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ACTIVACIÓN DE PROTEASAS Y SU PAPEL EN LA MUERTE NEURONAL EXCITOTÓXICA EN AUSENCIA Y PRESENCIA DE INHIBICIÓN MITOCONDRIAL EN EL ESTRIADO DE LA RATA *IN VIVO*

T E S I S

QUE PARA OBTENER EL GRADO ACADÉMICO DE

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P R E S E N T A

M. EN C. PERLA MARIANA DEL RÍO ESTRADA

DIRECTORA DE TESIS
DRA. MA DE LOURDES MASSIEU TRIGO

COMITÉ TUTORIAL
DR. JULIO MORÁN ANDRADE
DR. JOSÉ PEDRAZA CHAVERRI

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RESUMEN

El glutamato (Glu), principal neurotransmisor del sistema nervioso central de los mamíferos, puede tener efectos tóxicos a través de un mecanismo dependiente de Ca^{2+} llamado excitotoxicidad. La toxicidad del Glu se facilita cuando el metabolismo energético de la célula se encuentra inhibido y se ha asociado con patologías en las cuales hay una falla del metabolismo energético. Estudios hechos en cultivos de neuronas demostraron que la toxicidad del Glu está relacionada con el daño mitocondrial, la disminución de los niveles de ATP y la pérdida de la homeostasis de Ca^{2+}. Recientemente, en un estudio *in vivo*, demostramos que la facilitación de la toxicidad del glutamato cuando hay una inhibición mitocondrial previa, no está relacionada con una disminución drástica en los niveles de ATP. Esto sugirió que otros factores, como la activación de las proteasas, están relacionados con el daño neuronal inducido por Glu. La caspasa-3 y la calpaína son dos proteasas asociadas a la muerte apoptótica y necrótica, respectivamente. La activación de estas proteasas durante un proceso excitotóxico se ha relacionado con el daño mitocondrial y con la activación de receptores glutamatérgicos. El objetivo de esta tesis fue evaluar si, *in vivo*, estas proteasas están involucradas en el daño inducido por Glu, al administrarse en presencia o ausencia de una inhibición mitocondrial previa. También evaluamos el papel que tienen los receptores ionotrópicos glutamatérgicos en la activación de estas proteasas y en la inducción de la lesión inducida por Glu en el estriado de ratas. Los resultados muestran que la facilitación de la toxicidad del Glu, debida al tratamiento previo con un inhibidor mitocondrial, es independiente de la activación de la caspasa-3 y está relacionada con una activación fuerte de la calpaína. Los receptores NMDA y no-NMDA contribuyen en igual magnitud a la activación de la calpaína y a la producción de la lesión inducida por Glu. Ante un estímulo tóxico de Glu en ausencia de inhibición mitocondrial previa, también existe una activación fuerte de la calpaína y no de la caspasa-3. En este modelo de excitotoxidad severa el receptor NMDA contribuye fuertemente a la inducción de la lesión pero no a la activación de la calpaína. Esto sugiere que otros factores, relacionados con el receptor a NMDA están involucrados en el proceso de muerte celular. El conocimiento de éstos será de gran importancia para poder proponer tratamientos eficaces de las patologías relacionadas la toxicidad del Glu.

ABSTRACT

Glutamate (Glu), the major excitatory neurotransmitter of the mammalian central nervous system, can be neurotoxic by a calcium-dependent mechanism named excitotoxicity. Glu toxicity is exacerbated when energy metabolism is impaired and has been related to pathological conditions associated with failure of energy metabolism. Studies derived from neuron cultures have shown that neuronal death in these conditions is related to mitochondrial dysfunction, ATP depletion, and loss of calcium homeostasis. We have recently observed that, *in vivo*, enhancement of glutamate toxicity elicited by previous mitochondrial inhibition does not involve severe ATP depletion. This suggests that other factors such as proteases activation are involved in the *in vivo* excitotoxic process. Proteases such as calpain and caspases have been involved in necrotic and apoptotic cell death phenotypes, respectively. During excitotoxicity, activation of these proteases is related to mitochondrial damage or the activation of a particular glutamate receptor subtype. In the present study we aimed to investigate whether neuronal damage due to Glu intrastriatal injection during moderate inhibition of mitochondrial metabolism, or by an acute Glu stimulus, is related to caspase-3 or calpain activation. We also investigated the contribution of ionotropic glutamate receptors to proteases activation and neuronal death induced by Glu in the striatum of the treated rats. Results show that enhancement of glutamate toxicity due to inhibition of mitochondrial metabolism *in vivo*, do not recruit caspase-dependent apoptosis but favors calpain activation through the stimulation of both subtypes of glutamate ionotropic receptors. In these conditions, NMDA and non-NMDA receptors contributed equally to calpain activation and to the induction of neuronal death. On the other hand, neuronal damage after acute Glu stimulus in intact rats is also caspase-3-independent. However, although neuronal damage is mediated mainly by the NMDA receptor subtype, it can not be attributed solely to calpain activity, suggesting the involvement of other factors that should be studied in order to propose therapies for pathological conditions in which Glu toxicity is involved.

ORGANIZACIÓN DE LA TESIS

Esta tesis esta dividida en secciones que incluyen: Introducción, antecedentes, hipótesis y objetivos, materiales y métodos, resultados, discusión, conclusiones y apéndice.

En la introducción se revisa de manera general el papel del glutamato como neurotransmisor y se hace una revisión breve de sus receptores. Se menciona el papel tóxico que puede tener el glutamato cuando su concentración aumenta en el espacio sináptico o bien, en condiciones de deficiencia energética. Se revisan también los mecanismos celulares que intervienen en la toxicidad de este neurotransmisor y el papel que tiene la mitocondria en ésta. Al final de la introducción se describen los tipos de muerte celular así como los mecanismos involucrados en cada uno de ellos. Finalmente se describe el papel que tienen algunas proteasas en la muerte neuronal (Artículo 1).

En los antecedentes directos del trabajo se describen algunos estudios realizados por nuestro laboratorio en cultivos neuronales, en los cuales se encontró una relación entre la disminución de la concentración de ATP y la muerte neuronal inducida por glutamato cuando existe una inhibición mitocondrial previa.

En la sección de materiales y métodos se describen los modelos animales que utilizamos para evaluar el papel de la caspasa-3 y de la calpaína en la muerte excitotóxica. Se describe brevemente los materiales y métodos utilizados a lo largo de esta tesis. La descripción detallada de los procedimientos experimentales se describe en los artículos incluidos en los antecedentes y resultados.

En los resultados se incluyen dos artículos.

El primero, titulado “Mild mitochondrial inhibition in vivo enhances glutamate-induced neuronal damage through calpain but not caspase activation: Role of ionotropic glutamate receptors.” Aceptado para publicación en la revista: Experimental Neurology 212, 179–188.

El segundo artículo presentado en esta sección se titula “Contribution of NMDA and Non-NMDA receptors to in vivo glutamate-induced calpain activation in the rat striatum. Relation to neuronal damage.” Este artículo se publicó en la revista Neurochemical Research 33, 1475-1483.

En la discusión se resume e integra el papel de la activación de las proteasas estudiadas ante la estimulación de los receptores glutamatérgicos. Se incluye también una discusión acerca de las posibles vías de activación de la calpaína, haciendo énfasis en el papel que los diferentes receptores glutamatérgicos tienen en éstas.

En el apéndice se anexa el artículo titulado: “Exacerbation of excitotoxic neuronal death induced during mitochondrial inhibition in vivo: relation to energy imbalance or ATP depletion”, publicado en la revista Neuroscience. Este artículo formó parte de mi trabajo de maestría y constituye un antecedente directo de la presente tesis.

LISTA DE ABREVIATURAS

3-NP	ácido 3-nitropropiónico
AMPA	D-amino-3-hidroxi-5-metil-4-isoxazol propiónico
ERN	Especies reactivas de nitrógeno
ERO	Especies reactivas de oxígeno
KA	Kainato
MK-801	(+)-5-metil-10,11-dihidroxi-5-dibenzo(a,d)cicloheptano-5,10-imina maleato
mPTP	Poro de transición de la permeabilidad mitocondrial
NBQX	2,3-dihidroxi-6-nitro-7-sulfamoil-benzo[f]quinoxalina-2,3-diona
NMDA	N-metil-D-aspartato
RE	Retículo endoplásmico
SNC	Sistema nervioso central
$\Delta\Psi_m$	Potencial de membrana mitocondrial

INTRODUCCIÓN

El glutamato es un aminoácido no esencial que podemos encontrar a altas concentraciones en el cerebro, encontrándose principalmente en proteínas y en vesículas sinápticas (Schousboe, 1981). Este aminoácido es el principal neurotransmisor excitador en el sistema nervioso central (SNC) de los mamíferos. La potencialidad excitadora del glutamato en el tejido nervioso fue descubierta por Curtis y Watkins (1960). Trabajos publicados en las siguientes décadas mostraron que el glutamato tiene un papel fisiológico al participar en procesos como la migración, la diferenciación y la muerte celular durante el desarrollo del SNC, así como en procesos de memoria y aprendizaje (Mayer y Westbrook, 1987; Fujii et al., 2003; Cull-Candy et al., 2006; Neyman y Manahan-Vauhan, 2008). De manera conjunta al descubrimiento del glutamato como neurotransmisor también se describió la potencialidad neurotóxica del mismo (Lucas y Newhouse 1957). Olney (1971) postuló que los aminoácidos excitadores, en especial el glutamato, producen muerte neuronal por la activación continua de sus receptores, proponiendo la hipótesis excitotóxica. Actualmente se considera que la excitotoxicidad del glutamato participa en algunas enfermedades neurodegenerativas como la enfermedad de Parkinson, la de Huntington y la de Alzheimer, así como en la muerte neuronal subsiguiente a los accidentes vasculares y periodos isquémicos (Benveniste et al., 1984; Sandberg et al., 1986; Lees, 1993; Massieu y García, 1998; Salinska et al., 2005). Los mecanismos de la muerte neuronal excitotóxica se han estudiado extensivamente en modelos *in vitro*. Éstos involucran como principales ejecutores de la muerte, la activación continua de los receptores a glutamato, el estado metabólico y oxidativo de la célula, así como la falla en la regulación de la concentración de Ca^{2+} intracelular. En conjunto, estos factores llevan a la muerte neuronal, la cual presenta características tanto apoptóticas como necróticas. Los estudios realizados *in vivo,* concernientes a estos mecanismos, son escasos y principalmente se han realizado utilizando agonistas glutamatérgicos. En

esta tesis se estudia el papel de uno de estos factores, la activación de las proteasas, como ejecutores de la muerte neuronal en dos modelos de muerte excitotóxica *in vivo.*

La sinapsis glutamatérgica

En el SNC de los mamíferos las sinapsis excitadoras son principalmente glutamatérgicas, éstas incorporan alrededor del 80% del contenido total de glutamato en el cerebro (Storm-Mathisen et al., 1983; Ottersen et al., 1992). El glutamato es sintetizado en el SNC a través de dos procesos: 1) a partir de la glucosa por medio de la transaminación del α-cetoglutarato, un intermediario del ciclo de Krebs (Peng et al., 1991; Yudkoff, 1997), o 2) a partir de la glutamina por la acción de la enzima glutaminasa (Hassel et al., 1997; Danbolt, 2001). En la sinapsis glutamatérgica, el glutamato es introducido a las vesículas a través de transportadores vesiculares (VGLUT) (Takamori et al., 2000). La incorporación del glutamato hacia las vesículas se favorece por el gradiente de protones generado por una ATPasa de H^+ localizada en la membrana vesicular y que bombea H^+ hacia el interior de la vesícula (Tabb et al., 1992). De esta manera, el transportador vesicular de glutamato trabaja como un antiportador protón-glutamato que introduce el neurotransmisor a la vesícula sináptica (Danbolt, 2001). Cuando llega un potencial de acción, la membrana plasmática se despolariza lo que causa la apertura de los canales de Ca^{2+} sensibles a voltaje y aumenta la concentración de este ión en el interior celular. Como consecuencia, las vesículas sinápticas se fusionan con la membrana plasmática y liberan el glutamato, lo que aumenta su concentración en el espacio sináptico a concentraciones milimolares (alrededor de 1-2 mM; Danbolt, 2001). Una vez liberado al espacio extracelular, el glutamato ejerce su acción al activar a sus receptores localizados principalmente en la membrana post-sináptica (Nakanishi, 1992; Michaelis, 1998; Ozawa et al., 1998). Para evitar la activación continua de sus receptores, el glutamato debe ser eliminado del espacio sináptico, proceso realizado por transportadores específicos que se

encuentran tanto en la glia como en las neuronas (Nicholls y Artwell, 1990). Dentro de las células, el glutamato puede ser convertido a α-cetoglutarato (Yu et al., 1982); o bien, puede ser convertido a glutamina, por una reacción dependiente de ATP realizada por la enzima glutamina sintetasa (Norenberg y Martínez-Hernández, 1979; Ottersen et al., 1992). Esta última reacción solo ocurre en astrocitos (Norenberg y Martínez-Hernández, 1979; Laake et al., 1995). Finalmente, la glutamina sintetizada en los astrocitos puede ser liberada y tomada por neuronas vecinas donde se utiliza como sustrato para sintetizar glutamato. El glutamato sintetizado a partir de glutamina se almacena en las vesículas sinápticas para volver a liberarse. A esta forma de reciclar el glutamato se le conoce como ciclo del glutamina-glutamato (Fig. 1) (revisado en Hassel y Dingledine, 2006).

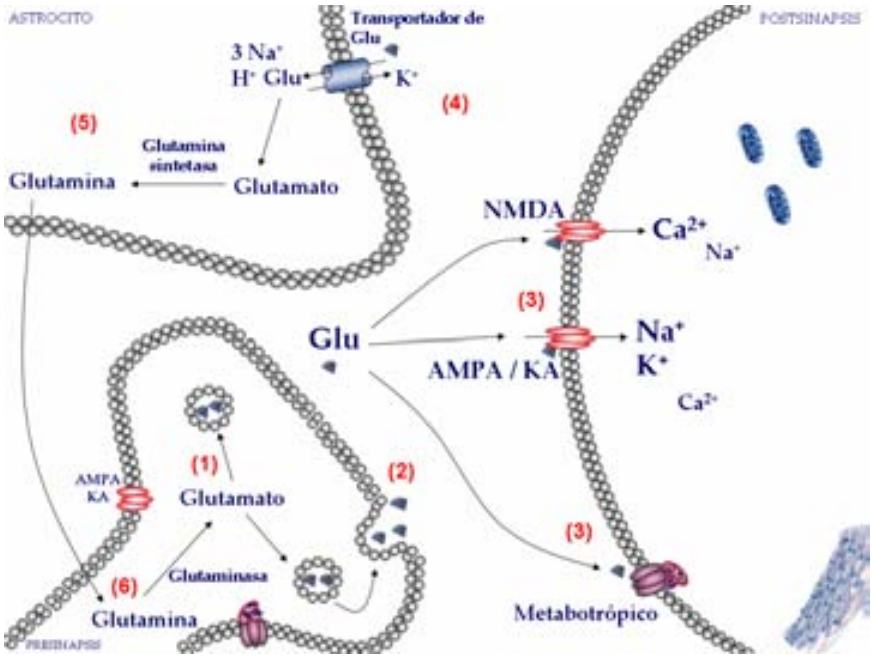

Figura 1. Esquema que representa los componentes de una sinapsis glutamatérgica. (1) El glutamato es vesiculizado con la ayuda de transportadores vesiculares y posteriormente liberado al espacio sináptico (2) donde activa a sus receptores (3). Para terminar la señalización glutamatérgica, el glutamato es eliminado del espacio sináptico por medio de transportadores membranales, los cuales se encuentran en los astrocitos (4). Dentro de los astrocitos el glutamato sirve como precursor de glutamina (5), la cual es liberada y capturada por neuronas pre-sinápticas. Dentro de la pre-sinapsis, la glutamina es convertida a glutamato (6) y éste es de nuevo vesiculizado. A este ciclo se le llama ciclo de la glutamina-glutamato.

Receptores a glutamato

Los receptores a glutamato pueden dividirse en dos grandes categorías, los metabotrópicos y los ionotrópicos (Nakanishi, 1992; Hollman y Heinemann, 1994; Dingledine et al., 1999). Los receptores ionotrópicos están acoplados a canales iónicos y permiten el paso de Ca^{2+}, Na^{+} o K^{+}, mientras que los receptores metabotrópicos transmiten su señal mediante el acoplamiento a proteínas G (Fig. 2) (Pin y Duvioson, 1995; Conn y Pin, 1997).

Receptores ionotrópicos.

Estos receptores se distinguen farmacológicamente en base a sus interacciones con agonistas no fisiológicos y se pueden agrupar en dos grandes grupos: los que responden al N-metil-D-aspartato (NMDA), y los que son insensibles a éste (denominados receptores no-NMDA; Hollmann y Heinemann, 1994). Dentro de este último grupo se encuentran los receptores a Kainato (KA) y los receptores al ácido D-amino-3-hidroxi-5-metil-4-isoxazol propiónico (AMPA) (Nakanishi, 1992; Nakanishi y Masu, 1994). Las técnicas de clonación han dado a conocer que existen subtipos de cada uno de estos receptores formados por diferentes subunidades (Boulter et al., 1990; Nakanishi, 1992). Las diferentes combinaciones de subunidades les confieren características electrofisiológicas y farmacológicas diversas como el tiempo de apertura del canal y la selectividad iónica del mismo (Nakanishi, 1992; Wisden y Seeburg, 1993).

Receptores no-NMDA

Los receptores a AMPA y a KA, conocidos como receptores no-NMDA, son mediadores de la transmisión glutamatérgica en un gran número de sinapsis excitadoras y los canales que forman son principalmente permeables a Na^{+} y K^{+} (Cull-Candy y Usowicz, 1987; Jahr y Stevens, 1987).

Receptores a AMPA.

Estos receptores median la mayoría de las sinapsis excitadoras del SNC, y tienen un papel muy importante en funciones del cerebro como la memoria y el aprendizaje (Dingledine et al., 1999; Derkach et al., 2007). Hasta la fecha se han encontrado cuatro subunidades del receptor a AMPA, denominadas GluR1 a GluR4 (Keinänen et al., 1990). Los receptores a AMPA funcionan como complejos pentaméricos y presentan conductancias a Na^+ y K^+ principalmente (Cull-Candy y Usowicz, 1987; Jahr y Stevens, 1987). La conductancia al Ca^{2+} depende de la presencia de la subunidad GluR2, por lo que los receptores heteroméricos que contienen a la subunidad GluR2 tienen baja conductancia al Ca^{2+} (Burnashev, 1996; Cull-Candy et al., 2006). Los receptores a AMPA se localizan predominantemente en la membrana plasmática de los cuerpos y dendritas de las neuronas post-sinápticas (Bernard et al., 1997; Chen et al., 1998). Estos receptores tienen una distribución amplia en el SNC, las regiones CA1 y CA3 del hipocampo y las capas más superficiales de la corteza cerebral son las zonas con mayor expresión de éstos (Petralia y Wenthold, 1992). Otras regiones como el caudado-putamen y las capas internas de la corteza cerebral también cuentan con la presencia de estos receptores, aunque con en menor proporción; mientras que las regiones con menos expresión de estos receptores son el diencéfalo, el cerebro medio y el tallo cerebral (Petralia y Wenthold, 1992; Ozawa et al., 1998).

Receptores a KA.

Los receptores a KA fueron descritos originalmente por Watkins y colaboradores (Davies et al., 1979; Watkins y Evans, 1981) y se conocen 5 subunidades diferentes que forman un receptor funcional (Glu_{K1}, Glu_{K2}, Glu_{K5}, Glu_{K6} y Glu_{K7}; Hollmann y Heinemann, 1994; Huettner, 2003). Estos receptores, como todos los receptores glutamatérgicos, se localizan en todo el SNC (Michaelis, 1998; Huettner, 2003). Estudios de hibridación *in situ* demostraron que las subunidades Glu_{K5}, Glu_{K6}, Glu_{K7} y Glu_{K2} se localizan principalmente en la corteza cerebral, el

estriado, el hipocampo y el cerebelo (Gall et al., 1990); mientras que la subunidad Glu_{K1} se encuentra principalmente en las regiones del giro dentado y CA3 del hipocampo (Huettner, 2003; Hirbec et al., 2003). Estos receptores son permeables principalmente a Na^+ y K^+; sin embargo, la permeabilidad del poro cambia si los ARNm de las subunidades Glu_{K5} y Glu_{K6} son procesados y editados para generar un cambio en la región del poro de una arginina por una glutamina (Sommer et al., 1991). Este cambio de aminoácidos, consecuencia de la edición del ARNm, determina la conductancia al Ca^{2+}; de esta manera los receptores que no han sido modificados presentan una alta permeabilidad al Ca^{2+} (Hollmann et al., 1991; Köhler et al., 1993). Se sabe que la mayor parte de los ARNm de las subunidades Glu_{K5} y Glu_{K6} identificadas en el SNC presentan esta modificación, lo que indica que la mayoría de los receptores a KA en el SNC no son permeables a Ca^{2+} (Ozawa et al., 1998). Las diferencias funcionales y características farmacológicas de estos receptores se deben a la formación de complejos heteroméricos con las diferentes subunidades, al procesamiento alternativo del ARNm y a su interacción intracelular con proteínas accesorias que contienen dominios PDZ (Hollmann et al., 1991; Wilding y Huettner, 1997; Hirbec et al., 2003).

Estos receptores se localizan en la post-sinapsis y participan en la respuesta post-sináptica (Vigne y Collingridge, 1997; Frerking et al., 1998); sin embargo, también se han encontrado localizados en la pre-sinapsis donde pueden regular la liberación de algunos neurotransmisores (Lerma, 2003). El mecanismo por el cual los receptores a KA pre-sinápticos modulan la liberación de los neurotransmisores no se conoce con exactitud, algunos experimentos han propuesto que estos receptores pueden estar acoplados a proteínas G (Lerma, 2006) y se propone que la modulación del neurotransmisor depende del tipo de proteína G que se asocie al receptor y por lo tanto, a la vía de señalización que se active (Rodríguez-Moreno y Sihra, 2004; Melyan et al., 2004).

Receptores a NMDA

Los receptores a NMDA median la neurotransmisión glutamatérgica de manera distinta a los receptores de tipo no-NMDA ya que son dependientes de voltaje y para su activación completa requieren tanto de la unión simultánea de glutamato y glicina, más la remoción del ión Mg^{2+}, el cual se encuentra bloqueando el canal del receptor y cuya liberación es inducida por la despolarización de la membrana (Nowak et al., 1984; Mayer et al., 1984; Cull-Candy et al., 2001). Este es el receptor que tiene más afinidad por el glutamato y se desensibiliza lentamente después de la exposición a su agonista (Waxman y Lynch, 2005). La apertura de su canal permite el paso de Ca^{2+}, K^{+} y Na^{+} al interior de la célula; la proporción para Ca^{2+} y Na^{+} es de 10:1, respectivamente (Nicholls, 1995). Los receptores a NMDA difieren en sus características funcionales, como es el tiempo de apertura del canal y su respuesta a ciertos agonistas o antagonistas, dependiendo de las subunidades que los conformen (Ishii et al., 1993; Chen et al., 1999; Cull-Candy et al., 2001). Se han descrito varios sitios de unión para diferentes moléculas en este receptor, la unión de estas moléculas modulan positiva o negativamente la actividad de éste (Waxman y Lynch, 2005). Por ejemplo, la unión de Zn^{2+} a su sitio de unión en este receptor disminuye la actividad de este receptor (Lynch y Guttmann, 2001), mientras que la unión de poliaminas a este receptor genera un efecto facilitador (Ransom y Stec, 1988). Se conoce también que la actividad de este receptor puede modularse positivamente y negativametne por agentes reductores y oxidantes, respectivamente (Aizenman et al., 1989); esto dada la existencia de cisteínas susceptibles a oxidación-reducción que se localizan en la subunidad NR1 (Sullivan et al., 1994).

Este receptor es un tetrámero compuesto por diversas subunidades; para que este receptor sea funcional se requiere de la subunidad NR1 (donde se encuentra el sitio de unión a la glicina) y cualquiera de las isoformas de la subunidad NMDAR2 (NMDAR2A–D), que tienen el sitio de unión al glutamato (Michaelis, 1998; Waxman y

Lynch, 2005). Se conoce una tercera subunidad, la NR3, que aparentemente inhibe la funcionalidad de este receptor y está expresada altamente durante el desarrollo (Das et al., 1998). La expresión de las subunidades de este receptor cambia durante el desarrollo (Monyer et al., 1994; Cull Candy et al., 2001). En la etapa embrionaria, la subunidad NMDAR2B se encuentra en casi todas las regiones, mientras que la subunidad NMDAR2D se encuentra en el diencéfalo y el tallo cerebral (Monyer et al., 1994). Después del nacimiento, la subunidad NMDAR2A se encuentra de manera ubicua, mientras que la NMDA2C se expresa prominentemente en el cerebelo (Monyer et al., 1994; Cull Candy et al., 2001). En el adulto, las subunidades del receptor NMDA están distribuidas en todo el SNC, de estas, la subunidad NMDAR1 es la que tiene la distribución más homogénea (Michaellis, 1998). Las regiones que más expresan la subunidad NMDAR2A son la corteza cerebral, el hipocampo y el bulbo olfatorio (Ishii et al., 1993). La subunidad NMDAR2B se expresa preferentemente en el telencéfalo; la subunidad NMDAR2C en las células granulares de corteza cerebelar; y la subunidad NMDAR2D en el diencéfalo y el tallo cerebral (Ishii et al., 1993; Michaellis, 1998).

En cuanto a su papel en el SNC, se sabe que el Ca^{2+} que entra a través de estos receptores se necesita para la formación de sinapsis nuevas y en procesos de plasticidad sináptica (Maren y Baudry, 1995; Asztély y Gustafsson, 1996; MacDonald et al., 2006). Por otro lado, este receptor también está involucrado en ciertas patologías ya que su activación desencadena un proceso de muerte excitotóxica por la entrada excesiva de Ca^{2+}, la producción de especies reactivas de oxígeno (ERO), entre otras cosas (Michaelis, 1998), fenómenos que se explican más adelante.

Receptores metabotrópicos

Los receptores a glutamato de tipo metabotrópico (mGluR, por sus siglas en inglés) se descubrieron en 1985 (Sladeczek et al., 1985). Dichos receptores pertenecen a la familia de los receptores de siete dominios transmembranales y están acoplados intracelularmente a una proteína G (Nakanishi, 1994; Pin y Duvoisin, 1995).

Existen ocho subtipos de estos receptores ($mGluR_1$-$mGluR_8$) que están agrupados en tres grupos, dependiendo de la cascada de señalización que activan y su selectividad por ciertos agonistas (Nakanishi, 1994; Pin y Duvoisin, 1995). Los receptores del grupo I ($mGluR_1$ y $mGluR_5$) están acoplados a una proteína G_q, la cual activa a la fosfolipasa C y provoca la hidrólisis de fosfolípidos de inositol (Aramori y Nakanishi, 1992). Los segundos mensajeros producidos en esta cascada de señalización son el diacilglicerol (DAG) y el IP_3; el DAG activa a la proteína cinasa C, mientras que el IP_3 moviliza Ca^{2+} del retículo endoplásmico (ER) (Abe et al., 1992). Los receptores del grupo I se expresan en la mayoría de las sinapsis glutamatérgicas, principalmente en el componente post-sináptico (Nakanishi, 1994). Los receptores del grupo II ($mGluR_2$ y $mGluR_3$) están asociados a las proteínas $G_{i/o}$ e inhiben a la proteína adenilato ciclasa y por lo tanto la producción de AMP cíclico (Ozawa et al., 1998). Los receptores pertenecientes al grupo III ($mGluR_4$, $mGluR_{6-8}$) también están acoplados a la proteína $G_{i/o}$ sin embrago éstos difieren de los del grupo II en la sensibilidad a varios agonistas y en la fuerza con la que inhiben a la adenilato ciclasa (Nakanishi et al., 1998; Swanson et al., 2005). Los sitios de expresión de estos receptores son variados, se encuentran en las células de Purkinje, en las células piramidales de la región CA1 y CA3 del hipocampo, en el estriado, el bulbo olfatorio y en la corteza cerebral (Martin et al., 1992; Molnar et al., 1993; Baude et al., 1993). Los receptores metabotrópicos de tipo $mGluR_1$, $mGluR_2$ y $mGluR_3$ tienen una alta expresión en el hipocampo, el cerebelo, el bulbo olfatorio, la amígdala y en los ganglios basales (Michaelis, 1998). El papel fisiológico de estos receptores se ha estudiado extensamente mediante la utilización de agonistas y antagonistas. Por ejemplo, se conoce que los receptores $mGluR_6$ participan en la transmisión glutamatérgica entre los fotorreceptores y las células bipolares de la retina (Nakanishi et al., 1998). Se ha propuesto que el papel de los receptores metabotrópicos es el de modular la transmisión sináptica, tal vez por esta razón su localización es tanto pre-sináptica como post-sináptica (Basyks y Malenka, 1991; Forsythe y Clemmens, 1990; Conn y Pin, 1997). También se ha

encontrado que algunos receptores metabotrópicos suprimen la transmisión sináptica inhibidora o excitadora mediante mecanismos pre-sinápticos como la disminución de la liberación de glutamato provocada por la inhibición de los canales de Ca^{2+} pre-sinápticos (Chavis et al., 1994), mecanismo que se ha demostrado en células del bulbo olfatorio y en las regiones CA1 y CA3 del hipocampo (Ozawa et al., 1998).

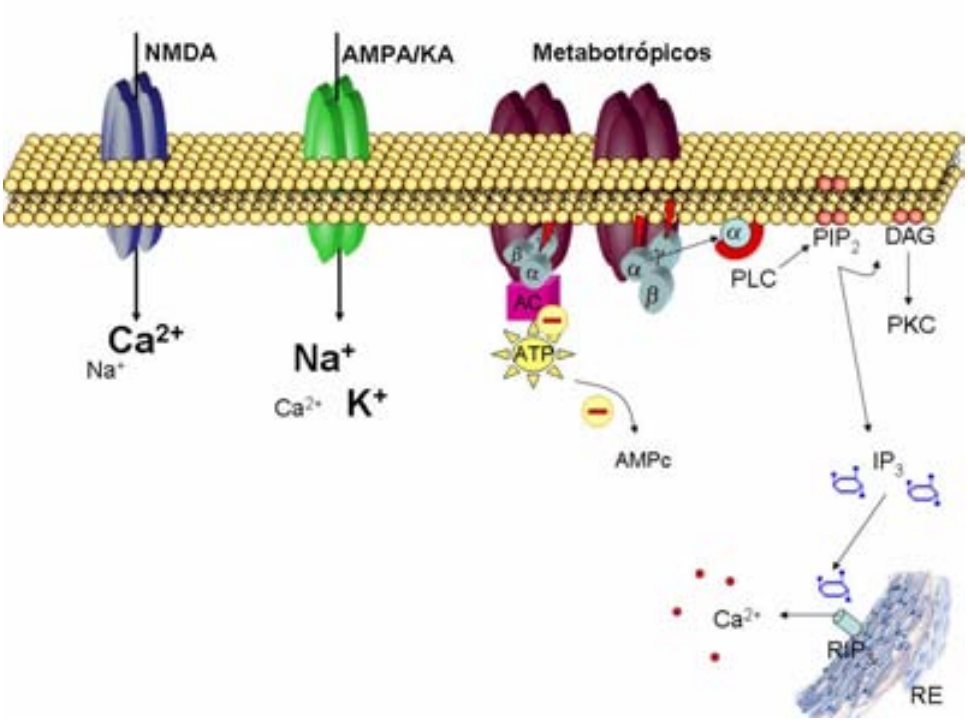

Figura 2. Esquema de los receptores a glutamato presentes en la post-sinapsis. Se muestran los receptores a NMDA y los receptores a AMPA o KA (que forman parte de los receptores de tipo no-NMDA), así como los receptores metabotrópicos, los cuales se encuentran acoplados a una proteína G intracelular.

Transportadores de glutamato.

Para que el glutamato termine su señalización en la sinapsis glutamatérgica éste debe ser removido del espacio sináptico mediante transportadores membranales así como por difusión simple (Danbolt, 2001). El buen funcionamiento de estos transportadores debe mantener la concentración extracelular de glutamato lo suficientemente baja como para no mantener activos a sus receptores por tiempos prolongados ya que, la activación continua de los receptores puede resultar dañina para las neuronas por un mecanismo denominado excitotoxicidad (ver mas adelante).

El mecanismo para la recaptura de glutamato involucra el co-transporte de 3 iones de Na^+ y un H^+, así como la salida de un ion K^+ (Zerangue y Kavanaugh 1996; Tzingounis y Wadiche, 2007).

Se conocen cinco tipos diferentes de transportadores de glutamato: dos transportadores gliales, GLT-1 y GLAST (transportador de glutamato-1 y transportador de glutamato/aspartato, respectivamente (Pines et al., 1992; Storck et al., 1992), un transportador neuronal EAAC1 (acarreador de aminoácidos excitadores-1) (Kanai y Heideger, 1992), el EAAT4 (transportador de aminoácidos excitadores-4) que se encuentra principalmente en células de Purkinje (Fairman et al., 1995), y los transportadores EAAT5 (transportador de aminoácidos excitadores-5) localizados en la retina (Danbolt et al., 1992; Rothstein et al., 1994; Lehre et al., 1995; Gadea y López-Colomé, 2001). De los 5 subtipos de transportadores de glutamato, el GLT1 es el más abundante en todo el cerebro (Danbolt et al., 1990; Danbolt, 2001). En la rata adulta, el GLT1 se encuentra principalmente en los astrocitos de la neocorteza, en el estriado, tálamo e hipocampo (Lehre et al., 1995). El GLAST es el transportador que más se expresa en el cerebelo y es un transportador que también se expresa en algunos órganos periféricos como el oído interno (Lehre et al., 1995). La principal función de los transportadores, tanto gliales como neuronales, es la remoción del glutamato del espacio sináptico (Robinson, 1998; Rothstein et al., 1996). Su papel en el SNC se ha estudiado en ratones a los cuales se les ha inhibido la expresión de estos transportadores. Por ejemplo, ratones carentes de los transportadores GLT1 y GLAST evidenciaron el papel de estas proteínas en la remoción del glutamato extracelular ya que estos animales muestran signos de excitotoxicidad (Tanaka et al., 1997). Esta función de los transportadores de glutamato se hace todavía más evidente durante periodos isquémicos, ya que durante éstos se observa un incremento de los niveles de glutamato asociado al funcionamiento inverso de los transportadores de glutamato dependientes de Na^+, con la subsiguiente muerte celular (Benveniste et al., 1984; Seki et al., 1999; Mitani y Tanaka, 2003). Se ha propuesto que estos transportadores

participan en la modulación de la liberación del mismo neurotransmisor, ya que, de no remover eficientemente el glutamato extracelular éste puede difundir y activar receptores pre-sinápticos (Nicholls y Artwell, 1990; Danbolt, 2001). Experimentos con ratones deficientes de GLT-1 indican que este subtipo de transportador también tiene un papel importante en la inducción de la potenciación a largo plazo (PLP) a través de la regulación de los niveles extracelulares de glutamato (Tanaka et al., 1997).

Excitotoxicidad

El término excitotoxicidad se refiere al proceso por el que los aminoácidos excitadores, como el glutamato, producen daño neuronal debido a la actividad prolongada de sus receptores (Olney, 1971). La propiedad neurotóxica del glutamato se describió de forma paralela a su acción excitadora. Lucas y Newhouse (1957), fueron los primeros en demostrar que la inyección sistémica del glutamato produce degeneración en la retina de ratones inmaduros. Tiempo después se demostró en ratones en desarrollo que los aminoácidos excitadores, en especial el aspartato y el glutamato, son capaces de generar la muerte de neuronas que se encuentran en regiones del cerebro que no están protegidas por la barrera hematoencefálica (Olney, 1971). Otros estudios demostraron que compuestos con capacidad despolarizante y efectos excitadores como los análogos del glutamato mimetizan la toxicidad de éste (Watkins et al., 1990; Choi, 1992). El glutamato ahora es reconocido como neurotransmisor y a su vez como una potente neurotoxina (Salinska et al., 2005). La toxicidad del glutamato se considera la causa principal de la muerte neuronal desencadenada durante periodos de isquemia y de hipoglucemia, ya que en éstos ocurre un incremento de los niveles extracelulares de glutamato (Benveniste et al, 1984; Sandberg et al, 1986). Por otro lado, la muerte neuronal excitotóxica también ha sido vinculada a la muerte celular que se observa en algunas enfermedades neurodegenerativas como la enfermedad de Huntington, la enfermedad de Parkinson y

la enfermedad de Alzheimer (Beal, 1992; Massieu y García, 1998). Existe una gran variedad de artículos acerca de los mecanismos por los que el glutamato genera muerte neuronal y se involucra principalmente al receptor NMDA dado que éste es permeable al Ca^{2+} (Choi et al., 1988; Sattler et al., 1998). También se reconoce que el Ca^{2+} y el estrés oxidativo son componentes importantes de la vía de muerte excitotóxica (Fig. 3, ver más adelante).

El concepto de excitotoxicidad involucra la activación excesiva y constante de los receptores glutamatérgicos (Fig.3.1), los eventos celulares relacionados con la muerte excitotóxica han sido ampliamente estudiados en cultivos neuronales (Choi, 1987, 1988; Arundine y Tymiansky, 2003**;** Sattler et al, 1998). Los primeros estudios acerca de la toxicidad del glutamato mostraron que la muerte excitotóxica es un fenómeno que afecta preferentemente a las neuronas y en menor medida a la glia (Choi et al., 1987). La constante activación de los receptores glutamatérgicos conduce a la despolarización membranal y a flujos iónicos que llevan al daño neuronal (Choi, 1987; Kiedrowski, 1999). El proceso excitotóxico empieza por el influjo de Na^{+}, Cl^{-} y agua (Rothman, 1985; Choi, 1987; Kiedrowsky et al., 1994). Este proceso inicial genera hinchamiento de las estructuras post-sinápticas (dendritas y cuerpos neuronales) el cual es transitorio y es regulado por las células sin producir daño celular (Choi, 1987; Dietz et al., 2007). Experimentos pioneros que estudiaban el potencial neurotóxico del glutamato mostraron que si se elimina el Ca^{2+} del medio de cultivo, las neuronas son más resistentes al daño por glutamato (Choi, 1987; Rothman et al., 1987). Muchos estudios sugieren que la excitotoxicidad está asociada a una falla en la regulación de la concentración de Ca^{2+} intracelular (Choi et al, 1987; Randall y Thayer, 1992; Tymianski et al., 1993b; Nicholls y Budd, 1998; Kristián y Siesjö, 1998). Actualmente se sabe que las neuronas que tienen una desregulación de la homeostasis de Ca^{2+} intracelular mueren inevitablemente.

Calcio y excitotoxicidad

El Ca^{2+} es un ión que tiene un papel fisiológico importante; interviene en procesos como la contracción muscular y la fusión de vesículas con la membrana plasmática (exocitosis) (Burnashev y Rozov, 2005), participa en cascadas de señalización intracelular como segundo mensajero (Ghosh y Greenberg, 1995) y en la expresión de ciertos genes (Berridge et al., 1998), y facilita la activación de varias proteínas como la calmodulina, la calpaína, fosfatasas, proteínas cinasas (Dawson et al., 1992; Schulz et al., 1995b; Gunasekar et al., 1995; Hongpaisan et al., 2004) y endonucleasas (Ghosh y Greenberg, 1995; Burnashev y Rozov, 2005; Hartmann y Konnerth, 2005; Hernández-Fonseca et al., 2007). La concentración de Ca^{2+} en el interior celular se encuentra regulada rigurosamente por sistemas que controlan el influjo y la extrusión del ión (Kristán y Siesjö, 1998), así como por mecanismos intracelulares que involucran organelos y proteínas de unión a calcio. Generalmente la concentración de Ca^{2+} intracelular es aproximadamente de 10^{-7} M, mientras que en el espacio extracelular es de 10^{-3}M (Salinska et al., 2005). Esto genera un gradiente de concentración que se mantiene por la acción de la ATPasa de Ca^{2+} de la membrana plasmática (PMCA, por sus siglas en inglés) (Pottorf et al., 2006) y del intercambiador de Na^{+}/Ca^{2+} (Kristan y Siesjö, 1998; Blaustein y Lederer, 1999). En condiciones fisiológicas la concentración de Ca^{2+} intracelular puede aumentar de manera transitoria (Kristan y Siesjö, 1998); el influjo de Ca^{2+} en estas condiciones ocurre principalmente a través de la activación de canales de Ca^{2+} dependientes de voltaje (Walter y De Waard, 1998), o de receptores acoplados a canales iónicos, como los receptores glutamatérgicos (Burnasehv, 1996; Kristan y Siesjö, 1998). El control de la concentración de Ca^{2+} intracelular en las neuronas es complejo e involucra proteínas de unión a Ca^{2+} (Pochet et al., 1990; Mattson et al, 1991), la captura de Ca^{2+} por la mitocondria y el RE (Kostyuk y Verkhraty, 1994; Nicholls y Akerman, 1982; Nicholls y Scout, 1980; Duchen, 2000; Nicholls et al., 2003), así como mecanismos de extrusión de Ca^{2+} mediados por el intercambiador Na^{+}/Ca^{2+} y por la PMCA (Miller, 1991;

Blaustein et al., 1996; Blaustein y Lederer, 1999). La mitocondria puede capturar Ca^{2+} en su interior a través de un uniportador, el cual depende del potencial de membrana mitocondrial ($\Delta\Psi_m$) y permite capturar el Ca^{2+} si éste aumenta a concentraciones mayores de 1 µM (Gunter y Gunter, 1994; Nicholls y Ward, 2000). En el caso del RE, éste captura Ca^{2+} por la acción de una ATPasa de Ca^{2+} denominada SERCA que mantiene la concentración de Ca^{2+} en el lumen del RE a concentraciones de alrededor de 0.5mM (Vangheluwe et al., 2005).

Una falla en la regulación de la concentración intracelular de Ca^{2+} puede ser dañina para la célula (Fig. 3.2) (Choi et al., 1988; Tymiansky et al., 1993a; Sattler et al., 1998; Bano y Nicotera, 2007). En el caso de la muerte excitotóxica, se ha observado un aumento en la concentración de este ión y se ha documentado que la principal vía de entrada de Ca^{2+} es el receptor a NMDA (Choi et al., 1988; Sattler et al., 1998). Varios estudios han demostrado que después de la activación de los receptores glutamatérgicos hay un aumento de Ca^{2+} citosólico que es asociado con la disminución en los niveles de ATP y la producción de ERO (Gunter y Gunter, 1994; Budd y Nicholls, 1996; Schinder et al., 1996; White y Reynolds, 1996a; Stout et al., 1998; Vesce et al., 2004). El incremento de Ca^{2+} durante un estímulo excitotóxico también está relacionado con el aumento de Ca^{2+} en el interior de la mitocondria el cual, entre otras cosas, daña a la mitocondria o dificulta su funcionamiento (Fig. 3.4) (Khodorov et al, 1996a, b; Schinder et al., 1996; Peng et al., 1998; Vergun et al, 1999; Puka-Sundvall et al., 2000a; Schild et al., 2003). Actualmente no se conoce con exactitud la procedencia del Ca^{2+} que provoca el aumento en la concentración de Ca^{2+} intracelular, pues algunas fuentes indican que éste puede provenir del exterior celular o bien, deberse a su liberación de las pozas intracelulares (Reynolds, 1998; Hernández-Fonseca et al., 2007). Por un lado se ha visto que la excitotoxicidad está acompañada de una despolarización y disminución del gradiente de sodio provocando el funcionamiento inverso del intercambiador de Na^+/Ca^{2+}, siendo éste la principal fuente de internalización de Ca^{2+} (Andreeva, et al., 1991; Kiedrowski et al., 2004). Así mismo

se ha visto que el receptor a NMDA es una de las principales vías de entrada de Ca^{2+} hacia la célula, por lo que una condición que lo mantenga activo favorece el aumento de este ión en el interior celular (Tymiansky et al., 1993a; Sattler et al., 1998). Por otro lado, también se tiene evidencia de que durante el proceso excitotóxico se inhibe el transporte de ATPasas como la PMCA y la SERCA del RE, posiblemente debido a la disminución en la concentración de ATP (Lehotsky, 2002; Paschen, 2003), lo que contribuye al aumento de la concentración de Ca^{2+} intracelular. La consecuencia final del aumento de Ca^{2+} intracelular sería un daño celular irremediable degradando componentes importantes para la sobrevivencia de la célula (Paschen, 2003; Salinska et al., 2005; Hernández-Fonseca et al., 2007).

Estrés oxidativo y excitotoxicidad

Otro componente determinante de la muerte excitotóxica es el estrés oxidativo (Fig. 3.5) (Bondy y LeBel, 1993; Lewén et al., 2000; Sugawara y Chan, 2003). La producción moderada de ERO sirve como señalización durante procesos como la regulación de la actividad de proteínas y la expresión de genes (Martin y Barret, 2002). La producción de ERO después de un estímulo excitotóxico se ha demostrado *in vitro* (Dykens et al., 1987; Lafon-Cazal et al., 1993; Bondy y LeBel, 1993). Los estudios *in vivo* son más limitados, pero se ha demostrado protección contra el daño excitotóxico al utilizar compuestos antioxidantes (Clough-Helfman y Phillis, 1991; Schulz et al., 1995a, b). La activación de los receptores NMDA produce un incremento en la concentración de Ca^{2+} intracelular, el cual puede activar enzimas que incrementen la producción de radicales libres como la fosfolipasa A2 (PLA2), la xantina oxidasa y la sintasa del óxido nítrico (Dawson et al., 1992; Schulz et al., 1995b; Gunasekar et al., 1995). Otra fuente de producción de ERO es la mitocondria, la cual se asocia con la captura de Ca^{2+} por parte de este organelo y al daño mitocondrial (Casthilo et al., 1999; Luetjens et al., 2000; Vergun et al., 2001; Vesce et al., 2004; Sas et al., 2007). Estudios recientes muestran que la captura de Ca^{2+} por la mitocondria es necesaria

para la producción de ERO y la muerte neuronal mediada por NMDA (Stout et al., 1998; Duan et al., 2007). Dentro de las ERO generadas más comunmente por la mitocondria está el anión superóxido, el cual se produce si un electrón escapa de la cadena transportadora de electrones y reacciona con el oxígeno (Liang et al., 2000; Hongpaisan et al., 2004; Vesce et al., 2004; Sas et al., 2007). Durante un estímulo excitotóxico también se generan especies reactivas de nitrógeno (ERN), derivadas de la producción de $NO^{\cdot}$ por la sintasa del $NO^{\cdot}$ (NOS) neuronal y endotelial (Knowles y Moncada, 1994; Moncada y Bolaños, 2006). El $NO^{\cdot}$ formado puede reaccionar con el anión superóxido y formar peroxinitrito, el cual induce la peroxidación de los lípidos de la membrana (Coyle y Puttfarcken, 1993). Como consecuencia de la producción exagerada de ERO y ERN, la célula experimenta daños considerables que la llevan a una muerte inevitable (revisado en Hernández-Fonseca et al., 2007).

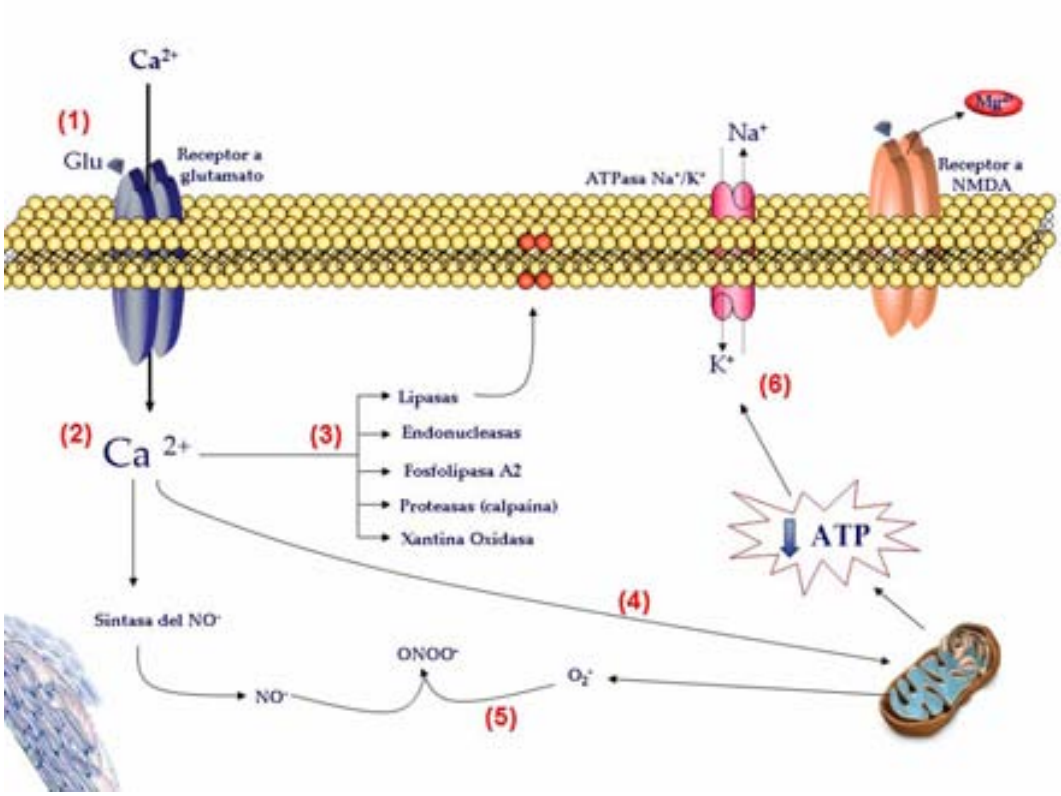

Figura 3. Eventos que se desencadenan después de la activación de los receptores glutamatérgicos y que están relacionados con un proceso excitotóxico. La excitotoxicidad comienza por la activación continua de los receptores glutamatérgicos (1) la cual esta asociada con el influjo de Ca^{2+}. El aumento en la concentración intracelular de Ca^{2+} (2) activa una serie de enzimas incluyendo proteasas que dañan componentes celulares como la membrana plasmática (3) y que favorecen la producción de especies reactivas de oxígeno. El Ca^{2+} también puede dañar a la mitocondria, si ésta lo acumula en exceso (4). El daño mitocondrial está asociado con la producción de ERO, como puede ser el anión superóxido (5), y con la disminución en la producción de ATP. Como consecuencia, todos los procesos dependientes de ATP se interrumpen, entre ellos la actividad de la ATPasa de Na^+/K^+ (6), lo cual generaría despolarización de la membrana plasmática y facilitación de la activación del receptor a NMDA, exacerbando la cascada excitotóxica y el daño celular.

Excitotoxicidad secundaria

La hipótesis excitotóxica implica la estimulación constante de los receptores glutamatérgicos debido al aumento en la concentración extracelular de su ligando (Olney, 1971). Sin embargo, en los últimos años se ha observado que el incremento de la concentración extracelular de glutamato no es suficiente para generar la muerte de

las neuronas (Massieu et al., 1995). De hecho, aunque se acepta que existe un componente excitotóxico en la muerte neuronal en las enfermedades neurodegenerativas crónicas, la elevación de glutamato en el líquido cefalorraquídeo de los pacientes con estas enfermedades no se ha observado con claridad (Mally et al, 1997; Kuiper et al, 2000). Ahora se sabe que la toxicidad del glutamato depende del estado energético celular, de tal manera que el umbral tóxico del glutamato disminuye cuando existe una deficiencia energética, aún cuando ésta sea parcial (Novelli et al, 1988, Simpson e Isacson, 1993; Green y Greenamyre, 1996; Massieu et al., 2000, 2001). Por lo tanto, la excitotoxicidad se induce por condiciones que favorecen la acumulación de glutamato en el espacio extracelular, pero también se facilita en condiciones de deficiencia energética (Choi, 1992; Novelli et al, 1988; Greene et al., 1998; García y Massieu, 2003; Sánchez-Carbente y Massieu, 1999). A esta condición se le ha llamado excitotoxicidad secundaria; y se postula que es la responsable de la muerte neuronal durante las enfermedades neurodegenerativas crónicas (Greenamyre et al., 1988; Schimdt et al., 1990; Beal, 1992; Green y Greenamyre, 1996). Estudios que han reportado fallas en el metabolismo energético de pacientes con estas enfermedades apoyan esta hipótesis; por ejemplo, se ha observado una disminución en la actividad del complejo I mitocondrial en pacientes con la enfermedad de Parkinson (Schapira, 1999), del complejo II mitocondrial en pacientes con la enfermedad de Huntington (Brouillet et al., 1995) y del complejo IV en pacientes con la enfermedad de Alzheimer (Parker et al, 1994). Asimismo, se ha demostrado que la deficiencia en el metabolismo energético da lugar a una neurodegeneración parecida a la excitotóxica (Storey et al. 1992; Beal et al. 1993a; Green et al. 1993; Brouillet et al., 1995, 1999). El estudio histológico de las lesiones producidas por inhibidores del metabolismo mitocondrial muestra características citológicas y neuroquímicas parecidas a las que se presentan después de la administración de agonistas glutamatérgicos (Beal et al. 1993a; Green et al. 1993). Por ejemplo, el tratamiento crónico o la inyección intraestriatal de la toxina mitocondrial ácido 3-nitropropiónico (3-NP) produce una lesión en el estriado de la rata con un patrón

histoquímico comparable al producido por la inyección de AMPA o KA (Beal et al. 1993a; Brouillet et al., 1995, 1999). El 3-NP es una toxina que inhibe a la enzima deshidrogenasa de ácido succínico (SDH, por sus siglas en inglés), la cual forma parte del ciclo de Krebs y del complejo II de la cadena de electrones (Coles et al., 1979). Estas observaciones se extendieron con modelos que mostraron que la neurodegeneración inducida por el 3-NP es de tipo excitotóxica ya que se previene cuando se utilizan antagonistas del receptor NMDA o cuando se remueven las aferencias glutamatérgicas que llegan al estriado de la rata (Beal et al., 1993a, b; Wüller et al., 1994, Greene et al., 1993; Henshaw et al., 1994). Observaciones similares se han descrito con otros inhibidores metabólicos como el MPP^+ (inhibidor del complejo I mitocondrial), el cual se utiliza para generar modelos animales de la enfermedad de Parkinson (Storey et al. 1992).

Los mecanismos por los cuales la deficiencia metabólica incrementa la vulnerabilidad neuronal a concentraciones subtóxicas de glutamato han sido objeto de estudio (Green y Greenamyre, 1996; Henneberry, 1997). Se sabe que si existe una deficiencia energética, asociada a la disminución de los niveles de ATP, la ATPasa de Na^+/K^+ disminuye su actividad (Fig. 3.6), lo que origina un desequilibrio iónico y como consecuencia la despolarización de la membrana (Silver et al., 1997). A su vez, el estado despolarizado de la membrana facilita la activación del receptor NMDA, ya que el ión Mg^{2+} que se encuentra bloqueando el canal se libera de manera dependiente del voltaje. Una vez que el Mg^{2+} se ha liberado, la activación del receptor NMDA puede darse de manera continua incluso en presencia de concentraciones fisiológicas de glutamato extracelular (Novelli et al., 1988; Cox et al. 1989; Zeevalk y Nicklas, 1992). De esta manera, el proceso de muerte excitotóxica también puede iniciarse si la célula tiene su metabolismo energético disminuido ó inhibido ya que, la probabilidad de que el receptor NMDA se active es mayor en estas condiciones (Novelli et al., 1988; Cox et al. 1989; Zeevalk y Nicklas, 1992).

La mitocondria y su papel como determinante de la muerte celular.

La principal función de la mitocondria es la producción de ATP, ésta genera el 95% del ATP que consume una célula (Darley-Usmar et al., 1987). La mitocondria también lleva a cabo otras funciones como la síntesis de hormonas esteroideas, el ciclo de la urea, el catabolismo de aminoácidos; así como el amortiguamiento del Ca^{2+} intracelular, la producción de ERO y participa en procesos de muerte celular (Zorov et al., 1997; Gunter y Gunter, 2001). La membrana externa de la mitocondria es permeable a iones y moléculas no mayores a 1000Da, mientras que su membrana interna es impermeable a éstos (Crompton, 1999). Por esta razón, existen transportadores necesarios para el buen funcionamiento de la mitocondria; en el caso de la captura de Ca^{2+}, existe un uniportador que trabaja de manera independiente de ATP pero que depende del $\Delta\Psi m$ (Pozzan y Rizzuto, 2000; Bianchi et al., 2004). Cuando la concentración de Ca^{2+} en el citosol aumenta por arriba de 300nM, la mitocondria inicia la captura de Ca^{2+}, en ciertas células las mitocondrias inician esta captura solo si la concentración de Ca^{2+} aumenta a 1µm (Gunter y Gunter, 2001; Jacobson y Duchen, 2004). Una vez dentro de la mitocondria el Ca^{2+} favorece el metabolismo mitocondrial, estimula la producción de NADH y ATP e induce el consumo de oxígeno (Hajnockzy et al., 1995; Jouaville et al., 1999; Pozzan y Rizzuto, 2000). Asimismo, favorece la actividad de algunas deshidrogenasas mitocondriales como la piruvato deshidrogenasa, la isocitrato deshidrogenasa y la α-cetoglutarato deshidrogenasa (Jouaville et al., 1999; Campanella et al., 2004; Bianchi et al., 2004). La salida de Ca^{2+} de la mitocondria hacia el citosol se da a través de intercambiadores de Na^{+}/Ca^{2+} y Ca^{2+}/H^{+} (Bernardi, 1999; Campanella et al., 2004); un segundo mecanismo de liberación de Ca^{2+} por la mitocondria involucra un complejo de proteínas de las membranas interna y externa de la mitocondria que recibe el nombre de poro de transición de la permeabilidad mitocondrial (conocido como mPTP, por sus siglas en inglés; Crompton, 1999; Pozzan y Rizzuto, 2000; Kim et al., 2003a).

Actualmente, la apertura del mPTP se asocia con la acumulación de Ca^{2+} dentro de la mitocondria, situación que ocurre en varias patologías (Crompton, 1999). Dada la capacidad de la mitocondria por capturar Ca^{2+} citosólico, ésta se considera un componente importante de la homeostasis de Ca^{2+} intracelular (Hernández-Fonseca et al., 2007). No obstante, un aumento excesivo de Ca^{2+} en el interior mitocondrial produce su hinchamiento y daño, lo cual inicia un proceso de muerte celular (Vergun et al., 1999). Asimismo, el daño mitocondrial se asocia con la pérdida del $\Delta\Psi_m$ (Nicholls y Ward, 2000) y de la capacidad de síntesis de ATP (Khodorov et al., 1996b; Schinder et al., 1996), con la liberación de Ca^{2+} y otros factores pro-apoptóticos hacia el citoplasma (Brustovetsky et al., 2002; Kim et al, 2003*a*, *b*; van Gurp et al, 2003), con la sobreproducción de ERO y con un aumento en la probabilidad de apertura del poro mPTP (Dugan et al., 1995; Keelan et al., 1999; Ward et al., 2000; Alano et al., 2002). La producción de ERO por la mitocondria involucra a los componentes de la cadena de electrones (McLennan y Esposti, 2000). Las principales especies reactivas de oxígeno producidas por la mitocondria son el anión superóxido y el peróxido de hidrógeno (Turrens, 2003; Rego et al., 2003; Adam-Vizi, 2005; Duan et al., 2007).

Por otro lado, la mitocondria, además de su papel en el metabolismo energético, también puede controlar el inicio y desarrollo de la muerte celular apoptótica o necrótica (Campanella et al., 2004). Se sabe que, en ocasiones, cuando hay daño mitocondrial ocurre un fenómeno en el que la permeabilidad de la membrana externa de la mitocondria incrementa, lo que permite la liberación de moléculas que generalmente residen en el espacio intermembranal (Brustovetsky et al., 2002; Kim et al, 2003*a*, *b*; van Gurp et al, 2003). Entre las moléculas que se liberan de la mitocondria están: 1) el citocromo c, el cual es necesario para la activación de la caspasa 9 (Liu et al., 1996); 2) el factor inductor de la apoptosis, que es una flavoproteína que, al ser liberada, se trasloca al núcleo e induce condensación nuclear (Daugas et al., 2000); 3) las proteínas Smac/Diablo, las cuales son proteínas pro-apoptóticas; y 4) la endonucleasa G, la cual es una endonucleasa mitocondrial que al

traslocarse al núcleo participa en la condensación y fragmentación de éste (van Gurp et al., 2003). No hay que olvidar que el daño mitocondrial también está asociado con la liberación del Ca^{2+} mitocondrial, el cual, además de lo revisado anteriormente, puede contribuir a la activación de la proteasa llamada calpaína (Jing et al., 2008). Aunque la vía de liberación de estas moléculas no se conoce con exactitud, se propone que éstas pasan a través del mPTP (Friberg et al., 1998; Kim et al, 2003a). La apertura de este poro puede ser consecuencia del aumento de Ca^{2+} en la mitocondria (Panov et al., 2005), un exceso en las ERO (Chalmers y Nicholls, 2003), condiciones que disminuyan el $\Delta\Psi_m$ (Li et al., 2000). El mPTP se ha asociado con la toxicidad mediada por glutamato después de periodos de isquemia/reperfusión y por la activación del receptor NMDA (Friberg et al., 1998; Budd et al., 2000; Li et al., 2000; Alano et al., 2002).

Actualmente se reconoce que durante el proceso excitotóxico el daño mitocondrial es un factor importante (Schinder et al, 1996; White y Reynolds, 1996b; Nichols et al, 1999; Ly et al, 2003) y que una de las principales consecuencias de esta disfunción mitocondrial es el aumento en la concentración de Ca^{2+} intracelular, lo que a su vez desencadenaría una serie de eventos que culminarían en la muerte celular (Budd y Nichols, 1996; Peng et al., 1998; Stout et al., 1998; Puka-Sundvall et al., 2000b; Schild et al., 2003). El papel de la mitocondria en la muerte excitotóxica se ha corroborado con estudios que muestran que la inhibición mitocondrial, ya sea parcial o completa, facilita la toxicidad de glutamato (Sánchez-Carbente y Massieu, 1999; Massieu et al,. 2001; Del Río et al., 2007). Así mismo, en nuestro laboratorio hemos demostrado que si se administran substratos energéticos mitocondriales, como el piruvato, el acetoacetato y el β-hidroxibutirato, se reduce del daño excitotóxico (Massieu et al., 2001; García y Massieu, 2003; Massieu et al., 2003).

Glutamato y tipo de muerte celular.

Queda claro que la activación continua de los receptores a glutamato induce daño mitocondrial con la consecuente muerte neuronal (Choi, 1987, 1988; Arundine y Tymiansky, 2003**;** Sattler et al, 1998). A pesar de que se conocen muchos de los mecanismos celulares que llevan a ella, aún existe un gran debate en cuanto al tipo de muerte que produce el glutamato ya que se ha observado que la muerte neuronal inducida por glutamato puede tener características tanto necróticas como apoptóticas (Dessi et al, 1993; Sohn et al, 1998; Kure et al, 1991; Bonfoco et al, 1995). La muerte celular suele clasificarse en dos tipos, la necrótica y la apoptótica, ambos tipos de muerte son diferenciados por sus características morfológicas y bioquímicas (McConkey, 1998). Estudios recientes han identificado un tercer tipo de muerte celular, la autofagia, el cual también podría estar involucrada en condiciones patológicas (Del Río y Massieu, 2007; Yasuo et al., 2008). La muerte necrótica generalmente es iniciada por un estímulo agudo; su principal característica es que la célula presenta hinchamiento generalizado, que incluye a los organelos, y ruptura de la membrana celular que trae como consecuencia la liberación de su contenido intracelular al espacio extracelular generando una respuesta inflamatoria (Edinger y Thompson, 2004). En general, se considera que la muerte necrótica es un proceso desordenado donde los niveles de ATP disminuyen rápidamente y existe una producción exagerada de ERO (Wyllie et al, 1980; Arends y Wyllie, 1991). Por otro lado, la apoptosis es un proceso activo y organizado que está caracterizado por presentar condensación del citoplasma y reducción del volumen celular, acompañado de cambios en el núcleo y ruptura internucleosomal del material genético (Hengartner, 2000). Durante el proceso apoptótico no hay ruptura de la membrana celular y por lo tanto tampoco una respuesta inflamatoria (Arends y Wyllie, 1991). Los niveles de ATP disminuyen gradualmente y poca producción de ERO (McConkey, 1998). Durante el proceso apoptótico se activan una serie de enzimas llamadas caspasas (Hengartner, 2000; Denault y Salvensen, 2002), las cuales, son proteasas que rompen sus sustratos en

residuos de aspartato y se consideran las principales ejecutoras de la muerte apoptótica (Donepudi y Grütter, 2002; Denault y Salvensen, 2002). Al final del proceso apoptótico se da la formación de cuerpos apoptóticos, los cuales son eliminados de los tejidos por los macrófagos (Kerr et al., 1972; Lossi y Merighi, 2003). Algunos factores que determinan el fenotipo de muerte celular pueden ser: la duración e intensidad del estímulo tóxico, el grado de daño mitocondrial, los niveles energéticos, la cantidad de Ca^{2+} intracelular, y las diferentes proteasas activadas durante el proceso de muerte (Ankarcrona et al., 1995; Gwag et al., 1995; Bonfoco et al., 1995; Pang y Geddes, 1997; Nasr et al., 2003). Con respecto a la muerte neuronal, se sabe que concentraciones bajas de glutamato generan un fenotipo de muerte apoptótico, mientras que concentraciones altas de glutamato generan un fenotipo necrótico (Bonfoco et al, 1995; Pang y Geddes, 1997). Finalmente, la gran variedad de proteasas que se activan durante los diferentes procesos de muerte celular también pueden determinar el fenotipo de ésta, como se ha mencionado anteriormente, la activación de las caspasas está relacionada con la muerte apoptótica, mientras que otras proteasas como la calpaína se relacionan con la muerte necrótica y la muerte autofágica (Del Río y Massieu, 2007). El primer artículo presentado en esta tesis revisa el papel que tienen las caspasas y la calpaína en el proceso de muerte excitotóxica. En éste se describen los tipos de muerte celular, las principales características de las caspasas y la calpaína, y se revisa el posible papel que tienen éstas en la muerte excitotóxica. Este artículo se publicó como capítulo en el libro “The Neurochemistry of Neuronal Death”.

ARTÍCULO 1

“Caspase-3 and Calpain activation as key step of the cell death phenotype”

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3 Caspase-3 and calpain activation as key step of the cell death phenotype

Perla del Río and Lourdes Massieu
Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México D.F. AP 70-253, CP 04510, Mexico

Abstract

Apoptosis and necrosis are two well-known cell death pathways. Apoptosis is a type of physiological cell death, usually present during development to eliminate unwanted neurons; necrosis, on the other hand, generally results from acute damage. The biochemical aspects that determine which death program a cell will follow include: the intensity of the cell death stimulus, ATP levels, and the different proteases activated during the process. Proteases such as caspases have been linked to an apoptotic phenotype, while calpains have been related to a necrotic one; however, recent evidence suggests a controversial role of these proteases in determining

Correspondence/Reprint request: Dr. Perla Del Río Estrada, Depto. de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México, D.F. CP 04510, Mexico
E-mail: pdelrio@ifc.unam.mx

the phenotype of the cell death. One of such examples is neuronal death induced by overactivation of glutamate receptors (excitotoxicity). Although excitotoxic neuronal death associated with cerebral ischemia and hypoglycemia has been considered necrotic in nature, evidence for apoptosis has also been found. In vitro studies have shown that excitotoxicity triggers a cell death cascade leading to apoptosis or necrosis, depending on the intensity of the initial stimulus, and it has been established that the activation of distinct families of proteases, such as calpains and caspases, can determine the neuronal death phenotype. Moreover, evidence indicates that excitotoxic death is influenced by calpain, due to excessive Ca^{2+} influx, and that calpain activation might determine the phenotype by modulating caspase-3 activity, leading to either a necrotic or apoptotic death phenotype. This review will focus on the role of caspase-3 and calpain as determinants of the cell death phenotype, and its role in excitotoxic neuronal death.

Introduction

Apoptosis and necrosis are considered as distinct forms of cell death. Nevertheless, there is increasing evidence suggesting that classical apoptosis and necrosis are the extreme ends of all the possible morphological and biochemical types of cell death. Apoptosis and necrosis are frequently distinguished by morphological and biochemical criteria; however recent evidence shows that both types of death share some features. Inhibition of the death program responsible for the apoptotic morphology does not necessarily result in cell survival; rather, it changes the cell death phenotype from apoptosis to necrosis. The phenotype of cell death can be modulated by some endogenous mediators such as the metabolic status of the cell, the activation or inactivation of individual subroutines, the severity of the insult, or the speed of the execution of the cell death cascade. It is also known that the execution of different patterns of cell death can involve distinct families of enzymes, and that the predominance of a given protease family depends on the type and intensity of the insult. A wide variety of proteases are engaged in the cell death process, these include cytosolic cysteine and aspartyl proteases, lysosomal proteases, and the ubiquitin proteosome system. Moreover, proteases can interact with each other in self-amplifying loops. This review will focus on two families of well characterized proteases: caspases and calpains. Caspase activation seems to be the predominant execution pathway in physiological cell death and death associated with moderate insults; meanwhile calpains are associated with intense insults and a necrotic phenotype.

Major forms of cell death

The term apoptosis was proposed by Kerr and colleagues (1972) to describe a specific morphological pattern of cell death during embryonic

development and normal cell turnover in healthy adult tissue [1]. The word apoptosis connotes a genetically and controlled form of cell death that is important throughout physiologic processes for removing individual components of an organism without causing damage to the surrounding tissue or cells. Thus, apoptosis is crucial for the maintenance of tissue homeostasis in the adult organism. The morphological features of apoptosis include nuclear and cytoplasmic condensation, internucleosomal DNA cleavage and packaging of the cell into apoptotic bodies, which are engulfed by phagocytes, preventing the release of the intracellular content [2,3] The morphologic features of apoptosis result from the activation of specific proteases called caspases (described later). Such activation is initiated either by the stimulation of a death receptor (extrinsic pathway) or by the release of apoptotic mediators from the mitochondria (intrinsic pathway). In cells dying by apoptosis, ATP levels slowly fall, $[Ca^{2+}]_i$ increases up to 200 – 400 nM, and there is a moderate production of reactive oxygen species (ROS) [4].

On the other hand, necrosis is not a programmed type of cell death; instead, necrosis occurs by deregulation of normal cell homeostasis when cells are exposed to conditions of extreme stress, and is the end product of a bioenergetic catastrophe resulting from ATP depletion, significant elevation of $[Ca^{2+}]_i$ (>1 μM), and excessive production of ROS [4]. Necrosis is morphologically characterized by an irreversible and generalized swelling of the cytoplasm and organelles accompanied by extensive vacuolation of the cytoplasm. Loss of membrane integrity results in the release of the intracellular content with the consequent inflammatory response. Cells dying by necrosis frequently exhibit changes in nuclear morphology, but not the organized chromatin condensation and DNA fragmentation into 180 bp fragments, characteristic of apoptotic cell death.

Other forms of cell death are known, autophagic cell death has been classified as a distinct form of non-apoptotic death separate from necrosis. Morphological features of autophagy include the formation of double membrane vesicles in the cytosol that encapsulate entire organelles and bulk cytoplasm. This autophagosome then fuses with the lysosome where its contents are degraded and recycled. The precise mechanism leading to autophagy is unknown; it is suggested that a family of cysteine proteases named autophagins are involved in the regulation and execution of this type of death. The death-associated protein (DAP) kinase and the DAP kinase-related protein (DRP)-1, which belong to the family of Ca^{2+}/calmodulin-regulated kinases, mediate the formation of autophagic vesicles as well as membrane blebbing during this type of cell death [5,6]. There are recent examples of the release of lysosomal proteins during the cell death process that leads to an apoptotic phenotype; however the exact mechanism is still unknown [7].

While necrosis and apoptosis can be clearly distinguished in some situations, certain cells show distinctive features of both apoptosis and necrosis. Thus, the suggestion of a continuum of responses ranging from apoptosis to necrosis has emerged [8,9]. The commitment of the cell to die by either necrosis or apoptosis is thought to be influenced by the nature and intensity of the insult [10], ATP availability [11,12], the mitochondrial integrity and functionality [13], and the type of proteases activated during the death process. Studies have focused on caspase activation, known as the classical executors of an apoptotic type of cell death, but there is considerable evidence that other proteases such as serine proteases and members of the calpain family are also involved in the cell death process [14,2]. Caspase activation is considered a hallmark of apoptosis while calpain activation is associated with necrotic cell death due to its dependence of Ca^{2+}.

Caspases

Caspases are a well characterized group of proteases that have been implicated in the initiation and execution of apoptotic cell death. They belong to a group of proteases containing a cysteine residue in their active site, which is essential for their activation. Caspases are a family of conserved proteases that usually cleave polypeptides on the carboxyl side of aspartate residues present in their substrates [15]. Inactive caspases exist as latent zymogens which contain a N-terminal pro-domain followed by a region forming the two-subunit catalytic domain. Activated caspases are constituted as tetramers composed of two 17-20 KDa and two 10-12 KDa subunits [16,17]. Caspases can be broadly divided into two groups: those involved in apoptosis and those related to caspase-1, whose primary role appears to be the processing of cytokine during inflammatory responses [18]. Caspases implicated in apoptosis can be further divided in two subgroups based on their structure and time-course of activation during cell death [19]. Initiator caspases (capase-2, -8, and -9) contain an extended N-terminal prodomain (>90 amino acids) essential for its actitvity, and are primarily responsible for initiating the caspase activation cascades. Effector caspases (caspase-3, -6, and -7) generally contain only a small pro-domain (20–30 residues) and are responsible for the dismantling of the cell through the cleavage of cellular components, although recently the role of caspase-6 and -7 during the execution phase of apoptosis has been questioned [20].

An initiator caspase is auto-activated during apoptosis; this process usually requires or is facilitated by the formation of protein complexes such as the apoptosome, responsible for the activation of caspase-9 [21,22]. The apoptosome is a multimeric complex formed by cytochrome *c* (released from the intermembrane space of mitochondria into the cytoplasm), apoptotic protease activating factor 1 (Apaf-1), dATP or ATP, and procaspase-9 [23]

(Fig 1). The *Induced proximity model* proposes that initiator caspases auto-process themselves when they are brought into close proximity to each other [24]. In this context, dimerization of initiator caspases, such as caspases-8 and -9, was proposed to be the driving force for their activation [25-27]. Hereby, the activation of the initiator caspase is a process facilitated by binding their pro-domains to adaptor molecules through the caspase-recruitment domain or the death-effector domain motifs [26]. Based on this model, the function of the apoptosome is to promote the homodimerization and the consequent activation of caspase-9. Until now, the only known function of the apoptosome is to recruit and facilitate the activation of caspase-9. Although an initiator caspase undergoes an autocatalytic intra-chain cleavage, this cleavage appears to exert only a modest effect on its catalytic activity [28], it has been demonstrated that

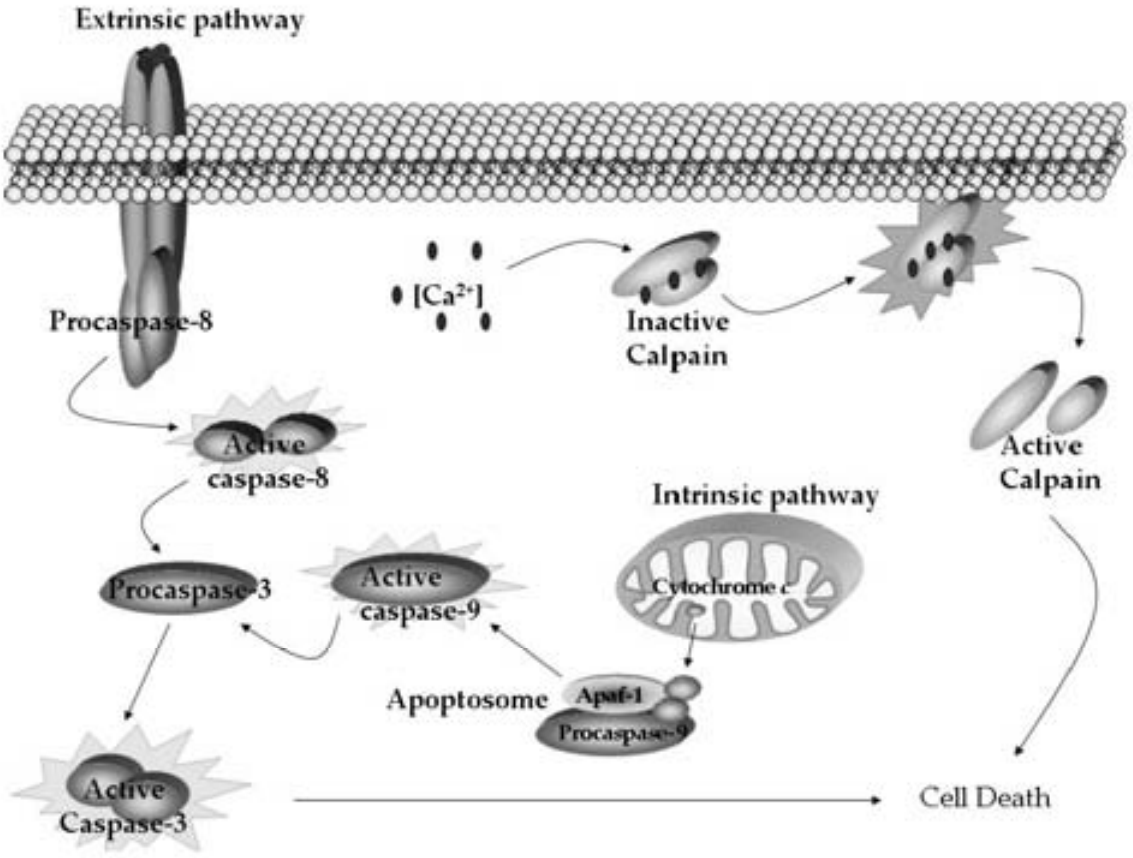

Figure 1. Caspase and calpain activation. Initiator caspases can be activated by membrane receptors (extrinsic pathways) that contain domains that allow their recruitment, or by intracellular signals such as cytochrome c release from the mitochondria (intrinsic pathway). Once the initiator caspases are activated they induce the activation of effector caspases such as caspase-3. On the other hand, calpain activation is Ca^{2+}-dependent, calpain translocates to the membrane immediately after Ca^{2+} binding, and then calpain subunits dissociate allowing the catalytic site to be in contact with its substrates.

caspase 9 association with the apoptosome leads to an enhancement of three orders of magnitude in its catalytic activity [29]. On the other hand, activation of an effector caspase occurs mainly through its cleavage by an inititator caspase at internal aspartate residues, allowing the assembly of heterotetramers. The catalytic activity of effector caspases, known to be present constitutively as homodimers, increases several orders of magnitude after its cleavage [24].

The participation of caspases in the apoptotic death cascade is well documented; once they are activated they promote apoptosis by activating other enzymes, or by degrading structural proteins of the cell. As an example, breakdown of nuclear lamin by caspase-6 is related to nuclear fragmentation and packaging of DNA fragments into apoptotic bodies [30]. Inhibition of the DNA repair system ocurrs due to cleavage of poly(ADP-ribose) polymerase (PARP) and DNA-PK by caspase-3 and -7 [31,32], internucleosomal DNA breakdown results from the activation of the nuclease CAD (caspase-activated deoxyribonuclease) due to caspase-3-mediated rupture of its inhibitor, ICAD (inhibitor of CAD) [33], and changes in cell shape might be the consequence of the breakdown of gelsolin and the p21-activated kinase, PAK2 [34,35].

Further support of the role of caspases in the apoptotic death program comes from pharmacological studies showing that treatment with caspase inhibitors, such as Z-VAD-fluoromethylketone, inhibits apoptotic death induced by different stimuli. Although the role of caspases in apoptotic cell death is well documented, it is important to keep in mind that other proteases also play an important role in this process. Studies using protease inhibitors have implicated calpains in the apoptotic death program [36].

Calpain

Seemingly, caspases have a central role in transducing the apoptosis signal, and caspase-3 activation appears to be a hallmark of apoptosis. In contrast, necrotic cell death is associated with massive Na^+ and Ca^{2+} influxes and, thus, calpain activation.

Calpains (calcium-dependent protease with papain-like activity) are a family of cytoplasmic cysteine proteases that contain several calcium-binding sites, which affect allosterically the enzyme activity [37]. Calpains are widely expressed in higher organisms, with both ubiquitous and tissue-specific isoforms. Fifteen gene products of the calpain family are known in mammals; and they are commonly classified as typical (9 members) and atypical (6 members) calpains. In mammals two ‘‘conventional’’ calpains, m-calpain and μ-calpain, are constitutively expressed in all tissues. They differ in the concentration of Ca^{2+} required for half-maximal activation *in vitro*, which is around 50 μM and 200 μM for μ-calpain and m-calpain, respectively.

In resting cells, these two ubiquitous calpains are present as a pro-enzyme heterodimer (80 KDa and 30 KDa) that contains multiple calcium-binding

sites. Calcium-induced structural changes are a pre-requisite for the formation of a functional catalytic center. In turn, the protease is activated by autolytic processing and the following dissociation of the small subunit from the large one [38]. Generally, calpain is present in the cytosol in its inactive form and translocates to membranes in response to Ca^{2+} influx [39,40]. Activated calpain hydrolyzes proteins in membranes or in cytosol after its release from the membrane [41]. In addition to Ca^{2+}, several factors modulate, either directly or indirectly, the activity of conventional calpains: 1) the interaction between the constituents' subunits; 2) the endogenous inhibitor protein calpastatin; 3) post-translational modifications like phosphorylation by protein kinase A, which inhibits m-calpain; and 4) the association of the protease with specific membrane phospholipids [42-44]. Calpains operate as calcium-dependent modulators which remove limited portions of protein substrates. Unlike caspases, calpains recognize either specific primary structures or global structural elements of the substrate proteins. Generally, amino acid preferences extend over 11 residues around the scissile bond, conventional calpains preferred residues are Lys, Tyr and Arg in P1 position; Leu, Thr, Val in P2 position, and Pro in P3 [45,46]. Calpain substrates are cleaved into limited fragments without further degradation [47,48]. Calpain substrates include cytoskeletal proteins, plasma membrane-associated proteins, signal transduction proteins, calmodulin-dependent proteins, and transcription factors.

The physiological role of calpain is still unclear, deletion of the 30 KDa subunit in mice results in embryonic lethality, indicating that this subunit of calpain has an important role during development [49]. Mice with a deletion of the 80 KDa subunit of μ-calpain are viable and only show a decrease in platelet aggregation [50]. Activated calpains respond to Ca^{2+} signals by cleaving specific proteins that are frequently components of signaling cascades, indicating that physiological processes like cell proliferation, cell cycle progression, differentiation, cytoskeletal reorganization, motility, and apoptosis, might involve calpain activation [38,51-54]. Specific functions of calpain such as cell motility, growth cone motility and guidance in neurons, have been described [55].

It is thought that limited calpain activation might be involved in plenty of cellular signals however; overactivation of calpain might be detrimental. The role of calpain in necrotic cell death is related to the increase in the $[Ca^{2+}]_i$. The involvement of calpain in necrosis is based on the finding that cells treated with maitotoxin, a potent marine toxin that opens voltage and ligand-gated Ca^{2+} channels, induced calpain overactivation with subsequent DNA degradation rather than condensation, features associated with necrosis [56]. Uncontrolled cytoskeletal disruption by calpain has been documented and associated with cell death. Calpain-mediated proteolysis of fodrin, a cytoskeletal component, has been observed during necrotic cell death induced by hypoxia in rat cardiomyocytes [57]. Myosin Va, a protein involved in

vesicle transport, is cleaved by calpain in cerebellar granule neurons after an excitotoxic stimulus, and calpain inhibitors prevent its cleavage favoring the preservation of the cytoskeleton integrity [58]. Another characteristic of necrotic cell death is ion dishomeostasis. It has been reported that calpain contributes to cell death by cleaving the membrane Na^{+}/Ca^{2+} exchanger, responsible of the regulation of the $[Ca^{2+}]_i$ [59]. In addition, calpain has recently been reported to cleave the endogenous calcineurin inhibitor which results in the activation of calcineurin and the promotion of Ca^{2+}-triggered cell death [60]. Altogether, these studies suggest an important role of calpain in necrotic cell death; however, calpain has also been related to apoptosis because calpain inhibitors prevent cell death in well known models of apoptosis, such as apoptosis in immune cells, apoptosis induced by Nerve Growth Factor deprivation in PC12 cells, and staurosporine-induced apoptosis in neurons [61,62]. Finally, calpains also have a role in the regulation of apoptotic death by cleaving regulatory proteins such as apoptotic protease activating factor 1 (APAF-1), and the BID protein (member of the Bcl-2 family proteins) [62-65].

Caspase and calpain crosstalk

Elucidating the precise role of calpains and caspases, especially that of caspase-3, in apoptotic or necrotic cell death is a challenging question; first because both proteases share common substrates and, second, because a cross-talk between both proteases exists.

Common substrates

Calpain and caspase-3 share a number of substrates suggesting that both proteases have important roles in cellular death. The vulnerability of the same substrates to proteolysis in both necrosis and apoptosis suggests that proteolysis mediated by the caspase and calpain systems might converge in common roles during the cell death process. Substrates shared by both proteases include: components of the cytoskeleton such as non-erythroid β- and α-spectrin, vimentin, keratin and actin; the Tau protein (a microtubule binding protein), the ADP-ribosyltransferase [poly(ADP) ribose polymerase (PARP)], the calmodulin dependent protein kinase IV (CaMKIV), the focal-adhesion kinase (FAK, a non-receptor tyrosine kinase implicated in integrin-mediated signal transduction) (reviewed in 66). Modulators of cell death that can be cleaved by both caspases and calpains include procaspase-3, -7, -9 and -12, Bcl-2 family proteins (Bid, Bcl-XL, and Bcl-2), and DNA repair and cell cycle regulatory proteins such as PARP [62-65].

Cross-talk

At present, it has been postulated that the morphological changes associated with cell death are determined by the type of protease activated, and

it is reasonable to state that caspase activation triggers an apoptotic phenotype while calpain activation a necrotic one. However, it has been shown that caspase-mediated proteolysis appears to have a role in necrosis, and that calpain is also activated in apoptotic cell death [67-69]. A study by Neumar et al. [70] demonstrated that calpain activity slows the execution phase of nuclear

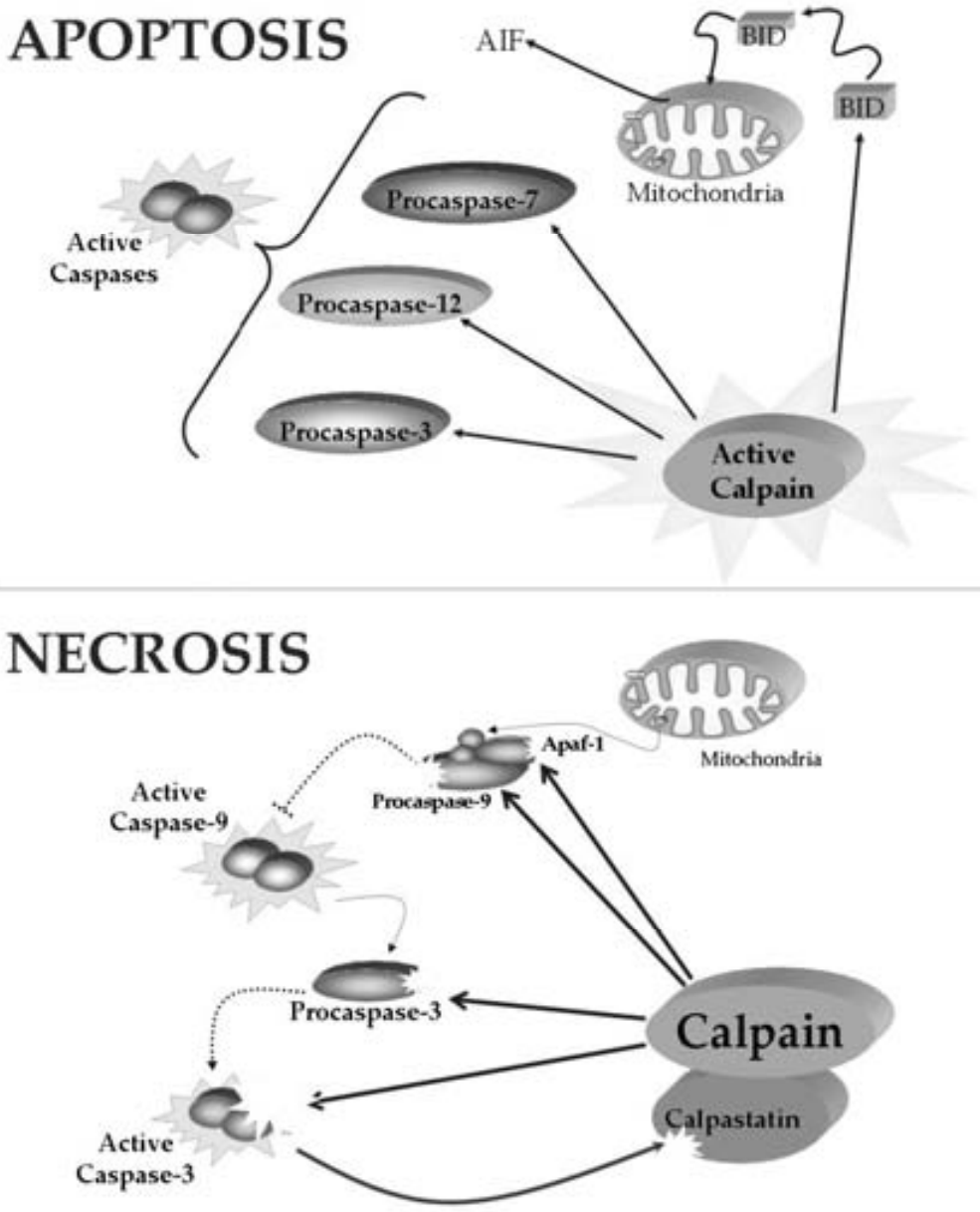

Figure 2. Protease interaction during cell death. Cell death, necrosis or apoptosis, can be regulated by calpains and caspases. An apoptotic phenotype is preserved if caspases are activated. Active calpain also favors an apoptotic phenotype by breaking procaspase-3, -7, -12, and BID. On the other hand, a necrotic phenotype is favored if calpain inhibits caspase-3 or -9 activation by direct cleavage or by processing APAF-1; or by calpastatin breakdown induced by caspase-3.

apoptosis but is necessary for plasma membrane disruption and secondary necrosis. This is because there is a significant cross-talk between the caspase and calpain proteolytic pathways, which defines distinct roles for calpain in the nucleus and plasma membrane during apoptosis. Calpain inhibition by calpastatin overexpression, up-regulates caspase-3 activity and accelerates apoptotic nuclear changes but promotes the preservation of the integrity of the plasma membrane [70]. Thus, the final phenotype of cell death could be the consequence of protease cross-talk.

Several studies demonstrate calpain-caspase cross-talk; however they do not clarify the role of these proteases in an apoptotic or necrotic death. It has been observed that calpain can cleave caspases-3, -7, -8, -9, and -12 with different functional consequences [71,72]. Calpain-induced procaspase-3 cleavage produces a 30 KDa fragment that has been shown either to enhance or inhibit caspase-3 activation [72,73]; truncation of procaspase-9 by calpain inhibits its ability to activate caspase-3 [71,74,75]. A recent study demonstrated that calpain can also cleave the active fragment of casapase-3, thus inhibiting its activity [71]. Calpain-mediated cleavage of the apoptosome components, caspase-9 and APAF-1, has also been demonstrated to indirectly inhibit subsequent caspase-3 activation [73,76]. In contrast to these studies, it has been reported that calpain can activate caspase-3, -7 and -12 [77]. On the other side of the story, there is also evidence showing that caspase can regulate calpain activation mainly through the processing of calpastatin, the only endogenous calpain inhibitor known at the moment [70], suggesting that caspases can up-regulate calpain activation in some cell death models. The ability of caspases to cleave calpastatin is well documented and has been reported to cause a 2-fold decrease in the activity of this calpain inhibitor [78].

Role of proteases in neurodegeneration

Acute and chronic neurodegeneration have been associated with both, necrotic and apoptotic cell death. Excitotoxicity is a mechanism of cell death associated with several pathological conditions including ischemia, hypoglycemia and some neurodegenerative diseases, such as Huntignton's, Parkinson's, and Alzheimer's disease (HD, PD, and AD, respectively). Excitotoxicity is the consequence of the continuous activation of glutamate receptors, due to an increase in glutamate extracellular levels or to a deficit in energy metabolism [79,80]. The elevation of intracellular Ca^{2+} concentration is the key-step of excitotoxic neuronal death, leading either to apoptosis or necrosis. Ca^{2+} overload triggers lethal downstream events, including oxidative stress, mitochondrial dysfunction and Ca^{2+} dependent protease activation. The elucidation of the role of caspases and calpains in excitotoxic neuronal death is of relevance due to its ubiquitous participation in neuropathological conditions. Caspase and calpain activation has been assessed by the recognition of the

products of spectrin-breakdown produced by the activity of caspase-3 and calpain. The presence of the calpain and caspase-3 breakdown products of spectrin have been observed in cerebrocortical neurons challenged with excitotoxic insults such as NMDA, Kainate (KA), and glucose–oxygen-deprivation [81].

Since the raise in $(Ca^{2+})_i$ is the most ubiquitous feature in excitotoxic neurodegeneration, calpain activation might be a critical step in neuronal death. Substantial evidence supports the role of calpain activation in acute cell injury and necrotic cell death triggered by Ca^{2+} influx. Apparently, the phenotype of cell death in adult neurons occurring after ischemia is mainly necrotic due to the increase in intracellular Ca^{2+}, and is caspase-independent, [82]. The role of calpains in neuronal death has been examined in several neuropathological conditions, and calpain activation has been demonstrated in postmortem tissue from PD and HD patients, and its inhibition can protect cells from death in a mouse model of PD [83,84]. Calpain has been shown to cleave the NR2B subunit of the glutamate NMDA receptor during an excitotoxic insult *in vitro* and *in vivo*, although the contribution of this process to excitotoxicity has not been determined [85]. Recent studies in mutant mice lacking or overexpressing the endogenous calpain inhibitor, calpastatin, suggest that calpain mediates excitotoxic signals through the mobilization of pro-apoptotic factors such as the apoptosis inducing factor (AIF), and endonuclease G from the mitochondria [65]. KA excitotoxicity is also influenced by calpain, KA-evoked caspase-3 activation was suppressed in calpastatin overexpressing mice, suggesting a role of calpain in caspase-3 activation. Moreover, hippocampal neurons from mice overexpressing calpastatin are spared from neuritic cytoskeleton disruption induced by KA [86]. During anoxia and ischemia, degradation of neurofilaments can be attenuated by Ca^{2+} removal or by inhibition of calpains, in isolated rat optic nerve [87]. Recently it was shown that calpain cleaves the plasma membrane Na^+/Ca^{2+} exchanger during brain ischemia in neurons undergoing excitotoxicity, inhibition of calpains by overexpression of calpastatin prevents secondary Ca^{2+} overload and rescues neurons from excitotoxic death [59].

On the other hand, mitochondrial damage, resulting from mitochondrial Ca^{2+} overload, induces the release of apoptotic inducing factors such as cytochrome *c* and AIF, suggesting a possible apoptotic phenotype. *In vitro* experiments in isolated liver and brain mitochondria showed that the release of AIF requires active calpain whereas the release of cytochrome *c* does not [88]. On the other hand, cytochrome *c* release might favor caspase-3 activation. In accordance with this observation, i*n vitro* and *in vivo* models of excitotoxicity have suggested an important role of caspase-3. Caspase 3 activation has been shown to occur after an excitotoxic insult [89-91], as well as after transient cerebral ischemia [92]. Caspase activation has been implicated in excitotoxic

cell death [93,94] and chronic neurodegenerative diseases such as Alzheimer's and Huntington's (HD) [95,96]. Levels of caspase-3 in brain varies depending on the age, young animals have relatively high levels of caspase-3 whereas adult animals have much lower levels. Thus, young animals can develop classical apoptotic morphology while adults are less likely to show classic apoptotic morphology following excitotoxic and ischemic insults [97,98].

It is conceivable that the degree of Ca^{2+} elevation, with the consequent calpain activation, will determine whether cells dye by apoptosis or necrosis, however an apoptosis-necrosis continuum has also been observed after glutamate exposure in cortical neurons, making the scenario even more complicated [9]. Mild Ca^{2+} elevation favors apoptosis, whereas acute calpain activation precipitates necrosis probably via catastrophic cleavage of regulatory and structural proteins [99]. The molecular pathways leading to the activation of the different proteases involved in neurodegeneration are certainly more complex due to the existence of a cross-talk between proteolytic mechanisms in brain [71]. Moreover, activating pathways are likely to differ depending on the neuronal population and the nature or severity of the insult, as well as on the subtype of activated glutamate receptor [8,10,13,100].

Conclusion

Overall, relative activity of calpains and caspases during the cell death cascade are likely to determine the morphologic features of cell death. It is reasonable to postulate that the degree of calpain activation could determine the ultimate morphology of cell death. Evidence for calpain activation during apoptosis supports either a proapoptotic or antiapototic role, depending on the type and severity of the apoptotic stimulus. On the other hand, severe insults associated with increased cytosolic Ca^{2+}, sufficient to trigger early calpain activation, may prevent or delay the execution of apoptosis through the suppression of caspase-3 activity; leading to necrosis before an apoptotic pathway can be executed. In contrast, milder insults not associated with cytosolic Ca^{2+} elevations, or with elevations inadequate to activate calpains, would be more likely to result in apoptotic cell death as long as apoptotic mediators are adequately expressed. Therefore, although caspase-3 activity is modulated by calpain, its role during apoptosis is not questionable.

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ANTECEDENTES Y PLANTEAMINETO DEL PROBLEMA

Dada la importancia del estado energético celular en la toxicidad al glutamato, en nuestro laboratorio existe un interés especial en conocer los mecanismos celulares responsables de la facilitación de la muerte excitotóxica cuando hay una falla en el metabolismo energético. Se ha demostrado con anterioridad que el aumento de la concentración extracelular de glutamato, dada por la inhibición de su recaptura, no produce daño neuronal a menos que el metabolismo energético esté inhibido (Sánchez-Carbente y Massieu, 1999; Massieu et al., 2001) y se ha enfatizado la importancia del metabolismo mitocondrial en estas condiciones (Massieu et al., 2001; García y Massieu, 2003). Actualmente no se conoce con exactitud cómo es que la inhibición mitocondrial, previa a un estímulo excitotóxico, modifica la cascada de eventos iniciada por el glutamato. Mientras que la gran mayoría de estos estudios se han realizado en cultivos de neuronas, los estudios *in vivo* son escasos. Los reportes hechos en cultivos neuronales indican que la muerte neuronal mediada por glutamato, cuando existe una inhibición mitocondrial previa, esta asociada a un daño mitocondrial severo. Se ha demostrado *in vitro* que la co-exposición de un inhibidor del metabolismo mitocondrial (3-NP) y de un inhibidor de la recaptura de glutamato induce muerte neuronal excitotóxica (García y Massieu, 2001, 2003) asociada a una disminución rápida y severa de los niveles de ATP, a la pérdida del potencial de membrana mitocondrial, a la producción excesiva de ERO, y al aumento en la concentración de Ca^{2+} citosólico y mitocondrial (Pang y Geddes, 1997; García y Massieu, 2001, 2003; Nasr et al., 2003; García et al., 2005). Estas observaciones sugieren que en estas condiciones la muerte ocurre por un mecanismo principalmente necrótico (Pang y Geddes, 1997).

En la actualidad se reconoce que los astrocitos tienen una comunicación estrecha con las neuronas a las que proveen de nutrientes y de un medio extracelular adecuado (Hertz et al., 1999; Newman, 2003). Dado que en los cultivos neuronales el componente astrocítico es reducido, desarrollamos un modelo experimental *in vivo*

para estudiar los mecanismos celulares que facilitan la muerte inducida por glutamato cuando existe una inhibición metabólica previa, condición que favorece la disfunción mitocondrial. El modelo utilizado consta de inhibir el metabolismo mitocondrial con la toxina 3-NP, la cual inhibe a la enzima deshidrogenasa del ácido succínico (SDH, por sus siglas en inglés), y posteriormente aplicar un estímulo excitotóxico moderado como lo es la inyección intraestriatal de una dosis subtóxica de glutamato. Con este modelo quisimos conocer cómo se afectan los niveles de ATP después de un estímulo excitotóxico en el estriado de ratas tratadas previamente con 3-NP. De manera diferente a lo que se había demostrado en los reportes en cultivos neuronales, nosotros observamos que los niveles de ATP no disminuyen drásticamente. De manera interesante, se observó que los niveles de ATP no disminuyen más del 35 % de los niveles control, a pesar de que en estas condiciones hay una lesión importante en el estriado de la rata (ver apéndice). Los resultados de este artículo nos sugirieron que otros factores, distintos a los niveles energéticos de la célula, pueden contribuir a la facilitación de la muerte inducida por glutamato en condiciones de inhibición mitocondrial. Es posible que dicha facilitación esté mediada ya sea por una mayor activación de ambos tipos de proteasas (calpaínas y caspasa-3), ó por la activación preferencial de alguna de ellas. Por un lado, dado que los niveles energéticos no disminuyen drásticamente aún en presencia del inhibidor mitocondrial, es posible la activación de caspasas. Por otro lado, es posible que el daño mitocondrial favorezca la pérdida del la homeostasis de calcio intracelular, contribuyendo a la activación de la calpaína.

HIPOTESIS

La toxicidad del glutamato *in vivo* podrá estar relacionada con la activación de calpaínas y de caspasa-3. La exacerbación de la muerte neuronal inducida por glutamato durante la inhibición mitocondrial, estará relacionada con una mayor activación de ambas proteasas.

Los receptores ionotrópicos a glutamato participarán en la activación de estas proteasas y en el daño inducido por glutamato.

OBJETIVO GENERAL

El objetivo principal de este proyecto es determinar la participación de la caspasa-3 y de la calpaína en el daño excitotóxico inducido en ausencia y presencia de una inhibición mitocondrial previa.

Además, se quiere conocer el papel de la activación de los diferentes receptores glutamatérgicos de tipo ionotrópico en la activación de proteasas, y en la consecuente muerte neuronal.

MATERIALES Y MÉTODOS

Todos los experimentos presentados en esta tesis se realizaron en ratas de la cepa Wistar de 250 a 300g de peso, las cuales se mantuvieron en condiciones controladas de temperatura y con un ciclo de luz-oscuridad de 12 h. Los animales tuvieron agua y alimento *ad libitum*. Los métodos están descritos detalladamente en los artículos presentados en los antecedentes y en la sección de resultados.

Modelos experimentales

En la presente tesis se muestran los resultados obtenidos en dos modelos experimentales de muerte excitotóxica producida por glutamato en el estriado de ratas (Fig.4). Dada la importancia del estado energético celular, el primer modelo de muerte excitotóxica combina la inhibición parcial del metabolismo mitocondrial, inducida por una dosis subtóxica de la toxina 3-NP, en conjunto con la inyección intraestriatal de una dosis de glutamato que, inyectado en el estriado de ratas intactas, produce una lesión similar a la producida con la inyección de su vehículo (500 nmolas totales; Del Río et al., 2007). Con este modelo se quiere enfatizar la importancia del estado energético celular en la facilitación de la muerte celular por glutamato, ya que, cuando se combinan los dos factores se observa un incremento significativo en el volumen de la lesión generado por la inyección intraestriatal de glutamato (Del Río et al., 2007). Para este primer modelo las ratas se inyectaron intraperitonealmente (i.p.) con el 3-NP durante dos días (15 mg/kg/día); la segunda inyección 24 h después de la primera. Después de 4 h de la segunda inyección de 3-NP, las ratas recibieron una inyección intraestriatal de glutamato (500 nmolas en un µl). Los resultados obtenidos de este modelo, se presentan en el tercer artículo titulado “Mild mitochondrial inhibition in *vivo* enhances glutamate-induced neuronal damage through calpain but not caspase activation: role of ionotropic glutamate receptors”, el cual fue aceptado para su publicación en la revista Experimental Neurology.

El segundo modelo experimental de daño excitotóxico se indujo mediante la inyección intraestriatal de una dosis tóxica de glutamato (2 µmolas en un µl), en el estriado de ratas intactas. En este modelo se quiere enfatizar la toxicidad del glutamato sin la influencia de una disfunción mitocondrial previa. Los resultados concernientes a este modelo se presentan en el cuarto artículo de esta tesis, el cual se titula “Contribution of NMDA and non-NMDA receptors to *in vivo* glutamate-induced calpain activation in the rat striatum. Relation to neuronal damage”, y se publicó en la revista Neurochemical Research.

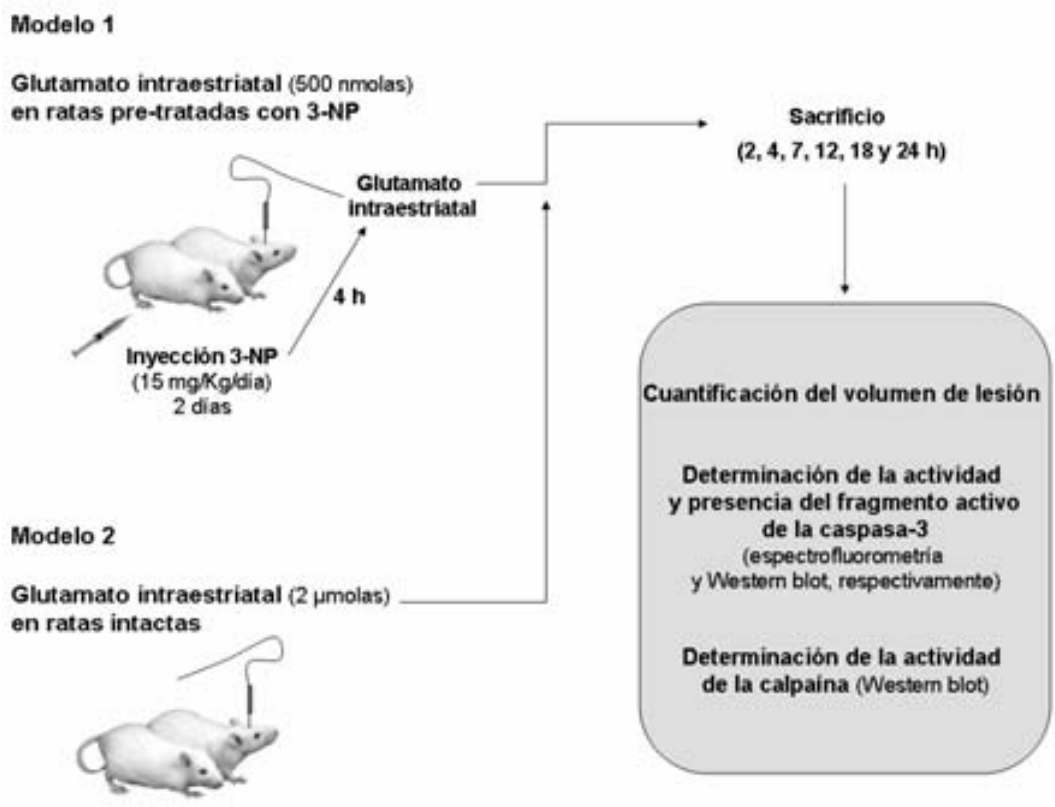

Figura 4. Esquema de los modelos experimentales utilizados en esta tesis. Éstos son modelos in *vivo* utilizando ratas de la cepa Wistar. El primer modelo combina el tratamiento con 2 inyecciones del inhibidor mitocondrial 3-NP (dosis que inhibe a la SDH pero que no genera daño en el estriado) y una inyección intraestriatal de una dosis subtóxica de glutamato (inyectado 4 horas después de la última inyección de 3-NP). El segundo modelo consta de una inyección intraestriatal de una dosis tóxica de glutamato en ratas intactas. Diferentes grupos de ratas fueron sacrificadas a diferentes tiempos después de la inyección de glutamato o de 3-NP (en el caso del primer modelo). Los cerebros de las ratas fueron procesados para evaluar el daño neuronal y la activación de la caspasa-3 o de la calpaína.

Inyecciones intraestriatales de glutamato

Las inyecciones intraestriatales de glutamato se realizaron en ratas anestesiadas con halotano. Para esto, las ratas se montaron en un aparato estereotáxico y se fijaron de los espacios interaurales con la barra dental en la posición -3.3. Las coordenadas utilizadas fueron: anteroposterior: + 0.7, lateral: + 2.8 y vertical: -4; de acuerdo al atlas de Paxinos y Watson (1986). Dichas coordenadas permiten inyectar el glutamato directamente al estriado de la rata. Como grupo control, algunas ratas fueron inyectadas con solución salina en lugar de glutamato. Los cerebros de las ratas fueron procesados para evaluar el daño inducido por la inyección de glutamato o para evaluar la activación de la caspasa-3 y la calpaína. Para esto, se sacrificaron diferentes grupos de ratas a diferentes tiempos después de la inyección de glutamato (2, 4, 7, 12, 18 y 24 h).

Tratamientos con antagonistas glutamatérgicos e inhibidor de la calpaína

Para conocer el papel que tienen los receptores glutamatérgicos de tipo ionotrópico en la inducción de la lesión excitotóxica así como su importancia en la activación de la calpaína, las ratas fueron tratadas con antagonistas glutamatérgicos. Un grupo de ratas se inyectó vía i.p. con un antagonista del receptor a NMDA, el (+)-5-methyl-10,11-dihydroxy-5-dibenzo(a,d)cycloheptene-5,10-imine maleate (MK-801). Un segundo grupo de ratas fue inyectado con 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), que es un antagonista de receptores de tipo no-NMDA. Un tercer grupo de ratas se pre-trató con ambos antagonistas. Los esquemas de inyección de estos antagonistas se basaron en reportes previos de nuestro laboratorio en los que se ha demostrado que dichos antagonistas protegen contra el daño neuronal en otros modelos de muerte excitotóxica *in vivo* (Massieu y Tapia, 1994; Massieu et al., 2001; Montiel et al., 2005; Camacho, et al., 2006). Un grupo independiente de ratas se trató con el inhibidor de la calpaína, el MDL-28170, el cual es un inhibidor potente de la calpaína que penetra rápidamente al cerebro

después de ser inyectado sistémicamente (Markgraff, 1998). Se sabe que este inhibidor penetra en el cerebro desde la media hora después de su administración sistémica y es capaz de inhibir significativamente a la calpaína (Markgraff, 1998). Los esquemas de inyección de los antagonistas glutamatérgicos y del inhibidor de la calpaína se describen en detalle en los artículos incluidos en los resultados.

Cuantificación del volumen de la lesión.

Para cuantificar el volumen de lesión producido por el glutamato, las ratas fueron sacrificadas 24 h después de la inyección intraestriatal de éste y se realizaron cortes coronales (40 µm) a nivel del estriado, los cuales fueron procesados para hacer tinción con violeta de cresilo. Para cuantificar el volumen de lesión los cortes fueron analizados en un microscopio óptico, el área lesionada se observa como una zona pálida debida al encogimiento ó desaparición de las células, y por lo tanto a la ausencia de tinción con el violeta de cresilo. Para hacer el cálculo del volumen de lesión, el área lesionada se delineó manualmente y se cuantificó con la ayuda de una cámara acoplada a un sistema de análisis de imágenes (NIH para Macintosh versión 1.6). El volumen de lesión se calculó haciendo la sumatoria del área lesionada (mm^2) en cada corte por el grosor (0.04 mm) en todos los cortes donde se observó lesión.

Determinación de la actividad y presencia de la caspasa-3 activa.

Para conocer si la caspasa-3 se activa después de un estímulo excitotóxico se utilizaron dos metodologías distintas. La primera es un método fluorométrico en el cual se utiliza un sustrato específico para la caspasa-3 que está acoplado a un fluoróforo (metil cumarina). Cuando la caspasa-3 activa corta este sustrato se emite fluorescencia, la cual se detecta con un espectrofluorómetro (Massieu et al., 2004). La segunda metodología detecta la presencia del fragmento activo de la caspasa 3 mediante la utlización de un anticuerpo policlonal que reconoce tanto al precursor (32 KDa) como al fragmento activo

de ésta (20 ó 10 KDa). La presencia del fragmento activo es indicativa de que la caspasa-3 ha sido cortada y por lo tanto activada (Massieu et al., 2004).

Determinación de la presencia de calpaína activa

La activación de la calpaína se determinó mediante inmunoblot utilizando un anticuerpo monoclonal que reconoce a la α-espectrina y a los productos de su rompimiento. La α-espectrina es un componente del citoesqueleto que es sustrato tanto de la μ/m-calpaína como de la caspasa-3. Cuando la calpaína rompe a la α-espectrina se producen fragmentos de 145 y 150 KDa, mientras que la caspasa-3 produce fragmentos de 120 y 150 KDa (Nasr et al, 2003). Estos fragmentos son reconocidos por el anticuerpo utilizado, y mediante el reconocimiento del peso molecular de los diferentes fragmentos se puede distinguir entre la actividad de ambas proteasas.

Estadística

Los resultados fueron analizados con una prueba de ANOVA de comparación múltiple seguida de una prueba de comparación múltiple de Fisher, utilizando el programa de estadística Statview 4.5. Todos los resultados se presentan como promedios ± error estándar.

DISCUSIÓN

Dada la importancia de la muerte neuronal excitotóxica en la progresión de algunas enfermedades neurodegenerativas crónicas, así como en el daño cerebral resultante de accidentes vasculares y periodos isquémicos, ésta ha sido objeto de gran interés. El hecho de que la muerte neuronal excitotóxica presente características morfológicas tanto necróticas como apoptóticas hace necesario investigar los posibles mecanismos moleculares que la gobiernan. El conocimiento de los eventos celulares que llevan a la muerte celular, más que evitar el daño desde su inicio, puede permitir el desarrollo de estrategias alternas para poder interrumpirla o retrasarla. A pesar de que se conocen algunos de los eventos de la cascada excitotóxica (Frasden, 1989; Shinder et al., 1996; Sapolsky, 2001; Schubert y Piasecki, 2001), todavía existe controversia en cuanto a la señalización intracelular responsable del fenotipo de la muerte en condiciones de deficiencia energética (Bonfoco et al., 1995; Ankarcrona et al., 1995; Portera-Caillau et al., 1997; Galas et al., 2004). Existen varios reportes acerca de la activación de la caspasa-3 y de la calpaína después de un estímulo excitotóxico; sin embargo, su papel en ésta aún no queda claro posiblemente debido a la gran variedad de modelos y de estímulos excitotóxicos existentes (Siman y Nozeck, 1988; Siman et al., 1989; Tenneti y Lipton, 2000; Bretch et al., 2001; Zhang et al., 2002; Benchoua et al., 2001; Ferrer et al. 2003; Paucaurd et al., 2004). Por un lado, la mayor parte de los trabajos se han realizado con agonistas selectivos de los receptores glutamatérgicos (Siman y Nozeck, 1988; Tenneti y Lipton, 2000; Jourdi et al., 2005; Yuen et al., 2007a), mientras que los estudios *in vivo* que utilizan glutamato son escasos (Benchoua et al., 2001, Tomioka et al. 2002). Dado que los agonistas glutamatérgicos son mucho más tóxicos que el glutamato (Obrenovitch et al., 1994), nosotros decidimos utilizar el glutamato como estímulo tóxico, pues es el neurotransmisor endógeno. Por otro lado, se ha visto que los estudios *in vitro* difieren de los resultados mostrados *in vivo* (Pang y Geddes, 1997; Garcia y Massieu, 2003; Del Río et al., 2007). Estos últimos son principalmente estudios que evalúan los mecanismos de muerte neuronal después de

periodos isquémicos (Zhang et al., 2002; Davoli et al., 2002; Ferrer et al., 2003; Yokota et al., 2003; Kambe et al., 2005; Gascón et al., 2008). La diferencia entre los estudios *in vivo* e *in vitro* puede radicar en la interacción entre los diferentes tipos celulares presentes en el tejido, situación más limitada en los cultivos neuronales; aparentemente la interacción entre astrocitos y neuronas influye en los mecanismos de muerte celular y se piensa que los astrocitos tienen un papel protector ante estímulos excitotóxicos (Ohgoh et al., 2000; Newman, 2003). Por otra parte, a pesar de que los astrocitos son más resistentes a la toxicidad del glutamato, sí son susceptibles a ésta (Sas et al., 1993; Zhao et al., 2000). La importancia de los modelos presentados en esta tesis radica en que son estudios *in vivo* realizados en el estriado de ratas, y a que el estímulo excitotóxico utilizado es el ligando endógeno, el glutamato. En la presente tesis, se utilizaron dos modelos de muerte excitotóxica *in vivo*, uno involucra la inhibición mitocondrial previa a la administración del glutamato, y el segundo, la inyección intraestriatal de una dosis tóxica de glutamato. Se utilizó la dosis de 2 µmolas de glutamato, ya que hemos demostrado con anterioridad que para producir daño con dosis de glutamato mas bajas, se necesita combinar su administración con la inhibición metabólica (Mejía-Toiber et al., 2006; Del Río et al., 2007). Estudios previos sugirieron que para producir lesiones administrando únicamente glutamato es necesario utilizar dosis mayores a 1 µmola (Mejía-Toiber et al., 2006; Del Río et al., 2007).

El daño inducido por glutamato no involucra la activación de la caspasa-3

Las caspasas son proteasas involucradas con los procesos de muerte celular durante el desarrollo del SNC y se les considera como los principales ejecutores de la muerte apoptótica (Donepudi y Grütter, 2002; Denault y Salvensen, 2002). Éstas se dividen en iniciadoras y efectoras y su activación puede ser influenciada por la mitocondria (Liu et al., 1996; Del Río et al., 2007) o por receptores membranales (Ashkenazi y Dixit, 1998; Del Río et al., 2007); en esta tesis decidimos evaluar la

activación de la caspasa-3, la cual es una caspasa efectora (Denault y Salvensen, 2002; Rield y Shi, 2004). A pesar de que se ha demostrado la activación de esta proteasa después de un estímulo excitotóxico tanto *in vitro* (Bonfoco et al., 1995; Tenneti y Lipton, 2000; Bretch et al., 2001) como *in vivo* (Zhang et al., 2002; Benchoua et al., 2001; Paucaurd et al., 2004), nosotros no observamos su activación después de la inyección de glutamato, a pesar de observar una lesión evidente en ambos modelos de excitotoxicidad *in vivo* (Fig. 1, artículo3; Fig. 1, artículo 4). La activación de las caspasas está altamente regulada y se han planteado varias formas en que las células, incluyendo las del SNC, regulan su activación (Nicotera et al., 2000; Davoli, et al., 2002; Ferrer et al., 2003; De Ridder et al., 2006). Por ejemplo, se conoce que la activación de la caspasa-9, como consecuencia de la actividad de la caspasa-3, es dependiente de energía (Nicotera et al., 2000). Nosotros hemos demostrado con anterioridad que los niveles de ATP *in vivo* no disminuyen más del 35 % en las ratas tratadas con 3-NP más glutamato, a pesar de observar una lesión evidente en el estriado de las ratas (Del Río et al., 2007). Por esta razón suponemos que la concentración de ATP no es un factor limitante para la activación de la caspasa-3 en nuestras condiciones. La activación de la caspasa-3 también se ha relacionado con la severidad del estímulo tóxico (De Ridder et al., 2006). Aparentemente esta proteasa se activa cuando el estímulo tóxico es moderado (Bonfoco et al, 1995; Pang y Geddes, 1997; Davoli, et al., 2002; Ferrer et al., 2003; DeRidder et al., 2006). Se ha visto que en la isquemia cerebral la caspasa-3 se activa sólo en el área de penumbra (región del cerebro donde el flujo sanguíneo se reduce parcialmente y que se considera la región rescatable del daño isquémico) y no en el centro isquémico (región donde el flujo sanguíneo disminuye en su totalidad y el daño neuronal es severo e irreversible; Davoli, et al., 2002; Ferrer et al., 2003). En concordancia, el fenotipo de muerte neuronal también depende de la severidad del estímulo tóxico, se considera que la apoptosis está relacionada con periodos cortos de isquemia cerebral (Endres et al., 1998) y con el daño neuronal que ocurre en la zona de penumbra isquémica (Ferrer et

al., 2003); mientras que la muerte celular necrótica se asocia con el centro isquémico (Benchoua et al., 2001; Ferrer et al., 2003). Dado que nosotros utilizamos métodos bioquímicos que involucran el homogenado del estriado completo, no descartamos que existan células en el borde de la lesión en las que se active la caspasa-3. Por ello sugerimos que estudios de inmunohistoquímica podrían responder esta pregunta. Por otro lado, la activación de las caspasas ha sido considerada por algunos autores como dependiente del tipo neuronal (Bizat et al., 2003a; Galas et al., 2004). Estos estudios sugieren que los mecanismos de ejecución de la muerte de las neuronas estriatales difieren de los mecanismos ejecutados por neuronas corticales ante el mismo estímulo excitotóxico, demostrando que las caspasa-3 se activa fácilmente en las neuronas corticales y no en las estriatales (Bizat et al., 2003a; Galas et al., 2004). Aunado a esto, existen varios reportes que sugieren que en el cerebro de ratas adultas, los mecanismos de muerte neuronal son independientes de caspasas, siendo éstas más importantes en eventos que ocurren durante el desarrollo o en los primeros días después del nacimiento (Takano et al., 2005; Zhu et al., 2005). Se ha demostrado que la cantidad de proteína precursora de la caspasa-3 (32 kDa) disminuye en ratas adultas y como consecuencia no se observa su activación después de un periodo de hipoxia-isquemia (Zhu et al., 2005). Otros factores relacionados con la activación de la caspasa-3, como BAX y APAF-1, también disminuyen con la edad, haciendo más difícil la activación de esta proteasa después de un estímulo tóxico (Vekrellis et al., 1997; Yakovlev et al., 2001; Stoka et al., 2006). Los cambios en la expresión de estos factores en ciertas poblaciones neuronales pueden estar relacionados con la incapacidad del estriado de rata adulta para activar a la caspasa-3 después de ciertos estímulos tóxicos.

El daño inducido por glutamato es dependiente de la activación de la calpaína

La falta de activación de la caspasa-3, así como estudios donde se muestra la co-existencia de características necróticas y apoptóticas en las mismas regiones

cerebrales después de un estímulo excitotóxico (Benchoua et al., 2001; Tomioka et al., 2002; Galas et al., 2004), hace pensar en la participación de otras proteasas en los procesos de muerte excitotóxica. Otra proteasa involucrada en la muerte celular es la calpaína, la cual se puede activar con pequeños cambios en la concentración intracelular de Ca^{2+}, pues es muy sensible a los cambios en la concentración de este ión (Czogalla y Sikorski, 2005). La calpaína se considera una de las principales proteasas activadas por Ca^{2+} en el SNC (Saido et al., 1993; Wang, 2000). Dado que, durante la excitotoxicidad hay una falla en la homeostasis del Ca^{2+}, decidimos estudiar la activación de esta proteasa y su relación con la muerte neuronal *in vivo*.

En esta tesis se muestra que la calpaína se puede activar sin necesidad de asociarse al daño mediado por glutamato (Fig. 1C y E, artículo 3). Demostramos la activación de la calpaína después de la inyección intraestriatal de una dosis subtóxica de glutamato (500 nmolas en ratas intactas), la cual no genera daño mayor a la producida por la inyección de salina. Los residuos de espectrina generados por la activación de la calpaína (145/150 kDa) se observaron a partir de las dos horas y hasta las 24 horas después de la inyección de glutamato (Fig. 1C y E, artículo 3). Los residuos de espectrina derivados del corte por la calpaína son muy estables (Vanderklish y Bahr, 2000; Czogalla y Sikorski, 2005), razón por la cual seguimos detectando los fragmentos 24 horas después de la inyección de glutamato. Desconocemos si la activación de la calpaína es transitoria o sostenida, un ensayo que permita determinar directamente si la calpaína se encuentra activa en los homogenados obtenidos del estriado de las ratas, a diferentes tiempos después de la inyección de glutamato, podría resolver esta pregunta. No conocemos las consecuencias funcionales de esta activación moderada de la calpaína pero la actividad fisiológica de esta proteasa se ha asociado con procesos de potenciación de largo plazo (LTP, por sus siglas en inglés) (Lynch y Baudry, 1987), remodelación sináptica (Vanderklish et al., 1995) y transcripción de genes (Liu et al., 2008). También se sugiere que la activación moderada de la calpaína puede modular la

neurotransmisión glutamatérgica, ya que al cortar al receptor a AMPA induce su internalización y de esta manera evita la sobre-activación de las neuronas (Yuen et al., 2007b).

Otra observación importante de esta tesis es que el daño inducido ~~sólo~~ por glutamato (2 μmolas, sin inhibición mitocondrial previa) se asocia con una fuerte activación de la calpaína y no de la caspasa-3 (Fig. 1, artículo 4). Diversos estudios han mostrado que la calpaína se activa rápidamente después de la exposición a agonistas glutamatérgicos y que su activación es proporcional al número de receptores activados (del Cerro et al., 1994; Bahr et al., 1995; Vanderklish et al., 1995). Los resultados mostrados en esta tesis apoyan estos estudios pues mostramos que la inyección de 2 μmolas de glutamato produce una lesión extensa en el estriado de ratas intactas, mientras que la lesión generada por la inyección de 500 nmolas es similar a la que produce la inyección de su vehículo. En concordancia con la severidad de la lesión, se demostró que la inyección de 2 μmolas de glutamato produce bandas de 145/150 kDa de mayor intensidad que las generadas por la inyección de 500 nmolas de glutamato, lo cual confirma los datos observados en otros reportes que asocian la activación patológica de la calpaína con la severidad del estímulo tóxico (Siman et al., 1989; Manev et al., 1991; Saido et al., 1993; Roberts-Lewis et al., 1994; Bahr et al., 1995; De Ridder et al., 2006). La cuantificación de la actividad de la calpaína se hace necesaria para determinar si esta proteasa permanece activa por periodos largos de tiempo y para definir si el daño celular observado se debe más a su activación prolongada o solo a la fuerte activación de esta proteasa. La activación de esta proteasa como parte de un proceso excitotóxico *in vivo*, evidencia que durante éste existe un aumento en la concentración de Ca^{2+} intracelular. Actualmente se acepta que la sobre-activación de los receptores glutamatérgicos genera una sobrecarga de Ca^{2+} como un factor importante en la excitotoxicidad (Peng y Greenamyre, 1998; Peng et al., 1998; Alano et al., 2002). Diversos estudios han relacionado a la calpaína con el daño inducido por el tratamiento o inyección de agonistas glutamatérgicos (Takano et

al., 2005). En nuestro caso, nosotros analizamos la activación de la calpaína después de la administración del ligando endógeno de los receptores glutamatérgicos y evaluamos la participación de los receptores ionotrópicos en la activación de esta proteasa, así como su relación con la producción del daño celular. Observamos que, si el estímulo de glutamato es severo (2 µmolas), los receptores a NMDA son más importantes en la inducción de la lesión que los no-NMDA (Fig. 3 y 4, artículo 4), pero no así en la activación de la calpaína (Fig. 2, artículo 4). Mostramos también que los receptores no-NMDA son importantes para la activación de la calpaína, sin embargo, su inhibición no previene del daño producido por una dosis tóxica de glutamato. Aunque los receptores a AMPA y KA tienen una conductancia preferente por cationes monovalentes, el glutamato puede inducir eventos dependientes de Ca^{2+} a través de receptores no-NMDA, si estos son permeables a Ca^{2+} (Araujo et al., 2004; Beart et al., 2007). Dado que, al utilizar los antagonistas glutamatérgicos, no se previno totalmente el daño ni la activación de la calpaína no descartamos que otros mecanismos independientes de los receptores ionotrópicos estén contribuyendo al daño inducido por un estímulo glutamatérgico severo. La toxicidad del glutamato en estas condiciones también puede deberse a la inhibición de la captura de cisteína, la cual ocurre por un transportador que también reconoce al glutamato, y que está asociada a procesos de estrés oxidativo (Murphy et al., 1990; Schubert y Piasecki, 2001). Por otra parte, la liberación de Ca^{2+} de pozas intracelulares (Lei et al., 1992; Khodorov et al., 1996a, 2002) o el influjo de este ión a través de canales de Ca^{2+} dependientes de voltaje (Walter y De Waard, 1998) pueden contribuir a la activación de otros procesos como la activación de lipasas y fosfatasas (Dawson et al., 1992; Schulz et al., 1995b; Gunasekar et al., 1995; Hongpaisan et al., 2004), la liberación de factores pro-apoptóticos independientes de caspasas (Boehning et al., 2003; Beart et al., 2007), y el daño oxidativo, (Lo et al., 2003; Ouyang et al., 2007).

La inhibición mitocondrial previa al estímulo glutamatérgico subtóxico exacerba el tamaño de la lesión a través de un mecanismo dependiente de la activación de la calpaína.

Conociendo que el daño asociado con el glutamato es dependiente de la actividad de la calpaína y no de la caspasa-3, quisimos saber si la inhibición mitocondrial previa al estímulo glutamatérgico modifica la activación de éstas. Para esto utilizamos un modelo animal donde primero se inhibe el metabolismo mitocondrial con la toxina 3-NP y después se induce el estímulo excitotóxico. El 3-NP es una toxina mitocondrial que inhibe a la enzima succinato deshidrogenasa (SDH) y por lo tanto el ciclo de Krebs y el complejo II de la cadena de electrones. El tratamiento crónico con el 3-NP se ha utilizado para generar modelos animales de la enfermedad de Huntington (Brouillet et al., 1999) ya que, como se mencionó en la introducción, su tratamiento crónico (50 mg/kg por día por más de 7 días) produce un patrón histopatológico similar al de pacientes con esta enfermedad (Beal, 1994; Brouillet et al., 1995). Se sugiere que la toxicidad de este inhibidor es a través de un mecanismo excitotóxico (Beal et al., 1993b; Kim et al., 2000; Lee et al., 2002a, b), y se ha asociado a la disminución severa de los niveles de ATP (Ludolph et al., 1992; Beal et al., 1993a; Matthews et al., 1998) y a la producción de ERO (Schulz et al., 1996; Kim y Chan, 2002). Cuando esta toxina es utilizada crónicamente produce lesiones bilaterales en el estriado de la rata que están asociadas a la inhibición constante de la SDH de más del 70% (Brouillet et al., 1998). En nuestro modelo, la dosis utilizada de 3-NP inhibe transitoriamente la actividad de la SDH (60 %) y disminuye parcialmente la carga energética del tejido sin dañar el estriado de la rata (Del Río et al., 2007). En esta tesis, se muestra que el tratamiento sólo con 3-NP no activa a la caspasa-3 ni a la calpaína (Fig.1 y 2, artículo 3), lo cual concuerda con el hecho de que la dosis utilizada no es tóxica. Sin embargo, nosotros observamos que esta dosis subumbral de 3-NP facilita la toxicidad del glutamato ya que, la inyección intraestriatal de 500 nmolas de glutamato sólo es neurotóxica cuando las ratas han sido pre-tratadas con 3-NP (Del

Río et al., 2007). Por lo tanto, esta toxina es una buena herramienta para demostrar que la inhibición parcial de la mitocondria es suficiente para que las neuronas se vuelvan susceptibles al daño por glutamato. Esta situación podría estar ocurriendo en pacientes con la enfermedad de Parkinson o Huntington, ya que se han demostrado deficiencias mitocondriales en pacientes con estas enfermedades (Browne et al., 1997; Tabrizi et al., 1999) y, en el caso de la enfermedad de Huntington, una disminución en la recaptura de glutamato que contribuye posiblemente al daño excitotóxico (Cross et al., 1986; Arzberger et al., 1997; Hassel et al., 2008).

Las consecuencias de la inhibición mitocondrial sobre la activación de factores ejecutores de la muerte celular, como es la activación de proteasas, no se conoce con exactitud. Estudios previos, realizados en cultivos neuronales, habían demostrado que la muerte neuronal subsecuente a la co-incubación con 3-NP y glutamato, tiene características necróticas y se sugiere una posible participación de la calpaína (Pang y Geddes, 1997; Nasr et al., 2003; Pang et al., 2003). Sin embargo, en estos trabajos no se estudió si la inhibición mitocondrial modifica la cascada de muerte inducida por el glutamato. Nosotros observamos que el pre-tratamiento con 3-NP no cambia el tipo de proteasas activadas sino que hace más fuerte la activación de la calpaína (Fig. 2D y G, artículo 3). La contribución de la calpaína a la facilitación de la toxicidad del glutamato durante la inhibición mitocondrial se evidencía por el hecho de que el tratamiento con el inhibidor de la calpaína, el MDL 28170, previene el daño neuronal y la activación de la calpaína en la misma proporción (Fig. 3 y 4, artículo 3). El MDL 28170 es un inhibidor que tiene alta afinidad por la calpaína y penetra al cerebro rápidamente después de su administración sistémica (Markgraf et al., 1998; Kawamura et al., 2005). Su utilización ha mostrado protección contra el daño en rebanadas de hipocampo incubadas con KA (López-Picon et al., 2006) y después de periodos de hipoxia (Chen et al., 1997). En modelos de muerte excitotóxica *in vivo* también se ha demostrado la protección contra el daño neuronal utilizando este inhibidor (Li et al., 1998; Markgraff et al., 1998; Wu et al., 2004; Higuchi et al., 2005; Kawamura et al., 2005), al igual que *in*

vitro (Araujo et al., 2004). Los resultados mostrados en esta tesis, indican que la activación de la calpaína es un evento temprano del proceso de muerte neuronal cuando la mitocondria esta inhibida. Recientemente mostramos que la lesión en el estriado de la rata se hace evidente a las 4 horas después de la inyección de glutamato (Del Río et al., 2007). En el presente trabajo, mostramos que la activación de la calpaína ocurre desde las 2 horas después de la inyección. Aunque no conocemos si, en nuestras condiciones, la calpaína se activa específicamente en neuronas y/o glia, se sabe que la calpaína existe en ambos tipos celulares (Manev et al., 1991; Yokota et al., 2003). Asimismo, se conoce que el requerimiento de Ca^{2+} para su activación es de concentraciones milimolares para la glia y de concentraciones micromolares para las neuronas (Manev et al., 1991; Yokota et al., 2003). En nuestras condiciones, sabemos que dentro de la lesión no se observan células vivas, solo se observan núcleos picnóticos. En un estudio previo demostramos, mediante inmuohostoquímica contra GFAP (marcador glial), que la glia reactiva se encuentra rodeando la lesión (Del Río et al., 2007). La ausencia de células dentro de la lesión sugiere que tanto las neuronas como la glia son susceptibles al daño por glutamato en nuestras condiciones y que la actividad de la calpaína posiblemente ocurra en las células gliales y en neuronas. Ya que ambos tipos celulares expresan receptores a AMPA y a NMDA se considera que son susceptibles al daño por glutamato (Matute et al., 2002; Krebs et al., 2003; Karadottir et al., 2005).

Los resultados mostrados en esta tesis concuerdan con otros reportes donde se ha mostrado que una alteración en la mitocondria favorece la muerte celular por un mecanismo independiente de caspasas (Chang y Jonson, 2002; Cregan et al., 2002; Takano et al., 2005). Esto se ve apoyado por estudios que han mostrado que el tratamiento con inhibidores de caspasas no disminuye el tamaño de la lesión inducida por diversos estímulos excitotóxicos (Tomioka et al., 2002). Se sugiere que la activación de la calpaína favorece la muerte independiente de caspasas (Lankiewicz et

al., 2000). Esto puede deberse a que, por un lado, la calpaína puede cortar tanto al precursor de la caspasa-3 como a su fragmento activo en el extremo carboxilo terminal (Bizat et al., 2003b) modulando la activación de la caspasa-3 y favoreciendo un mecanismo independiente de esta y un fenotipo necrótico (Lankiewicz et al., 2000). Otro tipo de muerte celular independiente de caspasas es la muerte autofágica, la cual se caracteriza por la presencia de vacuolas que en su interior contienen organelos (Uchiyama et al., 2008). La muerte autofágica se ha relacionado últimamente con la muerte neuronal que ocurre durante periodos de hipoxia-isquemia (Koike et al., 20008), así como en algunas enfermedades neurodegenerativas (Kegel et al., 2000; Chu, 2006; Nixon, 2006). El papel de la calpaína en este tipo de muerte celular no se conoce con exactitud, sin embargo su activación prolongada se vincula con la ruptura de lisosomas y la consecuente muerte autofágica (Posner et al., 1995; Yashamira, 2003; Yashamira et al., 2003; Demarchi et al., 2006). En este trabajo no se realizaron estudios concernientes al fenotipo de muerte celular en nuestras condiciones, estudios de microscopía electrónica serían necesarios para resolver esta pregunta. Finalmente, se ha mostrado que la activación de la calpaína favorece la liberación de factores pro-apoptóticos mitocondriales (Polster et al., 2005; Takano et al., 2005); dado que demostramos que la toxicidad del glutamato es un proceso dependiente de la activación de calpaína (Del Río y Massieu, 2008; Del Río et al., 2008), es interesante estudiar si esta activación está relacionada con la liberación de los factores mitocondriales y si éstos favorecen algún fenotipo de muerte celular, necrosis o autofagia.

El papel de los receptores ionotrópicos glutamatérgicos en la activación de la calpaína y su relación con el daño excitotóxico.

La gran variedad en los modelos de muerte excitotóxica ha dado a conocer una gran diversidad de mecanismos a través de los cuales las neuronas mueren después de un estímulo excitotóxico. Muchos de éstos dependen de los receptores a AMPA

(Brorson et al., 1994). En otros modelos se enfatiza la entrada de Ca^{2+} a través de los receptores a NMDA (Tymiansky et al., 1993a) y la captura de este Ca^{2+} por parte de las mitocondrias (Stout et al., 1998). Dado que nuestro estímulo tóxico es el glutamato, nosotros decidimos estudiar el papel de sus receptores ionotrópicos utilizando antagonistas glutamatérgicos, a dosis que previamente habían mostrado proteger en otros modelos de excitotoxicidad *in vivo* (Massieu y Tapia, 1994; Montiel et al., 2005; Camacho et al., 2006). Estudios pioneros que asocian estos receptores a la activación de la calpaína muestran que la inhibición selectiva de los receptores a NMDA o no-NMDA, con sus antagonistas, evita el corte de la espectrina (Jourdi et al., 2005), otro estudio indica que si las células no expresan receptores a glutamato la calpaína no se activa (Di Stasi et al., 1991). Los principales receptores involucrados en la muerte excitotóxica, dada su alta permeabilidad al Ca^{2+}, son los receptores a NMDA (Choi et al., 1988; Tymiansky et al., 1993a; Sattler et al., 1998). Esto se confirma en nuestra investigación ya que el tratamiento con MK-801 previene del daño inducido por la dosis tóxica de glutamato en ratas intactas (Fig. 4, artículo 4). El papel de los receptores ionotrópicos glutamatérgicos en la activación de la calpaína no se ha estudiado con detalle. Se sabe que la activación de los receptores a NMDA que contienen las subunidades NR2B permiten una mayor entrada de Ca^{2+} que los que contienen las subunidades NR2A y NR2C (Lynch y Guttmann 2001, 2002). Si tomamos en cuenta que la calpaína es muy sensible a los cambios en la concentración de Ca^{2+}, se puede especular que los receptores que contienen a las subunidades NR2B son los más importantes en la activación de esta proteasa. Esto se podría averiguar utilizando ifenprodil, un inhibidor de los receptores a NMDA que contienen la subunidad NR2B (Williams, 1993). Otros estudios han demostrado que los receptores a NMDA que contienen a la subunidad NR2A contribuyen a la toxicidad del glutamato, no así a la activación de la calpaína; mientras que los que tienen la subunidad NR2B contribuyen a la activación de la calpaína y al daño excitotóxico (Simpkins et al., 2003; De Ridder et al., 2006; Centonze et al., 2006).

El papel de los receptores no-NMDA en la muerte excitotóxica involucra principalmente su capacidad despolarizante; ahora se conoce que del 8 al 15% de las neuronas del SNC contienen receptores a AMPA permeables a Ca^{2+} (Pruss et al., 1991; Turetsky et al., 1994), y que el Ca^{2+} entrante por esta vía puede activar a la calpaína (Bi et al., 1994; Jourdi et al., 2005). Neuronas que expresan receptores no-NMDA permeables al Ca^{2+} mueren después de su exposición a KA en un periodo de tiempo similar a la muerte generada por su incubación con NMDA (Brorson et al., 1994; Weiss et al., 1994; Lu et al., 1996). En esta tesis mostramos que ambos tipos de receptores ionotrópicos glutamatérgicos contribuyen de igual manera a la muerte y activación de la calpaína cuando las ratas son pre-tratadas con 3-NP (Fig.5 y 6, artículo 3); no así en el modelo de muerte excitotóxica severa en donde los receptores no-NMDA no tienen una contribución al daño aunque sí a la activación de la calpaína (Fig. 2 y 4, artículo 4). Aunque por el momento no podemos sugerir un mecanismo que explique este resultado, sugerimos que en presencia de la inhibición mitocondrial, la falla en los sistemas reguladores de la concentración de calcio interno, resultará en la muerte de las neuronas independientemente del receptor involucrado en la entrada de éste catión. Es decir, las neuronas se harán más vulnerables a la activación de los receptores no-NMDA.

La participación de los receptores a NMDA en la activación de la calpaína y en la facilitación de la toxicidad del glutamato en presencia de 3-NP, se evidencia con el hecho de que el tratamiento con MK-801 previene el daño y la activación de la calpaína en la misma proporción (Fig. 5 y 6, artículo 3). Además, la inhibición de la calpaína con el MDL-28170 disminuye la extensión de la lesión y la activación de la calpaína en proporción similar (Fig. 3 y 4, artículo 3). El MK-801 es un inhibidor no competitivo que no tiene selectividad por una subunidad del receptor a NMDA en específico, por lo que desconocemos la contribución de un subtipo particular de receptor a NMDA en este modelo de muerte excitotóxica.

¿Cómo puede contribuir la calpaína al daño excitotóxico?

Conocemos que existe una asociación entre la activación de la calpaína y el daño neuronal inducido por glutamato en las ratas pre-tratadas con 3-NP. De hecho, la activación de la calpaína se ha implicado en la enfermedad de Alzheimer, Parkinson y Huntington (Saito et al., 1993; Gafni y Ellerby, 2002). También se conoce que el incremento sostenido de la concentración de Ca^{2+} intracelular conlleva a la activación sostenida de la calpaína; por lo tanto, al no estar regulada, puede contribuir al proceso neurotóxico al romper componentes estructurales necesarios para la sobrevivencia de la célula (Lynch y Baudry, 1987; Pant, 1988; Siman et al.; 1988; Greenwood et al., 1993; Brana et al., 1999; Rami, 2003). Varios son los mecanismos por los que la calpaína puede favorecer el proceso de muerte celular (Fig. 5). Primeramente, corta substratos haciendo que éstos se activen o inactiven. Por ejemplo, prolonga la activación de la cinasa dependiente de ciclina 5 (CDK5) al cortar a la proteína p35, la cual es un activador de ésta (Lee et al., 2000). Corta a la proteína cinasa C (PKC; Kishimoto et al. 1989), a la cinasa dependiente de calmodulina II (CaMK-IIa) (Hajimohammadreza et al., 1997) y a la calcineurina (Wu et al. 2004). Otras proteínas cortadas por la calpaína son: la sintasa del óxido nítrico, la proteína asociada a microtúbulos 2 (MAP2) (Creed et al., 1996; Zhang et al., 2007) y la espectrina (Wang et al., 1998). De manera importante, la activación excesiva de la calpaína puede estar relacionada con el daño excitotóxico, ya que se considera que ésta amplifica la cascada de muerte neuronal mediante el rompimiento de proteínas que contribuyen a la falla de la homeostasis del Ca^{2+} intracelular (Bano et al., 2005; Pottorff et al., 2006; Bano y Nicotera, 2007; Araújo et al., 2007). La calpaína corta al subtipo NCX3 del intercambiador de Na^{+}/Ca^{2+}, favoreciendo el aumento de Ca^{2+} intracelular relacionado con la desregulación de la concentración de Ca^{2+} intracelular (Bano et al., 2005; Bano y Nicotera, 2007; Araújo et al., 2007). También corta a la PMCA provocando su internalización y como consecuencia la extrusión de Ca^{2+} de la célula se hace más lenta (Pottorf et al., 2006).

Finalmente, se conoce que la activación continua de la calpaína produce cortes sobre los mismos receptores glutamatérgicos aumentando el efecto tóxico del neurotransmisor (Bi et al., 1996, 1997; Pineda et al., 2007; Xu et al., 2007; Yuen et al., 2007a, b). La subunidad mGluR1 de los receptores metabotrópicos de glutamato es cortada por la calpaína en su extremo carboxilo terminal y genera una retroalimentación positiva de sobrecarga de Ca^{2+} (Pineda et al., 2007; Xu et al., 2007). La estimulación continua de los receptores a NMDA activa a la calpaína, la cual puede a su vez cortar a la subunidad GluR1 de los receptores a AMPA en su extremo carboxilo terminal, reduciendo la actividad de este receptor (Bi et al., 1996, 1997; Yuen et al., 2007a, b). Una vez activa, la calpaína también corta a los receptores NMDA que contienen la subunidad NR2B; después de su corte, estos receptores permanecen en la membrana plasmática pero pierden sitios de regulación por la PKC y otras cinasas de tirosina (Simpkins et al., 2003). La activación de la calpaína desorganiza a las proteínas de la post-sinapsis ya que corta a la proteína PSD95, la cual se une a los receptores NMDA post-sinápticos, así como a las subunidades NR2A y B; este corte se asocia con la activación tóxica de estos receptores (Gascón et al., 2008).

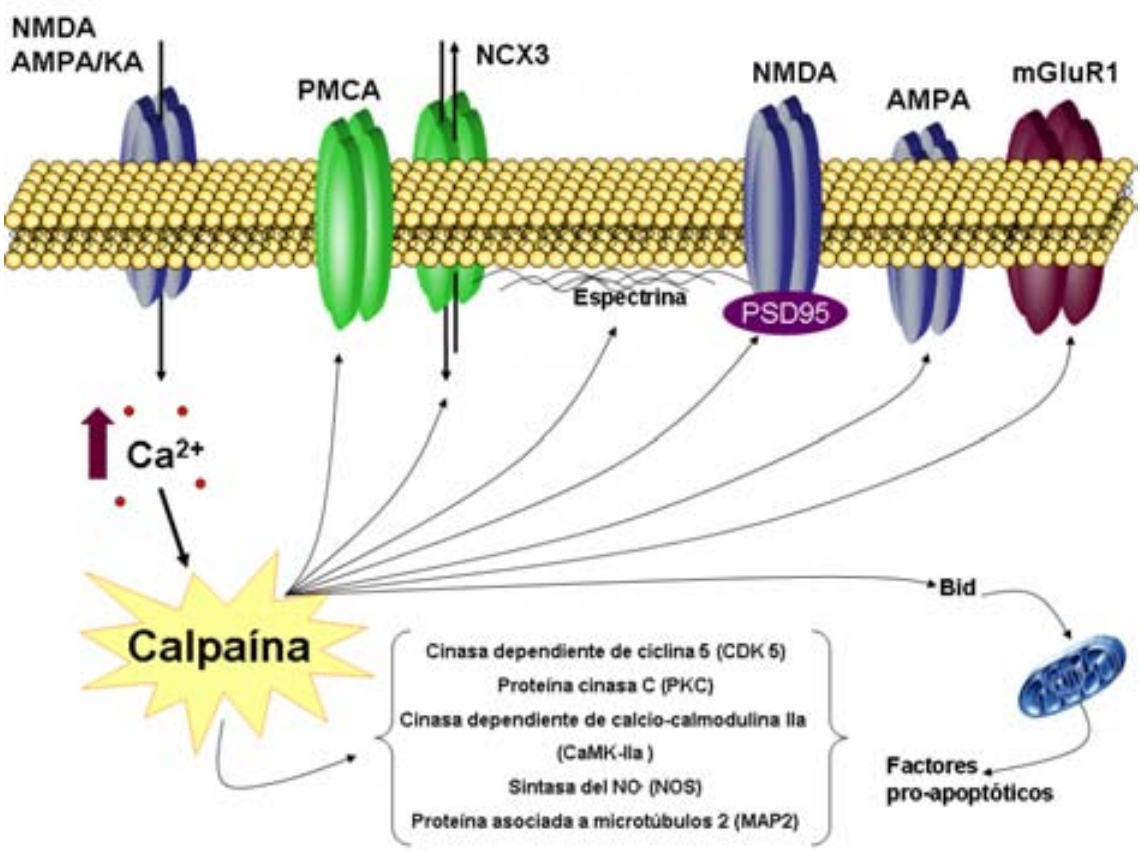

Figura 5. Substratos de la calpaína que pueden contribuir al daño excitotóxico. La calpaína es activada por el aumento en la concentración de Ca^{2+} intracelular, si la activación de la calpaína es intensa y por periodos de tiempo prolongados, ésta puede cortar componentes celulares importantes para la sobreviencia celular.

RESULTADOS

Articulo 2

"Mild mitochondrial inhibition in *vivo* enhances glutamate-induced neuronal damage through calpain but not caspase activation: Role of ionotropic glutamate receptors"

Del Río P y Massieu L. 2008

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Este artículo muestra los datos concernientes a la participación de la calpaína y de los receptores ionotrópicos glutamatérgicos en el daño neuronal inducido por glutamato cuando existe una inhibición mitocondrial previa. Los resultados más importantes presentados en este artículo son:

1) La exacerbación del daño producido por glutamato cuando hay una inhibición mitocondrial previa no está asociada a la activación de la caspasa-3. En este artículo se muestra que, a pesar de la presencia de una lesión de tamaño considerable, la caspasa-3 no está activada, lo que sugiere que éste es un proceso independiente de caspasas.

2) El glutamato activa de manera fisiológica a la calpaína y para que esta activación se asocie con la presencia de una lesión, es necesaria una activación intensa. Si se evita la activación de la calpaína, la lesión inducida por el glutamato es de menor tamaño.

3) Los receptores glutamatérgicos de tipo NMDA y los no-NMDA contribuyen de igual manera a la activación de la calpaína y el establecimiento de la lesión en estas condiciones. La inhibición de estos receptores previene del daño inducido por glutamato.

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Mild mitochondrial inhibition *in vivo* enhances glutamate-induced neuronal damage through calpain but not caspase activation: Role of ionotropic glutamate receptors

Perla Del Río, Lourdes Massieu *

Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México. México, D.F. Mexico

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Proteases

ABSTRACT

Glutamate neurotoxicity is exacerbated when energy metabolism is impaired. *In vitro* studies show that neuronal death in these conditions is related to mitochondrial dysfunction, ATP depletion, and the loss of calcium homeostasis. We have recently observed that, *in vivo*, enhancement of glutamate toxicity elicited by previous mitochondrial inhibition does not involve severe ATP depletion, suggesting the involvement of other processes. Factors such as the activation of different proteases may determine the extent and type of cell death. Protease activation might be triggered by internal or external factors, such as mitochondrial damage or the activation of a particular glutamate receptor subtype. In the present study we aimed to investigate whether moderate inhibition of mitochondrial metabolism facilitates glutamate toxicity through caspase-3 or calpain activation, as well as the contribution of NMDA and non-NMDA glutamate ionotropic receptors to this activation. Rats were pre-treated with a subtoxic dose of 3-NP and 4 h later intrastriatally injected with glutamate. Results show that neither of these treatments alone (3-NP or Glu) or in combination (3-NP+Glu) activated caspase-3. Conversely, calpain activity is induced after glutamate injection both in intact and 3-NP pre-treated rats. Inhibition of calpain activity by MDL-28170 significantly prevented striatal damage. NMDA and non-NMDA receptors contributed equally to calpain activation and to the induction of neuronal death. Results suggest that enhancement of glutamate toxicity due to inhibition of mitochondrial metabolism *in vivo*, does not recruit caspase-dependent apoptosis but favors calpain activation through the stimulation of both subtypes of glutamate ionotropic receptors.

Introduction

L-glutamate (Glu) is the major excitatory neurotransmitter in the mammalian central nervous system (Fonnum, 1984), however over activation of its receptors produces excitotoxic neuronal death. Excitotoxicity has been related to neuronal damage associated with ischemic periods, hypoglycemia, cerebral trauma, and some neurodegenerative diseases such as Huntington's disease (Beneveniste et al., 1984; Wieloch, 1985; Arundine and Tymianski, 2004). It is known that the continuous activation of Glu receptors induces a large increase in intracellular calcium accompanied with mitochondrial dysfunction, as revealed by the loss of mitochondrial membrane potential, mitochondrial calcium overload, and ATP depletion (Ankarcrona et al., 1995; White and Reynolds, 1995; Budd and Nicholls, 1996; Schinder et al., 1996; García et al., 2005).

Glutamate-mediated neurotoxicity is facilitated whenever energy metabolism is inhibited (Novelli et al., 1988; Zeevalk and Nicklas, 1992; Simpson and Isacson, 1993; Massieu et al., 2001). The identification of some of the processes involved in the exacerbation of Glu toxicity during energy failure has been presumed mainly from *in vitro* studies in neuronal cultures. These studies have shown that enhancement of Glu toxicity during mitochondrial inhibition with 3-nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase of mitochondrial complex II, is strongly related to the failure of mitochondrial activity and a rapid and severe decline in ATP levels (Nasr et al., 2003; García and Massieu, 2003; García et al., 2005). However, we have recently demonstrated that facilitation of Glu toxicity in the striatum of rats pre-treated with 3-NP, does not involve a severe ATP depletion but a sustained metabolic impairment (Del Río et al., 2007). Due to the complexity of the entire brain, it is possible that, *in vivo*, other factors related to mitochondrial inhibition could influence the enhancement of Glu toxicity. *In vitro* studies have shown that, depending on the severity of the initial stimulus (Ankarcrona et al., 1995; Bonfoco et al., 1995; Cheung et al., 1998) and on the activation of a particular Glu receptor subtype (Portera-Cailliau, 1997), Glu can trigger a distinct cell death phenotype leading to apoptosis or necrosis. In

Abbreviations: 3-NP, 3-nitropropionic acid; MK-801, (+)-5-methyl-10,11-dihydroxy-5-dibenzo(a,d)cycloheptene-5,10-imine maleate; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate; DTT, dithiothreitol; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-(4-methylcoumaryl-7-amide).

* Corresponding author. Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, D.F. CP 04510, AP 70-253, Mexico. Fax: +52 55 56 22 56 07.
E-mail address: lmassieu@ifc.unam.mx (L. Massieu).

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addition, factors such as ATP levels, mitochondrial dysfunction and the differential activation of distinct proteases, calpains and caspases, can determine the extent and phenotype of neuronal death (Ankarcrona et al., 1995; Pang and Geddes 1997; Pang et al., 2003; Del Río and Massieu, 2007). Caspase activation has been associated with an apoptotic phenotype, while calpain activation resulting from increased $[Ca^{2+}]_i$ has been related to a necrotic phenotype (Siman et al., 1989; Wang, 2000). Elucidating the precise role of calpain and caspases, particularly of caspase-3, in excitotoxic cell death during mild mitochondrial inhibition is a challenging question; first because both proteases share common substrates and, second, because a cross-talk between both proteases exists.

The mechanisms underlying the facilitation of Glu toxicity when mitochondria are partially inhibited are not well understood; furthermore, *in vivo* studies concerning this phenomenon are scarce. As mitochondrial failure and the consequent loss of calcium homeostasis might favor caspase and/or calpain activation, we aimed to determine whether mitochondrial inhibition *in vivo* influences glutamate-induced protease activation, and if this process is related to the exacerbation of Glu toxicity. We also aimed to know the contribution of NMDA and non-NMDA Glu receptors on the activation of these proteases and neuronal damage. Caspase activity was studied fluorometrically and by Western blot, using antibodies recognizing the procaspase and its active fragments. Calpain activity was monitored by the cleavage of its well-known substrate, α-spectrin, through Western blot using antibodies against the complete protein and its breakdown products.

Materials and methods

Materials

3-NP, L-glutamate (sodium salt), Cresyl Violet, HEPES, sucrose, CHAPS, EDTA, DTT were purchased from Sigma (St. Louis, MO, USA). MDL-28170 was obtained from Biomol (Plymouth Meeting, PA, USA). Glu receptors antagonists (+)-5-methyl-10,11-dihydroxy-5-ibenzo(a,d) cycloheptene-5,10-imine maleate (MK-801) and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) were purchased from Tocris (Cookson Ltd, Avonmouth, UK). Proteases inhibitor cocktail was obtained from Roche (Meylan, France). Ac-DEVD-AMC was purchased from Peptide Institute (Louisville, KY, USA). Antibodies against spectrin and actin were obtained from Chemicon (Temecula, CA, USA). Antibody against caspase-3 was from Santa Cruz Biotechnology (CA. USA). Anti-mouse or anti-rabbit IgG were from Jackson Immunoresearch (West Grove, PA, USA). ECL reagent was purchased from Millipore (Billerica, MA, USA).

Animal treatment

Male Wistar rats (250–300 g) were used throughout the study. They were handled according to the National Institute 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. Animals received two intraperitoneal (i.p.) injections of 3-NP (15 mg/kg), the second one administered 24 h after the first one. Four hours after the second 3-NP administration, rats received an intrastriatal injection of Glu (500 nmol/μl). 3-NP was dissolved in 10 mM phosphate buffer and pH was adjusted to 7.4 with 1 M NaOH. Glu (sodium salt) was dissolved in water (pH 7.3). Control animals received two i.p. injections of vehicle (250–300 μl of phosphate buffer, pH 7.4) and an intrastriatal injection of Glu. An additional group of animals received two 3-NP i.p injections and 1 μl saline intrastriatal injection. For neuroprotection experiments rats were treated with the calpain inhibitor MDL-28170. MDL-28170 (25 mg/kg) was injected i.p. 30 min before and immediately after Glu injection (50 mg/kg in total). MDL-28170 was dissolved in DMSO (25 mg/ml) and injection volume was adjusted according to the rat's weight (500–600 μl). An independent group of animals was treated with the NMDA receptor antagonist MK-801, and the non-NMDA receptor antagonist NBQX, or with both antagonists. MK-801 was injected i.p. 30 min before Glu intrastriatal injection (2 mg/kg); it was dissolved in saline solution (2 mg/ml in NaCl 0.9%) and the injection volume was adjusted to the rat's weight (250–300 μl). NBQX was intrastriatally co-injected with Glu (a total of 50 nmol in 1 μl) and it was dissolved in the Glu-containing solution. Brains were processed for histological analysis or for caspase-3 and calpain activity determinations at the times indicated in figures.

Stereotaxic injections

Intrastriatal Glu injections were performed according to previously reported methodology in halothane-anesthetized rats (Massieu et al., 2001; Del Río et al., 2007). Briefly, animals were anesthetized with 4–5% halothane in a 95% O_2/5% CO_2 mixture and placed on a stereotaxic frame with the nose bar positioned at −3.3. A 1–2 mm hole was drilled and 1 μl of either Glu (500 nmol) or vehicle solution (0.9% saline) was injected at a rate of 0.5 μl/min via a 27-gauge stainless steel needle connected to a Hamilton syringe with the aid of a microinjection pump (Harvard apparatus pump model 55, South Natick, MA, USA). Coordinates used were: AP +0.7 mm anterior from bregma, L +2.8 mm from midline, and −4.0 mm from the dura, according to Paxinos and Watson (1986). Brains were obtained at different times after Glu administration and prepared for histological evaluation (24 h), Western blotting and caspase-3 activity (from 2–24 h) as described below.

Histological evaluation

For histological evaluation animals were anesthetized with sodium pentobarbital anaesthesia 24 h after Glu injection 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. Brains were then transferred to a 20% and 30% sucrose solution successively. Coronal sections (40 μm) were obtained in a cryostat and stained with Cresyl Violet. Lesion volume was calculated as described previously (Del Río et al., 2007). Briefly, lesion size was calculated by examination of all brain sections where neuronal damage was evident in each experimental animal (number of animals per group: 6 for 3-NP alone, 6 for 3-NP+ intrastriatal vehicle, 7 for Glu + i.p. vehicle, 6 for 3-NP+Glu, 6 for MK-801, 5 for NBQX, 6 for MK-801+NBQX, and 6 for MDL-28170). 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 multiplying the average damaged area by the distance between the first and the last tissue section where tissue damage was visible. Results are expressed as means ± S.E.M. of lesion volume per each animal group.

Caspase 3 activation

For caspase-3 activity determination, intact or 3-NP-treated rats intrastriatally injected with Glu were killed by decapitation 2, 4, 7, 12, 18 and 24 h after Glu. Because Glu was injected 4 h after 3-NP treatment, the animal group treated only with 3-NP was sacrificed 6, 8, 11, 16, 22 and 28 h later. After dissection, the striatum was homogenized in caspase buffer containing: 100 mM HEPES (pH 7.4), 10% (w/v) sucrose, 0.1% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate (CHAPS), 10 mM dithiothreitol (DTT), 1 mM EDTA, and proteases inhibitor cocktail. Caspase-3 activity was assayed by a fluorometric method in a luminescence spectrometer (Spectronic Instruments, SLM Aminco-Bowman, Rochester, NY, USA) using the tetrapeptide acetyl-

Asp-Glu-Val-Asp-(4-methylcoumaryl-7-amide) (Ac-DEVD-AMC) as substrate, as reported previously (Massieu et al., 2004). Activity was followed for 15 min after the addition of substrate (2.5 μM) and 50 μg of protein from striatal homogenates, to 2.0 ml of a standard solution containing: 100 mM HEPES (pH 7.4); 10% (w/v) sucrose; 0.1% (w/v) CHAPS; 10 mM DTT; 1 mM EDTA; and protease inhibitors. Results are expressed as the change in fluorescence intensity per hour per milligram of protein produced after substrate cleavage. For these experiments data from 4 animals per each time point and treatment were analyzed, with the exception of the 7 h time point from the 3-NP+Glu treatment where results of 7 animals were analyzed.

Western blotting

Caspase-3 and calpain activation was assessed by Western blotting. Caspase-3 activation was evaluated by employing an antibody that recognizes the procaspase (32 kDa) and the active fragments of caspase-3 (17 and 12 kDa). Calpain activation was assessed by the utilization of an antibody that recognizes the complete and breakdown products of α-spectrin. Spectrin is a cytoskeletal protein that is a substrate for both, calpain and caspase-3; calpain generates breakdown products of 145 and 150 kDa, while caspase-3 produces fragments of 150 and 120 kDa. Recognition of the different breakdown products has been used as an index of caspase-3 and calpain activation. With this purpose, rats were killed by decapitation, the striatum was dissected and homogenized in 300 μl of ice-cold buffer containing 25 mM HEPES–KOH (pH 7.4), 0.1% Triton X-100, 5 mM $MgCl_2$, 1.3 mM EDTA, 1 mM EGTA, and a cocktail of protease inhibitors, according to Bizat et al. (2003b). Homogenates were centrifuged at 15,000 *g* for 30 min, and the supernatant stored at −80 °C until analysis. Protein concentration was quantified by the Bradford method (Bradford, 1976). For Western blot, 30 or 7.5 μg of supernatant protein, for caspase-3 and spectrin respectively, were separated by SDS-PAGE acrilamide gels (10 and 7% for caspase-3 and calpain, respectively). Samples were boiled for 3 min, electrophoresed at 75 mA during 2.5 h and then transferred to nitrocellulose membranes during 24 h at 4 °C (30 mA). For caspase-3 immunobloting the membrane was incubated during 1.5 h in a solution containing 5% albumin in TBS/tween 20 (TBS-T; 100 mM Tris–HCl , pH 7.5; 150 mM NaCl; 0.05% Tween 20) and then incubated overnight at 4 °C with anti-caspase-3 antibody (1:100 diluted in 5% albumin containing TBS-T solution). Membranes were washed with TBS-T (10 min/3 times) and then incubated 2 h at room temperature with anti-rabbit horseradish peroxidase-conjugated antibody (1:1000). They were washed again with TBS-T (10 min/3 times) before peroxidase activity was detected. Membranes for spectrin and actin (control loading) immunoblotting were incubated

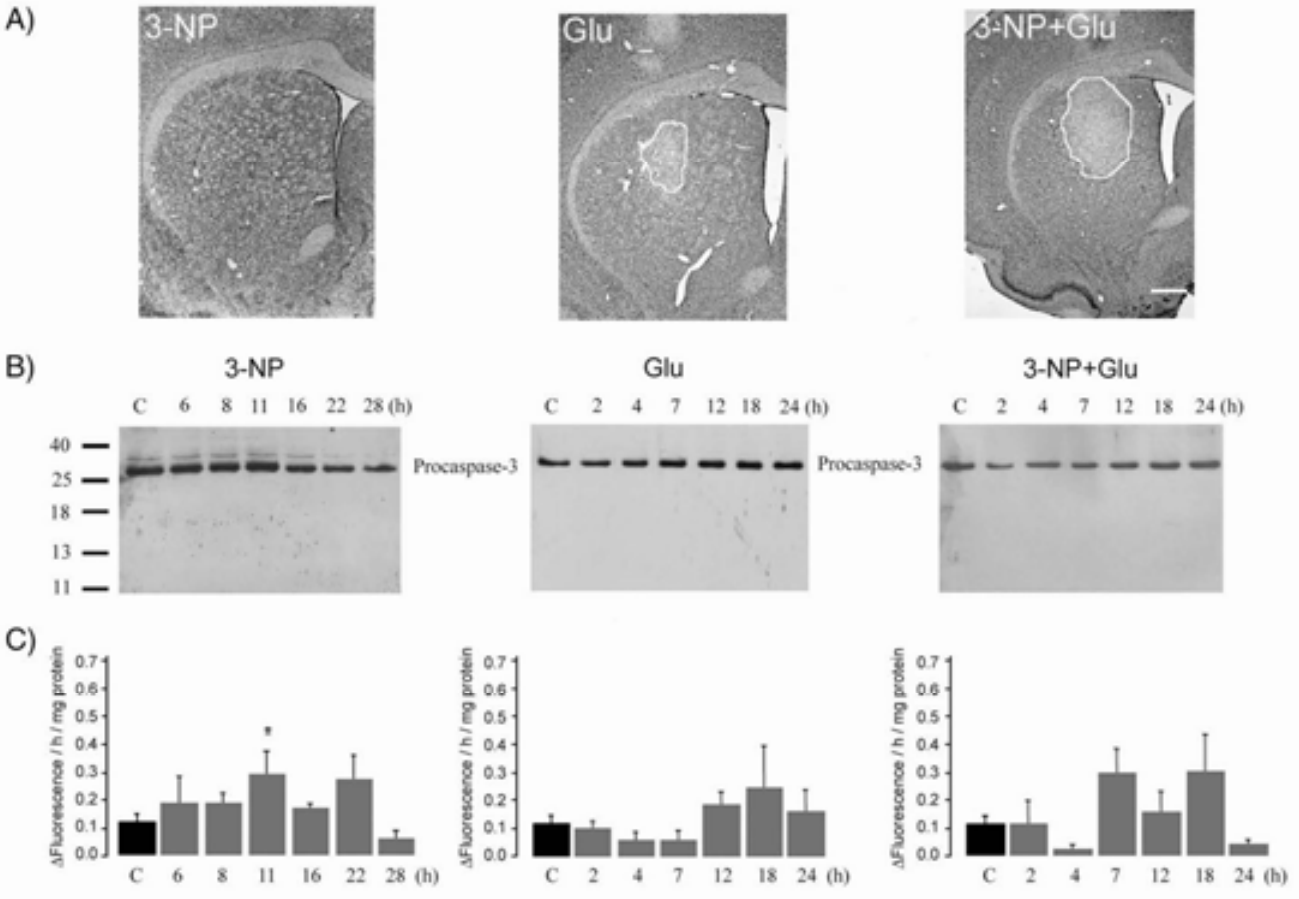

Fig. 1. A) Micrographs of representative tissue sections showing the lack of toxic effect of 3-NP treatment alone (3-NP, n = 6) and the lesions produced by glutamate (Glu, n = 7) in intact or 3-NP-pre-treated rats (3-NP+Glu, n = 7). In animals pre-treated with 3-NP+Glu an increase in the size of the lesion is clearly observed. Scale bar = 500 μm. B) Representative Western blots showing the precursor of caspase-3 (32 kDa) and the absence of its active fragment (12 kDa). Blots show that neither 3-NP treatment nor glutamate intrastriatal injection in intact or 3-NP pre-treated rats (Glu or 3-NP+Glu, respectively) activate caspase-3 at any of the times studied. C) Time-course of caspase-3 activation after the different treatments. Caspase activity was assayed by a fluorometric method using Ac-DEVD-AMC as substrate as described in the Materials and methods section. Results are expressed as the change in fluorescence intensity per hour per milligram protein. Data are means ± S.E.M. of 4 rats per time point. *$P \leq 0.05$ relative to control.

during 1.5 h in a solution containing 5% dry milk in TBS/tween 20 (TBS-T; 100 mM Tris–HCl , pH 7.5; 150 mM NaCl, 0.05% Tween 20) and then incubated overnight at 4 °C with anti-spectrin antibody (1:10,000) or anti-actin (1:12 000) (antibodies diluted in 5% dry milk containing TBS-T solution). Membranes were washed with TBS-T (10 min/3 times) and then incubated 2 h at room temperature with anti-rabbit horseradish peroxidase-conjugated antibody (1:10 000). They were washed in TBS-T (10 min/3 times) before peroxidase activity was detected. The peroxidase activity of all blots was detected using ECL reagent. Membranes were exposed to X-ray films and the resulting autoradiographies were scanned. The optical density (OD) values of delineated bands were obtained by means of the NIH Image/ Image J program. For this purpose, OD values of the 145 and 150 kDa bands were obtained as a single data because in the 3-NP+Glu group these two bands were seen as a single one. OD of actin bands was also obtained as loading control. Ratios of 145/150 kDa bands and actin were used for statistical analyses. We analyzed data of 4 animals per time point per treatment, with the exception of the 3-NP+Glu treatment where results of 5 animals were analyzed.

Statistics

All data are expressed as means ± SEM and were analyzed by one-way ANOVA. A post-hoc Fisher's least significant difference multiple comparison test was used to compare the differences between the activation times of caspase-3 and calpain (in Western blotting and fluorometry assays) within each individual treatment. The same test was used for comparisons between Glu receptor antagonist- and calpain

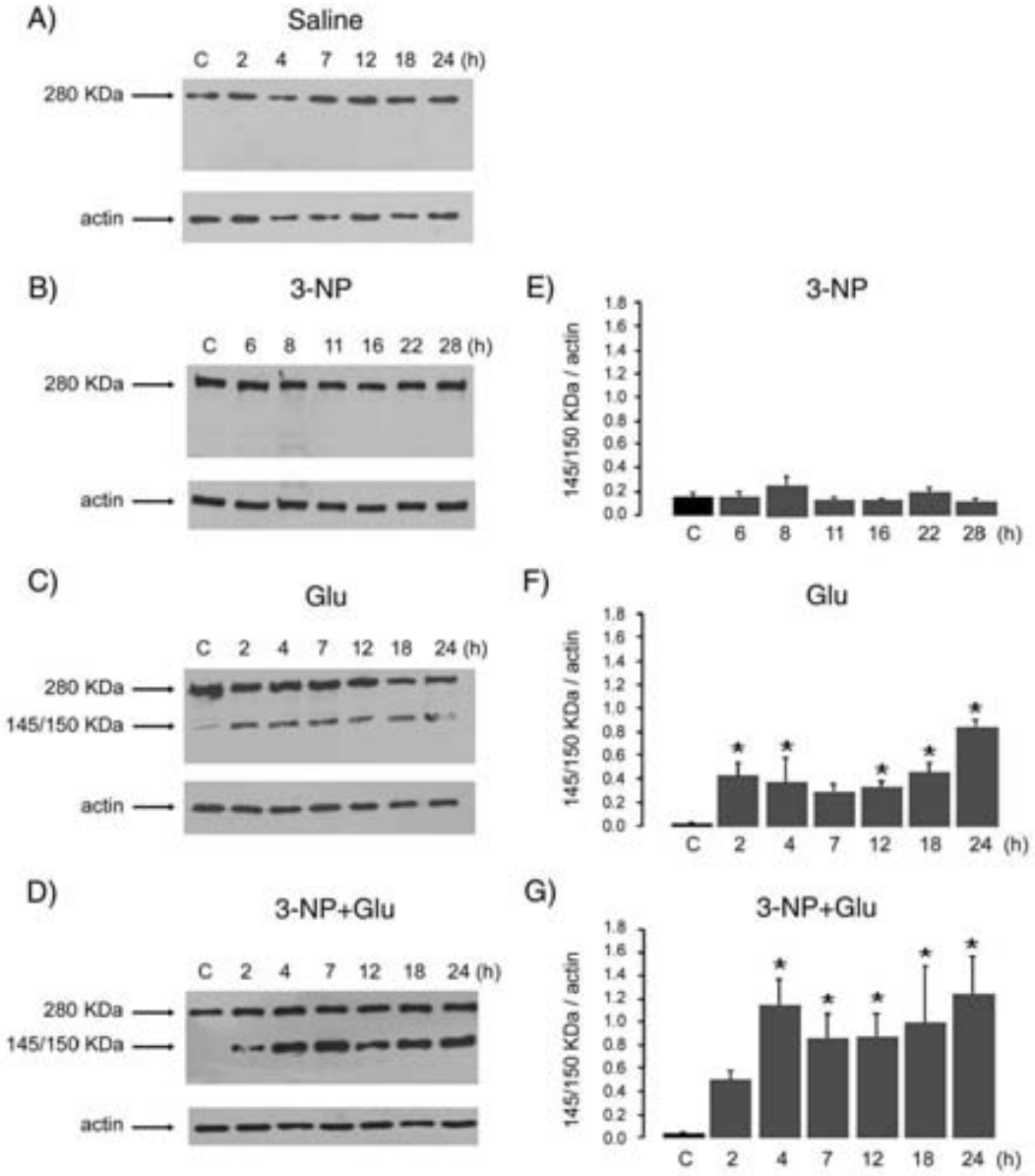

Fig. 2. Representative Western blots of spectrin and spectrin-breakdown products and densitometric analysis of the 145/150 kDa bands. Western blots (A–D) show the presence of the complete spectrin protein (280 kDa) and the bands produced by calpain (145 and 150 kDa). Rats were killed by decapitation at different times after 3-NP, Glu or saline injection. Graphs (E–G) show the densitometric analysis of the ratio of the densities of 145/150 kDa bands/actin. Data are means+S.E.M. of 4–5 rats per time point, per treatment. *$P \leq 0.05$ relative to control rats.

inhibitor-treatments relative to the 3-NP+Glu group (in calpain activity assay and lesion volume measurements). Differences were considered significant when $P \leq 0.05$ was obtained.

Results

Exacerbation of Glu neurotoxicity does not induce caspase-3 activation

In a recent report we have previously shown that the injection of 500 nmol of Glu into the striatum of intact rats does not injure the striatum beyond the damage induced by the intrastriatal administration of saline solution. If rats are systemically pre-treated with 3-NP, Glu becomes a potent toxin despite this mitochondrial inhibitor does not injure the striatum itself (Del Río et al., 2007). Fig. 1A shows representative micrographs of the striatum of rats treated only with 3-NP, intact rats injected with Glu, and rats pre-treated with 3-NP and intrastiatally injected with Glu (3-NP+Glu). Photographs illustrate that Glu intrastriatal injection in intact rats (Glu) induced a small lesion, shown as a pale area. When Glu is injected in 3-NP pre-treated rats (3-NP+Glu) the lesion increased in size. Damaged tissue appears as a pale region due to the lack of incorporation of cresyl violet and it is characterized by the presence of pyknotic nuclei and amorphous condensed cells. Quantification of the lesion volume showed that Glu-induced lesions in 3-NP pre-treated animals increases up to 4.9 fold compared to Glu injection in intact rats (Glu: 0.540 ± 0.059 mm^3; 3-NP+Glu: 2.675 ± 0.460 mm^3, $n = 6$–7, Fig. 6). These results agree with those published previously (Del Río et al., 2007). Rats treated only with 3-NP (Fig. 1A) showed no striatal lesion as previously reported (Del Río et al., 2007).

Since caspase-3 is one of the executioners of apoptotic cell death and its activation is promoted by mitochondrial dysfunction, we wanted to know if caspase-3 is related to the exacerbation of Glu toxicity during mitochondrial inhibition. With this purpose, we performed Western blot analyses using an antibody, which recognizes procaspase (32 kDa) and caspase-3 active fragments (17 and 12 kDa). Results in Fig. 1B show that neither 3-NP administration, nor Glu intrastriatal injection in intact rats (Glu) or in 3-NP pre-treated rats (3-NP+Glu), induce caspase activation at any of the times studied. Western blots show the presence of procaspase-3 (32 kDa) but the active fragments (17 or 12 kDa) were not observed (Fig. 1B). To corroborate the lack of activation of caspase-3 after the different treatments, we used a fluorometric method for measuring caspase-3 activity, by means of the fluorogenic substrate (Ac-DEVD-AMC), a specific substrate for caspase-3. Results corroborated that Glu injection does not activate caspase-3 neither in intact rats nor in 3-NP pre-treated rats at any of the times studied. A minor and transient activation of caspase-3 was observed at 11 h after the second administration of 3-NP (Fig 1C).

Glutamate induces calpain activation in intact and in 3-NP pre-treated rats

We next investigated whether calpain activation is related to Glu toxicity induced during mitochondrial inhibition. In immunoblotting studies an antibody was employed against α-spectrin, a well-known substrate for caspase-3 and calpain. Activated caspase-3 generates spectrin-breakdown products of 120 and 150 kDa; whereas calpain generates 145/150 kDa fragments. Fig. 2 shows representative Western blots of spectrin and spectrin-breakdown products at the times studied

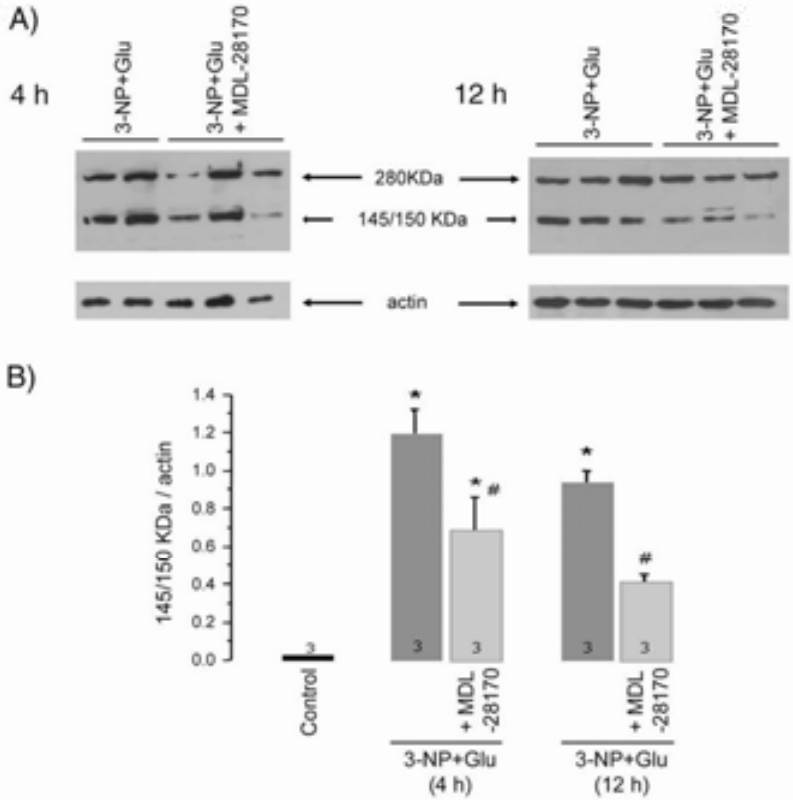

Fig. 3. Effect of the calpain inhibitor MDL-28170 on spectrin-breakdown products. Western blots (A) show the presence of the complete spectrin protein (280 kDa) and the bands produced by calpain (145 and 150 kDa) at 4 and 12 h after glutamate injection. Graph (B) shows the densitometric analysis of the ratio of the densities of 145/150 kDa bands/actin evaluated at 4 and 12 h after glutamate administration. The calpain inhibitor MDL-28170 was administered 30 min before and immediately after glutamate injection. *$P \leq 0.05$ relative to control; #$P \leq 0.05$ relative to 3-NP+Glu. Data are means±SEM from the number of animals indicated at the bottom of each bar.

after the different treatments, the graphs depicting the densitometric analysis of the ratio of the 145/150 kDa bands/actin band are also shown. None of the spectrin-breakdown products were observed after saline intrastriatal injection (Fig. 2A). Results illustrate the presence of the band corresponding to the complete spectrin protein (280 kDa) and the absence of the bands corresponding to the 120, 145 and 150 kDa fragments after 3-NP treatment alone (Fig. 2B, E), suggesting the lack of activation of caspase-3 and calpain in this condition.

Glu intrastriatal administration in intact rats produced a 145/150 kDa doublet from 2–24 h after the injection. The 120 kDa band was not observed in this condition, confirming that caspase-3 is not activated (Fig. 2C). When Glu was injected in 3-NP pre-treated rats, the 145/150 kDa bands were present from 2–24 h after the injection and the doublet appeared as one intense band, suggesting a stronger activation of calpain in this condition (Fig. 2D). Densitometric analysis shows that Glu in intact rats activated significantly calpain 2–24 h after the injection (Fig. 2F). The densitometric analysis of the 145/150 kDa bands produced by Glu in 3-NP pre-treated rats show that calpain is similarly activated at 2 h, but its activity is more than two fold enhanced 4 h after the injection (Fig. 2G). The 120 kDa band corresponding to the fragment produced by caspase-3 activity was not observed in this condition (Fig. 2D).

In order to know whether calpain activation is associated with the exacerbation of Glu toxicity induced by 3-NP, the calpain inhibitor MDL-28170 was administered 30 min before and immediately after Glu intrastriatal injection. Brains were processed for histological evaluation of the injured striatum and for calpain activation. Fig. 3 shows that administration of the calpain inhibitor significantly reduced the density of the 145/150 kDa bands, as assessed 4 and 12 h after Glu intrastriatal injection, times at which calpain was substantially activated. Treatment with MDL-28170 reduced the density of 145/150 kDa bands by 42 and 56% relative to 3-NP+Glu at 4 and 12 h, respectively (Fig. 3B). Reduction of calpain activation was accompanied by a significant 62% reduction in the lesion volume (Fig. 4D), suggesting that calpain activity is related to tissue damage induced by Glu in 3-NP pre-treated rats. Representative tissue sections from treated animals are shown in Fig. 4; it can be observed that the size of the lesion is substantially reduced in animals treated with the calpain inhibitor as compared to those treated with 3-NP+Glu (4B and 4C). The lesion volume was not reduced when rats were i.p. injected with DMSO (the MDL-28170 vehicle) (in mm^3, 3-NP+Glu = 3.709 ± 0.99; 3-NP+Glu+DMSO = 3.820 ± 1.185, $n = 4$).

Calpain activation after glutamate injection is partially dependent on glutamate ionotropic receptor activation

Activation of Glu receptors induces the influx of Ca^{2+} ions, which could in turn mediate calpain activation. Therefore, we next aimed to know if calpain activation after Glu injection was dependent on the stimulation of a particular receptor subtype. Rats were treated with the Glu NMDA and non-NMDA receptor antagonists, MK-801 and NBQX, respectively, and brains were processed for the quantification of the lesion volume at 24 h, or for the determination of calpain activation at 12 h. Animals treated either with MK-801 or NBQX showed a significant reduction of the lesion size, although those receiving both antagonists do not show a further reduction of the lesion volume (Fig. 5). Fig. 5A shows photographs of representative coronal sections at the level of the striatum from animals subjected to the different treatments. Injured tissue is observed as a pale region. Quantitative data shown in graph (Fig. 5B) indicates that the size of the lesion is partially but significantly (50%) reduced in animals receiving the Glu receptor antagonists.

We next aimed to know whether the protective effect of Glu receptor antagonists was due to diminished activation of calpain. Therefore, calpain activation was assessed 12 h after Glu injection in rats treated with or without Glu antagonists. Results show that treatment

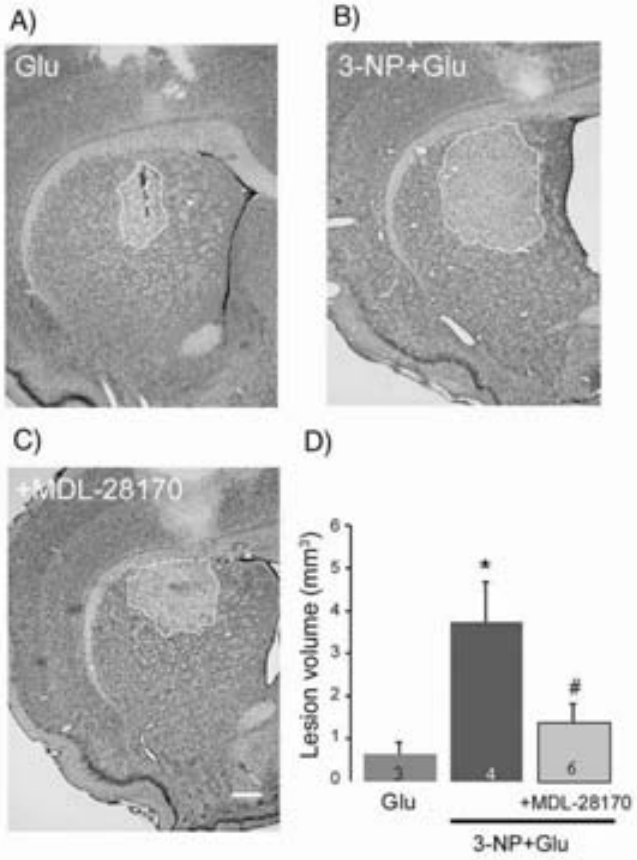

Fig. 4. Effect of the calpain inhibitor MDL-28170 on the lesion volume induced by glutamate in 3-NP pre-treated rats. A–C show micrographs of representative tissue sections of the striatum of rats subjected to the different treatments. Lesions are characterized by a pale area due to absence of incorporation of cresyl violet. Scale bar = 500 μm. The graph (D) shows the quantification of the lesion volume induced by glutamate in rats subjected to the different treatments. Volume lesion was quantified 24 h after glutamate injection. *$P \leq 0.05$ relative to Glu, #$P \leq 0.05$ relative to 3-NP+Glu. Data are means ± SEM from the number of animals indicated at the bottom of each bar.

with antagonists partially reduces calpain activation (close to 50%) in all cases. Fig. 6A illustrates representative Western blots showing the effect of the different treatments on the density of the 145/150 kDa bands. When MK-801 was administered before Glu in 3-NP pre-treated rats, calpain activation was evident due to the presence of the 145/150 kDa bands, but the density of these bands was clearly reduced. Similarly, when NBQX was co-administered with Glu in 3-NP-treated rats, the 145/150 kDa bands were reduced in density. When 3-NP-treated rats were pre-treated with MK-801 and co-injected with Glu and NBQX, the density of the 145/150 bands was similarly reduced as in rats treated individually with each antagonist alone (Fig. 6A,B). This result is in agreement with histological data showing that treatment with both Glu antagonists does not exert further protection than that elicited by each antagonist alone.

Discussion

The present findings agree with previous observations from our group showing that increasing the extracellular concentration of Glu through the inhibition of its reuptake or by the intrastriatal injection of a low concentration of Glu, does not lead to neuronal damage unless mitochondrial energy metabolism is disrupted with 3-NP (Sánchez-Carbente and Massieu, 1999; Massieu et al., 2001; García and Massieu, 2003; Del Río et al., 2007). Since mitochondrial dysfunction and augmented intracellular calcium favor the activation of proteases such

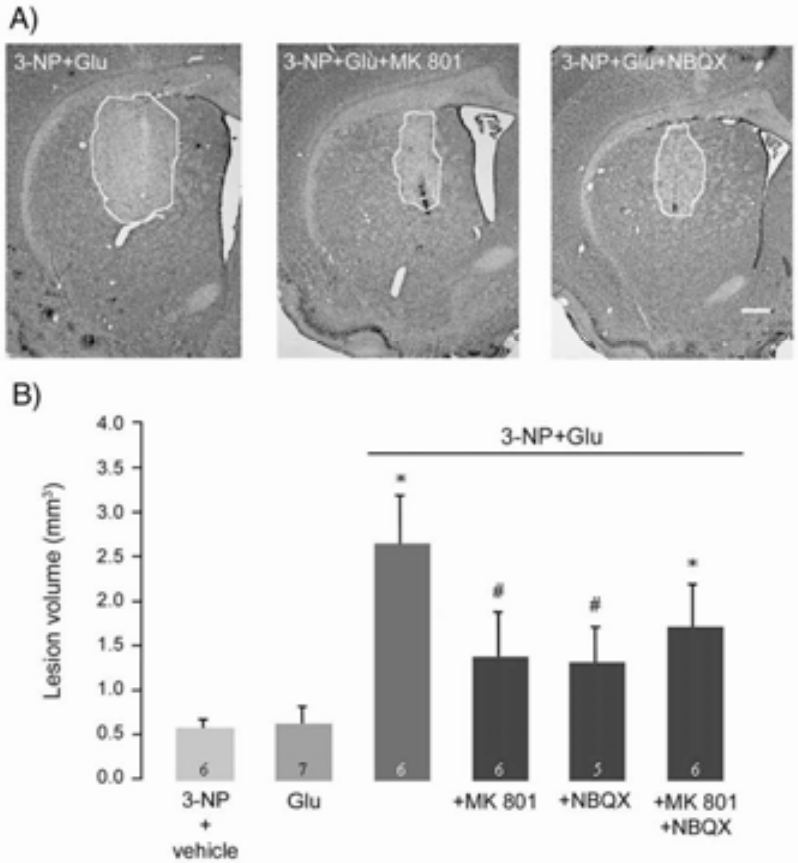

Fig. 5. Effect of glutamate receptor antagonists on tissue damage in rats subjected to the different treatments. Representative tissue sections of animals treated with MK-801 or NBQX are shown in A. Scale bar = 500 μm. Graph shows (B) that the lesion volume is partially reduced in animals treated with glutamate receptor antagonists as compared to those treated only with 3-NP+Glu. Data are means+SEM from the number of animals indicated at the bottom of each bar. *$P \leq 0.05$ relative to control rats, #$P \leq 0.05$ relative to 3-NP+Glu rats.

as caspases and calpains, we now studied the time-course of the activation of these proteases and its relation to exacerbated Glu toxicity.

Caspase-3 is known as one of the executioners of the apoptotic cascade; it has been shown to be activated after excitotoxic insults (Brecht et al., 2001; Tenneti and Lipton, 2000; Puig and Ferrer, 2002; Tomioka et al., 2002), ischemic periods (Benchoua et al., 2001; Davoli et al., 2002; Zhang et al., 2002; Ferrer et al., 2003), and chronic or acute treatments with SDH inhibitors (Bizat et al., 2003a; Paucard et al., 2004). We now show that the exacerbation of Glu toxicity during mitochondrial inhibition is not mediated by caspase-3. Although a transient caspase-3 activity was observed after 3-NP treatment by the fluorometric analysis, this activation was not confirmed by Western blot. Therefore, it is likely that this activation is not related to a cell death stimulus, because the dose used of 3-NP effectively inhibits SDH but does not injure the striatum (Del Río et al., 2007). Although caspase-3 activation has been reported after acute treatments with 3-NP, this activation is transitory and occurs only in the injured brain areas (Bizat et al., 2003a,b), a condition that is not present in our experimental conditions.

Calpain, a calcium-dependent protease, has been related to excitotoxic and necrotic cell death (Siman and Noszek, 1988; Seubert et al., 1989; Blomgren et al., 1995; Bednarski et al., 1995; Higuchi et al., 2005; Takano et al., 2005; Zhu et al., 2005). However, the contribution of NMDA and non-NMDA ionotropic receptors to calpain activity and to the exacerbation of Glu toxicity had not been studied; to our knowledge this is the first study addressing this question. Calpain activation was evaluated by the recognition of spectrin-breakdown products. Spectrin is a cytoskeletal protein that is a substrate for both caspase-3 and calpain (Siman et al., 1984; Nath et al., 2000; Wang, 2000), and has been widely used to detect the activation of these proteases (Bahr et al., 1995; Roberts-Lewis and Siman, 1993; Roberts-Lewis et al., 1994). The present results demonstrate that calpain is not activated in rats treated only with 3-NP. Results shown here demonstrate that calpain is activated after Glu injection, and most importantly, that its relation to neuronal damage is dependent on the magnitude of its activation. According to quantitative data, results show that after Glu injection the levels of calpain-generated spectrin-breakdown products are lower in intact rats as compared to 3-NP pre-treated. Calpain activation in intact rats is not related to Glu toxicity because at the dose injected Glu does not injure the tissue beyond the damage caused by vehicle injection. Calpain-mediated spectrin cleavage products are very stable and resistant to further degradation (Vanderklish and Bahr, 2000; Czogalla and Sikorski, 2005), this might explain the presence of the 145/150 kDa bands during the following 24 h after Glu injection. Transient disturbances in intracellular calcium concentrations after Glu administration are possibly related to the observed calpain activity in intact animals.

Results shown in this study lead us to conclude that only a strong and sustained calpain activation is related to the exacerbation of Glu toxicity during mild mitochondrial inhibition; first, because low calpain activation is not related to neuronal death in intact rats and second, because its inhibition reduces the extent of the lesions in 3-NP pre-treated rats. According to quantitative data the levels of the 145/150 kDa bands induced by Glu were about 3-fold higher in 3-NP-treated animals. In a recent study we have investigated the time-course of Glu-induced striatal injury in 3-NP pre-treated rats, and showed that tissue damage is

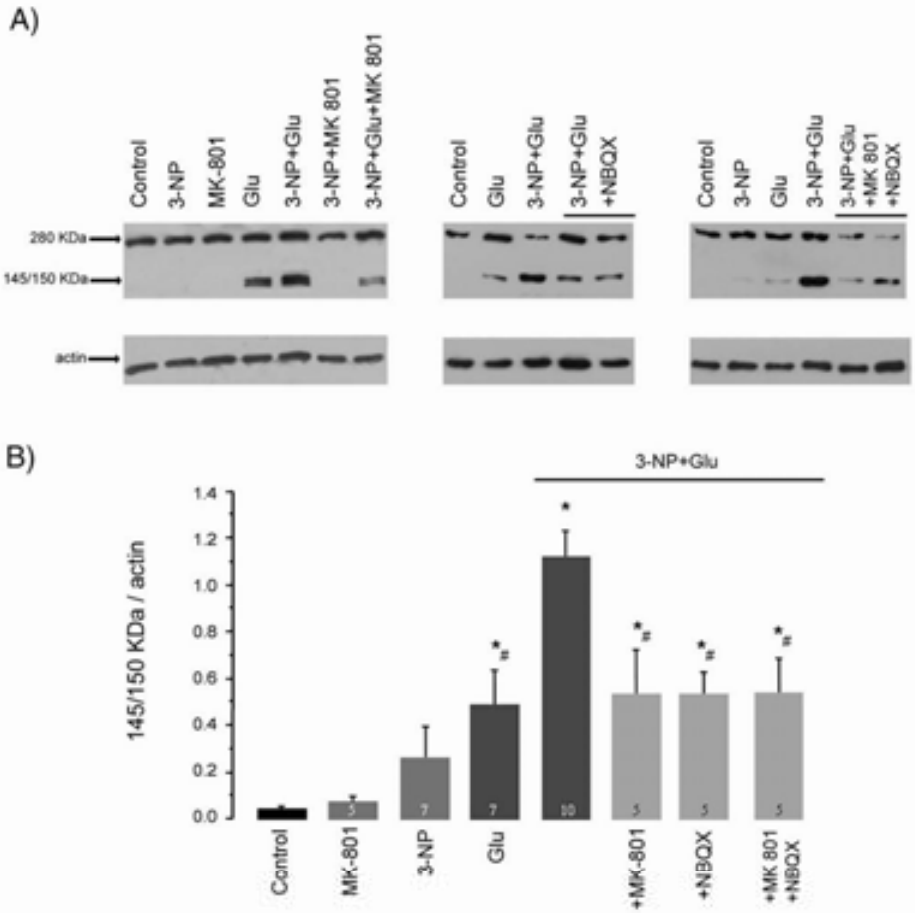

Fig. 6. Effect of glutamate receptor antagonists on the production of spectrin-breakdown products generated by calpain activation. Western blots (A) show the presence of the complete spectrin protein (280 kDa) and the bands produced by calpain (145 and 150 kDa). Rats were treated with the glutamate antagonists MK-801 and NBQX and brains obtained 12 h after glutamate injection. Graph shows the densitometric analysis of the ratio of the densities of 145/150 kDa bands/actin (B). Data are means+SEM from the number of animals indicated at the bottom of each bar. *$P \leq 0.05$ relative to control rats, #$P \leq 0.05$ relative to 3-NP+Glu rats.

already obvious 4 h after Glu injection, as judged by the presence of pyknotic cells (Del Río et al., 2007). Here we demonstrate that calpain is robustly activated 2 h after Glu administration, before cell damage is evident. Supporting this conclusion we observed that the administration of the calpain inhibitor MDL-28170, induced a sustained inhibition of calpain activity (from 4–12 h after Glu injection) and substantially reduced the size of the excitotoxic lesions. MDL-28170 is a potent inhibitor of μ and m calpain that enters the brain very rapidly and inhibits calpain activity 30 min after its administration (Markgraf et al., 1998). It has previously been shown that MDL-28170 protects from ischemic, hypoxic and excitotoxic damage induced by Glu agonists (Li et al., 1998; Markgraf et al., 1998; Kawamura et al., 2005). Furthermore, NMDA-induced calpain activation is proportional to the number of stimulated receptors and the duration of the stimulus (Bahr et al., 1995; Vanderklish et al., 1995; del Cerro et al., 1994). 3-NP might facilitate Glu receptors activity promoting calpain activation. Exposure of corticostriatal slices to 3-NP partially depolarizes the membrane, a condition that allows NMDA receptors to be easily activated (Saulle et al., 2004).

Robust calpain activation is related to neuronal death when trriggered after sustained stimulation of Glu receptors by its selective agonists (del Cerro et al., 1994; Bahr et al., 1995; Jourdi et al., 2005; Takano et al., 2005). NMDA and non-NMDA receptor antagonists have previously been shown to inhibit cell death and spectrin cleavage (Seubert et al., 1989; Siman et al., 1989; Jourdi et al., 2005; Del Río et al., in press). The present study shows that enhancement of Glu toxicity during mitochondrial inhibition is partly mediated by the activation of Glu ionotropic receptors because neither MK-801 nor NBQX, individually or in combination, reduced completely striatal lesions. Therefore, we can conclude that neuronal death is not only due to the stimulation of ionotropic Glu receptors. Calcium influx through Glu receptors occurs mainly through the NMDA receptor subtype; however, non-NMDA receptors can also be permeable to calcium, depending on their subunit combination (Ozawa et al., 1998). Consistent with the observation that calpain activation is highly vulnerable to intracellular calcium oscillations (Czogalla and Sikorski, 2005), we observed that both ionotropic receptor subtypes contributed equally to calpain activity. Furthermore, the protective effect of Glu receptor antagonists was closely related to calpain inhibition, because they reduced calpain activity to a similar extent as they reduced striatal lesions (close to 50%). Moreover, calpain inhibition by Glu receptor antagonists was very similar to that exerted by the calpain inhibitor, MDL-28170.

In conclusion, the facilitatory effect of mitochondrial inhibition on Glu neurotoxicity cannot be explained by the recruitment of a caspase-dependent apoptotic process but to the enhancement of calpain activity, possibly related to the loss of intracellular calcium homeostasis. Other recent evidence supports the importance of calpain, rather than caspase-mediated injury, in mature brain (Takano et al., 2005; Zhu et al., 2005). Calpain is suggested to contribute to excitotoxic neuronal death by targeting components essential for cell survival such as cytoskeletal proteins, enzymes, and membrane proteins (Pant, 1988; Siman et al., 1989; Brana et al., 1999; Rami, 2003; Bano et al., 2005; Lopez-Picon

et al., 2006; Xu et al., 2007). Further studies concerning the consequences of calpain overactivation during mitochondrial inhibition may be of relevance for the treatment of neurodegenerative diseases associated with alterations in energy metabolism and excitotoxicity.

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Artículo 3

"Contribution of NMDA and Non-NMDA receptors to in *vivo* glutamate-induced calpain activation in the rat striatum. Relation to neuronal damage."

Del Río P., Montiel T. y Massieu L 2008

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Este artículo muestra los datos concernientes a la participación de la calpaína y de los receptores ionotrópicos glutamatérgicos en el daño neuronal severo inducido por glutamato. La importancia de este artículo radica en que se muestran datos *in vivo* en donde se utilizó el ligando endógeno: el glutamato. Estudios previos relacionados con el papel de la caspasa-3 y la calpaína fueron realizados con agonistas glutamatérgicos. En resumen, los resultados más importantes son:

1) La muerte neuronal inducida por glutamato es independiente de la activación de la caspasa-3 y la principal proteasa involucrada en este proceso es la calpaína.

2) El daño severo inducido por glutamato está asociado con la activación intensa de calpaína. Aparentemente esta activación sobrepasa los mecanismos excitotóxicos, dado que la inhibición de los receptores glutamatérgicos previene parcialmente el daño inducido por el glutamato.

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ORIGINAL PAPER

Contribution of NMDA and Non-NMDA Receptors to In vivo Glutamate-Induced Calpain Activation in the Rat Striatum. Relation to Neuronal Damage

Perla Del Río · Teresa Montiel · Lourdes Massieu

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Abstract Glutamate, the major excitatory neurotransmitter, can cause the death of neurons by a mechanism known as excitotoxicity. This is a calcium-dependent process and activation of the NMDA receptor subtype contributes mainly to neuronal damage, due to its high permeability to calcium. Activation of calpain, a calcium-dependent cysteine protease, has been implicated in necrotic excitotoxic neuronal death. We have investigated the contribution of NMDA and non-NMDA ionotropic receptors to calpain activation and neuronal death induced by the acute administration of glutamate into the rat striatum. Calpain activity was assessed by the cleavage of the cytoskeletal protein, α-spectrin. Caspase-3 activity was also studied because glutamate can also lead to apoptosis. Results show no caspase-3 activity, but a strong calpain activation involving both NMDA and non-NMDA receptors. Although neuronal damage is mediated mainly by the NMDA receptor subtype, it can not be attributed solely to calpain activity.

Keywords NMDA · AMPA · Kainate · Caspase-3 · Calpain · Neurotoxicity

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P. Del Río · T. Montiel · L. Massieu
Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico, D.F 04510, Mexico

L. Massieu (✉)
Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, CP 04510, AP 70-253, Mexico, D.F, Mexico
e-mail: lmassieu@ifc.unam.mx

Introduction

Glutamate, the major excitatory neurotransmitter in the mammalian brain, leads to the death of neurons whenever its concentration rises and its receptors are active during prolonged periods of time. Stimulation of NMDA and non-NMDA glutamate receptors by selective agonists triggers neuronal death by a process known as excitotoxicity [1–3]. It has been widely accepted that the entry of calcium through the NMDA receptor subtype contributes substantially to the induction of excitotoxic neuronal death [4–6]. Calcium entry induces the activity of several enzymatic processes involved in the degradation of a number of cell components such as endonucleases, phopholipases and proteases, as well as enzymes involved in the production of reactive oxygen species [7, 8]. Calpains are calcium-activated cystein proteases involved in physiological and pathological processes. Calpain activity is required for the induction of long-term potentiation [9–12] and is involved in necrotic cell death through the degradation of several substrates essential for cell survival, including enzymes, transcription factors, receptors, transporters, channels and proteins of the cytoskeleton such as spectrin [13, 14]. Calpain activation has been implicated in excitotoxicity triggered during pathological conditions such as ischemia [15–17], or by the activation of NMDA and non-NMDA glutamate receptors by selective agonists in in vitro and in vivo conditions [10, 18–20]. The NMDA receptor subtype is particularly involved in glutamate-mediated neuronal damage due to its high permeability to calcium ions [21, 22]. Therefore, it was reasoned that NMDA receptors stimulation, rather than kainate or AMPA, would be particularly involved in calpain activation and neuronal death elicited by the acute administration of glutamate into the rat striatum. Using the selective antagonists of NMDA

and non-NMDA receptors, MK-801 and NBQX respectively, we investigated the contribution of each ionotropic receptor subtype to calpain activation induced by in vivo glutamate injection, and studied its relation to the induction of striatal lesions. The role of calpain in glutamate-mediated lesions was also studied using the potent inhibitor of μ and m calpain, MDL-28170, which rapidly penetrates into the brain and inhibits calpain activity after its systemic administration [23]. The potential role of caspase-3, an executor protease related to apoptotic cell death, on striatal lesions was also studied, since glutamate can lead to necrotic or apoptotic neuronal death depending on the severity of the stimulus [24–26]. While calpain activation resulting from increased $[Ca^{2+}]_i$ has been related to necrosis, caspase activity has been associated with an apoptotic phenotype [13, 27]. Results show that although calpain is the main protease activated by glutamate it is only partially implicated in the induction of neuronal death. Results also suggest that both ionotropic receptor subtypes are involved in calpain activation, but the NMDA receptor is mainly involved in neuronal death.

Experimental Procedures

Materials

Glutamate, cresyl violet, N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), sucrose, 3-[3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) and DL-Dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO, USA). MDL-28170 was obtained from Biomol (Plymouth Meeting, PA, USA). The glutamate receptors antagonists (+)-5-methyl-10,11,-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801) and 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo[f] quinoxaline-7-sulfonamide disodium (NBQX) were purchased from Tocris (Cookson Ltd., Avonmouth, UK). Protease inhibitor cocktail was obtained from Roche (Meylan, France). Ac-DEVD-AMC was purchased from Peptide Institute (Louisville, KY, USA). Antibodies against spectrin and actin were obtained from Chemicon (Temecula, CA, USA). Antibody against caspase-3 was from Santa Cruz Biotechnology (CA, USA). Anti-mouse or anti-rabbit IgG were from Jackson Immunoresearch (West Grove, PA, USA). ECL reagent was purchased from Millipore (Billerica, MA, USA).

Animal Treatment

Male Wistar rats (250–300 g) were used throughout the study. They were handled according to the National Institute 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. Intrastriatal glutamate (sodium salt) injections were performed according to previously reported methodology in halothane-anesthetized rats [28]. Briefly, 1 µl of either glutamate (2 µmol) or vehicle solution (0.9% saline) was injected at a rate of 0.5 µl/min via a 27-gauge stainless steel needle connected to a Hamilton syringe with the aid of a microinjection pump (Harvard apparatus pump model 55, South Natick, MA, USA). Coordinates used were: AP +0.7 mm anterior from bregma, L +2.8 mm from midline, and −4.0 mm from the dura, according to Paxinos and Watson [29]. Animals were sacrificed at different times after glutamate administration and brains were prepared for histological evaluation, Western blotting and caspase-3 activation (see below). Although we did not perform behavioural or electroencephalographic studies, we observed that approximately 30% of all rats treated with glutamate showed head weaving immediately after Glu injection. This behaviour remained no longer than 10 min, after that, rats did not show other behavioural disturbance.

For neuroprotection experiments rats were treated either with the NMDA receptor antagonist MK-801, the non-NMDA receptor antagonist NBQX, or with both antagonists. MK-801 (2 mg/kg) was intraperitoneally (i.p.) injected 30 min before glutamate intrastriatal injection. NBQX (50 nmol) was intrastriatally co-injected with 1 µl of glutamate solution. An additional group of animals was treated i.p. with NBQX (20 mg/kg) 15 min before, immediately after and 15 min after glutamate administration (60 mg/kg in total). MK-801 and NBQX were dissolved in saline solution (NaCl 0.9%). Two additional groups were treated with the calpain inhibitor MDL-28170, the first group was injected i.p. 15 min before (20 mg/kg), and 45 min and 1.45 h (15 mg/kg each one) after glutamate injection (50 mg/kg in total). The second group was injected i.p. 30 min before (30 mg/kg), immediately after, and 1 h (20 mg/kg each one) after glutamate injection (70 mg/kg in total). Brains were processed for histological analysis or for caspase-3 and calpain activity determinations.

Histological Evaluation

Animals were anesthetized with sodium pentobarbital anaesthesia 24 h after glutamate injection 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. Brains were then

transferred to a 20% and 30% sucrose solution successively. Coronal sections (40 µm) were obtained in a cryostat and stained with cresyl violet. Lesion volume was calculated as described previously [28]. Briefly, lesion size was calculated by examination of all brain sections where neuronal damage was evident in each experimental animal. Damaged area (recognized as a pale region containing pyknotic nuclei and shrinked soma) was delineated manually and measured with the aid of an image analyzer (NIH Macintosh Image 1.6). The lesion volume was calculated by adding the measured areas in all sections and multiplying the sum by the distance between the first and the last section where damage was visible. Results are expressed as means ± SEM of lesion volume per each animal group.

Caspase 3 Activation

For caspase-3 activity determination, intact or glutamate injected rats were sacrificed 2, 4, 7, 12, 18 and 24 h after glutamate. After dissection, the striatum was homogenized in caspase buffer containing: 100 mM HEPES, 10% (w/v) sucrose, 0.1% (w/v) CHAPS, 10 mM DTT, 1 mM EDTA, and protease inhibitor cocktail. Caspase-3 activity was assayed by a fluorometric method in a luminescence spectrometer (Spectronic Instruments, SLM Aminco-Bowman, Rochester, NY, USA) using the tetrapeptide acetyl-Asp-Glu-Val-Asp-(4-methylcoumaryl-7-amide) (Ac-DEVD-AMC) as substrate, as reported previously [30]. Activity was followed for 15 min after the addition of substrate (2.5 µM) and 50 µg of protein from striatal homogenates, to 2.0 ml of a standard solution containing: 100 mM HEPES, 10% (w/v) sucrose, 0.1% (w/v) CHAPS, 10 mM DTT, 1 mM EDTA, and protease inhibitors. Results are expressed as the change in fluorescence intensity per hour per milligram of protein produced after substrate cleavage.

Western Blotting

Caspase-3 and calpain activation was assessed by Western blotting. Caspase-3 activation was evaluated by means of an antibody that recognizes both pro-caspase (32 kDa) and the active fragments of caspase-3 (17 and 12 kDa). Calpain activation was assessed by the utilization of an antibody recognizing the complete protein and the breakdown products of α-spectrin. Spectrin is a substrate for calpain and caspase-3 leading to breakdown products of 145 and 150 kDa and of 150 and 120 kDa, for calpain and caspase-3, respectively. Recognition of the different breakdown products has been used as an index of caspase-3 and calpain activation [31–33]. At different times after glutamate injection the striatum was dissected and homogenized in 300 µl of ice-cold buffer containing 25 mM HEPES-KOH (pH 7.4), 0.1% Triton X-100, 5 mM $MgCl_2$, 1.3 mM EDTA, 1 mM EGTA, and a cocktail of protease inhibitors, according to Bizat et al. 2003 [34]. Homogenates were centrifuged at 15,000 g for 30 min, and the supernatant stored at −80°C until analysis. Protein concentration was quantified by the Bradford's method [35]. For Western blot, 30 or 10 µg of supernatant protein, for caspase-3 and spectrin respectively, were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes. The membranes were blocked for 1.5 h with 5% nonfat dry milk in TBS, for caspase-3; and with 5% bovine albumin in TBS for actin and spectrin. Blots were incubated overnight at 4°C with antibodies rose against spectrin (1:10,000) or caspase-3 (1:100). Actin was used as loading control (1:12,000). The blots were incubated with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:10,000) for 2 h, and peroxidase activity was detected using ECL reagent. The membranes were exposed to X-ray films and the resulting autoradiographies were scanned. Optical density (OD) values of bands were obtained by means of the NIH Image/ Image J program.

Statistics

All data are expressed as means ± SEM. Statistical analysis was carried out by one-way ANOVA followed by a Fisher's least significant difference multiple comparison test.

Results

Caspase-3 activity was determined at different times after glutamate administration by Western blot analysis using an antibody, which recognizes procaspase-3 (32 kDa) and caspase-3 active fragments (17 and 12 kDa). Representative Western blots in Fig. 1a show the presence of the procaspase-3. None of the active fragments were detected at any of the times studied. To corroborate the lack of activation of caspase-3, enzyme activity was measured fluorometrically by means of the fluorogenic substrate (Ac-DEVD-AMC), a specific substrate for caspase-3. Graph in Fig. 1b indicates that no activity of caspase-3 was present in striatal homogenates from rats injected with glutamate, at any of the times studied.

Calpain activity was studied at different times after glutamate administration as assessed by Western blot using an antibody against α-spectrin, which is a substrate for caspase-3 and calpain. Activated caspase-3 produces breakdown products of 120 and 150 kDa, whereas calpain generates 145/150 kDa fragments. Figure 1c shows

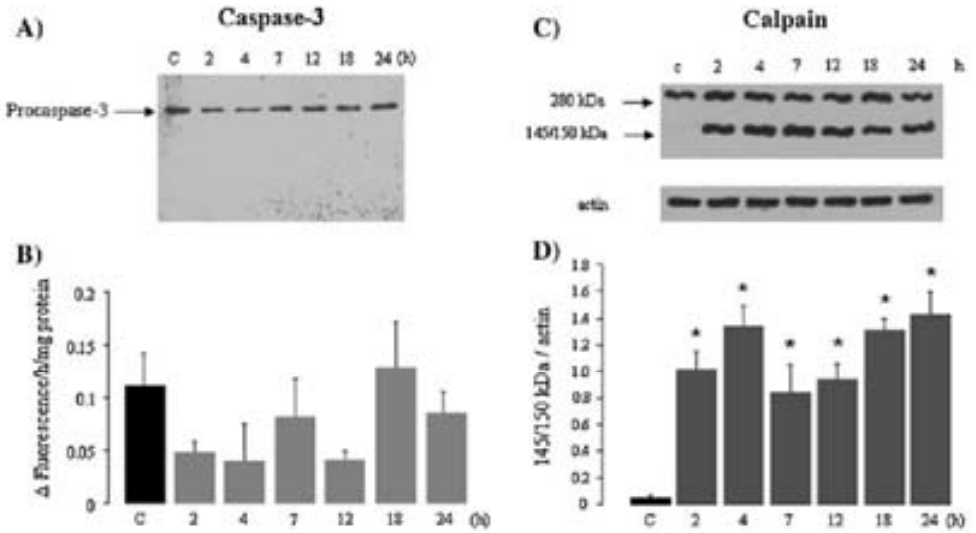

Fig. 1 Time-course of caspase-3 and calpain activation after intrastriatal injection of glutamate (2 μmol). (**a**) Representative Western blot showing the precursor of caspase-3 (32 kDa) and the absence of its active fragment (12 kDa). (**b**) Graph showing caspase-3 activity assayed by a fluorometric method using Ac-DEVD-AMC as substrate, as described in the methods section. Results are expressed as the change in fluorescence intensity per hour per milligram protein. Data are means ± S.E.M. of 4 rats per time point. (**c**) Representative Western blot of spectrin and spectrin breakdown-products and densitometric analysis of the 145/150 kDa bands. Western blot shows the presence of the complete spectrin protein (280 kDa) and the bands produced by calpain (145 and 150 kDa). (**d**) Graph shows the densitometric analysis of the ratio of the densities of 145/150 kDa bands/actin band. Data are means ± S.E.M. of 4 rats per time point, $^{*}P \leq 0.05$ relative to control rats

representative Western blots of spectrin and spectrin-breakdown products at the times studied after glutamate administration, and the graph shows the densitometric analysis of the ratio of the 145/150 kDa bands/actin band (Fig 1d). Results illustrate the presence of the bands corresponding to the complete spectrin protein (280 kDa) and the 145 and 150 kDa fragments, suggesting calpain activity. The presence of the 145/150 kDa bands was clearly observed 2 h after glutamate injection, the shortest time studied, and continued to be present during the following 24 h. The 120 kDa band was not detected at any of the times studied discarding the activation of caspase-3. None of the spectrin-breakdown products were observed after saline intrastriatal injection (not shown).

In order to study the role of ionotropic glutamate receptors on glutamate-mediated calpain activity, we administered the glutamate receptor antagonists MK-801 and NBQX and analyzed calpain activation. We used doses and administrations protocols of glutamate receptor antagonists based on previous studies showing they are effective to prevent excitotoxic lesions in vivo [36–39]. As shown in Fig. 2a and b, individual treatments with MK-801 and NBQX had no effect on calpain activity as assessed 12 h after glutamate administration, while treatment with both antagonists reduced significantly in 40% calpain activation (Fig. 2a and b).

We next sought to determine the contribution of the ionotropic glutamate receptor subtypes to striatal damage. For this purpose we determined the lesion volume in the striatum of rats injected with glutamate and with the different glutamate receptor antagonists. Micrographs of striatal tissue of rats subjected to the different treatments are shown in Fig. 3. Lesions are indicated as pale tissue areas due to the absence of cresyl violet incorporation. Magnification of an area in the vicinity of the injection site of a vehicle-injected animal, shows the presence of some pyknotic nuclei (black arrows) intermingled with many healthy cells similar to those observed in an intact control tissue (white arrows). In the case of glutamate-injected animals, magnification of the lesioned area shows the presence of mainly pyknotic nuclei, while no healthy cells are visible. When animals were treated with MK-801, the damaged area is considerably smaller, although inside the lesion many pyknotic cells are present and only a few healthy cells are visible (white arrows). In the case of animals treated with NBQX, the lesion size was not reduced significantly (Fig. 4) but large normal appearing cells are present inside the damaged area (white arrows in Fig. 3)

Quantification of the lesion volume shown in Fig. 4 indicates that glutamate administration induces an extensive lesion in the striatum up to 10-fold larger than that induced by saline injection (vehicle). Treatment with MK-801 reduced significantly in 47% the lesion volume while co-injection of glutamate with NBQX did not reduce the size of the lesion. In previous studies we have observed protection against in vivo induced excitotoxic lesions by the systemic treatment with NBQX [38, 39]. Therefore, the effect of this treatment on glutamate-mediated damage was

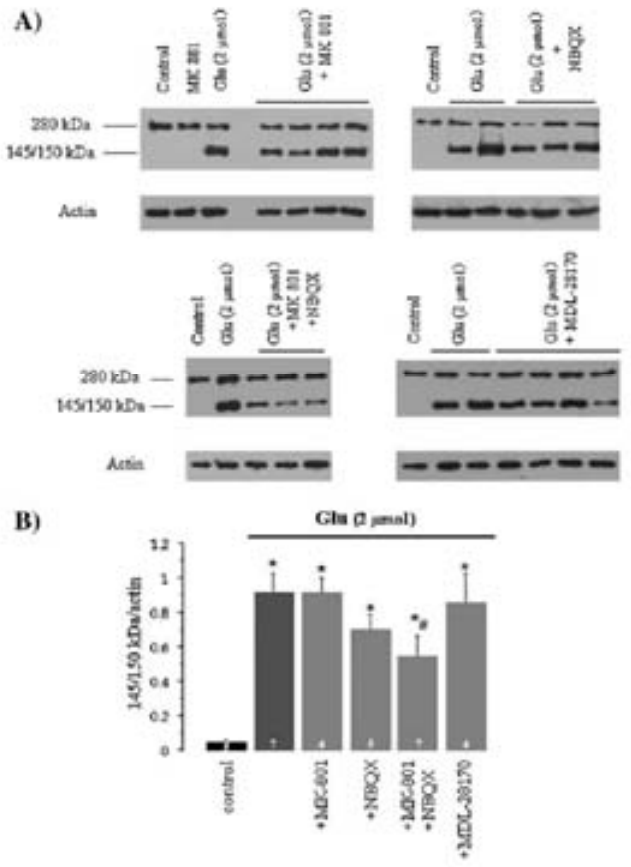

Fig. 2 Effect of glutamate receptor antagonists and of the calpain inhibitor MDL-28170 on the production of spectrin breakdown-products generated by calpain activation. (**a**) Representative Western blots showing the presence of the complete spectrin protein (280 kDa) and the bands produced by calpain (145 and 150 kDa) after the different treatments. Rats were treated with the glutamate antagonists MK-801 (2 mg/kg) and NBQX (50 nmol). The calpain inhibitor MDL-28170 was administered 30 min before (30 mg/kg), immediately, and 1 h (20 mg/kg each one) after glutamate injection (70 mg/kg in total). Rats were sacrificed 12 h after Glu injection and brains were processed as described in the methods section. (**b**) Graph showing the densitometric analysis of the ratio of the densities of the 145/150 kDa and the actin bands. $^{*}P \leq 0.05$ relative to control. $^{\#}P \leq 0.05$ relative to Glu. Data are means ± SEM from the number of animals indicated at the bottom of each bar

tested. No reduction in the lesion volume was observed supporting the lack of effect of this antagonist against glutamate neurotoxicity induced in the present conditions (Fig. 4). Treatment with both antagonists did not improve the protective effect of MK-801 (Fig. 4).

The effect of the calpain inhibitor MDL-28170, on the size of the striatal lesions was also studied. We have recently observed that treatment with 50 mg/kg of MDL-28170 inhibits significantly calpain activity and prevents the damage induced by 500 nmol of glutamate in rats pre-treated with a mitochondrial toxin (Del Río and Masssieu, submitted). However, in the present conditions this treatment did not reduce the size of the lesion (Fig. 4), although several healthy cells (white arrows) were observed inside the lesioned area (Fig. 3). In order to increase the inhibitor concentration in the brain before glutamate injection, a higher dose of MDL-28170 (70 mg/kg) was tested. Figure 4 shows that this dose of MDL-28170 caused a 25% reduction in the lesion volume, although, this effect was not statistically significant (Fig. 4). In order to know the extent of calpain inhibition by MDL-28170, we performed Western blot analysis. Figures 2a, b show that MDL-28170 did not prevent calpain activity as assessed 12 h after glutamate injection.

Discussion

Intracellular events involved in glutamate-induced neuronal death have been extensively studied; however there are controversies among the great number of studies possibly because of the different models, preparations, and stimulus utilized. The role of calpain and caspase on glutamate-induced death is also controversial. Previous studies showing that calpain activation is implicated in excitotoxicity have been performed utilizing selective glutamate agonists such as NMDA, AMPA and kainate [20, 40–43]. To our knowledge this is the first study investigating calpain activation and its relation to neuronal death in an in vivo model of neurotoxicity induced by the endogenous ligand glutamate, and not by glutamate receptor agonists. We sought to investigate the contribution of each receptor subtype to calpain activity and neuronal damage induced by glutamate. For this purpose, we injected 1 µl of a high concentration of glutamate (2 µmol) to induce a striatal lesion. A high concentration of glutamate was chosen because the damage induced by lower concentrations (500–600 nmol) is not different to that produced by the injection of saline solution. Only at concentrations as high as 1–2 µmol glutamate induces extensive striatal lesions [28, 44]. The lack of neurotoxicity of low concentrations of this amino acid is attributed to its rapid clearance from the extracellular space by its transporter proteins. On the other hand, inhibition of glutamate transporters, despite inducing the accumulation of extracellular glutamate, leads to minimal damage [37, 45]. The present study shows that the administration of 2 µmol of glutamate induces very extensive lesions enclosing almost the entire striatum, suggesting that glutamate removal by its uptake systems is overwhelmed. Glutamate neurotoxicity induced in these conditions was 47% prevented by pre-treatment with MK-801 and was not sensitive to blockade of non-NMDA receptors by NBQX. The lack of effect of NBQX on striatal lesions was corroborated by systemic and intrastriatal treatments with this antagonist. In previous studies we have

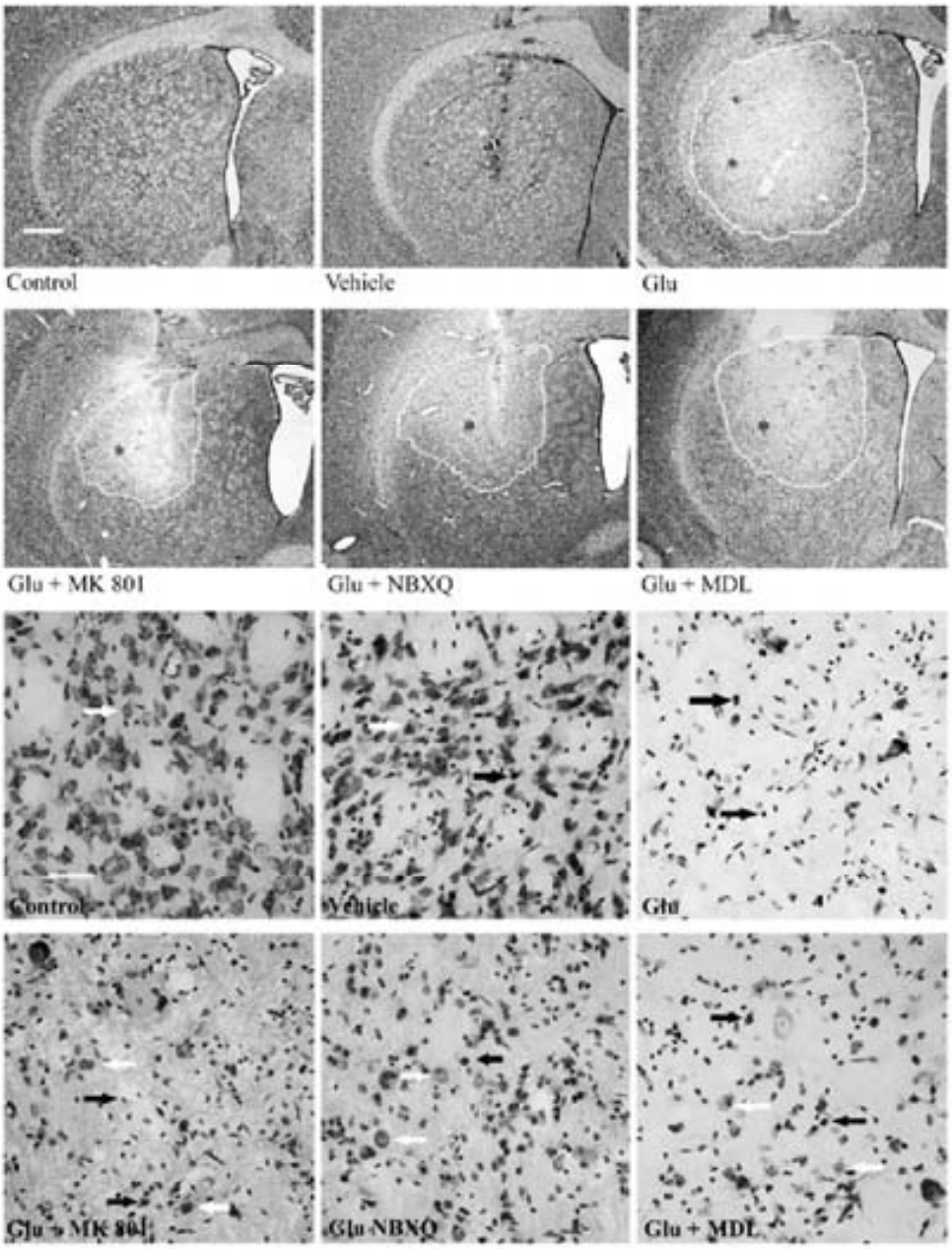

Fig. 3 Effect of the glutamate receptor antagonists, MK-801 and NBQX, and of the calpain inhibitor MDL-28170, on glutamate-induced striatal damage. Figure shows micrographs of representative tissue sections of the striatum of rats subjected to the different treatments. MK-801 (2 mg/kg) was administered i.p., NBQX was co-injected with Glu (2 µmol), and MDL-28170 (70 mg/kg) was administered i.p. Damaged areas are limited by a white line in the upper panels; they are characterized by a pale region due to absence of incorporation of cresyl violet. Bottom panels show magnification of areas inside the lesions (asterisks). White arrows indicate healthy cells and black arrows show pyknotic cells. Scale bar = 500 µm (upper panels) and 50 µm (bottom panels)

observed that the i.p administration of NBQX at the same dose used in this study, partially prevents neuronal damage induced by the blockade of glutamate transporters as well as excitotoxic lesions induced by glycolysis inhibition in vivo [38, 39]. In a different study we have shown that an intrastriatal injection of NBQX, at the same dose as that used in the present study, effectively prevents striatal damage induced by AMPA and partially reduces that triggered by kainate [36]. Similarly, AMPA-induced striatal lesions elicited during mitochondrial inhibition, are effectively reduced by the intrastriatal administration of NBQX [46]. Thus, the present results support the notion that glutamate-induced neuronal death involves mainly NMDA receptors activation [2]. In contrast to previous studies showing that MK-801 reduces completely glutamate excitotoxic damage induced during mitochondrial inhibition [37]; blockade of NMDA receptors only reduced partially glutamate-mediated neuronal death in the present experimental conditions. As we could not prevent completely glutamate damage by blocking the ionotropic glutamate receptors, these results suggest that glutamate-induced death involves additional factors that are ionotropic receptor-independent.

The precise role of calpain and caspase-3 in neuronal death induced by glutamate is still not completely understood. Here, we show that glutamate administration notably

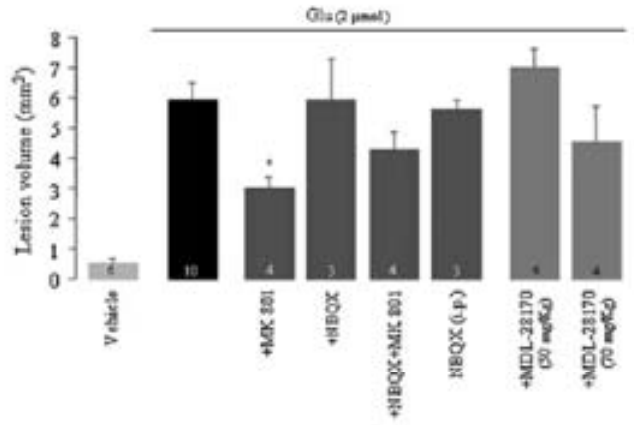

Fig. 4 Effect of the glutamate receptor antagonists MK-801 and NBQX, and of the calpain inhibitor MDL-28170 on the lesion volume induced by glutamate. MK-801 (2 mg/kg) was administered i.p., and NBQX was either co-injected with Glu (2 μmol) or i.p. injected (60 mg/kg total dose). Two different doses of MDL-28170 were tested (50 and 70 mg/kg) as described in the methods section. Volume lesion was quantified 24 h after glutamate injection. $^{*}P \leq 0.05$ relative to Glu. Data are means ± SEM from the number of animals indicated at the bottom of each bar

stimulated calpain activity as revealed by the presence of the 145/150 kDa spectrin fragments. According to the densitometric analysis calpain activity was increased up to 10-fold after 2 h of glutamate injection. The 145/150 kDa bands were still visible 24 h after glutamate administration possibly because of their high stability and slow rate of degradation [47, 48]. In contrast to calpain, caspase-3 was not activated at any of the times studied suggesting that necrosis rather than caspase-mediated apoptosis is involved in glutamate neurotoxicity. Previous in vitro studies show that activation of NMDA and non-NMDA receptors by selective agonists, induces calpain activation and the cleavage of several proteins including, nitric oxide synthase, the plasma membrane Ca^{2+}ATPase, the Na^{+}/Ca^{2+} exchanger and the GluR1 subunit of AMPA receptors [20, 49–52]. On the other hand, several studies have shown protection against excitotoxic injury by inhibition of this Ca-dependent protease [20, 23, 43, 53] To further evaluate the role of calpain on glutamate-induced neuronal death we treated the rats with the selective calpain inhibitor MDL-28170. Treatment with MDL-28170 has been shown to efficiently prevent calpain activity and neuronal damage induced by kainate in in vitro studies [20, 43] and by ischemia in in vivo models [23, 53]. However, in the present experimental conditions we could not detect calpain inhibition in rats receiving either 50 or 70 mg/kg of MDL-28170. Furthermore, we could not observe a significant reduction of glutamate-induced striatal lesions after MDL-28170 treatment. Due to the high concentration of glutamate used in the present experimental conditions, a continuous supply of the calpain inhibitor might be needed in order to prevent calpain activation.

Calpain activation might result from increased calcium influx through ionotropic glutamate receptors. Accordingly, we studied the role of ionotropic glutamate receptors in calpain activation and its relation to neuronal death. Results show that calpain activity is not prevented in rats treated individually either with MK-801 or NBQX, but rats treated with both antagonists showed a 38% significant reduction in the intensity of the 145/150 kDa bands. These results suggest that stimulation of either NMDA or non-NMDA receptors is sufficient to strongly stimulate calpain activation, and that blockade of both receptor subtypes is necessary to reduce enzyme activity. Results also suggest that, at least in the present conditions, other factors are involved in the induction of enzyme activity, because blockade of both receptor subtypes did not inhibit completely calpain activation. Moreover, results suggest that the protective effect of MK-801 involves the blockade of other calcium-dependent processes since treatment with this antagonist alone had no effect on calpain activation, but partially reduced the size of the lesions.

An important conclusion of the present study is that glutamate in vivo administration leads to calpain activation through both ionotropic receptor subtypes. Even though the NMDA receptor channel is more permeable to calcium ions than the AMPA/kainate receptor, and has a major contribution to glutamate toxicity, stimulation of either ionotropic receptor subtype activates calpain to same extent. Although AMPA/kainate receptors are mostly permeable to Na^{+} ions, they are also permeable to calcium depending on its subunit composition [54]. In addition, we can conceive that numerous AMPA/kainate receptors are stimulated in the present conditions, leading to the entry of calcium in sufficient amounts to trigger sustained calpain activity. These observations provide new data about the contribution of the different glutamate receptor subtypes to the activation of proteases, particularly calpain, as executioners of excitotoxic neuronal death induced by the administration of the endogenous ligand, glutamate. According to the results, we can conclude that calpain activity might not to be essential for neuronal death, and that apparently other Ca-dependent processes are involved; neuronal damage might result from the convergence of multiple factors.

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CONCLUSIONES

Los resultados mostrados en esta tesis indican que, contrario a lo propuesto en la hipótesis, la toxicidad inducida por glutamato (en ausencia o presencia de una inhibición mitocondrial previa) es independiente de caspasa-3 y dependiente de la activación de la calpaína. Sin embargo, la activación de la calpaína por el glutamato no siempre se asocia con una cascada de muerte celular, es necesario que la activación de la calpaína sea intensa y prolongada para que ésta se asocie con una cascada de muerte neuronal. Estos resultados confirman que la inhibición mitocondrial amplifica los procesos dependientes de Ca^{2+} relacionados con la muerte neuronal (Nasr et al., 2003; Jacquard et al., 2006) y que no modifica el patrón de activación de proteasas inducido por el glutamato. La contribución de los receptores ionotrópicos glutamatérgicos, tanto a la activación de la calpaína como a la generación del daño neuronal, es similar durante la facilitación de la muerte excitotóxica cuando hay una inhibición mitocondrial previa. Esto sugiere que la inhibición mitocondrial parcial hace que el aumento en la concentración de Ca^{2+} intracelular sea dañino para las células, independientemente de tipo de receptor (NMDA o no-NMDA) por el que entre este ión a la células. En cambio, cuando la concentración de glutamato es tóxica, el principal receptor involucrado en el daño celular es el NMDA. Es importante enfatizar que aunque el aumento en la concentración intracelular de Ca^{2+} y la activación de la calpaína son determinantes para la muerte excitotóxica, otros procesos, como la producción excesiva de ERO, pueden estar involucrados en el daño celular si el estímulo tóxico de glutamato es muy fuerte. El estudio de las consecuencias que tienen la activación de la calpaína durante la muerte excitotóxica es necesario para poder frenar el proceso de muerte neuronal y proponer tratamientos para las enfermedades relacionadas con este mecanismo de muerte.

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APÉNDICE

ARTÍCULO 4

"Exacerbation of excitotoxic neuronal death induced during mitochondrial inhibition in vivo: relation to energy imbalance or ATP depletion? Del Río P., Montiel T., Chagoya V. y Massieu L. 2007 Neuroscience 146, 1561-1570

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EXACERBATION OF EXCITOTOXIC NEURONAL DEATH INDUCED DURING MITOCHONDRIAL INHIBITION *IN VIVO*: RELATION TO ENERGY IMBALANCE OR ATP DEPLETION?

P. DEL RÍO,[a] T. MONTIEL,[a] V. CHAGOYA[b] AND L. MASSIEU[a]*

[a]*Depto. de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México, D.F. CP 04510, Mexico*

[b]*Depto. de Biología Celular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México, D.F. CP 04510, Mexico*

Abstract—During the past two decades a close relationship between the energy state of the cell and glutamate neurotoxicity has been suggested. We have previously shown that increasing the extracellular concentration of glutamate does not cause neuronal death unless a deficit in energy metabolism occurs. The mechanisms of glutamate-induced neuronal death have been extensively studied *in vitro* and it has been associated with a rapid and severe decrease in ATP levels, accompanied with mitochondrial dysfunction. In this study we aimed to investigate the time course of the changes in energy metabolites during glutamate-induced neuronal death, in the presence of a moderate inhibition of mitochondrial metabolism in the rat striatum *in vivo*. We also aimed to study whether or not, as reported *in vitro*, changes in ATP levels are related to the extension of neuronal death. Results show that glutamate-induced lesions are exacerbated when rats are previously treated with a subtoxic dose of the mitochondrial toxin 3-nitropropionic acid (3-NP). However, changes in nucleotide levels were similar in rats injected with glutamate alone and in rats injected with glutamate and previously treated with 3-NP. In spite of the presence of an extensive striatal lesion, nucleotide levels were recovered in 3-NP-treated rats 24 h after glutamate injection. Results show that 3-NP pre-treatment induced an imbalance in nucleotide levels that predisposed cells to glutamate toxicity; however it did not influence the bioenergetic changes induced by glutamate alone. Enhancement of glutamate neurotoxicity in 3-NP pre-treated rats is more related to a sustained nucleotide imbalance than just to a rapid decrease in ATP levels.

Key words: 3-nitropropionic acid, glutamate, ATP, energy charge, lactate.

Glutamate is the most abundant excitatory neurotransmitter in the mammalian CNS (Fonnum, 1984), however it becomes toxic under certain circumstances such as impairment of energy metabolism. Glutamate neurotoxicity, named excitotoxicity, has been implicated in neurodegeneration linked to pathological conditions associated with failure of energy metabolism, such as brain ischemia, hypoglycemia and cerebral trauma (Benveniste et al., 1984; Wieloch, 1985; Arundine and Tymianski, 2004), as well as with some chronic neurodegenerative diseases such as Huntington's disease and amyotrophic lateral sclerosis (Beal et al., 1986; DiFiglia, 1990; Ikonomidou and Turski, 1995; Massieu and García, 1998). Previous studies have demonstrated that metabolic poisoning induced by glycolysis and mitochondrial inhibition, elicits excitotoxic death (Zeevalk and Nicklas, 1991; Beal et al., 1993; Pang and Geddes, 1997), and that the threshold for glutamate toxicity is diminished during impairment of energy metabolism or glucose deprivation, inducing neuronal death even at low concentrations (Novelli et al., 1988; Zeevalk and Nicklas, 1992; Simpson and Isacson, 1993; Massieu et al., 2001). It has been demonstrated that metabolic inhibition favors glutamate NMDA receptor activation through the removal of Mg^{2+} block from the receptor channel, due to membrane depolarization as a consequence of the failure of membrane Na^+/K^+-ATPase activity (Cox et al., 1989; Zeevalk and Nicklas, 1992). On the other hand, disruption of energy metabolism has focused the attention of many studies because numerous reports in different types of cells have shown that a decline in ATP levels can lead to cell death, and that depending on the extent of this decrease, cell death will show an apoptotic or a necrotic phenotype (Bonfoco et al., 1995; Ankarcrona et al., 1995; Leist et al., 1997, 1999).

The mechanisms involved in glutamate-mediated neuronal damage during metabolic inhibition have been extensively studied *in vitro*. In cultured neurons exposure to 3-nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase (SDH), in combination with a low dose of glutamate, leads to a severe ATP decline and a rapid necrotic cell death (Pang and Geddes, 1997; García and Massieu, 2003). *In vitro* studies suggest that the main mechanism underlying the synergistic effect of metabolic inhibition and glutamate toxicity is the rapid loss of ATP levels and, as consequence, increased intracellular calcium and reactive oxygen species production (Pang and Geddes, 1997; Nasr et al., 2003; García and Massieu, 2003; García et al., 2005), however, *in vivo* evidence is still inconclusive.

In previous *in vivo* studies we have shown that increasing the extracellular concentration of glutamate by inhibition of its reuptake, does not lead to neuronal damage unless the energy metabolism is altered (Massieu et al., 2001, 2003). Subtoxic doses of 3-NP, that do not induce neuronal damage per se, facilitate glutamate-toxicity in the

*Corresponding author. Tel: +52-55-56-22-57-61; fax: +52-55-56-22-56-07.
E-mail address: lmassieu@ifc.unam.mx (L. Massieu).
Abbreviations: EC, energy charge; GFAP, glial fibrillary acidic protein; PDC, L-*trans*-pyrrolidine-2,4-dicarboxylate; SDH, succinate dehydrogenase; 3-NP, 3-nitropropionic acid.

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striatum and hippocampus of rats injected with L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC), a substrate inhibitor of glutamate transport (Massieu et al., 1995; Sánchez-Carbente and Massieu, 1999). In these conditions neuronal death is partially prevented by energy-supplementing substrates such as pyruvate or ketone bodies, supporting the role of the maintenance of energy metabolism in cell survival (García and Massieu, 2001; Massieu et al., 2001, 2003). The present investigation aimed at determining whether or not glutamate toxicity during mitochondrial inhibition is related to a severe drop in ATP levels similar to the situation *in vitro*. The results of this study are relevant to the understanding of the mechanisms involved in excitotoxic neuronal death induced during ischemia and hypoglycemia, two conditions associated with metabolic impairment and increased extracellular glutamate, where the progression of brain damage appears to be related to the extent of energy impairment (Kass and Lipton, 1989; Yager et al., 1994).

EXPERIMENTAL PROCEDURES

Animal treatment

Male Wistar rats (250–300 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. Animals received two i.p. injections of 3-NP (15 mg/kg) (Sigma, St. Louis, MO, USA), the second administered 24 h after the first. Rats received an intrastriatal administration of glutamate (500 nmol/μl) 4 h after the second 3-NP administration. 3-NP was dissolved in 10 mM phosphate buffer (pH adjusted to 7–7.5 with 1 M NaOH). Glutamate was dissolved in saline solution (0.9% NaCl). For intrastriatal injections, animals were anesthetized with 3.5–4% halothane in a 95% O_2/5% CO_2 mixture and placed on a stereotaxic frame. Rats were maintained under low anesthesia throughout the injection. A 1–2 mm hole was drilled and a 1 μl volume of either glutamate or vehicle was injected at a rate of 0.5 μl/min via a 27-gauge stainless steel needle connected to a Hamilton syringe (Hamilton, Reno, NV, USA) with the aid of a microinjection pump (model 55; Harvard Apparatus, South Nantick, MA, USA). Coordinates used were: anteroposterior +0.7 mm from Bregma, lateral +2.8 mm from midline, and ventral −4.0 mm below the dura mater, according to Paxinos and Watson (1986). Two minutes after the injection was completed, the needle was withdrawn and the skin was sutured. Animals were killed at different times after glutamate administration and brains were prepared for histological evaluation, glial fibrillary acidic protein (GFAP) immunohistochemical analysis, nucleotide determination, SDH activity or lactate measurements (see below). A group of control animals received two i.p. administrations of phosphate buffer instead of 3-NP and intrastriatal glutamate, and a second group of control animals received two i.p. administrations of 3-NP (15 mg/kg) and an intrastriatal injection of saline solution instead of glutamate.

Histological evaluation

For histological evaluation animals were anesthetized with sodium pentobarbital anesthesia at different times after glutamate injection (3, 4, 7, and 24 h), 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. Brains were then transferred to a 20% and 30% sucrose solution successively. Coronal sections (40 μm) were obtained 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. 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 multiplying the average damaged area by the distance between the first and the last tissue section where tissue damage was visible. Results are expressed as means±S.E.M. of lesion volume per each animal group.

SDH histochemistry

To study the time course of SDH inhibition after the second 3-NP administration, SDH activity was measured histochemically in tissue sections as previously reported by Brouillet et al. (1998). Succinate was used as the specific substrate and Nitro Blue Tetrazolium (NBT) (Sigma) as the electron acceptor which forms formazan. Rats were treated with 3-NP and killed at 1, 2, 4, 7, 11, and 24 h later. Control animals received two i.p. injections of 10 mM phosphate buffer instead of 3-NP. The optical density of the dark-blue stain produced by the formazan product was measured in tissue sections as previously reported (Massieu et al., 2001). Bilateral images from the striatum of five tissue sections per animal were chosen (taken between coordinates 0.2 and 1.0 mm anterior to bregma). An area containing the whole caudate-putamen was manually delineated and the optical density was measured. The optical density of both striata was averaged, and the mean optical density from each animal was calculated. The mean optical density in control animals was 15,846.7±71.693 (n=12) and was considered as 100% of activity. SDH activity was calculated as percent of optical density of control animals.

Nucelotide determination by HPLC

ATP, ADP and AMP levels were monitored by HPLC according to Delaney and Geiger (1996). Rats intrastriatally injected with glutamate and previously injected (i.p.) with vehicle or 3-NP were killed 4, 7, and 24 h after glutamate injection. Because glutamate was injected 4 h after the second 3-NP administration, the animal group treated only with 3-NP was killed 8, 11 and 28 h after 3-NP injection. Control values were measured from the striata of intact rats. All animals were killed by the freeze-clamp technique. Briefly, rats were anesthetized with halothane 3.5% and placed on a stereotaxic apparatus, the cortex was exposed by removing part of the skull, and a plastic funnel was placed firmly over the tissue. Liquid nitrogen was poured directly onto the tissue until the animal stopped breathing (3–5 min later), indicating that the medulla was frozen (Delaney and Geiger, 1996). Brains were removed, placed in liquid nitrogen for 10 min and stored at −70 °C. After dissection of the striatum on dry ice, tissue was homogenized in 0.8 M perchloric acid (10 μl/μg wet weight), and centrifuged (10,000 r.p.m. for 10 min at 4 °C). Supernatants were collected and kept frozen at −20 °C; 24 h later, supernatants were neutralized with 2 M K_2CO_3 (12% of total volume) and re-centrifuged. Supernatants were used for nucleotide determinations by HPLC. Twenty-five microliters of an 80-fold dilution of each sample was injected in the HPLC system using a μbondapack column (3.9×300 mm; Waters, Milford, MA, USA) and a mobile phase consisting of 0.1 M KH_2PO_4, 10% methanol (pH 6), at a flux of 1.5 ml/min. External standards were identically treated as brain homogenates; ATP, ADP, and AMP peaks were identified by retention time. Data are expressed as pmol/μg of frozen tissue weight. Energy charge (EC) was calculated as follows: EC=(ATP+0.5 ADP)/(ATP+ADP+AMP).

Lactate determinations

Lactate determinations were performed as reported previously (Massieu et al., 2001). For these determinations, rats were treated as described for nucleotides determination. Animals treated with 3-NP alone were anesthetized and killed 8, 11, and 28 h after the second injection of 3-NP. Rats treated with 3-NP or vehicle (i.p.) and injected intrastriatally with glutamate, were anesthetized and killed 4, 7, and 24 h after glutamate injection (8, 11, and 28 h after 3-NP). Brains were removed and the striata dissected. Tissue was homogenized in 10 volumes (w/v) cold water and 5% perchloric acid was added. Samples were centrifuged at 3000 r.p.m. for 5 min; the supernatants were collected and neutralized with 1 M KOH. Lactate concentration was measured using a lactate determination kit (Sigma), following the instructions from the suppliers, with the exception that 150 μl of the sample was used. Briefly, samples (150 μl) were added to 860 μl of the buffer provided by the manufacturers reconstituted in 10 ml distilled water. Absorbance was measured 5 min later at 540 nm. Lactate concentrations were calculated from readings obtained from a standard curve (2.5, 5, 10, 20, and 40 mg lactate). Data are expressed as μmol lactate/g wet weight.

GFAP immunohistochemistry

For immunohistochemistry, rats were killed 24 h after 3-NP (3-NP alone) or after glutamate administration in 3-NP-treated (3NP+glutamate) or control rats (glutamate alone). They were transcardially perfused with 250 ml of 0.9% saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). Brains were removed and transferred to sucrose solutions as previously described. Coronal sections (40 μm) were obtained in a cryostat, rinsed in 0.1 M PBS and incubated in 0.1 M PBS containing 0.3% Triton X-100 and 1% normal goat serum for 2 h. After a rinse with 0.1 M PBS, sections were incubated with primary anti-rabbit antibody against GFAP (Dako, Denmark) (1:2000 dilution in 0.1 M PBS containing 0.3% Triton X-100 and 1% normal goat serum) for 72 h at 4 °C. Tissue sections were incubated with biotinylated secondary antibody (diluted 1:200 in the same buffer as the primary antibody) for 2 h at room temperature. Immunohistochemistry was detected by incubation with the avidin–biotin–peroxidase complex (ABC, Vector Laboratories, Burlingame, CA, USA), prepared according to manufacturer's instructions, in PBS containing 0.1% bovine serum albumin (pH 7.2) for 2 h. Tissue sections were incubated for 5–10 min in 0.05% 3,3′-diaminobenzidine in 0.1 M Trizma (pH 7.2) with hydrogen peroxide (1 μl/ml). Sections were mounted on glass slides and covered with permount. Sections incubated without primary antibody showed no staining.

Statistics

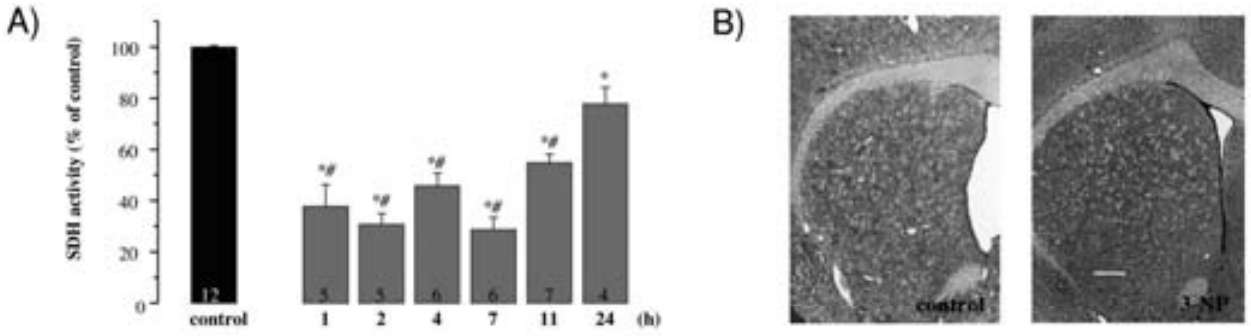
All data are expressed as means±S.E.M. Statistical analysis was carried out by one-way ANOVA followed by a Fisher's least significant difference multiple comparison test.

RESULTS

3-NP transiently inhibits SDH activity and decreases EC without injuring the striatum

3-NP inhibits the mitochondrial enzyme SDH, which is part of complex II of the mitochondrial electron transport chain and of the Krebs cycle. After a single dose, 3-NP inhibits brain SDH very rapidly (Brouillet et al., 1998; Massieu et al., 2001). In the present study we determined SDH activity in the rat striatum after two administrations of a subtoxic dose of 3-NP (15 mg/kg/day). According to this analysis SDH activity was significantly inhibited as soon as 1 h after the second administration of 3-NP to 37% of control activity, and remained significantly inhibited during the following 11 h. SDH activity recovered to 77% of control values 24 h after the treatment (Fig. 1A). Nucleotide levels were slightly modified by 3-NP treatment. ATP levels were not significantly altered although a moderate reduction was observed, as well as a significant decrease in the EC 11 h after the treatment. AMP levels increased significantly at 28 h after the second 3-NP administration and this was reflected in a reduction in the EC (Fig. 1C). It is worth noticing that the two-dose treatment of 3-NP employed in this study did not injure the striatum despite the bioenergetic changes it induced (Fig. 1B). None of the animals treated with 3-NP showed any behavioral changes or became recumbent as has been described after the chronic administration of the toxin (Hamilton and Gould, 1987). The term *recumbency* refers to the loss of body posture and the adoption of a severe dystonic ventral or lateral position with hind limbs rigidly extended. These alterations are related to severe striatal damage, a condition that was not observed under our experimental paradigm (Hamilton and Gould, 1987).

In previous studies we have demonstrated that neuronal damage induced by the glutamate transport blocker, PDC, is exacerbated in 3-NP-treated animals. The present study investigated the progress of glutamate-induced lesions in 3-NP-treated animals and its relation to changes in nucleotide levels. Thus, glutamate was intrastriatally injected 4 h after the second administration of 3-NP, time at which SDH activity is 55% inhibited. Rats were killed at 3, 4, 7 and 24 h after glutamate administration and the progression of the lesion was studied by determining the lesion volume in Cresyl Violet–stained sections. Damaged tissue appeared as a pale region in tissue sections due to the lack of incorporation of Cresyl Violet (Fig. 2). The extension of glutamate-induced lesions in animals not treated with 3-NP was similar at all times studied. In contrast, larger lesions were observed when glutamate was injected in 3-NP-treated animals, and tissue damage was evident as soon as 4 h after glutamate injection. The lesion core, in 3-NP pre-treated rats, was initially characterized by the presence of amorphous and condensed cells at 4 and 7 h (Fig. 2B–C, white arrows). Pyknotic nuclei were observed 24 h after glutamate injection (Fig. 2D, black arrows). Quantification of the lesion volume shows that, intrastriatal administration of 500 nmol glutamate to intact rats induces only small lesions that were not statistically different from vehicle injection (0.40±0.04 mm^3) as monitored 24 h after the administration. The lesion induced by glutamate was larger 24 h after its injection (1.03±0.27 mm^3), but it was not statistically different from that induced by vehicle solution. On the other hand, glutamate-induced lesions were exacerbated in 3-NP pre-treated rats, and this effect was statistically significant at 4 h. The lesion progressed thereafter and at 7 h its volume doubled the initial size (1.43±0.29 and 3.37±0.66 mm^3 at 3 and 7 h, respectively) (Fig. 3). These results are consis-



C) Effect of 3-NP on ATP, ADP, AMP and EC (pmol/μg of frozen tissue weight) in striatal homogenates of rats sacrificed at different times after the second 3-NP administration.

Control			
ATP	6.891 ± 0.607		
ADP	1.660 ± 0.143		
AMP	0.366 ± 0.065		
ATP / ADP	4.061 ± 0.383		
Total Nucleotides	8.816 ± 0.623		
EC	0.866 ± 0.007		
3-NP i.p. treatment	**8 h**	**11 h**	**28 h**
ATP	7.52 ± 1.192	5.091 ± 1.110	5.54 ± 0.458
ADP	1.811 ± 0.158	1.739 ± 0.471	1.860 ± 0.280
AMP	0.562 ± 0.148	0.476 ± 0.046	0.667 ± 0.336*
ATP / ADP	4.475 ± 0.213	3.221 ± 0.446	3.076 ± 0.557
Total Nucleotides	9.469 ± 1.802	7.075 ± 1.217	9.305 ± 0.760
EC	0.845 ± 0.016	0.812 ± 0.014 *	0.802± 0.037 *

Nucleotide levels were measured in striatal homogenates by HPLC after the second 3-NP i.p. administration. Data are mean ± SEM n = 3-6 * $p \leq 0.05$, significantly different compared to control.

Fig. 1. Changes in SDH activity induced by 3-NP (A), lack of effect on tissue integrity (B), and changes in nucleotide levels (C). (A) Time-course of SDH inhibition after the second i.p. administration of 3-NP (15 mg/kg). SDH activity was measured histochemically at different times in tissue sections from the rat striatum, as described in Experimental Procedures. Data are expressed as percent SDH activity relative to control animals. (B) Micrograph of a tissue section of the striatum of a control rat and a rat injected (i.p.) with 3-NP. Tissue was processed and stained with Cresyl Violet. Scale bar=400 μm. (C) Effect of 3-NP on ATP, ADP, AMP, and EC in striatal homogenates of rats killed at different times after the second 3-NP i.p. administration. Nucleotide levels were quantified at 8, 11, and 28 h after the second 3-NP administration. Nucleotides were monitored by HPLC as described in Experimental Procedures. Control values were obtained from striatal homogenates of intact rats killed in parallel to 3-NP treated rats. * $P \leq 0.05$ relative to control. # $P \leq 0.05$ relative to the 24 h point. Data are means±S.E.M. from the number of animals indicated at the bottom of each bar.

tent with the fact that the threshold to glutamate toxicity is reduced when energy metabolism is impaired.

Energy imbalance induced by glutamate is not enhanced in 3-NP pre-treated rats

Previous studies in neuronal cultures have shown that excitotoxicity induced during inhibition of mitochondrial metabolism is accompanied by an abrupt decline in ATP levels (Nasr et al., 2003; García and Massieu, 2001). We therefore aimed to study this phenomenon *in vivo*. Thus, rats of the different experimental groups were killed at different times after glutamate injection and nucleotide levels were measured in striatal homogenates. Because a progressive increase in the size of the lesion was observed when glutamate was injected in 3-NP-treated rats, a corresponding decrease in ATP levels was expected. As previously shown, 3-NP treatment induces a moderate energetic imbalance without injuring the striatum (Fig. 1C). A similar effect was observed when glutamate was intrastriatally injected in intact rats. Glutamate-induced changes in nucleotide levels were generally observed 7 h after its injection, but were statistically significant at 24 h as compared with control values in intact rats (Fig. 1C). Glutamate induced a decrease in ATP and a transient reduction in ADP at 7 h, followed by an increase in ADP and AMP levels and a significant reduction in the EC at 24 h as compared with control values (Fig. 1C). Despite these changes striatal tissue was not significantly damaged (Figs. 2 and 3). Contrary to what we expected, glutamate-induced changes in nucleotide levels were not enhanced

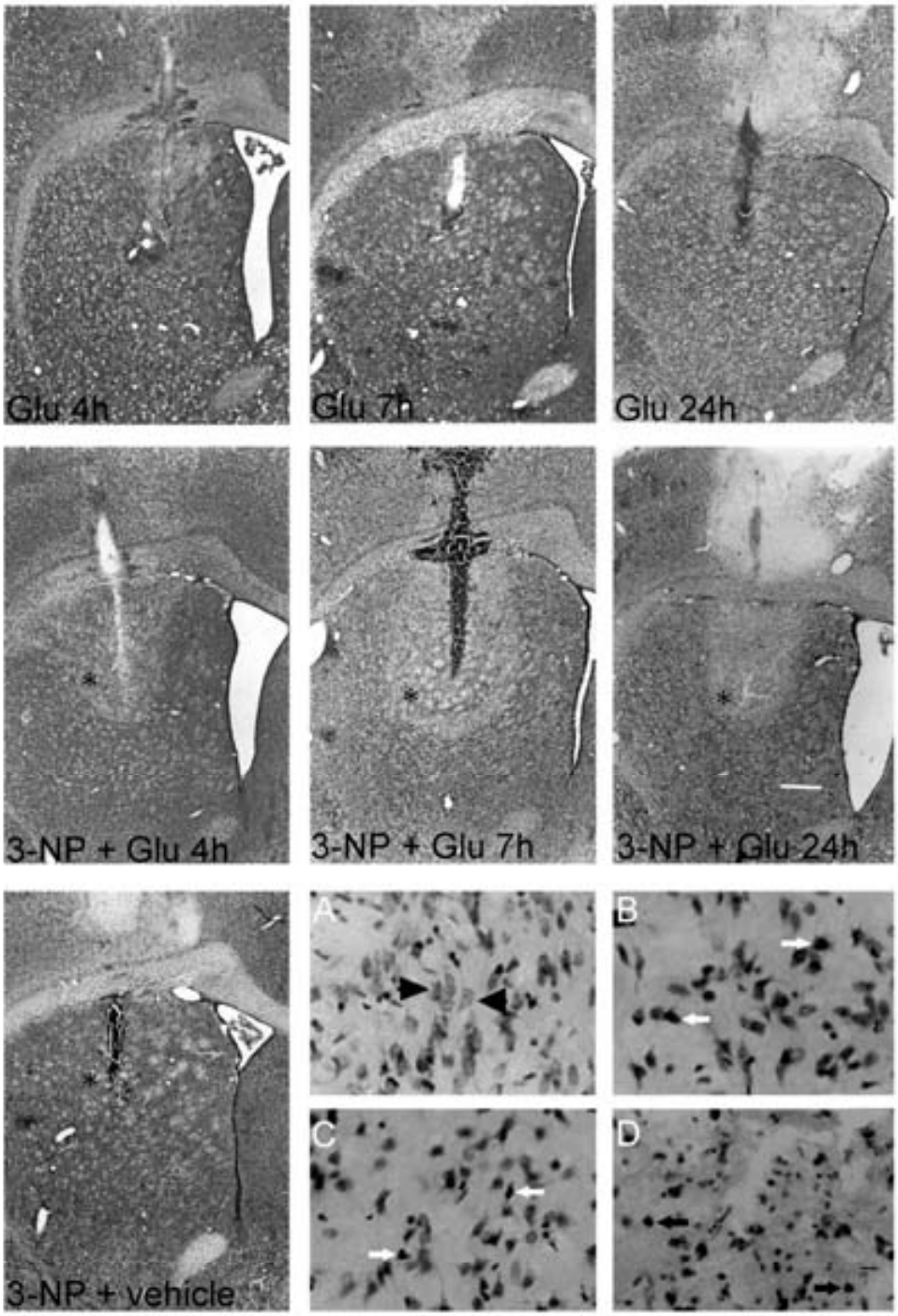


Fig. 2. Micrographs of representative tissue sections showing the progression of lesions produced by glutamate (Glu) in intact rats or rats previously treated with 3-NP. Lesions are observed as a pale region due to the absence of Cresyl Violet staining. Vehicle injection does not injure the striatum in 3-NP-pretreated rats (3-NP+vehicle). In vehicle-treated animals intrastriatal injection of Glu induced small lesions with a similar size at all times studied. In animals pre-treated with 3-NP (3-NP+Glu) an increase in the size of the lesions is clearly observed. Scale bar=400 μm. Magnifications of micrographs from 3-NP pre-treated rats injected with Glu are shown in A–D. Magnifications were taken from the lesion core (*). (A) Vehicle injection in a 3-NP-treated rat; (B) 3-NP+Glu at 4 h; (C) 3-NP+Glu at 7 h; (D) 3-NP+Glu at 24 h. Normal appearing cells are observed in 3-NP-treated rats injected with vehicle (black arrowheads), and the presence of dark and shrunken cells are visible at 4 and 7 h after Glu injection (white arrows). Twenty-four hours after Glu injection the lesion core is characterized by the presence of condensed nuclei (black arrows). Scale bar=10 μm.

by 3-NP pre-treatment despite the energy imbalance produced by the mitochondrial toxin and by the administration of glutamate itself. In 3-NP pre-treated animals glutamate induced a transient and not significant decrease in ATP,

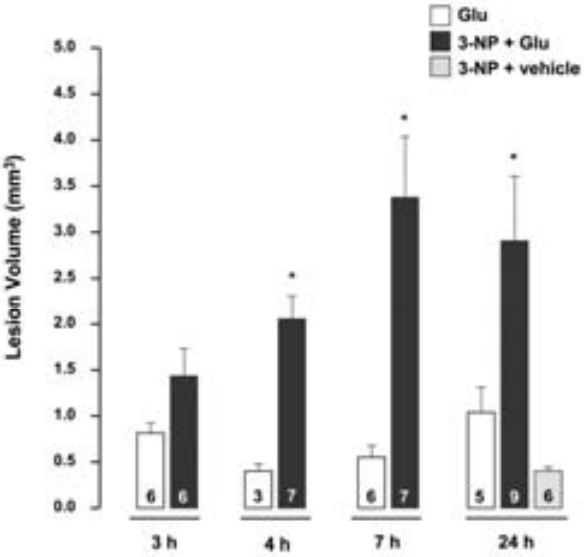


Fig. 3. Quantification of lesion volume (mm^3) at different times after intrastriatal glutamate injection. Rats were i.p. injected either with vehicle or 3-NP, and glutamate was injected 4 h after the second i.p. administration (Glu and 3-NP+Glu, respectively). One additional group of rats was treated with 3-NP and intrastriatally injected with phosphate buffer 4 h later (3-NP+vehicle). Lesion volume was evaluated at different times after glutamate injection, and was measured as described in Experimental Procedures. * $P \leq 0.05$ relative to Glu. Data are means±S.E.M. from the number of animals indicated at the bottom of each bar.

which recovered at 24 h (Table 1) as compared with intact rats (Fig. 1C). An increase in AMP levels was also observed at 7 h, which increased further and significantly at 24 h (Table 1) as compared with control levels (Fig. 1C).

Table 1. Effect of glutamate on ATP, ADP, AMP, and EC (pmol/μg of frozen tissue weight) in striatal homogenates of intact and 3-NP pre-treated rats killed at different times after intrastriatal Glu injection

Intrastriatal Glu injection	4 h	7 h	24 h
Intact rats (Glu)			
ATP	7.32±0.474	4.84±1.062	4.8±0.724*
ADP	1.439±0.150	1.072±0.083*	2.008±0.191
AMP	0.542±0.135	0.543±0.144	0.646±0.207
ATP/ADP	5.234±0.428	4.565±0.997	3.162±0.807
Total nucleotides	9.305±0.483	6.480±0.967	6.399±1.266
EC	0.862±0.014	0.792±0.024	0.78±0.019*
3-NP pre-treated rats (3-NP+Glu)			
ATP	6.066±1.121	4.761±0.837	6.961±0.679°
ADP	1.484±0.259	1.270±0.223	1.904±0.353
AMP	0.351±0.046	0.5±0.097	0.954±0.088*
ATP/ADP	4.419±1.01	4.244±0.608	4.135±0.801
Total nucleotides	7.901±1.199	6.207±0.768*	9.129±0.899
EC	0.852±0.025	0.817±0.027*	0.805±0.010*

Nucleotide levels were measured in striatal homogenates by HPLC as previously described in the Experimental Procedures section.
* $P \leq 0.05$ relative to control values presented on Fig. 1C.
° $P \leq 0.05$ relative to Glu injection in intact rats. Data are means±S.E.M., $n=3$–6.

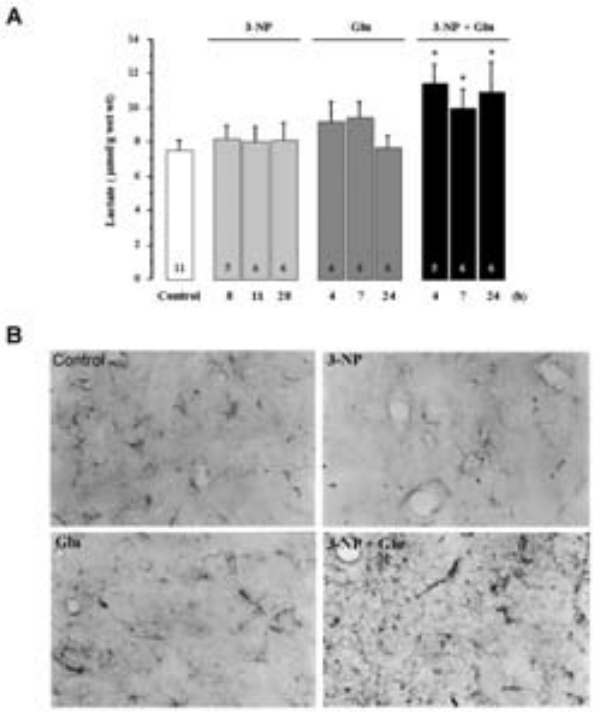


Fig. 4. (A) Lactate levels in the striatum of rats treated either with 3-NP or vehicle solution and intrastriatally injected with glutamate, and in rats treated only with 3-NP. Lactate determinations were made in striatal homogenates at different times after the second administration of 3-NP or after glutamate injection, as described in Experimental Procedures. * $P \leq 0.05$ relative to control levels. Data are means±S.E.M. from the number of animals indicated at the bottom of each bar. (B) Micrographs of representative striatal tissue sections showing immunoreactive cells to GFAP. Micrographs of glutamate- and 3-NP+glutamate-treated rats were taken from an area adjacent to the lesion core 24 h after glutamate or 3-NP injection. An increase in the number of GFAP-immunoreactive cells is observed in tissue sections from 3-NP+Glu-treated rats. Scale bar=40 μm.

Therefore, in spite of the recovery of ATP at 24 h tissue is still under energetic stress, as evidenced by the elevation of total nucleotide levels and the decline in the EC at this time. These results indicate that despite the lesions observed in animals treated with 3-NP+glutamate, nucleotide levels are recovered 24 h after glutamate injection but not in their correct proportion.

Metabolic compromise can be expressed in a variety of ways and not only by a fall in ATP. Decreased phosphocreatine and increased lactic acid production also indicate perturbations of the normal energy state within the tissue. We further aimed to know whether exacerbated glutamate toxicity in 3-NP-treated rats was related to an increase in lactate concentration. Lactate levels were measured in striatal homogenates 4, 7 and 24 h after glutamate injection in 3-NP-treated and intact rats. As reported previously (Massieu et al., 2001), 2-day 3-NP administration did not induce an increase in lactate levels at the concentration used (Fig. 4); glutamate induced a non-significant and transient elevation of lactate levels from 4 to 7 h (39% of control levels), which returned to basal values at 24 h. When glutamate was injected in the striatum of 3-NP-

treated rats, lactate levels significantly increased from 4 to 24 h after the injection (increases of 51, 47, and 61% relative to control levels were observed at 4, 7 and 24 h, respectively) (Fig. 4). Because in these conditions animals show a prominent lesion in the striatum, as soon as 4 h after glutamate injection, it was reasoned that possibly surviving cells together with reactive astrocytes surrounding the lesion were the main lactate and ATP producers probably by means of a glycolytic up-regulation. Accordingly, the presence of reactive glia was analyzed by immunohistochemistry using an antibody against the GFAP. Animals were killed 24 h after glutamate injection, time at which ATP levels were recovered, and lactate levels were still augmented in 3-NP+glutamate-treated rats. Photomicrographs shown in Fig. 4B demonstrate the presence of numerous astrocytic cells in the periphery of the lesion.

DISCUSSION

The mechanisms of neuronal death associated with chronic 3-NP treatment have been widely studied and it is suggested that an excitotoxic mechanism is involved (Beal et al., 1993; Kim et al., 2000; Lee et al., 2002a,b). *In vivo* treatment with repetitive doses of 3-NP induces a decrease in ATP levels (Ludolph et al., 1992; Beal et al., 1993; Matthews et al., 1998), an increase in lactate concentrations (Beal et al., 1993; Massieu et al., 2001; Vis et al., 2002) and the production of free radicals in the rat striatum (Schulz et al., 1996; Kim and Chan, 2002). On the other hand, *in vitro* studies have shown that 3-NP increases cytosolic and mitochondrial calcium concentration (Lee et al., 2002b; Nasr et al., 2003). The administration protocol of 3-NP used in the present study partially inhibits SDH activity and decreases the EC, but induces no neuronal death. This result is in accordance with other reports, and suggests that after one or two systemic administrations of 3-NP, SDH activity apparently does not remain permanently inhibited during the following days, and no neuronal damage is produced (Brouillet et al., 1998; Massieu et al., 2001). Recovery of SDH activity might be related to newly synthesized enzyme since 3-NP shows a short half-life time in plasma (Schulz et al., 1996), and therefore no free 3-NP would be available to bind to newly synthesized enzyme molecules. 3-NP toxicity is related to SDH inhibition damaging tissue only when it is continuously inhibited in more than 70% (Brouillet et al., 1998). The present data agree with this observation and also demonstrate that even a partial inhibition of SDH induces bioenergetic changes without damaging the striatum, but facilitating the neurotoxic activity of glutamate.

On the other hand, a decline in ATP levels after the exposure to toxic concentrations of glutamate or its analogs has been reported in several studies *in vitro* and *in vivo* (Biziere and Coyle, 1978; Retz and Coyle, 1982; Espanol et al., 1994; Tsuji et al., 1994; Marcaida et al., 1995; Ankarcrona et al., 1995). Glutamate-induced neuronal death is concentration-dependent and seems to be related to the extent of ATP decrease (Ankarcrona et al., 1995; Yu et al., 2002; García and Massieu, 2003). In the present study we show that a low dose of glutamate administered into the striatum induces a moderate decline in ATP levels and the EC, which is sustained even 24 h after its administration. This reduction in ATP levels might be the consequence of an increased ATP demand leading to an energy compromise, but without the sufficient intensity to alter cellular survival in intact rats.

The present findings agree with previous observations from our group showing that increasing the extracellular concentration of glutamate through inhibition of its reuptake, leads to neuronal damage only if energy metabolism is disrupted (Sánchez-Carbente and Massieu, 1999; Massieu et al., 2001, 2003; García and Massieu, 2003). In agreement, results show that a low concentration of glutamate is able to induce extensive lesions in the striatum when the animals are under a moderate metabolic stress induced by 3-NP. Decreased ATP production has been shown to be critical for cell viability under conditions of energy stress not only in neurons (Kass and Lipton, 1989) but also in other cellular types such as hepatocytes (Nieminen et al., 1994). In this study we aimed to test whether ATP depletion precedes neuronal death, as has been shown in *in vitro* studies (García and Massieu, 2003; Nasr et al., 2003). Results indicate that glutamate-mediated damage under mild metabolic compromise is evident as soon as 4 h after its administration. However, at this time no changes in nucleotide levels were observed in striatal tissue, although lactate concentration is already significantly elevated suggesting a metabolic alteration. In contrast to *in vitro* observations, the present results show that, nucleotide concentration was similarly altered in the striatum after glutamate injection in intact rats and in rats receiving a subtoxic treatment of 3-NP, 7 h after glutamate injection. Despite that glutamate injection in 3-NP-treated rats did not induce an additional decay in the EC, tissue damage was enhanced in this condition. Moreover, AMP and total nucleotide levels were augmented at 24 h after glutamate administration, with the consequent reduction in EC, suggesting that under these circumstances an energy imbalance is present. This suggestion is further supported by the observation that lactate concentration in striatal homogenates is still enhanced at this time. Nucleotide accumulation might suggest their lack of utilization possibly as a part of an adaptive metabolic response of the recovering tissue. Reactive astrocytes surrounding the lesion might also contribute to these metabolic adaptations possibly by augmenting their glycolytic metabolism. *In vitro* experiments have shown that, during metabolic stress, neurons prevent energy depletion through the reduction of energy utilization and/or the stimulation of the glycolytic pathway. This mechanism has been proposed as a protective strategy against cell death during a short period of anoxia or ischemia (Sims and Zaidan, 1995; Schurr et al., 1997a,b; Izumi et al., 1997; Munns et al., 2003).

Due to the difficulty of a fine dissection in nitrogen-frozen brains, nucleotide determinations were made in homogenates obtained after the dissection of the entire striatum. Therefore, nucleotide levels reflect the state of the energy metabolism in damaged cells as well as in

surviving cells surrounding the lesion. For this reason, we do not discard the possibility of a severe decrease of the EC and ATP levels inside the lesion core. In fact, others and we have demonstrated that when culture neurons were exposed to 3-NP and glutamate, or to 3-NP and an inhibitor of glutamate uptake, ATP levels decrease 30 min after the exposure. This decay is followed by partial loss of mitochondrial membrane potential and mitochondrial activity, suggesting an impairment of oxidative phosphorylation (García and Massieu, 2003; Nasr et al., 2003). These events are accompanied by the loss of intracellular calcium homeostasis and excessive free radical production (Schinder et al., 1996; Pang and Geddes, 1997; Saulle et al., 2004; Nasr et al., 2003; García and Massieu, 2005; Vesce et al., 2004). On the other hand, we have also reported *in vitro* and *in vivo* that, in spite of the partial inhibition of SHD by 3-NP and the toxic effects of glutamate during SDH inhibition, anabolic substrates such as acetoacetate and pyruvate exert neuroprotection (Massieu et al., 2001; García and Massieu, 2003). Protection by these energy substrates is mediated at least in part by the maintenance of ATP levels according to *in vitro* studies (García and Massieu, 2003), indicating that mitochondria are still able to utilize alternative substrates. Such processes could be occurring in the cells inside the lesion core.

The vast majority of the mentioned studies have been performed in neuron-enriched cultures, however it is known that glial cells influence neuronal energy metabolism (Pellerin and Magistretti, 1994; Dringen et al., 1993a,b; Schurr et al., 1997a; Voutsinos-Porche et al., 2003; Kasischke et al., 2004) and vulnerability of neurons to toxic insults (Zeevalk and Nicklas, 1997; Xiao-Yuan and Stringer, 2004). It is also known that glial cells are vulnerable to glutamate and 3-NP toxicity (Deshpande et al., 1997; Fukuda et al., 1998; Chen et al., 2000; Ryu et al., 2003), and although they are more resistant than neurons to toxic insults, specially microglia, they might also be damaged (Kahlert and Reiser, 2004; Szydlowska et al., 2006). Since astrocytes represent up to 90% of cells in the CNS they might have a substantial contribution to the observed metabolic changes. At the moment we ignore whether there is a selective vulnerability of neurons and astrocytes to glutamate toxicity in the present experimental conditions, and the specific contribution of the different types of glial cells to the metabolic changes. Further experiments are needed in order to clarify this issue.

Lactate is commonly accepted as a marker of impaired oxidative metabolism, although during the past years, a metabolic coupling between astrocytes and neurons through lactate production has been described (Pellerin and Magistretti, 1994; Schurr et al., 1999; Pellerin, 2003; Voutsinos-Porche et al., 2003). It has been also suggested that oxidative metabolism is sustained by the astrocyte–neuron lactate shuttle during neuronal activity (Kasischke et al., 2004). In addition, lactate has been proposed as an alternative substrate during hypoxic conditions (Schurr et al., 1997b). In the present experimental conditions lactate accumulation might be the result of the metabolic coupling between astrocytes and neurons and its lack of utilization by damaged neurons. Alternatively, lactate can be produced by metabolically compromised cells due to the increased energy demand and the impairment of oxidative phosphorylation.

Altogether, results indicate that, in the *in vivo* condition, enhancement of glutamate toxicity in striatal tissue is related to a metabolic disturbance as evidenced by an increase in lactate levels and the accumulation of total nucleotides at the expense of an increase in AMP levels. These metabolic changes might be the consequence of an enhanced energy demand at early times, and of an adaptive metabolic response of the recovering tissue, at later times. Further investigation of additional mechanisms involved in glutamate neurotoxicity during *in vivo* metabolic impairment is needed, in order to have a comprehensive picture of the complex interaction between energy metabolism and glutamate neurotoxicity.

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