activation of NMDA receptors, because of the lack of correlation between the binding affinity to NMDA receptors and the ED_{50} for inducing convulsions (Shimamoto et al., 2004).

The effects of DL-TBOA in the striatum might be attributed to activation of both NMDA and non-NMDA receptors by released glutamate, since treatment with MK-801 and NBQX significantly prevented DL-TBOA neurotoxicity. Administration of PDC did not produce any behavioral alterations or changes in electroencephalographic activity. These results agree with previous studies showing that PDC intrahippocampal or intrastriatal administration fails to induce large depolarizations such as spreading depression or anoxic depolarization, which are associated with neuronal damage during cerebral ischemia (Obrenovitch et al., 1997).

The differential effects of DL-TBOA and PDC on neuronal damage and EEG activity are not likely attributed to the different inhibitor concentrations used. In previous studies we have tested higher concentrations of PDC (400 and 500 nmol) and observed hippocampal and striatal lesions larger but not statistically different to those induced by vehicle injection (Massieu et al., 2001, 2003). The size of the lesions induced at these concentrations are similar to those observed in the present study using 300 nmol PDC (0.1 and 9.7 mm^3 for hippocampus and striatum, respectively). In contrast, DL-TBOA at concentrations 12-40-fold lower (7.5–25 nmol) induced extensive lesions in both regions (14–100 mm^3 and 45–300 mm^3 in hippocampus and striatum, respectively). Additionally, the reported IC_{50} value for PDC on the Na^+-dependent [^{3}H]L-aspartate binding in frozen tissue sections is close to 5.0 μM in different brain regions (Balcar et al., 1995). From these data, it might be predicted that 300 nmol/μl PDC directly administered into the brain would be sufficient to inhibit glutamate transporters.

Alternatively, the differential effects of these compounds might be attributed to the more moderate action of PDC on excitatory amino acids levels. However, in previous microdialysis studies using higher concentrations of PDC (25 and 100 mM) we have observed large elevations of glutamate and aspartate in the extracellular medium, achieving levels even higher than those observed after DL-TBOA perfusion. Despite this, only small lesions have been found (Massieu et al., 1995; Sánchez-Carbente and Massieu, 1999; Massieu and Tapia, 1997). These observations suggest that no simple correlation exists between glutamate accumulation by transport blockade and neuronal damage, as previously discussed (Obrenovitch and Urenjak, 1997). Additional factors might contribute to DL-TBOA neurotoxicity, such as a direct action on NMDA receptors *in vivo*. The lack of effect of PDC on glycine levels, its lack of binding affinity to glutamate receptors (Bridges et al., 1991; Isaacson and Nicholl, 1993; Balcar et al., 1995; Blitzblau et al., 1996), and/or its putative inhibitory action on presynaptic glutamate release, might contribute to its lower neurotoxic potency. Additionally, PDC and DL-TBOA might block different populations of glutamate transporters *in vivo*. *In vitro* experiments indicate that PDC and DL-TBOA show similar IC_{50} values for glutamate uptake inhibition in COS-1 cells transfected with GLAST, but the IC_{50} value for DL-TBOA is lower than that of PDC for GLT-1 (Shimamoto et al., 1998). Considering the great abundance of GLT-1 in the hippocampal formation and the important role of this transporter in removing extracellular glutamate in this region (Rothstein et al., 1996), its correct functioning, might be crucial for the control of glutamate toxicity. At the moment it is not possible to discriminate between the aforementioned alternatives.

The notable neurotoxic effect of DL-TBOA administration is of great relevance when studying its actions on excitatory amino acid release during conditions of energy depletion *in vivo*, such as ischemia. In a previous study it was observed that DL-TBOA partially prevented the release of glutamate and aspartate induced by a 20 min period of global ischemia; however, no histological data were provided (Phillis et al., 2000). On the other hand, recent evidence showed that DL-TBOA inhibits [^{14}C]-aspartate release during energy-depletion conditions (Anderson et al., 2001), and that at low concentrations (10 μM) it prevents neuronal death induced by oxygen and glucose deprivation in cultured hippocampal slices (Bonde et al., 2003). In the present study we observed that DL-TBOA perfusion at a low concentration (50 μM) *in vivo* induces the elevation of excitatory amino acids and glycine levels and neuronal damage. Therefore, further evaluation of the effects of glutamate uptake blockers *in vivo* during conditions of energy impairment is of relevance. The comprehension of the mechanisms of action of glutamate transport blockers might be useful to the understanding of the mechanisms involved in excitatory amino acids release during physiological and pathological conditions, and its relation to excitotoxic cell death.

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THE ANION CHANNEL BLOCKER, 4,4′-DINITROSTILBENE-2, 2′-DISULFONIC ACID PREVENTS NEURONAL DEATH AND EXCITATORY AMINO ACID RELEASE DURING GLYCOLYSIS INHIBITION IN THE HIPPOCAMPUS *IN VIVO*

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Abstract—Neuronal death associated with cerebral ischemia and hypoglycemia is related to increased release of excitatory amino acids (EAA) and energy failure. The intrahippocampal administration of the glycolysis inhibitor, iodoacetate (IOA), induces the accumulation of EAA and neuronal death. We have investigated by microdialysis the role of exocytosis, glutamate transporters and volume-sensitive organic anion channel (VSOAC) on IOA-induced EAA release. Results show that the early component of EAA release is inhibited by riluzole, a voltage-dependent sodium channel blocker, and by the VSOAC blocker, tamoxifen, while the early and late components are blocked by the glutamate transport inhibitors, L-trans-pyrrolidine 2,4-dicarboxylate (PDC) and DL-threo-beta-benzyloxyaspartate (DL-TBOA); and by the VSOAC blocker 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS). Riluzole, DL-TBOA and tamoxifen did not prevent IOA-induced neuronal death, while PDC and DNDS did. The VSOAC blockers 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) and phloretin had no effect either on EAA efflux or neuronal damage. Results suggest that acute inhibition of glycolytic metabolism promotes the accumulation of EAA by exocytosis, impairment or reverse action of glutamate transporters and activation of a DNDS-sensitive mechanism. The latest is substantially involved in the triggering of neuronal death. To our knowledge, this is the first study to show protection of neuronal death by DNDS in an *in vivo* model of neuronal damage, associated with deficient energy metabolism and EAA release, two conditions involved in some pathological states such as ischemia and hypoglycemia.

Key words: anion channels, exocytosis, glutamate transporters, iodoacetate, hypoglycemia, neuronal death.

The basal concentration of glutamate in the extracellular space is regulated by several release pathways including the spontaneous calcium-dependent release from synaptic terminals (Takahashi and Momiyama, 1993), the activity of the cystine-glutamate exchanger (Baker et al., 2002) and the activation of astrocytic hemichannels (Ye et al., 2003). An increase in glutamate extracellular levels and the consequent over-activation of its receptors induces excitotoxic neuronal death (Choi, 1988), a cell-death mechanism triggered during ischemia and hypoglycemia (Benveniste et al., 1984; Sandberg et al., 1985; Wieloch, 1985; Sandberg et al., 1986; Smith and Meldrum, 1992; Takagi et al., 1993; Auer, 2004). The extracellular accumulation of the excitatory amino acids (EAA), aspartate and glutamate, during ischemia involves an early calcium-dependent release, possibly from neurons (Drejer et al., 1985; Nelson et al., 2003). After exocytotic release glutamate removal from the synaptic cleft is carried out by glutamate transporters coupled to the sodium electrochemical gradient generated by the Na^+,K^+-ATPases. One glutamate or aspartate molecule is co-transported with three sodium ions while one potassium ion is countertransported to the extracellular medium (Huang and Bergles, 2004). Collapse of the sodium/potassium gradient during ischemia or hypoglycemia induces an intracellular Na^+ overload, promoting the reverse function of glutamate transporters and the increase in amino acid extracellular basal levels (Rossi et al., 2000; Mitani and Tanaka, 2003). This mechanism has been suggested to be involved in EAA release during energy failure associated with cerebral ischemia (Seki et al., 1999; Phillis et al., 2000). An additional release pathway for EAA during ischemia involves the action of volume-sensitive organic anion channels (VSOAC) activated in response to cell swelling (Seki et al., 1999; Kimelberg et al., 2004). Although several studies suggest the participation of these routes in glutamate and aspartate release during ischemia (Drejer et al., 1985; Phillis et al., 1994, 1998, 2000; Seki et al., 1999; Dawson et al., 2000), their relation to neuronal death is still unclear. On the other hand, the role of these pathways on amino acid efflux and cell damage during hypoglycemia has received less attention.

Glucose oxidation provides the energy necessary for the regulation of ambient glutamate concentration *in vivo* (Voutsinos-Porche et al., 2003; Shulman et al., 2004). Impairment of the glycolytic pathway induces excitotoxic cell death, possibly resulting from increased EAA release and failure of its removal. *In vitro* inhibition of the glycolytic enzyme, glyceraldehyde 3-phosphate dehydro-

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Abbreviations: DIDS, diisothiocyanostilbene-2,2′-disulfonic acid; DL-TBOA, DL-threo-beta-benzyloxyaspartate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; EAA, excitatory amino acids; IOA, iodoacetate; MK801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; NBQX, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo[f]quinoxaline-7-sulfonamide disodium; NMDA, *N*-methyl-D-aspartate; NPPB, 5-nitro-2-(3-phenylpropyl-amino) benzoic acid; PDC, L-trans-pyrrolidine 2,4-dicarboxylate; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; VSOAC, volume-sensitive organic anion channel.

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genase (GAPDH) by iodoacetate (IOA), promotes the elevation of extracellular glutamate through the activation of exocytotic release, the inverse function of glutamate transporters and hemichannel permeation (Ogata et al., 1995; Zeevalk et al., 1998; Kulik et al., 2000; Contreras et al., 2002; Sundstrom and Mo, 2002). In previous *in vivo* studies we have demonstrated that IOA perfusion through a microdialysis probe into the rat hippocampus induces the elevation of extracellular EAA levels and neuronal death (Massieu et al., 2000), suggesting an important role of glycolysis in the control of glutamatergic neurotransmission and neuronal survival. In the present study we have investigated whether EAA release during IOA perfusion into the hippocampus is related to exocytotic release, the inverse action of glutamate transporters, or VSOAC activation. To test these alternatives we have used the voltage-dependent sodium channel blocker, riluzole, to inhibit exocytotic EAA release, the substrate and no-substrate glutamate uptake blockers, L-trans-pyrrolidine-2,4-dicarboxylate (PDC) and DL-threo-β-benzyloxyaspartate (DL-TBOA), respectively, and the VSOAC blockers, 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS), diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), tamoxifen and phloretin. We have also studied the involvement of each one of these release pathways on the induction of neuronal death.

EXPERIMENTAL PROCEDURES

Chemicals

IOA, riluzole, DIDS, phloretin and tamoxifen were purchased from Sigma Chemical Co. (St. Louis, MO, USA); PDC, DL-TBOA, NPPB, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK801), and 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo[f]quinoxaline-7-sulfonamide disodium (NBQX) were obtained from Tocris (Ellisville, MO, USA), and DNDS from Molecular Probes, Inc. (Eugene, OR, USA).

Microdialysis experiments

Male Wistar rats (250–300 g) were used throughout the study. They were handled according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and the local Animal Care Committee approved all animal treatments. All efforts were made to minimize the number of animals used and their suffering. Rats were anesthetized with halothane (1.5%) and fixed on a stereotaxic frame. Microdialysis probes, 2 mm long and 0.5 mm in diameter (CMA12; Bioanalytical Systems, West Lafayette, IN, USA, were implanted in the CA1 region of the hippocampus using the following coordinates: AP −3.6 mm from Bregma, L +2.0 mm from midline, and V −4.0 mm from dura (Paxinos and Watson, 1986) and animals were maintained under low anesthesia (0.5% halothane) throughout the experiment. Body temperature was kept at 36.5–37.5 °C by means of a heating pad throughout the implantation of dialysis probes and the duration of the microdialysis experiment.

Microdialysis probes were continuously perfused with a Ringer–Krebs medium containing (in mM): NaCl 118, KCl 4.5, $MgSO_4$ 2.5, NaH_2PO_4 4.0, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 10, at a flux rate of 2 μl/min via a microinjection pump (BAS MD 1001, West Lafayette, IN, USA), during a 90-min period in order to obtain the stabilization of the probe. Animals were assigned to four experimental groups: the first group was perfused with 1 mM IOA; the second with 1 mM IOA+1 mM Riluzole; the third group received 1 mM IOA+either one of the glutamate uptake blockers studied (100 μM or 500 μM DL-TBOA; or 1 mM or 5 mM PDC); and animals in the fourth group were perfused with 1 mM IOA+either one of the VSOAC inhibitors tested (10 mM DNDS, 250 μM tamoxifen, 1 mM phloretin or 1 mM NPPB). The doses used of the different anion channel blockers were chosen according to previous studies showing their effectiveness in blocking ischemia-induced amino acid release (Singh et al., 1991; Rutledge et al., 1998; Seki et al., 1999; Fan et al., 2001; Franco et al., 2001; Al-Nakkash et al., 2004; Haskew-Layton et al., 2005; Abdullaev et al., 2006). Since the real concentration of the different compounds in the tissue is not known, we assumed a 10% delivery of the drugs according to the recovery of amino acids by the microdialysis probe previously calculated (Massieu et al., 1995).

After the stabilization of the probes 10 fractions of 25 μl were continuously collected. When the effect of IOA alone was studied, the three first fractions were perfused with Ringer–Krebs alone, then IOA was administered in Ringer–Krebs medium during the four following fractions, and during the last three fractions Ringer–Krebs medium alone was perfused again. When the effect of the different inhibitors on IOA-induced EAA release was studied, basal levels were monitored during the first collected fraction, afterward the corresponding inhibitor was perfused alone in Ringer–Krebs medium for two fractions, and during the next four fractions perfusion media contained inhibitor+IOA. The last three fractions corresponded to Ringer–Krebs medium alone. In a series of experiments 100 mM KCl was administered after IOA perfusion in order to discard amino acid depletion from releasable pools.

DNDS is a stilbene disulfonic acid related to SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid) and DIDS and was used instead of SITS or DIDS because they were found to interfere with the fluorescence emission of amino acid o-phthaldialdehyde-derivatives studied by high-performance liquid chromatography (Seki et al., 1999).

The effects of PDC and DL-TBOA on basal amino acid levels, have been previously studied (Montiel et al., 2005). In the present work the effect of DNDS (10 mM) and NPPB (1 mM) on EAA basal concentrations was studied. In these experiments Ringer–Krebs was perfused during the first fraction, during the six following fractions DNDS or NPPB was added to Ringer–Krebs medium, and Ringer alone was perfused again during the three last fractions.

Twenty-four hours after the end of the microdialysis experiment rats were anesthetized with sodium pentobarbital anesthesia and transcardially perfused with 250 ml of 0.9% saline solution followed by 250 ml of 5% formaldehyde in 0.1 M phosphate buffer (pH 7.3). Brains were removed and placed in fixative solution for additional 24 h. They were then transferred successively to a 20% and 30% sucrose solution and coronal sections (40 μm) were made in a cryostat and stained with Cresyl Violet. Lesion size was calculated by examination of all brain sections where neuronal damage was evident in each experimental animal. Tissue damage was determined by the presence of pyknotic nuclei and the absence of cells with a visible cytoplasm. The damaged area was delineated manually and measured with the aid of an image analyzer (NIH Macintosh Image 1.6). The lesion volume was calculated by the sum of measured areas in all sections multiplied by the slice thickness. Results are expressed as means±S.E.M. of lesion volume per each animal group.

Quantification of amino acid levels

Amino acid content in dialysates was determined by HPLC as previously reported by (Antoine et al., 1999), with slight modifications. Briefly, the 25-μl fractions were derivatized with the same volume of *o*-phthaldialdehyde and 3 min later a 10-μl volume of this mixture was injected into an HPLC system (Waters 600; Waters, Millford, MA, USA) equipped with an ODS column

(25 cm×4 mm internal diameter, Supelco, Inc., Bellefonte, PA, USA). The mobile phase consisted of 18% methanol: 22% acetonitrile: 14% isopropanol: 46% phosphate buffer (60 mM, pH=6.65)/phosphate buffer (60 mM pH=6.65) and a flux rate of 1 ml/min was used in a linear gradient of 33 min duration from 10 to 90% solvent mixture. Amino acid concentrations were calculated by comparison with those obtained from a standard mixture of amino acids identically processed. Data represent means±S.E.M. of the number of animals indicated in figure legends.

Effect of glutamate receptor antagonists on IOA-induced hippocampal lesions

The effect of the *N*-methyl-D-aspartate (NMDA) receptor antagonist, MK801, and the non-NMDA receptor antagonist, NBQX, on the lesions induced by IOA was tested. For these experiments, IOA (10 nmol/μl, 1 μl total volume) was administered directly into the hippocampus through an injection needle at a rate of 0.5 μl/min during two minutes. DIDS (15 nmol/μl) was co-administered with 10 nmol IOA. It was intrahippocampally injected instead of intraperitoneally because it does not permeate through the blood–brain barrier, while MK801 and NBQX, are effective when administered systemically (Wong et al., 1986; Foster et al., 1987; Massieu et al., 1993; Gill, 1994).

In these series of experiments, animals were anesthetized with 2% halothane in a 95% O_2/5% CO_2 mixture and placed on a stereotaxic frame. A Hamilton needle was positioned into the hippocampus (coordinates: AP −3.6 mm from bregma, L +2.0 mm from midline, and V −2.5 mm from dura, according to Paxinos and Watson (1986), and 10 nmol/μl IOA (with or without DIDS) was injected at a rate of 0.5 μl/min (1 μl final volume) with the aid of a microinjection pump (Harvard Apparatus; model 55, South Natick, MA, USA). Two minutes after the injection was completed the needle was withdrawn and the skin sutured. MK801 (2 mg/kg) was administered intraperitoneally 30 min before the intracerebral injection of IOA, and NBQX (25 mg/kg) was administered 15 min before, immediately after and 15 min after the intracerebral injection of IOA. Twenty-four hours after the injection animals were anesthetized, transcardially perfused and brains prepared for histological analysis. Lesion volume was determined as described above.

Statistical analysis

Results are expressed as means±standard error of the mean (S.E.M.). A one-way ANOVA followed by a Fisher's least significant difference multiple comparison test was used for comparisons between the treatments.

RESULTS

Pathways involved in EAA release during glycolysis inhibition

IOA perfusion into the CA1 hippocampal region promoted the elevation of the extracellular levels of EAA as reported previously (Massieu et al., 2000). Aspartate and glutamate basal levels were increased up to 150% and 100%, respectively, during the administration of 1 mM IOA (Fig. 1A). Amino acids remained elevated after removal of the glycolytic inhibitor in agreement with the irreversibility of glyceraldehyde 3-phosphate dehydrogenase (GADPH) inhibition by IOA. The extracellular levels of taurine, glycine, glutamine and alanine were not altered (Table 1), suggesting that at this concentration the effect of IOA is specific for EAA. Because GABA levels are not reliably detected with the methodology used either in control or in IOA-treated animals, they are not reported.

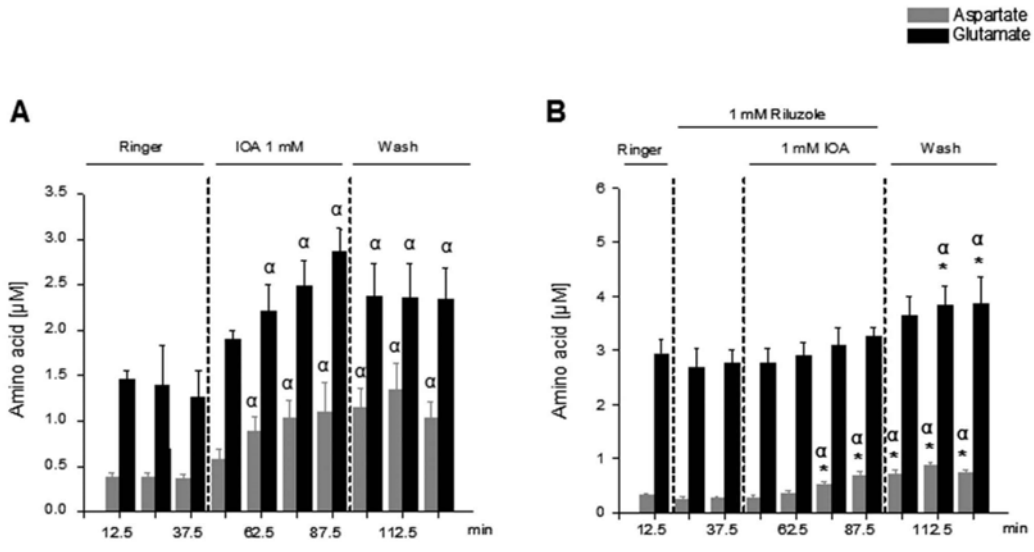

Fig. 1. Effect of IOA on the extracellular concentration of EAA (A) and its modification by riluzole (B). Microdialysis experiments were performed as described in Experimental Procedures and amino acid concentrations were determined by HPLC. Each fraction corresponds to 12.5 min. Data represent means±S.E.M. from five (A) and four (B) independent experiments. $^{\alpha}$ $P \leq 0.05$ relative to basal levels and * $P \leq 0.05$ relative to riluzole alone (amino acid levels collected at 37.5 min).

Table 1. Basal levels (μM) of glutamine, taurine, alanine and glycine in the hippocampus and their lack of change induced by IOA perfusion

Amino acid	Basal levels	Peak concentration during IOA treatment	Levels after wash
Glutamine	43.06±1.15	49.98±2.86	44.65±4.91
Taurine	3.95±0.51	3.14±0.045	4.33±0.40
Alanine	12.06±0.61	12.16±1.03	12.41±0.54
Glycine	10.09±0.64	13.25±1.43	15.53±2.43

Amino acid levels were determined in collected microdialysis by HPLC as described in Experimental Procedures. Levels at peak represent the maximal amino acid concentration observed during IOA administration. Data are means±SEM from five animals.

Riluzole was used to study the participation of exocytotic release in IOA-induced EAA accumulation. In the presence of this compound the release of glutamate was completely abolished, while aspartate release was delayed (Fig. 1B). EAA levels slowly increased after riluzole removal, and its perfusion alone had no effect on amino acid basal levels (Fig. 1B).

To study the involvement of glutamate/aspartate transporters on IOA-induced EAA release, the substrate and non-substrate uptake blockers, PDC and DL-TBOA, respectively, were tested. PDC at a 1 mM concentration significantly increased glutamate and aspartate basal levels as previously reported (Massieu et al., 1995). In the presence of PDC, IOA perfusion induced a non-significant increase in glutamate levels, while it delayed aspartate release (Fig. 2A). When the concentration of PDC was increased to 5 mM, glutamate and aspartate basal levels were substantially increased but the effect of IOA on glutamate levels was completely abolished. Nevertheless, in these conditions IOA still induced an increase in aspartate levels, which was significant 37 min after IOA perfusion (Fig. 2B). Similarly to PDC, inhibition of glutamate transporters by the non-substrate inhibitor DL-TBOA induced a dose-dependent increase in glutamate and aspartate extracellular levels, as reported previously (Montiel et al., 2005). In the presence of DL-TBOA (100 μM) IOA-induced EAA release was partially inhibited, while at 500 μM DL-TBOA, the effect of IOA was completely blocked (Fig. 3A–B).

Blockade of IOA-induced release of EAA by glutamate uptake inhibitors could be the result of amino acid depletion from intracellular pools and the reduction of their availability for release. To discard this possibility high potassium (100 mM) was perfused after PDC+IOA or DL-TBOA+IOA administration, to promote EAA release by depolarization. As shown in Fig. 2C–D and Fig. 3C–D blockade of the EAA transporters with 5 mM PDC or 500 μM DL-TBOA does not deplete the amino acid pool available for release, since glutamate and aspartate extracellular levels were increased after depolarization with KCl.

To study the participation of VSOAC activation in EAA release, four blockers of these channels was tested, DNDS, NPPB, tamoxifen and phloretin. When DNDS was perfused before IOA, release by IOA was completely abolished (Fig. 4A). DNDS after IOA appeared to significantly increase glutamate release (Fig. 5A). To further study the role of VSOAC on EAA release we perfused 1 mM NPPB, a widely used blocker of volume-sensitive channels (Evanko et al., 2004; Abdullaev et al., 2006). As shown in Fig. 4B NPPB did not prevent EAA release during IOA perfusion. The lack of effect of NPPB could be due to a deficient permeation of NPPB through the dialysis membrane. To discard this possibility the effect of this compound on amino acid release was studied under hyposmotic conditions (decreasing NaCl concentration by 50%). In these conditions a 66 and 50% increase in taurine and glycine levels, respectively was observed. NPPB inhibited almost completely taurine and glycine release corroborating that NPPB permeates through the microdialysis probe (data not show). To further study the participation of VSOAC we used two additional very effective blockers of these channels, tamoxifen and phloretin (Zhang et al., 1994; Fan et al., 2001; Abdullaev et al., 2006). Results show that 250 μM tamoxifen perfusion delayed EAA release but did not prevent the late accumulation of EAA induced by IOA (Fig. 4C). In addition, 1 mM phloretin perfusion did not prevent IOA-induced release (Fig. 4D). Since a decrease in EAA extracellular levels was observed when DNDS was administered before IOA perfusion, the effect of this blocker on basal amino acid levels was tested. As shown in Fig. 5B 10 mM DNDS reduced significantly glutamate basal levels. In contrast to DNDS, NPPB had no effect on the basal concentrations of glutamate and aspartate (Fig. 5C).

Prevention of neuronal damage by PDC and DNDS

The consequences of EAA release through exocytosis, glutamate transporters and activation of VSOAC on neuronal survival were then studied. Inhibition of exocytotic release of EAA by riluzole did not prevent neuronal death. Lesions induced by IOA in the hippocampus were of a similar extent when it was administered in the presence or the absence of riluzole (Fig. 6A, B and L). Despite the fact that glutamate and aspartate levels were increased under PDC perfusion, IOA-induced lesions were 50.0% reduced when glutamate transporters were blocked by 5 mM PDC before IOA administration (Fig. 6A, D and L). In contrast, DL-TBOA, which has been previously shown to be toxic during *in vivo* administration (Montiel et al., 2005), did not prevent IOA-induced damage at either of the concentrations tested (Fig. 6A, E, F and L). DNDS perfusion prior to IOA administration effectively prevented neuronal damage since hippocampal lesions were reduced significantly in 66% (Fig. 6A, H and L). However, when DNDS was administered after IOA perfusion the extent of hippocampal lesions was not significantly reduced (Fig. 6A, I and L). In contrast to DNDS, the VSOAC blockers NPPB, tamoxifen and phloretin showed no protection (Fig. 6A, G, J, K and L).

Effect of MK801, NBQX and DIDS on neuronal death elicited during glycolysis inhibition

To test the involvement of excitotoxicity on neuronal damage MK801 or NBQX was intraperitoneally injected before IOA intrahippocampal injection (Fig. 7). For these experi-

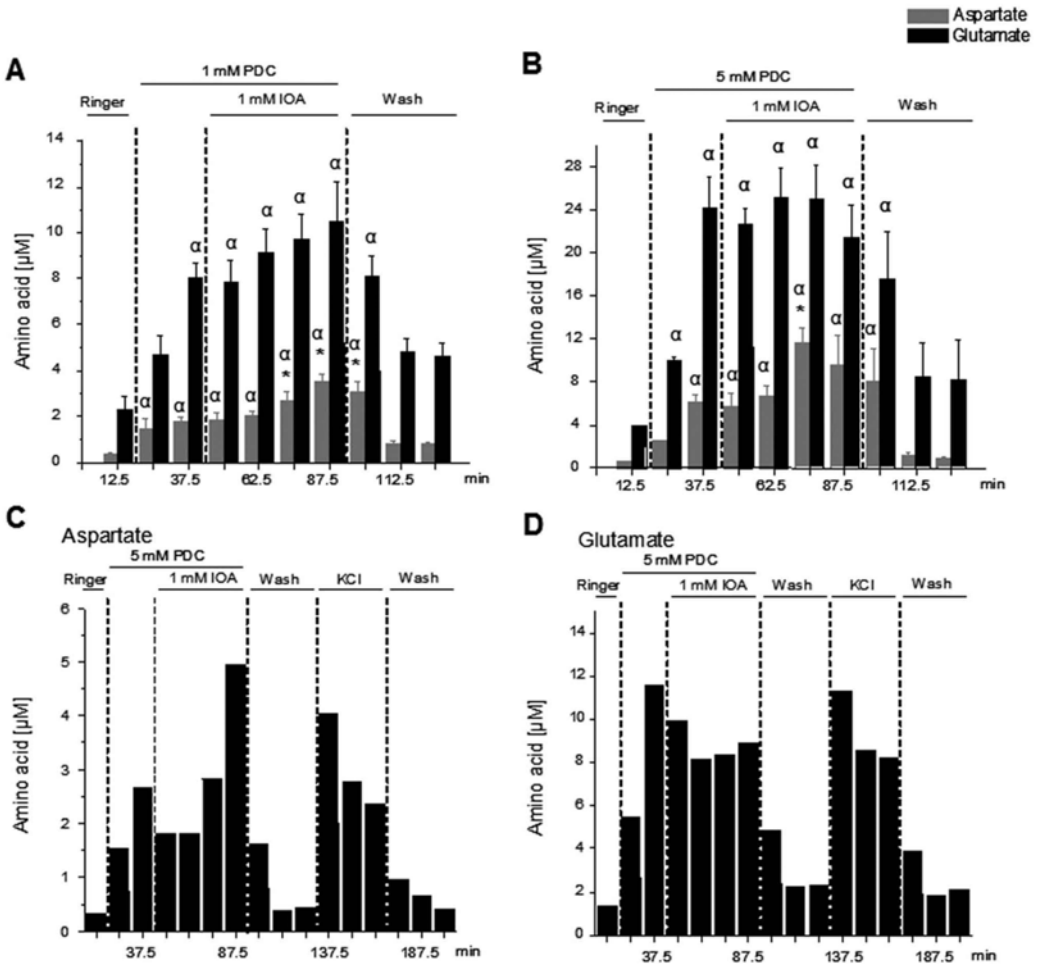

Fig. 2. Effect of the glutamate transport blocker, PDC 1 mM (A) or 5 mM (B) on IOA-induced amino acid release. (C, D) Representative experiment showing aspartate (C) and glutamate (D) release induced by depolarization with 100 mM KCl after 5 mM PDC+IOA administration. Data represent means±S.E.M. from seven (A) and six (B) independent experiments. $^{\alpha}$ $P \leq 0.05$ relative to basal amino acid levels; * $P < 0.05$ relative to PDC alone (amino acid levels collected at 37.5 min).

ments 10 nmol/μl IOA was directly injected into the hippocampus. In rats treated with MK801 hippocampal lesions were partially but significantly reduced in 40% (Fig. 7). Similarly, NBQX partially, although not significantly prevented cell death (Fig. 7). Similar to DNDS, when the VSOAC blocker, DIDS, was co-injected with IOA the extent of the hippocampal lesions was significantly reduced in 64% (Fig. 7).

DISCUSSION

The role of the glycolytic pathway in the control of glutamate ambient levels, is well documented (Shulman et al., 2004). When energy supply is impaired due to ischemia or hypoglycemia, glutamate and aspartate are released to the extracellular space causing the death of neurons (Benveniste et al., 1984; Sandberg et al., 1985, 1986). A previous *in vivo* study suggested that during insulin-induced hypoglycemia, glutamate and particularly aspartate, are released from transmitter pools in the rat striatum, since prior decortication significantly reduced the amount of released EAA (Butcher et al., 1987). Similarly, other studies have suggested glutamate synaptic release during ischemia (Drejer et al., 1985; Phillis et al., 1994; Dawson et al., 2000). Consistent with these observations, the present results suggest that exocytotic release contributes at least in part to the early EAA efflux during glycolysis inhibition,

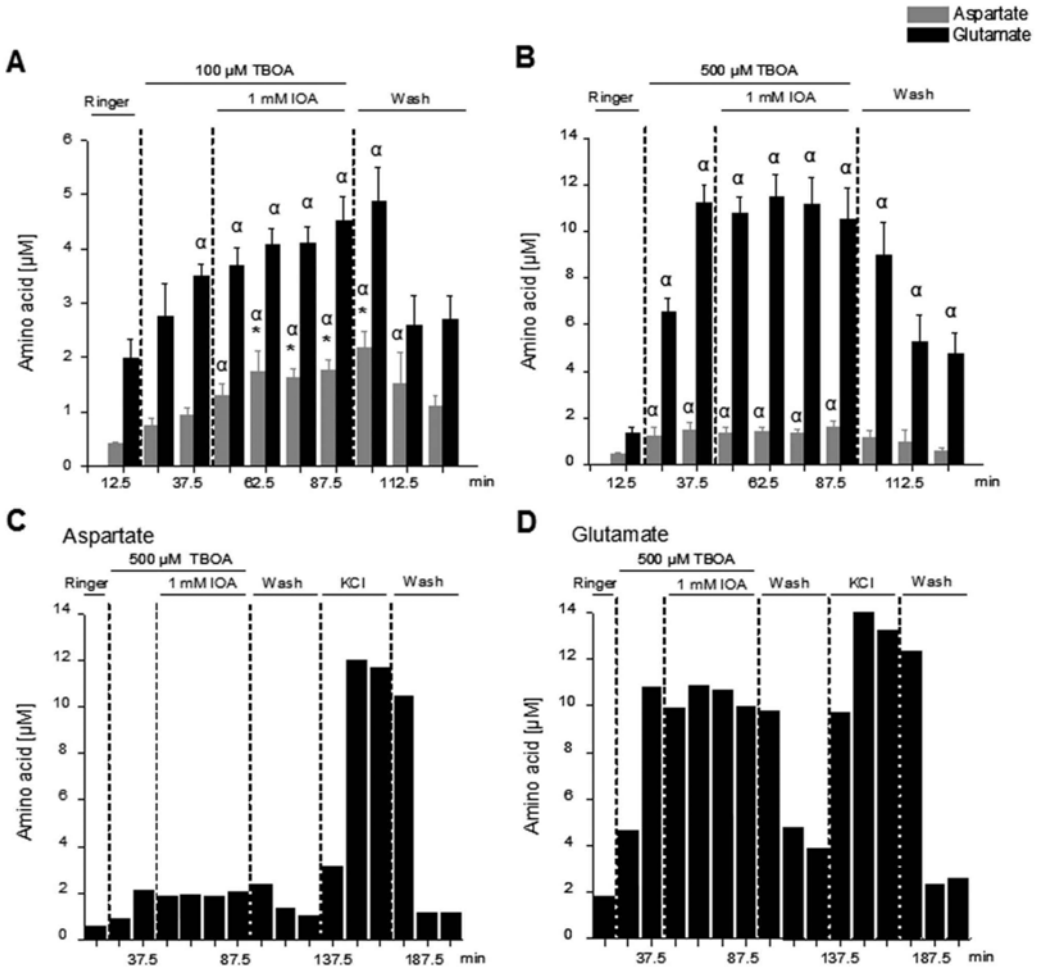

Fig. 3. Effect of the glutamate transport blocker, DL-TBOA 100 μM (A) or 500 μM (B) on IOA-induced amino acid release. (C, D) Representative experiment showing aspartate (C) and glutamate (D) release induced by depolarization with 100 mM KCl after 500 μM DL-TBOA+IOA administration. Data represent means±S.E.M. from six (A) and five (B) independent experiments. $^{\alpha}$ $P \leq 0.05$ relative to basal amino acid levels; * $P < 0.05$ relative to DL-TBOA alone (amino acid levels at 37.5 min).

since riluzole, a blocker of the voltage-dependent sodium channels, significantly inhibited the initial release. Riluzole has been shown to decrease glutamate efflux through the inhibition of sodium-dependent voltage channels and to prevent ischemic neuronal injury (Doble et al., 1992; Martin et al., 1993; Wang et al., 2004; Heurteaux et al., 2006). However, in the present experimental conditions no prevention of neuronal damage by riluzole was observed, suggesting that the early exocytotic EAA efflux, does not contribute to neuronal death. Consistent with these findings, Dawson et al. (2000) showed no reduction of the ischemic infarct volume despite EAA release being substantially reduced in the absence of calcium or in the presence of tetrodotoxin.

The present study strongly suggests the participation of glutamate transporters on the release of EAA during glycolysis inhibition, since both PDC and DL-TBOA prevented the IOA-induced glutamate and aspartate efflux. Previous *in vitro* studies show that inhibition of energy metabolism with high concentrations of IOA reduces EEA uptake and induces their release through the reversed activation of glutamate transporters (Madl and Burgesser, 1993; Gemba et al., 1994; Longuemare et al., 1999). *In vivo* studies have also shown that IOA perfusion into the

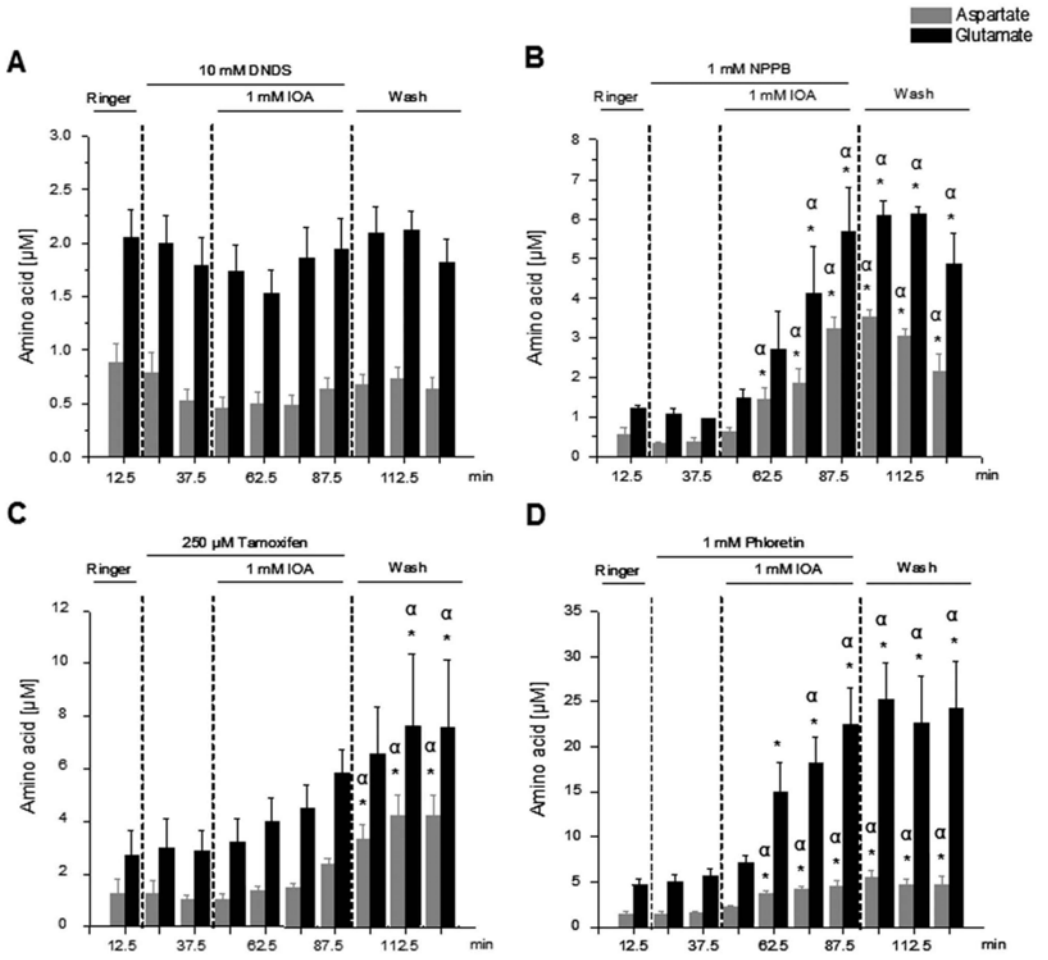

Fig. 4. Effect of the VSOACs blockers, DNDS (A), NPPB (B), tamoxifen (C) and phloretin (D) on IOA-induced release of amino acids. DNDS (10 mM), NPPB (1 mM), tamoxifen (250 μM) or phloretin (1 mM) was administered before IOA perfusion. Data represent means±S.E.M. from six (A), four (B) five (C) and four (D) independent experiments. " $P<0.05$ relative to basal values, * $P\leq0.05$ relative to DNDS, NPPB, tamoxifen, or phloretin alone (amino acid levels at 37.5 min).

hippocampus favors the release of EAA, particularly aspartate, as observed during insulin-induced hypoglycemia (Sandberg et al., 1985; Massieu et al., 2000), and significantly reduces ATP content (Massieu et al., 2003). Therefore, it is likely that an increase in the intracellular concentration of sodium due to inhibition of the Na^+,K^+-ATPase activity impairs glutamate transporters or promotes their reverse action. Results show that blockade of glutamate transporters by PDC and DL-TBOA prior to IOA perfusion, efficiently inhibited EAA efflux by IOA. In contrast, to these results mitochondrial inhibition induced by the direct administration of the toxin, 3-nitropropionic acid, does not alter the extracellular levels of EAA either in the rat striatum or hippocampus (Sanchez-Carbente and Massieu, 1999), or in cultured cerebellar granule neurons (Garcia and Massieu, 2001).

Both PDC and DL-TBOA increased the basal extracellular levels of glutamate and aspartate as reported by others (Phillis et al., 2000) and our group (Massieu et al., 1995; Montiel et al., 2005). The lack of effect of IOA after PDC or DL-TBOA perfusion was not due to the depletion of amino acid pools, since depolarization with KCl still induced the release of both amino acids. PDC at 5 mM completely prevented IOA-induced glutamate efflux and partially aspartate release. Although at this concentration PDC prevented neuronal damage, a clear correlation between this effect and the reduction of extracellular EAA could not be established. In previous studies we have

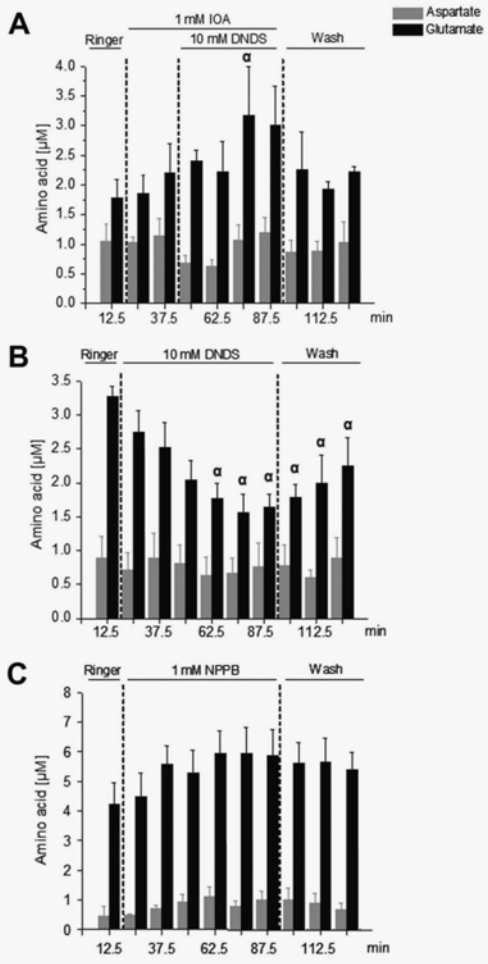

Fig. 5. Effect of DNDS perfusion after IOA administration on amino acids levels (A), and of DNDS (B) and NPPB (C) alone on glutamate and aspartate basal levels. (A) DNDS (10 mM) was administered after IOA perfusion. (B, C) DNDS (10 mM) or NPPB (10 mM) was perfused during six fractions (75 min) after perfusion of Ringer–Krebs during 12.5 min. After DNDS or NPPB administration Ringer–Krebs was administered again during three additional fractions. Data represent means±S.E.M. from six (A) and four (B–C) experiments. ^{a} $P<0.05$ relative to basal values.

observed that PDC-induced EAA release *in vivo* only slightly reduces cell survival (Massieu et al., 1995; Montiel et al., 2005). This effect might be due to additional actions of this compound on presynaptic glutamate transporters (Dudel and Schramm, 2003). DL-TBOA completely blocked glutamate and aspartate efflux but did not prevent neuronal damage. These results are in accordance with recent data from our group showing that *in vivo* administration of DL-TBOA in the rat hippocampus induces prominent neuronal damage and epileptic discharges (Montiel et al., 2005), possibly because of its relatively high binding affinity for NMDA receptors (Shimamoto et al., 1998). In a previous *in vivo* study it was shown that DL-TBOA inhibits significantly glutamate and aspartate transport during ischemia, but the effect of this inhibitor on tissue damage was not reported (Phillis et al., 2000). The present results suggest that reversal of glutamate transporters substantially contributes to EAA release, but its relation to neuronal damage is still not clear due to the effect of glutamate transport inhibitors on basal EAA levels.

Results suggest the participation of an excitotoxic mechanism on neuronal damage induced by IOA, since the administration of MK801 and NBQX partially reduced the size of the lesions. However, other factors besides EAA release might be involved in neuronal death, such as energy failure, since glycolysis inhibition will eventually lead to the impairment of mitochondrial metabolism due to the lack of pyruvate.

The role of EAA efflux through VSOAC was also investigated. The molecular identity of these channels has not been identified but several blockers of these channels have shown to inhibit amino acid release induced during *in vivo* ischemia (Phillis et al., 1994; Seki et al., 1999). All these blockers effectively block chloride channels and ischemia- or hyposmolarity-induced amino acid release at micromolar concentrations (Rutledge et al., 1998; Fan et al., 2001; Franco et al., 2001; Al-Nakkash et al., 2004; Haskew-Layton et al., 2005; Abdullaev et al., 2006). A 1 mM concentration of NPPB and phloretin was chosen in order to get an approximate concentration of 100 μM in the tissue, assuming a 10% recovery through the dialysis probe, as previously determined for amino acids (Massieu et al., 1995). DNDS at 10 mM was used since this concentration is more effective than 1 mM to prevent ischemia-induced EAA release (Seki et al., 1999). DNDS inhibited glutamate and aspartate release during IOA perfusion, while NPPB and phloretin did not. Since NPPB and phloretin are potent and highly efficient blockers of VSOAC (Fan et al., 2001; Abdullaev et al., 2006) we can conclude that the extracellular accumulation of EAA is not related to cell swelling. This suggestion is further favored by the lack of release of taurine, glycine and alanine, which are well-known osmolytes in the CNS (Pasantes-Morales et al., 2002), during IOA perfusion. In the presence of NPPB and phloretin IOA-induced release of EAA was potentiated. This effect might be related to an additional decrease in ATP levels to that induced by IOA, since NPPB has been shown to reduce ATP content in hippocampal cultured neurons and hepatocytes (Ballatori et al., 1995; Olson and Martinho, 2006). On the other hand, phloretin has shown to increase the fluidity of lipid bilayers (Auner et al., 2005), which might explain the exacerbation of EAA release.

Previous studies have shown that tamoxifen prevents amino acid release induced during ischemia (Phillis et al., 1998) and hyposmolarity *in vivo* (Estevez et al., 1999), and blocks chloride channels and swelling-activated EAA release *in vitro* (Zhang et al., 1994; Rutledge et al., 1998; Estevez et al., 1999; Abdullaev et al., 2006). The present

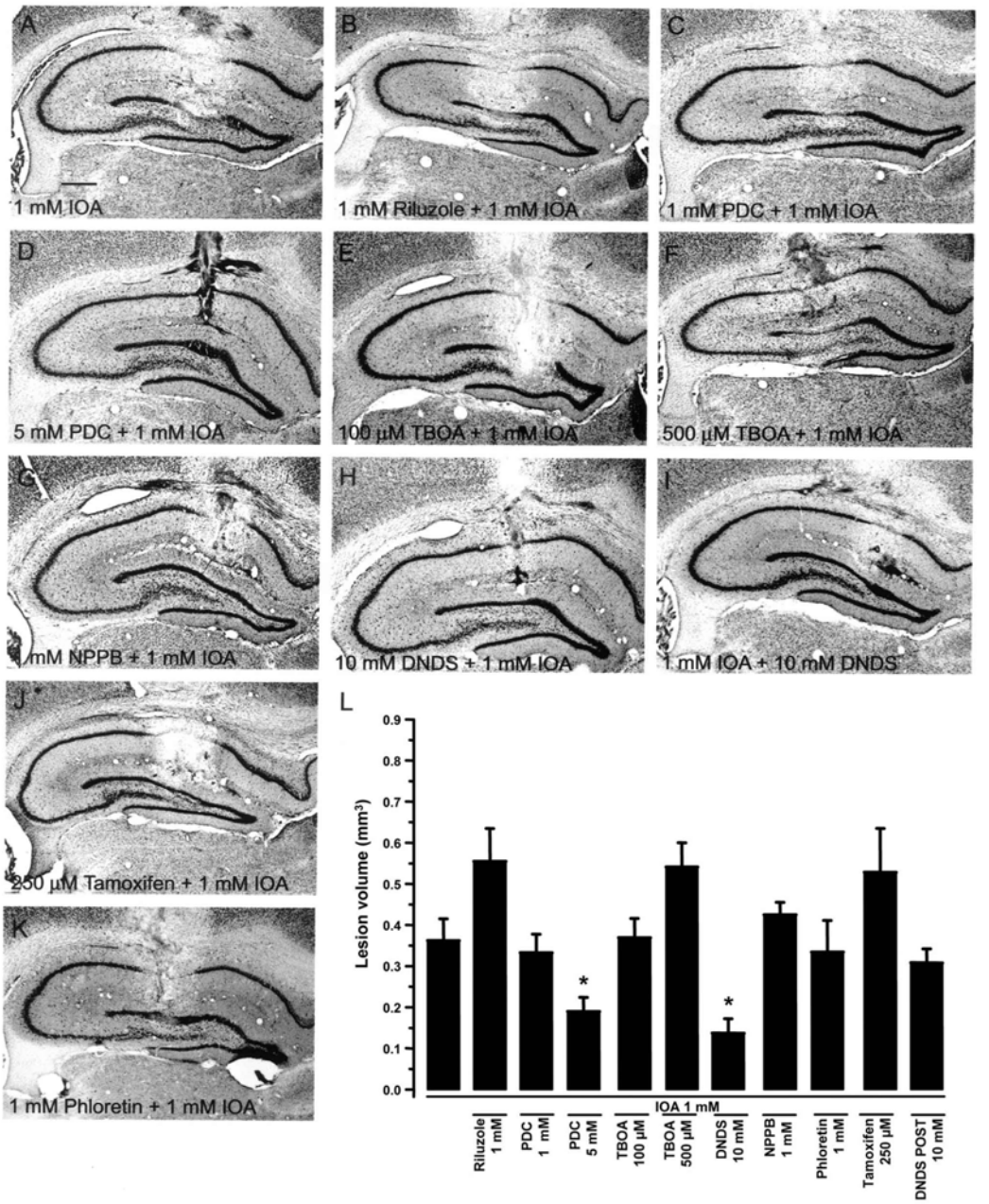

Fig. 6. Effect of the different compounds studied on neuronal damage induced by IOA administration into the hippocampus. (A–K) Micrographs of representative animals showing neuronal damage in the hippocampus as evaluated 24 h after the microdialysis experiment. Scale bar=500 μm. (L) Graph shows the volume (mm^3) of hippocampal lesions induced by IOA in the presence and the absence of the different inhibitors tested. Lesion volume was determined in histological sections 24 h after microdialysis experiments as described in Experimental Procedures. Data represent mean±S.E.M. from four to seven independent experiments. * $P<0.05$ relative to IOA alone.

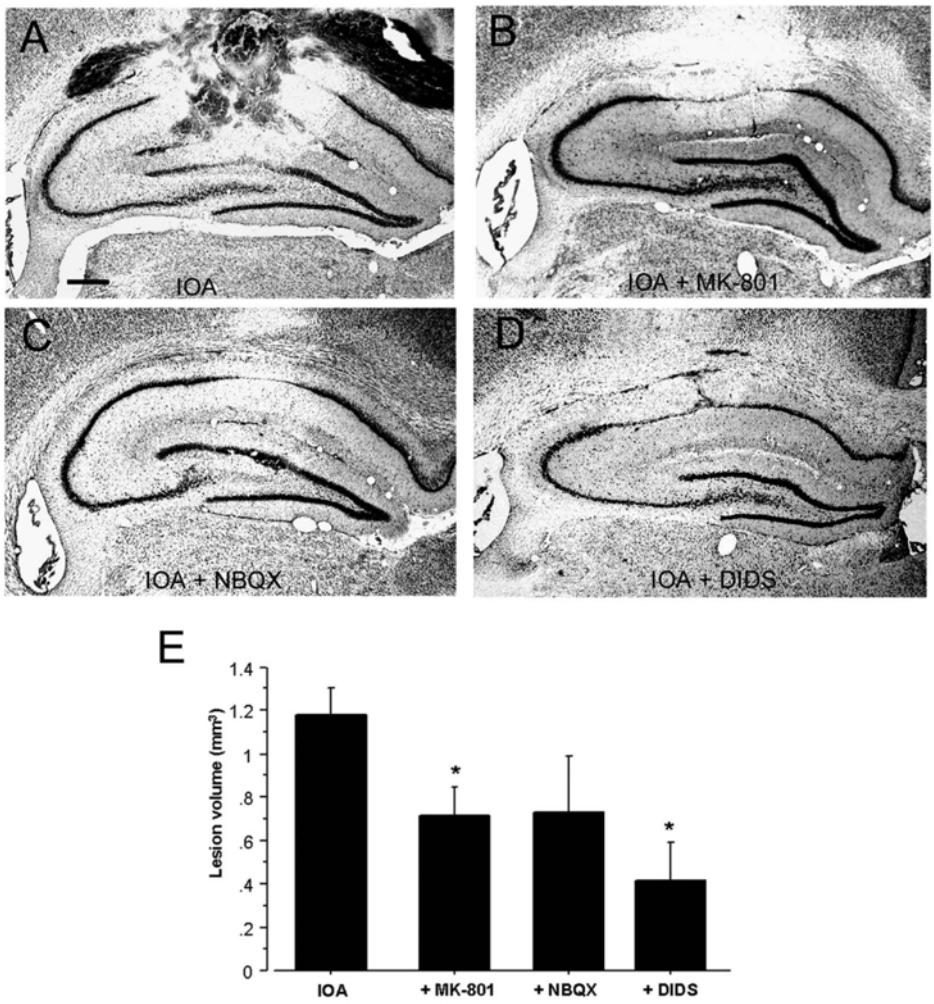

Fig. 7. Effect of MK801, NBQX and DIDS on hippocampal lesions induced by direct administration of IOA (10 nmol/μl) in the CA1 hippocampal region. Animals were treated with the different compounds as described in Experimental Procedures. (A–D) Micrographs of representative animals treated with MK801 (B), NBQX (C) and DIDS (D). Scale bar=500 μm. (E) Graph shows the volume (mm^3) of hippocampal lesions induced by the different treatments. Data are expressed as means±S.E.M. from four to eight independent experiments. * $P<0.05$ relative to IOA alone.

results show that tamoxifen blocks only the early component of EAA release.

On the other hand, from the four VSOAC blockers used, only DNDS showed protection from neuronal damage. This effect might be related to the inhibition of EAA release, since no significant reduction of the lesion volume was observed when DNDS was perfused after IOA administration, a condition which only partially inhibited glutamate release. Protection by DNDS might be related to additional effects of this compound, such as reduction of glutathione efflux after activation of glutamate receptors (Wallin et al., 2003), or inhibition of the release of reactive oxygen species from mitochondria, as suggested for DIDS in cardiomyocytes (Vanden Hoek et al., 1998). Previous studies have suggested the participation of oxidative stress in neuronal death induced by IOA *in vitro* (Uto et al., 1995; Malcolm et al., 2000), and Matthews et al. (1997) have shown hydroxyl radical formation after the intrastriatal injection of IOA. The protective effect of DNDS might not be attributable to an antagonistic action on NMDA receptors, as has been suggested for DIDS, since it shows no NMDA receptor antagonism and no protective effect against oxygen-glucose deprivation *in vitro* (Tauskela et al., 2003). The protective effect of DIDS might be attributed

to its antagonistic action on NMDA receptors (Tauskela et al., 2003). This might explain why the protective effect of DIDS was similar to that of MK801 and NBQX. It remains to be elucidated whether DIDS and DNDS share the same mechanisms of protection in the present experimental conditions.

Besides blocking IOA-induced EAA efflux, DNDS also significantly reduced the basal levels of EAA. Previous studies have shown inhibition of tonic glutamate release by DIDS in rat hippocampal slices (Cavelier and Attwell, 2005), possibly mediated by the inhibition of synexin-mediated membrane aggregation and fusion (Liu and Chander, 1995), or by blockade of $P2X_7$ purinergic receptors (Bultmann and Starke, 1994). Both mechanisms have been suggested to contribute to the maintenance of basal glutamate concentration (Bultmann and Starke, 1994; Liu and Chander, 1995). Since DIDS is a structurally related to DNDS, it is possible that DNDS shows similar effects.

CONCLUSION

In conclusion the present study emphasizes the role of the glycolytic pathway on the control of ambient EAA levels, and suggests that EAA efflux during glycolysis inhibition *in vivo* involves exocytotic release, the reversed action of glutamate transporters and a DNDS-sensitive mechanism. An important finding from the present study is that the delayed component of EAA release is apparently the pathway most importantly involved in neuronal death, since only the compounds which inhibited the delayed release, PDC and DNDS, showed protection. To our knowledge this is the first study showing histological evidence for the involvement of a DNDS-sensitive mechanism in neuronal damage induced in an *in vivo* model involving deficient energy metabolism and EAA release, two conditions associated to some pathological states such as ischemia and hypoglycemia. Additional experiments are necessary in order to elucidate the mechanisms of protection of DNDS.

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SUSTAINED METABOLIC INHIBITION INDUCES AN INCREASE IN THE CONTENT AND PHOSPHORYLATION OF THE NR2B SUBUNIT OF *N*-METHYL-D-ASPARTATE RECEPTORS AND A DECREASE IN GLUTAMATE TRANSPORT IN THE RAT HIPPOCAMPUS *IN VIVO*

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Abstract—The concentration of glutamate is regulated to ensure neurotransmission with a high temporal and local resolution. It is removed from the extracellular medium by high-affinity transporters, dependent on the maintenance of the Na^+ gradient through the activity of Na^+,K^+-ATPases. Failure of glutamate clearance can lead to neuronal damage, named excitotoxic damage, due to the prolonged activation of glutamate receptors. Severe impairment of glycolytic metabolism during ischemia and hypoglycemia, leads to glutamate transport dysfunction inducing the elevation of extracellular glutamate and aspartate, and neuronal damage. Altered glucose metabolism has also been associated with some neurodegenerative diseases such as Alzheimer's and Huntington's, and a role of excitotoxicity in the neuropathology of these disorders has been raised. Alterations in glutamate transporters and *N*-methyl-D-aspartate (NMDA) receptors have been observed in these patients, suggesting altered glutamatergic neurotransmission. We hypothesize that inhibition of glucose metabolism might induce changes in glutamatergic neurotransmission rendering neurons more vulnerable to excitotoxicity. We have previously reported that sustained glycolysis impairment *in vivo* induced by inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), facilitates glutamate-mediated neuronal damage. We have now investigated whether this facilitating effect involves altered glutamate uptake, and/or NMDA receptors in the rat hippocampus *in vivo*. Results indicate that metabolic inhibition leads to the progressive elevation of extracellular glutamate and aspartate levels in the hippocampus, which correlates with decreased content of the GLT-1 glutamate transporter and diminished glutamate uptake. In addition, we observed increased Tyr^{1472} phosphorylation and protein content of the NR2B subunit of the NMDA receptor. Results suggest that moderate sustained glycolysis inhibition alters glutamatergic neurotransmission.

Key words: excitotoxicity, glutamate uptake, glutamate receptors, glycolytic metabolism, neurodegenerative diseases.

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Abbreviations: DHK, dihydrokainic acid; EAA, excitatory amino acids; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IOA, iodoacetate; NMDA, *N*-methyl-D-aspartate; PBS-T, phosphate buffer 0.1 M, NaCl 0.9%, Tween 20 0.1%; RK, Ringer-Krebs; RKC, Ringer-Krebs containing choline chloride.

Glutamate uptake after its synaptic release stimulates glucose transport and its rate of oxidation in astrocytes (Hyder et al., 2006; Loaiza et al., 2003; Rothman et al., 2003; Swanson et al., 1994; Voutsinos-Porche et al., 2003). Administration of antisense oligonucleotides targeted to the glial glutamate transporter GLAST, or inhibition of glutamate transporters, prevents the stimulation of the glycolytic pathway mediated by excitatory amino acids (EAA) (Cholet et al., 2001; Debernardi et al., 1999). On the other hand, severe glycolysis inhibition impairs glutamate transport or even induces its reversed operation releasing glutamate to the extracellular space (Gemba et al., 1994; Ogata et al., 1995). These observations suggest that glycolytic metabolism is involved in the regulation of glutamate removal after its synaptic release, and as a consequence in the prevention of excitotoxicity.

We have shown previously that blockade of glutamate uptake *in vivo* does not induce neuronal death, despite increasing the extracellular levels of EAA (Massieu et al., 1995). However, when glycolytic metabolism is continuously inhibited by the systemic administration of iodoacetate (IOA), an inhibitor of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glutamate-mediated neuronal damage is enhanced (Massieu et al., 2000, 2003). In agreement, *in vitro* data show exacerbation of glutamate toxicity during glucose deprivation or glycolysis inhibition (Novelli et al., 1988; Zeevalk and Nicklas, 1992; Rego et al., 1999). However, the molecular mechanisms underlying this effect are not completely understood.

Decreased glucose metabolism is related to aging (Eberling et al., 1995; Moeller et al., 1996) and chronic neurodegenerative diseases (Mielke et al., 1998; Planel et al., 2004; Slosman et al., 2001; Stein et al., 1998). In addition, a moderate reduction in GAPDH activity is observed in brain and fibroblasts from Alzheimer's and Huntington's disease patients (Kish et al., 1998; Mazzola and Sirover, 2001). Moderate but progressive alterations in energy metabolism during the lifetime of patients might predispose neurons to excitotoxicity. In fact, an excitotoxic component has been suggested in the mechanism of neurodegeneration associated with these disorders (Schwarcz et al., 1983; Young et al., 1988; Csernansky et al., 1996). On the other hand, decreased glutamate uptake and expression levels of glutamate transporters have been observed in patients from Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (Arzberger et al., 1997; Ferrarese et al., 2000; Heath and Shaw, 2002; Zoia

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et al., 2004). Similar results have been observed in transgenic mice of Huntington's disease (Behrens et al., 2002; Lievens et al., 2001; Shin et al., 2005). In addition to impaired glutamate removal, changes in the expression levels of NMDA receptor subunits might contribute to excitotoxicity. The presence of the NR2B subunit might be particularly relevant, because receptors containing this subunit show slower deactivation kinetics and high calcium permeability (Cull-Candy and Leszkiewicz, 2004; Monyer et al., 1994; Vicini et al., 1998). Altered expression of NMDA receptor subunits has been reported in postmortem tissue from Alzheimer's and Huntington's patients (Arning et al., 2005; Arzberger et al., 1997; Bi and Sze, 2002; Mishizen-Eberz et al., 2004). Furthermore, in a transgenic mouse model of Huntington's disease, increased vulnerability to excitotoxicity and enhanced amplitude of NMDA-mediated currents was recently reported (Li et al., 2003; Zeron et al., 2002). Moreover, cells expressing Huntingtin and the NR1/NR2B subunits show increased excitotoxic cell death (Zeron et al., 2001).

We have investigated whether enhancement of excitotoxic neuronal death during sustained glycolysis inhibition *in vivo*, involves changes in the extracellular levels of EAA, glutamate uptake and protein content of glutamate transporters and/or NMDA receptor subunits, in the rat hippocampus.

EXPERIMENTAL PROCEDURES

Microdialysis

Male Wistar rats (250–300 g) were used throughout the study. They were handled according to the National Institutes of Health Guide for Care and Use of Laboratory animals and the local Animal Care Committee approved all animal treatments. All efforts were made to minimize the number of animals used and their suffering. Animals were slightly anesthetized with ether before kill, and all microdialysis experiments were performed in anesthetized animals. Eight days before initiating the experiments rats were anesthetized with halothane (1.5%) and fixed on a stereotaxic frame for unilateral implantation of a guide cannula into the CA1 hippocampal region (coordinates: AP −3.6 from bregma, L +2.0 from midline, and V −2.0 from dura, according to Paxinos and Watson, 1986). The guide cannula was fixed to the skull with dental acrylic cement.

Seven days later, microdialysis experiments were initiated and carried out during the five following days. On the first day rats received an i.p. vehicle injection (phosphate buffer 10 mM), they were anesthetized and the microdialysis probe was inserted into the hippocampus in order to record extracellular amino acids basal values. During the three following days rats received an i.p. injection of the glycolysis inhibitor, IOA (15 mg/kg per day), and on the fifth day rats received again an i.p. vehicle injection. Microdialysis was performed daily 1 h after the corresponding i.p. injection. Probes were continuously perfused with a Ringer-Krebs medium (RK) containing (in mM): NaCl 118, KCl 4.5, $MgSO_4$ 2.5, NaH_2PO_4 4.0, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 10, at a flux rate of 2 μl/min via a microinjection pump (BAS MD 1001, West Lafayette, IN, USA). After a 90-min equilibration period, three 25-μl fractions were collected and amino acids levels were determined as described below. At day 5, 24 hours after the end of the microdialysis experiment, animals were transcardially perfused for histological analysis.

In vitro amino acid membrane recovery was determined according to Massieu et al. (1995) and was calculated to be around 7–11%. Data represent values calculated from HPLC amino acid determination without correcting for membrane recovery since it was not determined *in vivo*.

Quantification of amino acid levels

Amino acid content in dialysates was determined by HPLC according to Antoine et al. (1999), with slight modifications as reported previously (Montiel et al., 2005). Briefly, the 25-μl collected fractions were derivatized with the same volume of *o*-ophthalaldehyde and 3 min later a 10-μl volume of this mixture was injected into an HPLC system (Waters 600, Milford, MA, USA) equipped with an ODS column (25 cm×4 mm internal diameter, Supelco, Inc., Bellefonte, PA, USA). The mobile phase consisted of 18% methanol: 22% acetonitrile: 14% isopropanol: 46% phosphate buffer (60 mM, pH=6.65)/phosphate buffer (60 mM pH=6.65) and a flux rate of 1 ml/min was used in a linear gradient of 33 min duration from 10 to 90% solvent mixture. Amino acid concentration was calculated by comparison with a standard mixture of amino acids equally processed. Values from the three fractions collected daily were pooled. Data in Table 1 and Fig. 2 represent means±S.E.M. of the number of animals indicated in figure legends.

Histological analysis

In order to verify the site of the probe insertion 24 hours after the end of the microdialysis experiment animals were transcardially perfused under deep anesthesia with 200 ml 0.9% saline followed by 200 ml 5% formaldehyde in 0.1 M phosphate buffer, pH 7.3. Brains were removed and left in 5% formaldehyde for additional 24 h. They were transferred to 10, 20 and 30% sucrose (24 h each) and 40-μm sections were obtained in a cryostat and stained with Cresyl Violet.

Determination of GAPDH activity

Rats were treated either with vehicle (phosphate buffer 10 mM) or IOA (15 mg/kg) during one, two or three consecutive days. Two hours after each injection and 24 h after the latest, the animals were ether-anesthetized, killed by decapitation and the hippocampus extracted. The left hippocampus was used for immunoblot analysis (see below) and the right hippocampus for the determination of GAPDH activity. This was determined as previously reported with minor modifications (Ikemoto et al., 2003; Montiel et al., 2006). Tissue was homogenized in 1:10 (wt/vol) Tris–HCl pH 8.5 and GAPDH activity was monitored in a reaction mixture (1 ml total volume) containing (in mM): 1.7 arsenic acid, 20 sodium

Table 1. Basal levels (μM) of glutamine, taurine, alanine and glycine in the hippocampus and their changes induced by IOA treatment

Amino acid	Basal level	Peak concentration during IOA treatment	Level 24 h after IOA treatment
Glutamine	58.55±4.32	64.14±7.14	61.98±6.69
Taurine	33.31±5.05	32.95±2.67	34.48±4.47
Alanine	18.41±1.93	24.16±3.39	18.56±1.88
Glycine	41.62±5.18	59.31±7.57	62.76±13.9
GABA	0.326±0.03	0.369±0.09	0.291±0.03

Amino acid levels were determined in collected microdialysates by HPLC as described in the experimental procedures. Levels at peak represent the maximal amino acid concentration observed during IOA treatment. Data are means±SEM from 5–7 animals.

fluoride, 1 NAD^+ and 5 KH_2PO_4. The reaction was initiated by the addition of glyceraldehyde 3-phosphate (final concentration 1 mM). GAPDH activity was determined by monitoring the amount of NADH formed at 340 nm during the first 30 s when the reaction is linear. A molar extinction coefficient of 2.07 was used to calculate the amount of NADH formed in μmol. Protein content was determined by the Bradford's method. Data are expressed as μmol NADH/min/mg protein. Specific activity is expressed as mean±S.E.M.

Immunoblot analysis

The following antibodies were used for immunoblot analysis. For NMDA receptor subunits goat polyclonal anti-NR1, anti-NR2A and anti-NR2B were used (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, catalog number sc-1467, sc-1468 and sc-1469, respectively). All antibodies were directed against the carboxy-terminus. For 1472 tyrosine phosphorylation of NR2B subunit we used mouse monoclonal antibody (Sigma-Aldrich, Toluca, Mexico, catalog number M5442). For glutamate transporters: guinea-pig polyclonal anti-GLAST and anti-GLT-1 transporters (Chemicon Int., Temecula, CA, USA, catalog number AB1782 and AB1783, respectively) and goat anti-EAAC1 transporter (Chemicon Int. catalog number AB1520) were used. All antibodies for glutamate transporters were directed against the carboxy-terminus of the rat proteins. Immunoblot experiments were carried out as previously described with slight modifications (Rao et al., 2001). Animals were chronically treated (i.p.) with vehicle or IOA as described above. Two or 24 h after IOA treatment rats were slightly ether-anesthetized and killed by decapitation. Because altered protein levels of NR2B subunit and GLT-1 transporter were observed 2 and 24 h after IOA treatment, a more detailed time-course of changes in these proteins was performed. For these purpose protein levels were analyzed 2 h after each daily IOA administration, and 24 and 72 h after the treatment was completed. The hippocampus was extracted and sonicated in 1:10 (wt/vol) phosphate buffer (25 mM) containing EDTA (2 mM) and protease inhibitors (Complete Mini, EDTA-free, Roche, Mannheim, Germany). Samples were centrifuged at 38,000 r.p.m. during 35 min and the pellets were resuspended in 1:5 (vol/vol) phosphate buffer (25 mM), EDTA (2 mM) and protease inhibitors. Protein concentration curves were performed in order to determine the amount of protein necessary for an adequate immunodetection with the different antibodies. Blots performed with different protein concentrations (1, 2, 5, 10, 15, 25 μg) showed a linear relation between the amount of protein loaded and the intensity of the optical density. From these curves a concentration of 15 μg was chosen for NR1, NR2A, NR2B and EAAC1 immunodetection and 5 μg for GLT-1 and GLAST. Samples were boiled for 3 min, electrophoresed at 25 mA during 2 h and then transferred to immobilon-P membrane (Millipore, Bedford, MA, USA) during 24 h at 4 °C. The membrane was incubated during 2 h in medium containing 5% dry milk in phosphate buffer saline/Tween 20 (PBS-T; phosphate buffer 0.1 M, NaCl 0.9%, Tween 20 0.1%). For NMDA receptor subunits immobilon-P membranes were then incubated with goat polyclonal antibody against NR1, NR2A, NR2B subunits, respectively (1:500; Santa Cruz Biotechnology) during 24 h at 4 °C. They were washed (4 times/10 min) in PBS-T, and incubated for 2 h with anti-goat horseradish peroxidase–conjugated antibody (1:500; Vector Laboratories, Burlingame, CA, USA). In the case of glutamate transporters immobilon-P membranes were incubated with anti-goat antibody against EAAC1 or anti-guinea-pig polyclonal antibodies against GLAST and GLT1 (1:100 for EAAC1; 1:5000 for GLAST and GLT1; Santa Cruz Biotechnology) during 24 h at 4 °C. They were washed (4 times/10 min) in PBS-T, and incubated for 2 h with anti-guinea-pig horseradish peroxidase–conjugated antibody (1:500; Vector Laboratories). Membranes were washed (4 times/10 min) in PBS-T, incubated with chemiluminescence's reagent (ECL™ Amersham Biosciences, Little Chalfont, UK) and exposed to X-ray film. Levels of immunoreactivity were quantified densitometrically in an image analyzer Bio Rad, Laser-Pix (Software 4.0.0.13). Negative controls were prepared by omission of primary antibodies. Densitometry analysis of NMDA receptor subunits or transporter proteins was corrected with respect to actin content (1:1000, Sigma-Aldrich). When the primary antibodies were omitted, no densitometric signal was present (data not shown). Data represent means±S.E.M. of the number of animals indicated in figure legends.

Immunoprecipitation

Immunoprecipitation studies were performed following the methodology described by Arias et al. (2002) with some modifications. The hippocampus of rats treated (i.p.) with vehicle or IOA was extracted and homogenized in ice-cold lysis buffer pH 7.5 (1:10 wt/vol) containing 50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P40, 0.5% SDS, 10 mM Na_3VO_4, 25 mM sodium fluoride and protease inhibitors (Complete, Roche). All procedures were made at 4 °C. Samples were centrifuged at 1500×*g* for 5 min. The supernatants were collected and protein concentrations determined by the Lowry assay. Equal amounts of protein (5000 μg) and 12 μl of polyclonal antibody were then used for NR2B immunoprecipitation overnight. Samples were precleared with protein G-sepharose (Amersham) for 2 h at 4 °C and the immune complexes were isolated by centrifugation at 1500×*g* during 2 min. The pellets were washed three times with lysis buffer and centrifuged at 1500×*g* during 2 min. The pellet was washed twice with buffer 50 mM Tris–HCl, 150 mM NaCl, (0.1% Nonidet P40, pH 7.5) and one more time with buffer 50 mM Tris–HCl pH 7.5. The samples were centrifuged at 12000×*g* during 3 min and the pellets were re-suspended in 30 μl of Laemmli buffer. The bound proteins were eluted by boiling during 5 min and centrifuged during 3 min at 2000×*g*. After centrifugation, 30 μl of the same sample buffer were used for Western blotting as described above. The immobilon-P membranes were incubated with phosphotyrosine 1472 monoclonal antibody (1:1000) during 24 h at 4 °C. They were washed (4 times/10 min) in PBS-T, and incubated for 2 h with anti-mouse horseradish peroxidase–conjugated antibody (1:3000; Santa Cruz Biotechnology). Levels of immunoreactivity were quantified as described above. Densitometry analysis of phosphotyrosine 1472 was corrected relative to NR2B content (1:500; Santa Cruz Biotechnology). Negative controls were prepared by omission of primary NR2B antibody and no densitometry signal was evident in this condition.

Immunohistochemistry

Rats were treated as described above and 2 h, 24 h, 72 h and 8 days after the treatment, they were transcardially perfused under deep pentobarbital anesthesia with 250 ml 0.9% saline followed by 250 ml 4% paraformaldehyde, 0.4% picric acid in 0.1 M phosphate buffer, pH 7.3. Brains were removed and left in 4% paraformaldehyde for additional 24 h. They were transferred to 10, 20 and 30% sucrose (24 h each) and 40 μm sections were obtained in a cryostat. Floating sections were blocked with anti-rabbit serum during 1 h and then incubated with goat polyclonal antibody against NR1 or NR2B subunit proteins (1:500; Santa Cruz Biotechnology) during 72 h at 4 °C. They were washed (4 times/10 min) in PBS, and incubated for 2 h with anti-goat horseradish peroxidase–conjugated antibody (1:500 for NR1; 1:300 for NR2B, Vector Laboratories). Sections were washed (4 times/10 min) in PBS, and incubated for 2 h with avidin/biotin complex. After washing, immunoreactivity was detected using 3,3′-diaminobenzidine 0.06% (Sigma-Aldrich) and 0.1% hydrogen peroxide. Immunoreactivity was quantified densitometrically in three sections of each one of the animals by means of an image analyzer (NIH Macintosh Image 1.6). Negative controls were prepared by omission of pri-

mary antibodies and no densitometry signal was evident in these conditions.

[^{3}H]-D-aspartate uptake in hippocampal slices

Twenty-four hours after treatment with vehicle solution or IOA, rats were slightly ether-anesthetized and killed by decapitation, the hippocampus was extracted and 250 μm slices were obtained in a tissue chopper at 4 °C (Brinkmann, Westbury, NY, USA). Slices were oxygenated for 20 min in a RK medium containing (in mM): NaCl 118, KCl 4.5, $MgSO_4$ 2.5, NaH_2PO_4 4.0, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 10, at 37 °C. Kinetic analysis was performed in slices incubated with RK containing [^{3}H]-D-aspartate (0.5 μCi, Amersham Biosciences) and different concentrations of unlabeled aspartate (5–1000 μM) at 37 °C during 5 min. Nonspecific uptake was determined in the presence of Ringer-Krebs containing choline chloride (RKC) instead of NaCl. Uptake was terminated by three ice-cold washes with RK or RKC for specific and nonspecific uptake, respectively, and immediately filtered through nitrocellulose filters (0.45 μm pore size; Millipore Corporation). Slices were digested in 500 μl 0.2 N NaOH during 24 h. Radioactivity was counted after the addition of 10 ml tritosol and 500 μl acetic acid in a liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA). Sodium-dependent transport was calculated by the subtraction of radioactivity accumulated in the choline-containing buffer from that in the sodium-containing buffer. In choline-containing buffer less than 10% of the substrate was accumulated in hippocampal slices. Radioactivity data were corrected by protein content determined by the method of Lowry. Data are expressed as nmol/min/mg protein. Saturation curves were performed from the data of control and IOA-treated animals and the best curve fittings were plotted using the GraphPad Prism 4 program (GraphPad, San Diego, CA, USA). The Lineweaver-Burk analysis was performed and K_m and Vmax values were calculated using the same program.

[^{3}H]-D-aspartate uptake was also analyzed in the presence of dihydrokainic acid (DHK), a GLT-1 selective transporter blocker (Kawahara et al., 2002) in order to determine the GLT-1-dependent uptake. Hippocampal slices were extracted as described above and pre-incubated during 1 h at 37 °C with 1 mM DHK (Tocris Bioscience, Ellisville, MO, USA) according to the previously reported studies (Shin et al., 2005). Hippocampal slices were incubated with RK containing [^{3}H]-D-aspartate (0.5 μCi, Amersham Biosciences) and different concentrations of unlabeled aspartate (50–750 μM), at 37 °C during 5 min. Radioactivity data analysis was made as described above. The difference between total [^{3}H]-D-aspartate uptake and uptake observed in the presence of DHK represents GLT-1-mediated glutamate uptake.

Statistics

Data were analyzed by one-way ANOVA followed by a Fisher's least significant multiple comparison test in all cases.

RESULTS

Chronic glycolysis inhibition increases the extracellular levels of aspartate and glutamate

Chronic treatment with IOA induced a decline in GAPDH activity in the hippocampus (Fig. 1). Two hours after the end of the treatment GAPDH activity was significantly inhibited in 33%, and inhibition was sustained during the following 24 h. Analysis of amino acid content in dialysates showed no significant alterations in the extracellular concentration of glutamine, taurine, alanine, glycine and GABA during or after IOA treatment (Table 1). Extracellular levels of these amino acids were very similar to those

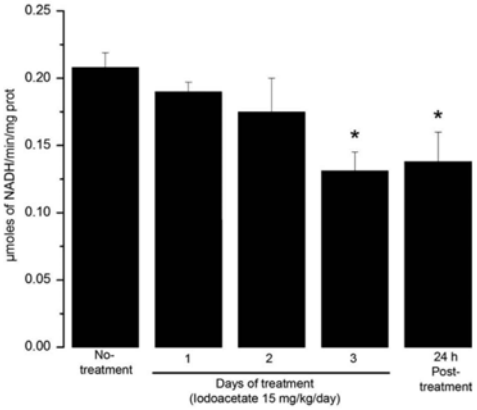

Fig. 1. Effect of IOA chronic administration on GAPDH activity in the hippocampus. GAPDH was determined in animals treated with vehicle or IOA during 3 days. Two hours after each administration and 24 h after the last injection, GAPDH activity was determined spectrophotometrically in hippocampal homogenates as described in Experimental Procedures. Values are means±S.E.M. from 6 to 8 animals. * $P<0.001$ relative to control animals.

determined in control rats chronically injected with vehicle (data not shown). Glutamate levels were progressively increased during IOA treatment and remained elevated during the following 24 h, reaching maximum values of 24.5 μM (Fig. 2). A more discrete increase in aspartate levels was observed during IOA treatment, which was not statistically significant. Twenty-four hours after the end of the treatment aspartate levels increased further reaching values significantly higher than basal levels (Fig. 2).

Despite that chronic glycolysis inhibition induced a progressive increase in the extracellular levels of glutamate and aspartate, no neuronal damage was observed in the hippocampus or any other brain region, as evaluated in Cresyl Violet–stained sections from rats killed 48 h after the treatment (data not shown) as previously observed (Massieu et al., 2000).

Increased aspartate and glutamate extracellular levels correlate with decreased glutamate transport

The increase in the extracellular levels of EAA during glycolysis inhibition might be the consequence of altered removal after synaptic release. We studied this possibility evaluating the changes in the protein content of glutamate transporters by immunoblot, and by monitoring [^{3}H]-D-aspartate uptake in hippocampal slices from control and IOA-treated rats.

No significant changes were observed in the protein levels of glutamate transporters GLAST and EEAC1, 2 or 24 hours after the last IOA administration (Fig. 3A–B). The protein content of GLT-1 glutamate transporter slightly decreased during IOA treatment and significantly diminished in 23% 24 h after the end of treatment (Fig. 3C). At 72 h GLT-1 protein levels recovered to control values (Fig. 3C). Based on these observations, [^{3}H]-D-aspartate uptake

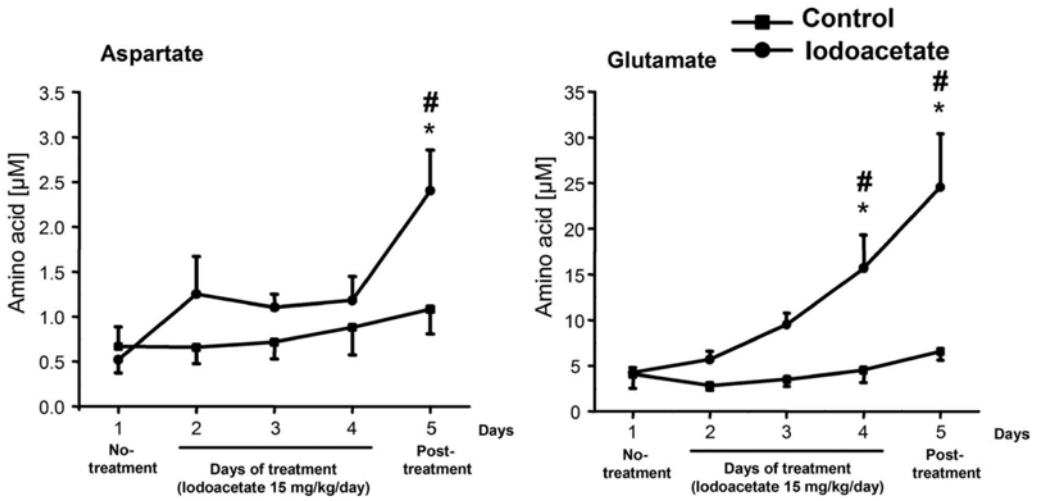

Fig. 2. Glycolysis inhibition increases the extracellular concentration of aspartate and glutamate in the hippocampus. Microdialysis experiments were performed as described in Experimental Procedures. Microdialysates (25 μl) were collected during five consecutive days. The first day (pre-treatment) dialysis was performed in control animals to determine basal amino acid levels. During the following 4 days dialysates were collected 2 h after IOA administration (days 2, 3 and 4), and 24 h after the last injection (day 5). Dialysis was performed in control animals treated daily with vehicle. Amino acid content was determined by HPLC as described in Experimental Procedures. Data represent means±S.E.M. from 6 to 7 animals. * $P<0.01$ relative to basal levels and # $P<0.01$ relative to control rats.

was studied in hippocampal slices from animals killed 24 h after IOA treatment. [^{3}H]-D-aspartate uptake was Na^+-dependent since less than 10% of the radioactivity was recovered in hippocampal slices incubated in the absence of Na^+ (choline-containing medium) (Fig. 4A). According to the saturation analysis, IOA-treated animals showed decreased glutamate uptake (Fig. 4B). Data were analyzed by the Lineweaver-Burke plot and a significant reduction in the Vmax was found in treated animals (controls Vmax=7.041±0.967 nmol/min/mg prot; IOA-treated Vmax=2.887±0.314 nmol/min/mg prot). Although not significant, a decrease in K_m was observed in treated animals (controls K_m=766±194 μM; IOA-treated 240±69 μM) (Fig. 4B). We then evaluated if the observed reduction in [^{3}H]-D-aspartate uptake in IOA-treated animals could involve GLT-1 transporter. Therefore, the [^{3}H]-D-aspartate uptake assay was performed in the presence of the GLT-1-selective blocker, DHK (Kawahara et al., 2002; Shin et al., 2005). Results show that DHK-sensitive [^{3}H]-D-aspartate uptake is decreased in animals treated with IOA, as analyzed 24 h after the treatment (Fig. 4C).

Sustained glycolysis inhibition correlates with increased protein levels and phosphorylation of the NR2B subunit of the NMDA receptor

The facilitating effect of glycolysis inhibition on glutamate neurotoxicity (Massieu et al., 2000, 2003), might be explained by changes in NMDA receptor subunit composition altering its activity. Protein content of the NR1, NR2A and NR2B subunits of the NMDA receptor was evaluated in control and IOA-treated animals. As shown in the Fig. 5A–B, protein levels of the NR1 and NR2A subunits did not change either 2 or 24 h after IOA treatment. In contrast, a slow progressive increase in NR2B subunit protein levels was observed during IOA treatment, which is significant at 24 h post-treatment (Fig. 5C). At 72 h NR2B protein levels were still slightly elevated.

Changes in the NR2B subunit were studied further by immunocytochemistry at different times after IOA chronic administration. Results show a progressive increase in NR2B immunoreactivity from 2 to 72 h after the treatment (Fig. 6). According to the densitometric analysis in the CA1 region, increased immunoreactivity was maximal and statistically significant at 24 h (Fig. 7). The observed changes were transient since NR2B immunoreactivity returned to basal values 8 days after the treatment (Figs. 6 and 7). Increased NR2B immunoreactivity was also observed in the CA3 region and the dentate gyrus, and was maximal or near maximal at 2 h, remaining elevated at 24 and 72 h, and returning to basal levels 8 days after the treatment (Figs. 6 and 7). Densitometric analysis revealed a maximal 2.3- and 5.5-fold increase in immunoreactivity in CA3 and dentate gyrus, respectively (Fig. 7), while the in CA1 a 40% elevation was observed. Magnification of the hippocampal CA1 region is depicted in Fig. 6 (right panel). As can be observed increased NR2B expression is mainly restricted to the apical dendrites of the CA1 pyramidal layer (arrowheads). In agreement with immunoblot analysis no changes in NR1 subunit were observed by immunocytochemistry in tissue sections of IOA-treated animals killed 2 h and 24 h after the treatment (data not shown).

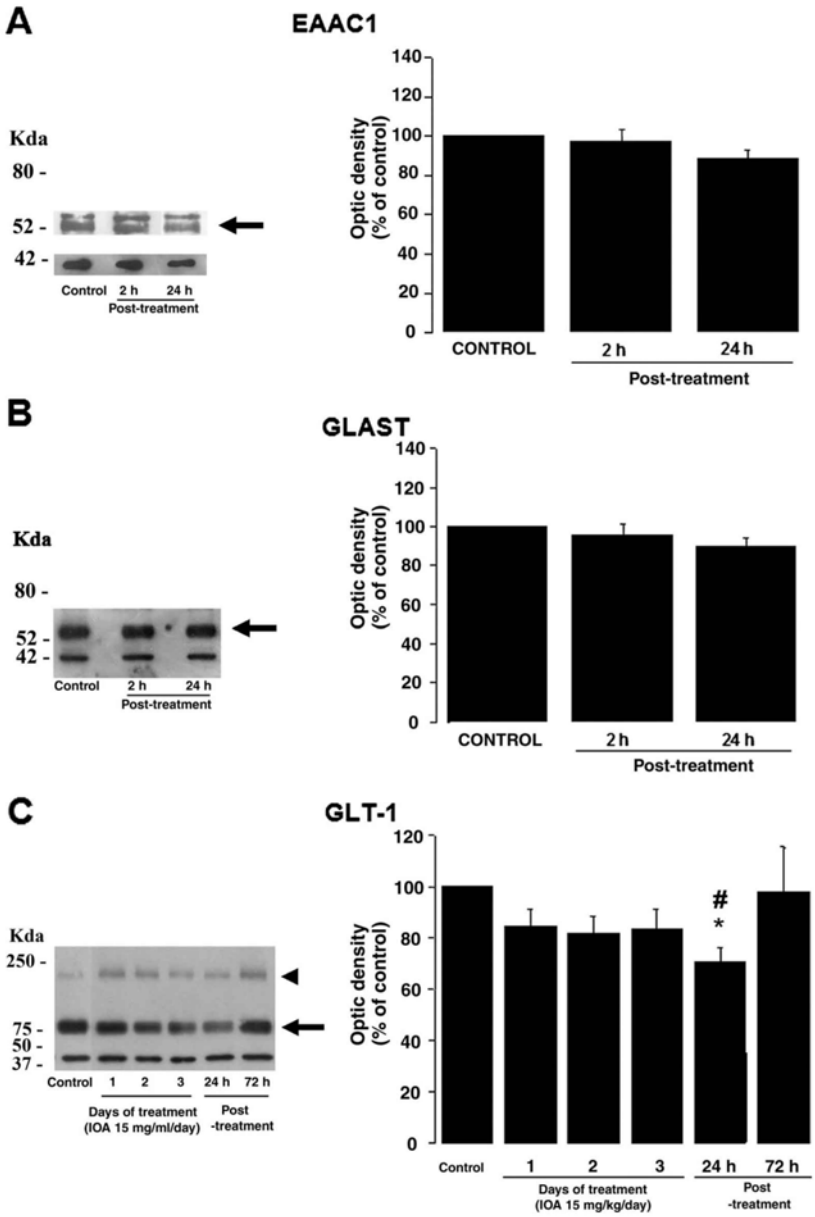

Fig. 3. Effect of GAPDH inhibition on protein levels of glutamate transporters. Rats received a daily injection of either phosphate buffer (10 mM) or IOA (15 mg/kg) for three consecutive days. (A, B) EAAC1 and GLAST protein levels were determined 2 and 24 h after IOA treatment, and (C) GLT-1 protein levels were analyzed 2 h after each IOA daily injection and 24 and 72 h post-treatment. Left panels show immunoblots of glutamate transporters of representative animals. Graphs show densitometric analysis of the different glutamate transporters determined from immunoblots. The arrows show the band corresponding to each transporter. In C arrowhead and arrow show the multimer and monomer conformations of GLT-1, respectively. Values are means±S.E.M. from 4 to 5 animals. The band at the bottom of each panel corresponds to actin. * $P<0.01$ relative to control values. # $P<0.01$ relative to 72 h.

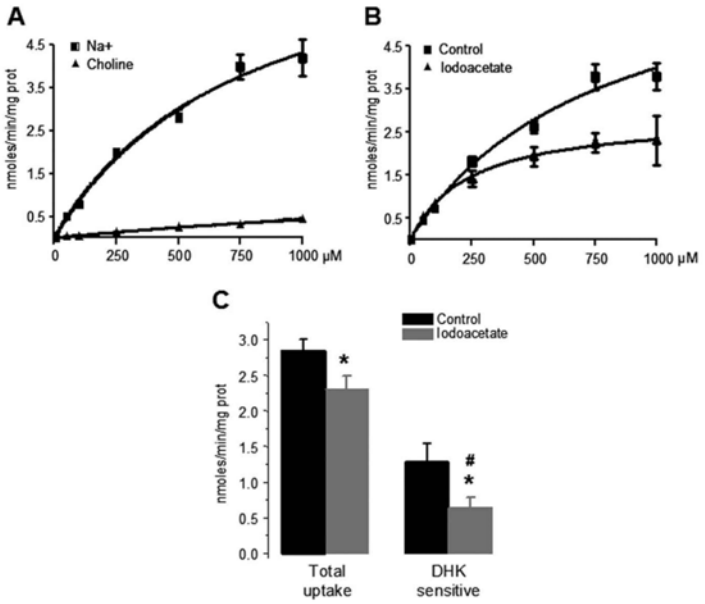

Fig. 4. Kinetic analysis of [^{3}H]-D-aspartate uptake during glycolysis inhibition. Animals were treated with IOA and killed 24 h after the last injection. Hippocampal slices were incubated with [^{3}H]-D-aspartate and unlabeled aspartate at the concentrations shown during 5 min, and radioactivity was quantified. (A) [^{3}H]-D-aspartate uptake was evaluated in hippocampal slices from control animals incubated in Na^+-containing and choline-containing medium. Na^+-dependent uptake was calculated by the subtraction of the uptake measured in choline-containing medium from that in the Na^+-containing medium. (B) Saturation curves of [^{3}H]-D-aspartate transport in hippocampal slices from control and IOA-treated animals. (C) Total and DHK-sensitive [^{3}H]-D-aspartate uptake was evaluated in hippocampal slices from control and treated-animals. 1 mM DHK was pre-incubated during 1 h and [^{3}H]-D-aspartate uptake (500 μM final concentration) determined. Uptake observed in the presence of DHK was subtracted from total uptake to determine GLT-1-mediated aspartate transport. Data are means±S.E.M. from 4 to 6 animals. * $P<0.05$ relative to total uptake, $^{\#}$ $P<0.05$ relative to control animals.

We then analyzed whether the increase in the NR2B protein content observed during metabolic impairment could be related to changes in phosphorylation. Twenty-four hours after IOA treatment the hippocampal slices were extracted and immunoprecipitation experiments performed. Results show a significant increase in the phosphorylation of Tyr^{1472} in rats treated with IOA (Fig. 8).

DISCUSSION

An important role of glucose metabolism on the regulation of the extracellular levels of EAA during physiological conditions, has been suggested previously (Bonvento et al., 2002; Hyder et al., 2006). Acute impairment of this metabolic route during ischemia or hypoglycemia, or during acute administration of IOA, leads to the release of EAA and neuronal damage (Benveniste et al., 1984; Camacho et al., 2006; Massieu et al., 2000; Matthews et al., 1997; Sandberg et al., 1985). However, very little is known about the control of the extracellular concentration of EAA during moderate but sustained metabolic inhibition. Such a condition might be associated with aging and some neurodegenerative diseases, since a reduction in glucose metabolism has been reported in aged individuals and Alzheimer's patients (Eberling et al., 1995; Mazzola and Sirover, 2001; Mielke et al., 1998; Planel et al., 2004; Slosman et al., 2001). Consistent with this idea, we monitored by microdialysis the extracellular levels of EAA in the hippocampus during continuous metabolic inhibition. A progressive increase in the extracellular concentration of EAA was observed during IOA administration, which was sustained during the following 24 h after the end of the treatment. This observation correlates with the inhibition of GAPDH activity, which was significant at day 3 of treatment and remained inhibited for the following 24 h.

In vitro studies have shown that glycolysis inhibition by IOA induces a decrease in [^{3}H]-glutamate uptake both in astrocytes and neurons (Gemba et al., 1994; Jabaudon et al., 2000; Longuemare et al., 1999); therefore we tested if EAA accumulation was related to deficient glutamate uptake or decreased levels of glutamate transporters. According to the kinetic analyses of [^{3}H]-D-aspartate uptake a significant reduction in the Vmax was observed in IOA-treated animals suggesting a reduction in the number of glutamate transporters. High K_m values for D-aspartate uptake were determined in the present study relative to those previously reported in other *in vitro* preparations, such as synaptosomes, cultured astrocytes and in *in vitro* expression systems of cloned glutamate transporters (Danbolt, 2001; Garlin et al., 1995; Kanai and Hediger, 1992; Robinson, 1998). In these preparations K_m reported

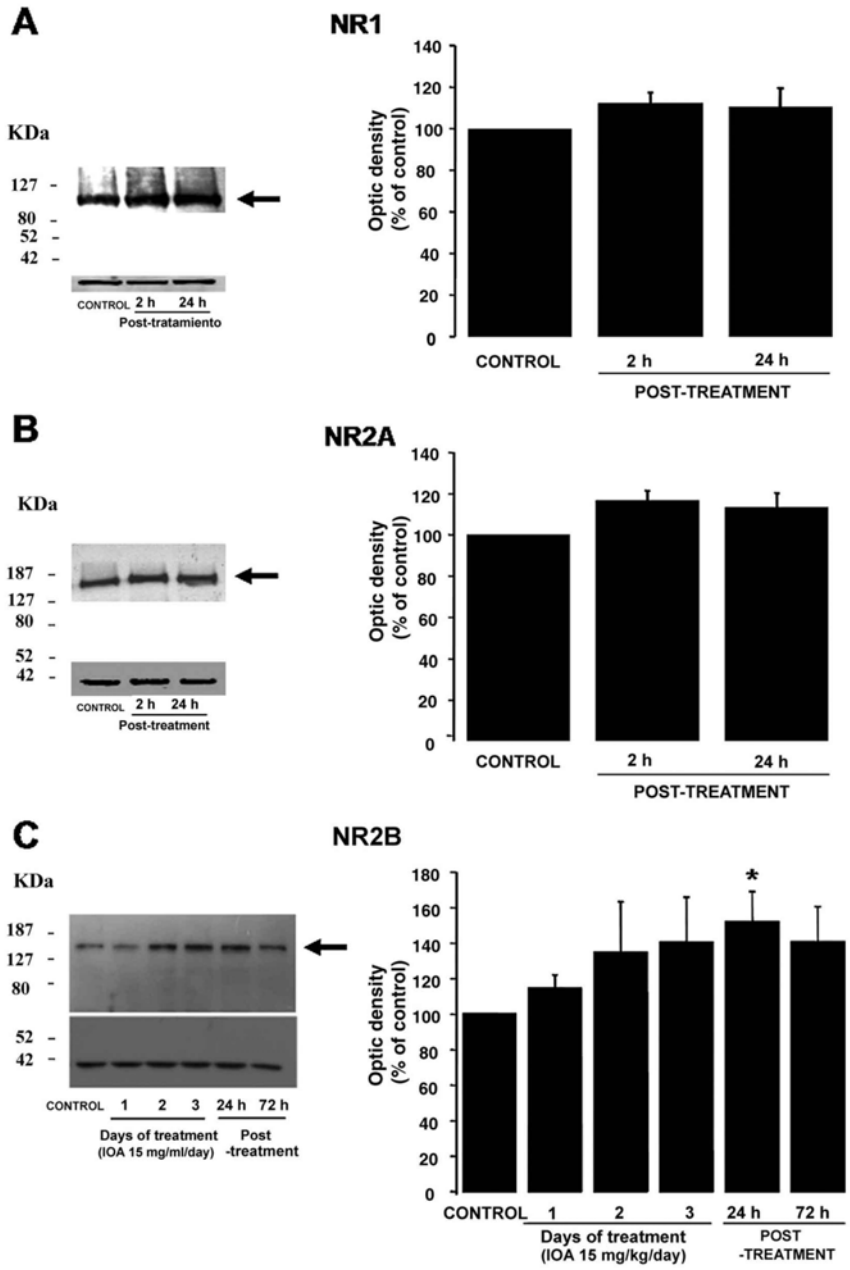

Fig. 5. Glycolysis inhibition increases the protein levels of the NR2B NMDA receptor subunit in the hippocampus. Animals were treated as described in legend to Fig. 3, and immunoblot analysis was performed as described in Experimental Procedures. Left panels show immunoblots of NR1, NR2A and NR2B of representative animals. The band at the bottom of each panel corresponds to actin. Graphs indicate densitometric analysis of the different NMDA receptor subunits determined from immunoblots. The arrows indicate the band corresponding to each subunit. Data are means±S.E.M. from 5 to 6 animals. * $P<0.01$ compared with control.

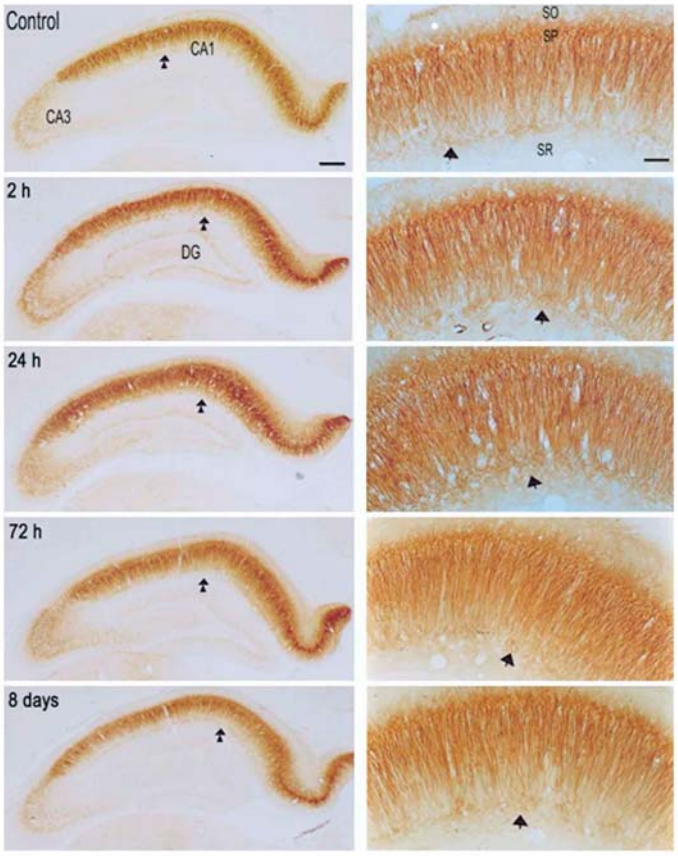

Fig. 6. Micrographs of representative animals showing the immunohistochemistry of NR2B NMDA receptor subunit in the hippocampus. Rats were killed at different times after IOA treatment, the brain was extracted and coronal tissue sections were obtained. Immunohistochemistry was performed in floating sections as described in Experimental Procedures. The observed increase in NR2B immunoreactivity is transient in all hippocampal regions, returning to basal levels 8 days after the treatment. DG (dentate gyrus). Scale bar=200 μm. Right panel shows magnifications of the CA1 region (arrow in micrographs on the left panel). Note that increased NR2B immunoreactivity is evident in the region of the apical dendrites of pyramidal cells in the CA1 region (arrowheads). SO (stratum oriens), SP (stratum pyramidale), SR (stratum radiatum). Scale bar=100 μm.

values vary between 1 and 100 μM. In brain slices higher K_m values for glutamate uptake have been described (around 250 μM) (Kuwahara et al., 1992; Heron et al., 1995), but they are still lower than those reported in the present study. This result might be related to the difficulty to observe a high affinity system in an intricate preparation with many tissue constituents affecting aspartate diffusion. This would cause an apparent increase in the K_m because the concentration of D-aspartate in the bulk environment required to half-maximally saturate the transporter would be higher relative to a more homogeneous system such as cultured cells or synaptosomes (Garthwaite and Garthwaite, 1985; Robinson, 2006). In addition, different subpopulations of glutamate transporters with different affinities for glutamate are present in the slice preparation, and a relatively long time (5 min) for D-aspartate uptake was used in saturation experiments. On the basis of the K_m obtained in the present study, D-aspartate uptake would correspond to a low affinity system. However, low affinity glutamate uptake has been described as sodium-independent (Anderson and Swanson, 2000; Benjamin and Quastel, 1976; Davies and Johnston, 1976; Kuwahara et al., 1992), and in the present conditions D-aspartate uptake was highly dependent on the presence of sodium. In addition, it was inhibited by the selective GLT-1 glutamate transporter blocker DHK, as well as by the non-selective blocker of high affinity transporters DL-TBOA (data not shown), suggesting that the uptake observed in the present conditions is at least in part mediated by sodium-dependent DHK-sensitive glutamate transporters.

In IOA-treated animals D-aspartate uptake sensitive to DHK was significantly reduced. This observation corre-

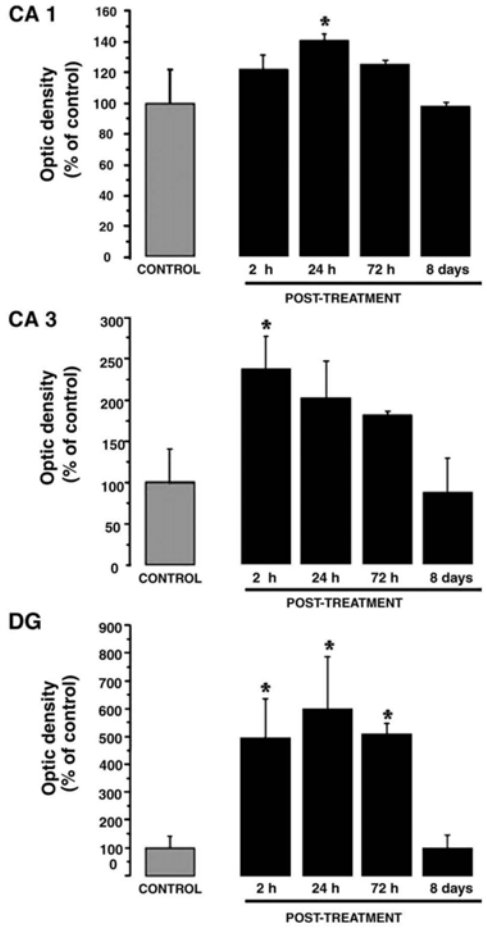

Fig. 7. Densitometric analysis of NR2B immunoreactivity in the different hippocampal regions. Increases in immunoreactivity are transient and return to basal levels 8 days after the treatment. DG (dentate gyrus). Data represent means±S.E.M. of four animals. * $P<0.05$ relative to control.

lates with the observed decrease in GLT-1 protein content, suggesting that decreased uptake during *in vivo* glycolysis inhibition might result at least in part, from the reduction in the content of the GLT-1 glutamate transporter. This reduction might be related to protein kinase inhibition. Expression of GLT-1 correlates with double phosphorylation of p42 and p44 MAP kinases inducing the activation of CREM and ATF-1 transcription factors (Danbolt, 2001; Gegelashvili et al., 2000). Recently, it has been reported that Akt induces the expression of GLT-1 through increased transcription in astrocytes (Li et al., 2006). On the other hand, activation of PKC decreases both the activity and cell surface expression of GLT-1 in a variety of preparations (Fang et al., 2002; Kalandadze et al., 2002; Zhou and Sutherland, 2004). The activation of different signaling pathways could be mediated by accumulated glutamate. Indeed, decreased levels of GLT-1 have been observed in human astrocytes after activation of metabotropic glutamate receptors (Aronica et al., 2003). However, additional experiments are needed in order to assess whether the observed decrease in GLT-1 protein is accompanied by a reduction in its mRNA, or if it is the result of a redistribution of GLT-1 transporter by protein kinases activation.

An increase in EAA levels has been observed in the CSF of Alzheimer's patients, which correlates with the disease severity (Masliah et al., 1996; Zoia et al., 2005). These changes might be related to decreased glucose metabolism (Hoyer and Nitsch, 1989; Csernansky et al., 1996; Jimenez-Jimenez et al., 1998), and/or a reduction in the number of glutamate transporters (Ferrarese et al., 2000; Li et al., 1997; Scott et al., 1995; Zoia et al., 2004). Similarly, in Huntington's disease patients decreased expression of glutamate transporters and glutamate uptake has been reported (Arzberger et al., 1997; Shin et al., 2005). Similar observations have been found in transgenic animal models of these diseases (Dabir et al., 2006; Lievens et al., 2001, 2005; Masliah et al., 2000; Shin et al., 2005). However, the relationship between decreased glucose metabolism, increased extracellular EAA and altered glutamate transport in these pathologies remains to be determined.

Because the different NR2 subunits in combination with the NR1 subunit, produce NMDA receptors with different affinity for agonists, sensitivity to Mg^{2+} blockade and response to antagonists (Cull-Candy and Leszkiewicz, 2004; Monyer et al., 1994; Vicini et al., 1998), changes in the protein content of the different subunits might influence neuronal excitability and vulnerability to excitotoxic cell death. Our data show that glycolysis inhibition does not alter the protein content of the NR1 and NR2A subunits while a transitory significant increase in the NR2B subunit levels was observed at 24 h. Immunohistochemistry of the NR2B subunit revealed that such an increase occurred in the CA1 and CA3 hippocampal regions as well as in the dentate gyrus. In the CA1 region this increase was particularly evident in the apical dendrites where many synaptic contacts are located.

The presence of the NR2B subunit might be particularly important, because NMDA receptors containing this subunit show a slower deactivation kinetic and an high calcium permeability (Cull-Candy and Leszkiewicz, 2004; McBain and Mayer, 1994; Monyer et al., 1994; Popescu, 2005). The lack of alteration of the NR1 and NR2A subunits and the increase in NR2B levels, suggest the presence of receptors composed of NR1/NR2B subunits.

In some studies up-regulation of NMDA receptor subunits has been observed after severe ischemia in the hippocampus (Besshoh et al., 2005; Heurteaux et al., 1994; Kang et al., 2001; Quintana et al., 2006; Sutcu et al., 2005; Won et al., 2001), and a role of glutamate in promoting the increase in the NR2B subunit has been suggested (Heurteaux et al., 1994). Expression of NR2B subunit is regulated by synaptic activity through CaM kinases, tyrosine kinases and CINAP protein (Corsi et al., 1998;

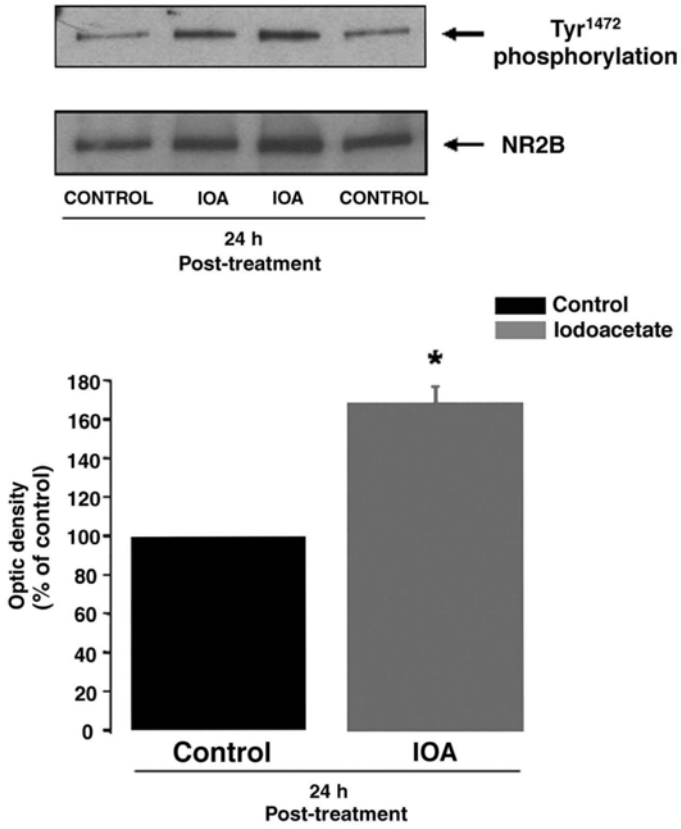

Fig. 8. Sustained glycolytic inhibition promotes Tyr^{1472} phosphorylation of the NR2B subunit. Immunoprecipitation and immunoblot blot analysis of the NR2B subunit was performed in hippocampal tissue from rats treated with IOA as described in Experimental Procedures. Graph indicates densitometric analysis of Tyr^{1472} phosphorylation of the NR2B subunit as expressed relative to NR2B total protein. Data are means±S.E.M. from five animals. * $P<0.0001$ compared with control.

Kalluri and Ticku, 1999; Wang et al., 2004). It is possible that in our experimental conditions, the observed increase in NR2B results from the progressive rise in the extracellular concentration of glutamate. NR2B is the dominant phosphoprotein found in the PSD (Moon et al., 1994), and its trafficking to the plasma membrane can be triggered by tyrosine phosphorylation promoting its synaptic stability (Lavezzari et al., 2003; Prybylowski et al., 2005). Results show that sustained metabolic inhibition promotes the phosphorylation of Tyr^{1472} of the NR2B subunit, which might promote its trafficking to plasma membrane. In addition, tyrosine phosphorylation of this subunit has been related with Fyn tyrosine kinase activation (Nakazawa et al., 2001), increasing the NMDA receptor channel activity (Xu et al., 2006). Although, from the present data it is not possible to conclude that the observed changes may lead to NMDA receptors with different functional properties, we hypothesized that the observed changes might alter the responsiveness of NMDA receptors to ambient glutamate rendering neurons more vulnerable to excitotoxicity, as recently suggested (Song et al., 2003; Viviani et al., 2006).

The present results might be relevant for the understanding of the mechanisms leading to cell death in chronic degenerative diseases such as Alzheimer's and Huntington's, which are associated with changes in glucose metabolism and brain energy.

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